Characterisation of deubiquitinase enzymes involved in androgen receptor regulation in prostate cancer

A thesis submitted in part requirement for

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In Loving Memory of Walter Whittaker

(1929 – 2008)

Declaration

I certify that this thesis is my own work, except where stated, and has not been previously submitted for a degree or any other qualification at this or any other university.

Abstract

The androgen receptor (AR) is a key transcription factor in prostate cancer (CaP) growth and metastases, and is the main target for CaP treatment via the use of anti-hormonal therapies. Unfortunately, this treatment is only effective in the short-term and re-growth of the tumour results in most cases, termed castrate-resistant CaP (CRCaP), this is refractory to additional chemotherapies and hence fatal. Expression and reactivation of the AR is commonly seen in CRCaP and acts as a driver of advanced CaP growth suggesting the receptor remains a suitable target for next generation CaP therapies.

Ubiquitination represents one of the numerous post-translational modifications that are vital for many cellular processes, including transcription and regulation of protein stability. The AR is a target for ubiquitination by several E3 ubiquitin ligase enzymes that can lead to either increased activity or degradation depending on the E3 ligase involved. Importantly, the persistence of the AR in CRCaP suggests that deciphering the mechanisms that regulate AR stability in this disease state may provide extremely useful and novel therapies. A major knowledge gap exists however, in our understanding of the reversal of ubiquitination by deubiquitinase enzymes (DUBs) within the AR signalling cascade that could be extremely important for the evidenced aberrant AR function in CRCaP. Therefore, the aim of this study was to characterise deubiquitinase enzymes (DUBs) involved in controlling AR activity and establishing potential roles of the enzymes in CaP development.

A comprehensive siRNA library screen investigating the role of each DUB enzyme in AR signalling using the androgen-dependent LNCaP CaP cell line was undertaken and ubiquitin specific protease (USP) 12 (USP12) and USP10 were identified as regulators of AR activity. USP12 depletion resulted in reduced expression of androgen-responsive genes, suggesting USP12 is an activator of AR-mediated transcription, a notion confirmed in luciferase reporter assays. USP12 depletion failed to affect AR protein levels, but attenuated receptor recruitment to target gene promoters suggesting it is required to facilitate activated AR promoter binding. Studies of the phenotypic effects of USP12 knockdown revealed reduced LNCaP proliferation, increased cell cycle arrest and apoptosis, suggesting that USP12 inhibition could hold therapeutic potential in prostate cancer.

In contrast, USP10 depletion resulted in increased expression of androgen-response genes, and repressed AR transcriptional activity in luciferase reporter assays. Unlike USP12, which interacted with AR when over-expressed in COS-7 cells, USP10 did not interact with the receptor, leading to the hypothesis that it may exert its effects through deubiquitination and stabilisation of the AR co-repressor, and E3 ubiquitin ligase, MDM2. A preliminary study to investigate the effects of MDM2 and USP10 co-transfection was unfortunately unsuccessful but further optimisation would assist in elucidating the role of the DUB in the AR signalling cascade.

In conclusion, two DUB enzymes, USP12 and USP10, were identified to be important in modulation of AR activity. USP12 acts as co-activator of the AR and may be a potential therapeutic target in CaP. USP10, on the other hand, is a co-repressor of the AR and may act through MDM2.

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A special thank you to Uncle Walter, who sadly passed away after a long battle with prostate cancer in 2008, you were my inspiration and I hope I have made you proud.

List of Abbreviations

2D	2 dimensional
AAT	Androgen ablation therapy
AF	Activation function
AMSH-LP	Associated molecule with the SH3 domain of STAM-like protein
APS	Ammonium persulphate
AR	Androgen receptor
ARE	Androgen responsive element
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5’-triphosphate
ATXN	Ataxin
β-gal	β-galactosidase
BCA	Bicinchoninic acid
bp	Base pairs
BPH	Benign prostate hyperplasia
BRCA	Breast cancer
BSA	Bovine serum albumin
CaP	Prostate cancer
cDNA	Complimentary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
ChIP	Chromatin immunoprecipitation
CHIP	C-terminus of Hsc70 interaction protein
CRCaP	Castrate resistant prostate cancer
Cys	Cysteine
DBD	DNA binding domain

DCC	Dextran coated charcoal
DEPC	Diethylpyrocarbonate
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DMWD	Dystrophia myotonic WD repeat-containing protein
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOD	Died of disease
DoxR	Doxorubicin
DTT	Dithiolthreitol
DUB	Deubiquitinase enzyme
E6-AP	E6 associated protein
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycol tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ENaC	Epithelial sodium channels
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FA	Fanconi anaemia
FAS	Fatty acid synthase
FCS	Foetal calf serum
G3BP	Ras-GAP SH3 binding protein

GST	Glutathione-S-transferase
H	Histone
HDAC	Histone deacetylase
HECT	Homologous to E6-AP carboxyl terminus
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSP	Heat-shock protein
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
IP	Immunoprecipitation
IPTG	Isopropyl-D-1-thiogalactopyranoside
JAMM/MPN+	Jab1/Mov34/MPr1/Pad1/N-terminal + domain
K	Lysine
kDa	Kilodalton
LBD	Ligand binding domain
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LTS	Long-term survival
luc	Luciferase
MDM2	Murine double minute 2
MMTV	Mouse mammary tumour virus
mRNA	Messenger RNA
NFκB	Nuclear factor kappa B
NLS	Nuclear localisation signal
NT	Non-transfected

NTD	N-terminal domain
Oligo	Oligonucleotide
ONPG	Ortho-nitropheyl-β-D-galactopyranoside
OTU	Ovarian tumour related
PAGE	Poly-acrylamide gel electrophoresis
PAP	Prostatic acid phosphatase
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PGS	Protein-G sepharose
PHD	Plant homeogene domain
PHLPP	PH domain and leucine rich protein phosphatase
PHLPPL	PHLPP-like
PI	Propidium iodide
PIN	Prostatic intra-epithelial neoplasia
PIRH2	P53-induced protein with a RING-H2 domain
PMSF	Phenylmethylsulfonyl fluoride
PSA	Prostate specific antigen
QPCR	Quantitative reverse transcriptase PCR
RCC	Renal cell carcinoma
RING	Really interesting new gene
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCR	Scrambled siRNA

SDS	Sodium dodecyl sulphate
SHBH	Sex hormone binding globulin
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNX3	Sorting nexin 3
SRB	Sulphorhodamine B
TAE	Tris acetate EDTA buffer
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N', N', N', N'-tetramethylethylenediamine
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TRAF	TNF-associated factor
TRUS	Transrectal ultrasound
TTBS	Tris buffered saline Tween20
TURP	Transurethral resection of prostate
UAF	USP1-associated factor
UBA	Ubiquitin-like modifier activating enzyme
UBE	Ubiquitin activating enzyme
UBC	Ubiquitin conjugating enzyme
UCH	Ubiquitin carboxyl-terminal hydrolase
USP	Ubiquitin specific protease
WDR	WD repeat-containing protein
X-gal	Bromo-chloro-indolyl-galactopyranoside

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Chapter 1: Introduction

1.1 The Prostate

1.1.1 Functions of the prostate

The prostate is a small, walnut sized gland important in the male reproductive system. It is situated at the neck of the bladder surrounding the urethra. The primary function of the prostate is to contribute secretary proteins to the seminal fluid thereby sustaining sperm motility and viability whilst maintaining the fluidity of the seminal fluid (Hamdy, 2002).

1.1.2 Zonal anatomy of the prostate

The human prostate is divided into four zones; the anterior fibromuscular structure, the peripheral zone, the central zone and the transition zone (McNeal, 1981) (Figure 1.1). The anterior fibromuscular structure encapsulates the entire prostate. The peripheral zone is adjacent to the anterior fibromuscular stroma and contains the central and transition zones. The peripheral zone makes up around 70% of the total glandular prostate and is the most common site of prostate cancer (CaP) (Coffey, 1993). The central zone surrounds the ejaculatory ducts and accounts for 25% of the glandular prostate. CaP is rare in this zone (Kirby, 2001). The transition zone consists of two symmetrical lobes situated on either side of the urethra. This region is small in young men but with age it enlarges to become a more dominant zone. Although only around 25% of CaP is found in this region it is the most common site of benign prostate hyperplasia (BPH) (McLaughlin *et al.*, 2005).

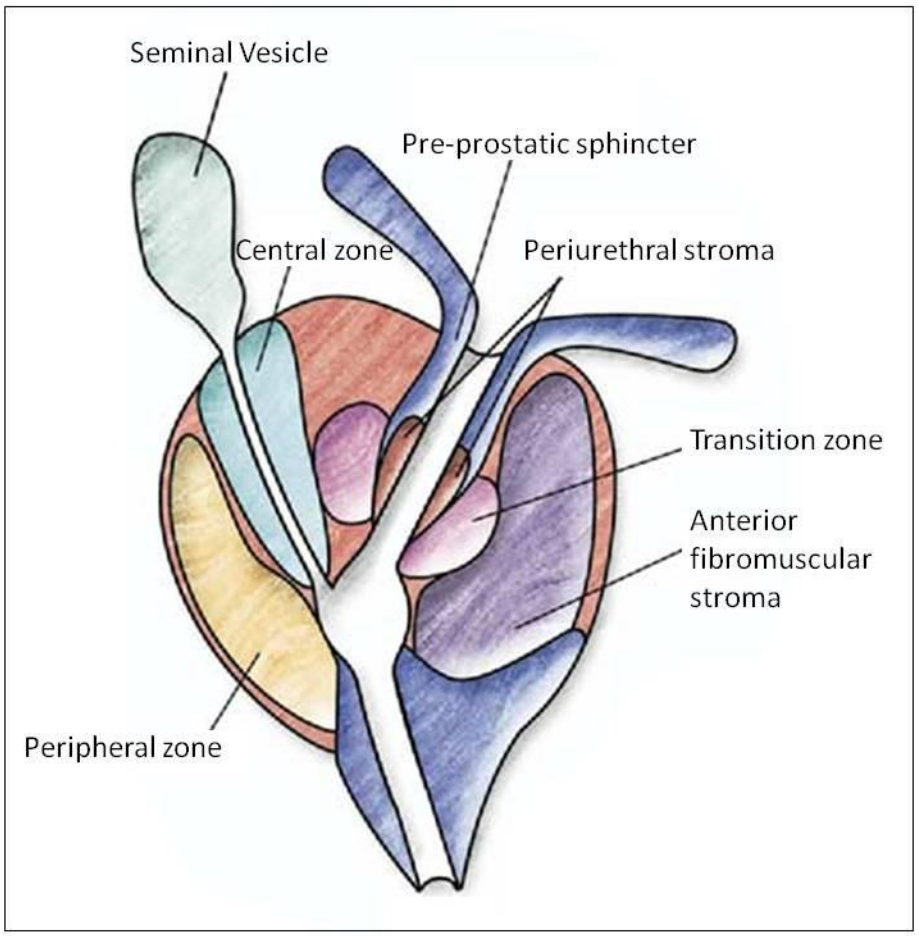

Figure 1.1. Zonal anatomy of the prostate.

The prostate consists of the anterior fibromuscular stroma, the peripheral zone, the central zone and the transition zone. Adapted from (McLaughlin et al., 2005).

1.1.3 Histology of the prostate

The prostate is a glandular organ and has a characteristic ductal structure. The ducts are lined with epithelial cells which are embedded in fibromuscular stromal tissue consisting of smooth muscle cells, fibroblasts and endothelium (Foster, 1998).

Three types of epithelial cell exist in the normal prostate (Lepor, 1993). The luminal cells are the principal epithelial cells which express androgen receptor (AR) and are dependent upon androgens for growth. These are the cells that produce the proteins that are subsequently secreted into the lumen of the prostate ducts. The basal cells are sandwiched between the luminal cells and the basement membrane. These cells do not contribute to the secretary function of the luminal cells (Lepor, 1993). Although the basal cells express functional AR they are not androgen dependent. It is thought that it is within this layer that the prostate cancer stem cell resides (Maitland and Collins, 2008). The prostate cancer stem cell is thought to be an androgen independent cell with stem cell-like properties that reconstitutes the tumour with differentiated androgen-dependent cells following androgen ablation therapy and regression of the tumour (Maitland and Collins, 2008). Finally the neuroendocrine epithelial cells are thought to provide the paracrine signalling which stimulates and regulates the normal growth of the prostate. These cells themselves are androgen independent (di Sant'Agnese, 1998).

1.1.4 Prostatic disease and prostate cancer

1.1.4.1 Benign prostate hyperplasia

Benign prostate hyperplasia (BPH) is the increased number of normal prostate cells which is the most common non-malignant disease of the prostate; with around 50% males over 60 and 90% over 85 years of age displaying disease (Lepor, 1993; Hamdy, 2002). The transition zone enlarges with increased age and can cause disturbance of the glandular structure of the prostate as well as causing urinary problems. The urethra can become compressed making urination difficult in males with BPH and there is an association with urinary infections (McLaughlin *et al.*, 2005).

BPH is treated with inhibitors of the enzyme 5-α-reductase, which is responsible for the conversion of testosterone to the more potent androgen dihydrotestosterone (DHT), thereby reducing the levels of androgens. Alternatively, inhibitors of α-

adrenergic receptor cause relaxing of the bladder tissue to relieve symptoms. Surgical trans-urethral resection of the prostate (TURP) can be used where other treatments are unsuccessful to decrease prostatic volume around the bladder also relieving symptoms (McLaughlin *et al.*, 2005).

1.1.4.2 Prostatic intra-epithelial neoplasia

Prostatic intra-epithelial neoplasia (PIN) is the abnormal expansion of prostate cells within a benign prostate gland. Atypical cells are often observed and the main region of PIN is the peripheral zone, where most cases of CaP occur. PIN is described as an intermediate stage between benign prostatic hyperplasia and invasive CaP. Like BPH, there is an increased incidence with increased age (Bostwick *et al.*, 2004).

1.1.4.3 Prostate cancer

Prostate cancer is an epithelial derived, androgen-dependent disease. Clinically localised disease is the most commonly diagnosed stage where the tumour is contained within the prostate. This is generally a low grade and highly differentiated tumour that is slow to progress (Catalona *et al.*, 1993; Nguyen and Jones, 2011). Expansion of cancer cells can lead to invasion through the basement membrane of the prostate, known as locally advanced disease, and metastasis to other tissues. Metastatic CaP is commonly found in the bone, lung and liver (Yoneda, 1998). It is thought that up to 90% of patients with advanced CaP will have some bone metastasis (Cooper *et al.*, 2003). Unfortunately many patients relapse after treatment with an androgen independent castrate resistant tumour (CRCaP). CRCaP is an aggressive form of CaP and is usually associated with increased tumour cell invasion and metastasis (Bonaccorsi *et al.*, 2003). The mechanisms of androgen independence are discussed in Section 1.2.1.3 (Feldman and Feldman, 2001).

1.1.4.3.1 Incidence and mortality

CaP is the most common male cancer in the UK accounting for almost a quarter of newly diagnosed cases with 35,000 cases diagnosed annually. It is also the fourth leading cause of cancer related deaths worldwide and the second leading cause of cancer deaths in men in the UK with approximately 10,000 deaths per year (Cancer Research UK, 2011) (Figure 1.2).

There has been a steady increase in the incidence of CaP in recent years, possibly due to better detection, although mortality rates have not been significantly affected. Interestingly, one year and five year survival rates have increased dramatically as more cases of CaP are being diagnosed at an earlier stage than previously. One year survival has increased from 65% in 1971-1975 to 93% in 2004-2006, five year survival in 2001-2006 was 77% compared to 31% in 1971-1975 (Cancer Research UK, 2011).

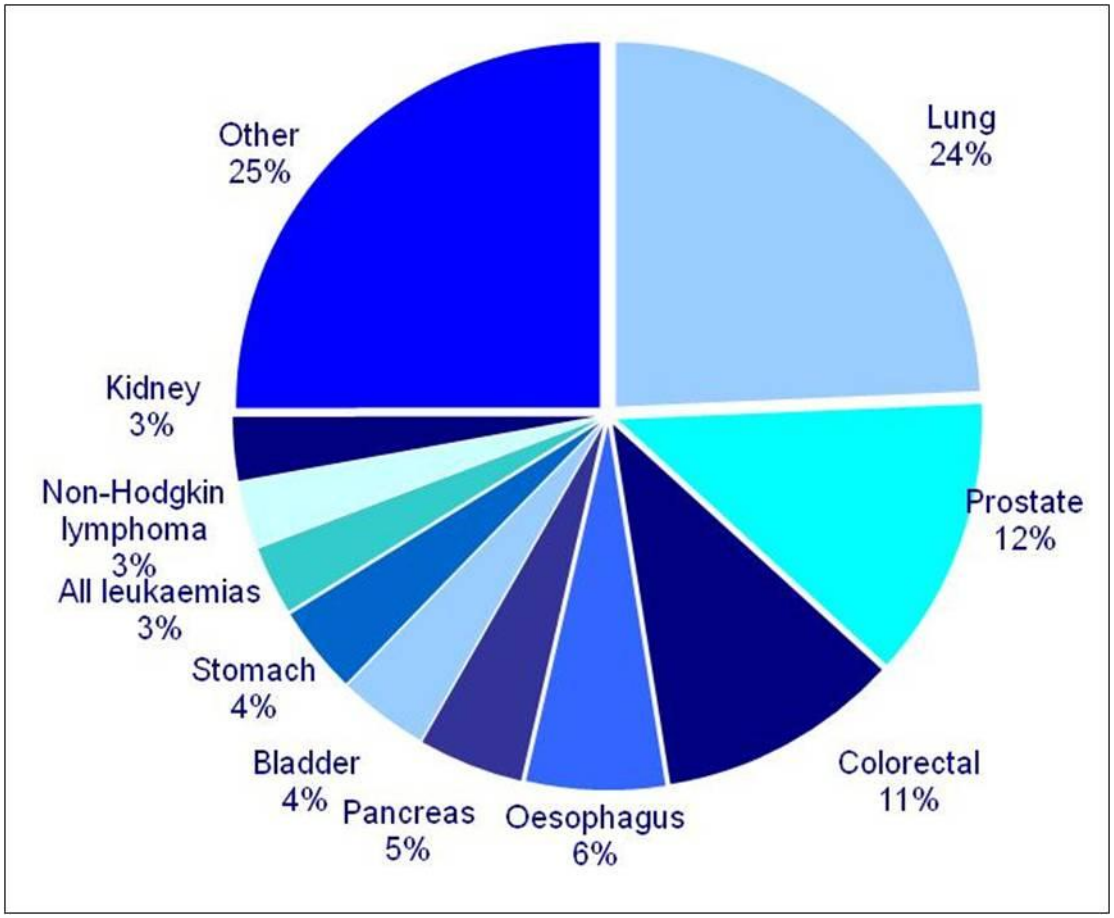

Figure 1.2. The most common cancer-related deaths in males in the UK.

Prostate cancer is the second most common cause of cancer-related death and accounts for approximately 10,000 deaths per year. Taken from (Cancer Research UK, 2011).

1.1.4.3.2 Risk factors

The single greatest risk factor in CaP is age. The mean age of disease onset is 72-75 years of age but latent CaP has been observed in men as young as 30 years old (Sakr *et al.*, 1996; Cancer Research UK, 2011) (Figure 1.3). 80% of men over the age of 80 years have signs of CaP upon autopsy (Sakr *et al.*, 1993). Around 5-10% of total CaP cases and 30-40% of early onset cases are thought to be due to familial genetic susceptibility (Bratt, 2002). Risk increases 2-fold if a first degree relative, i.e. father or brother, develops CaP and increases further if they are below 60 year old. Familial mutations within the BRCA1 and BRCA2 tumour suppressor genes, which are also associated with breast and ovarian cancer susceptibility, have been found to increase the rate of CaP diagnosis (Kirchhoff *et al.*, 2004; van Asperen *et al.*, 2005).

Race also plays an important role with major differences in incidence observed between the western world and African and Asian populations (Gronberg, 2003). African American men have been shown to have a higher incidence of CaP than Caucasian men whilst Asian populations have a low incidence (Sakr *et al.*, 1996; Gronberg, 2003). A study of Japanese men who immigrated to America showed that incidence of CaP increased although it was still 50% and 25% lower than Caucasian and African American men, respectively (Shimizu *et al.*, 1991). This study identified that environmental factors did have a role in risk of CaP however they were not sufficient to account for the differences in populations (Shimizu *et al.*, 1991).

Environmental factors influencing risk include diet and obesity. Diets high in fat, low in soy, green tea, vitamin D and E and lycopene have been shown to increase the risk of CaP. Soy and vitamin D have been shown *in vitro* to reduce proliferation of CaP cell lines and induce apoptosis (Hori *et al.*, 2011).

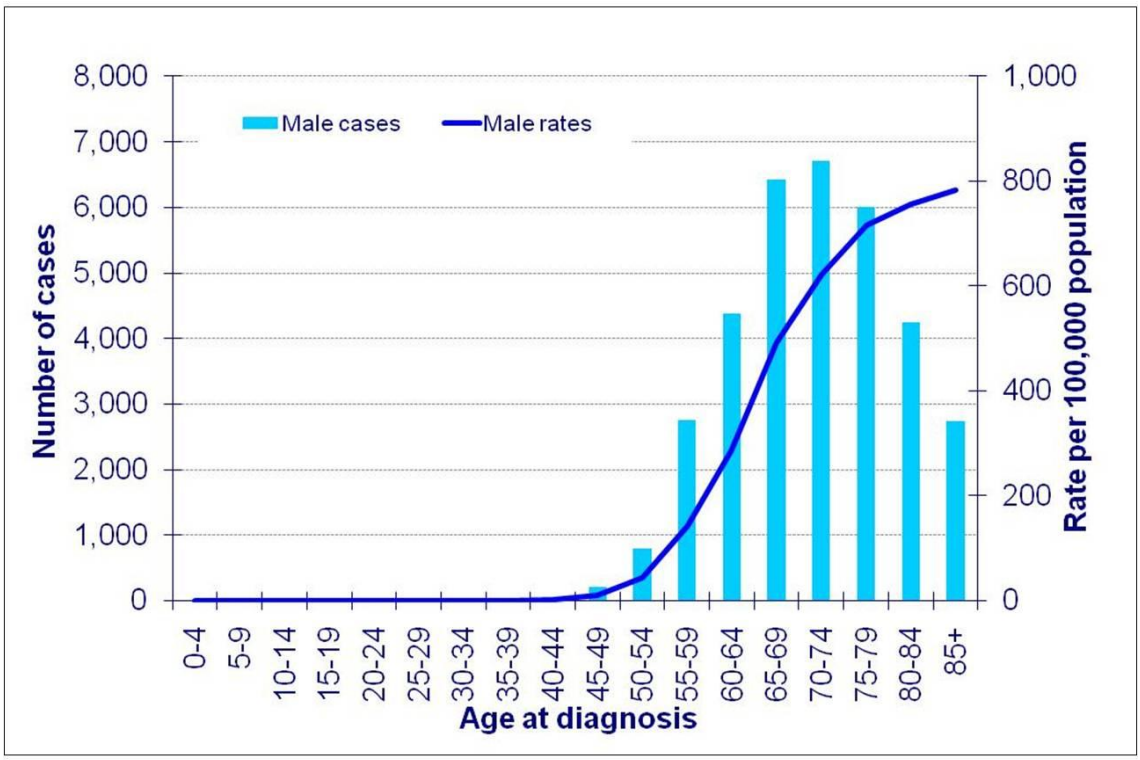

Figure 1.3. The rate of prostate cancer incidence increases with age.

The median age of onset is 72-75 years of age. Taken from (Cancer Research UK, 2011).

1.1.4.3.3 Detection of prostate cancer

Prostate cancer is usually diagnosed after histological analysis of 8-12 prostate needle core biopsies taken by trans-rectal ultrasound (TRUS) guided biopsy (Damber and Aus, 2008). However, surrogate biomarkers of CaP have been developed and allow a non-invasive method of detecting potential tumours (Bok and Small, 2002).(Seruga and Tannock, 2011)

Prostatic acid phosphatase (PAP) was the first marker to be developed, which could be detected in the prostatic fluids (Bok and Small, 2002). However, this was superseded by prostate specific antigen (PSA) which could detect potential CaP at an earlier stage (Stamey *et al.*, 1987; Catalona, 2004). *PSA* is an androgen regulated gene which encodes a secretary protein which is a major component of the seminal fluid. PSA detected at levels of greater than 2.5ng/ml in serum suggests possible CaP. Initially the threshold was set at 4ng/ml but a study of a large cohort of patients over 12 years discovered that many cases were being overlooked (Catalona *et al.*, 1991; D'Amico *et al.*, 2004). However, the lowering of the threshold has stimulated an argument that CaP is now being over-detected and over-treated as many men will not develop symptomatic disease (Welch and Albertsen, 2009; Nguyen and Jones, 2011).

1.1.4.3.4 Treatment of prostate cancer

The treatment of CaP can depend on stage of disease as well as the life expectancy and general health of the patient. Radical prostatectomy, in which the prostate is surgically removed, is commonly used in clinically localised disease. TURP can be used to relieve urinary tract symptoms as described for BPH (McLaughlin *et al.*, 2005).

Endocrine therapy is used to reduce the level of circulating androgens; commonly termed androgen ablation therapy (AAT). In the past, surgical removal of the testes (castration) was the gold standard of reducing testosterone levels with 60-80% of patients responding (Huggins and Hodges, 1941). However, this has now been superseded by the use of chemical agents which achieve similar results. Luteinising hormone releasing hormone (LHRH) agonists cause suppression of LHRH by desensitising and reducing the number of LHRH receptors. This in turn suppresses release of luteinising hormone (LH) and subsequently reduces testosterone levels (Hamdy, 2002). Similarly, estrogens can cause suppression of LHRH and LH by negative feedback on the hypothalamus (Lepor, 1993) Abiraterone is a small molecule inhibitor

that has been shown to have effects in CRCaP by blocking the CYP17 enzyme that is involved in androgen biosynthesis (Salem and Garcia, 2011).

An additional class of modulators of the AR signalling axis are the direct AR antagonists, such as flutamide and bicalutamide, which competitively bind to the receptor, disrupting androgen binding and attenuating transcriptional function of the AR (Goktas *et al.*, 1999).

Chemotherapy is used as a treatment in CRCaP as it no longer responds to AAT. Generally this is palliative treatment not curative treatment. Chemotherapy, although causing regression of disease and palliation of symptoms was shown not to significantly affect survival (Petrylak, 2005b). In 2004, the results of the TAX 327 and Southwest Oncology Group (SWOG) 99-16 trials showed for the first time that treatment of CRCaP patients with docetaxel increased survival whilst decreasing the risk of death compared to patients treated with mitoxantrone and prednisone (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). Docetaxel is a semi-synthetic taxane which disrupts the disassembly of microtubules during the G2-M phase of the cell cycle causing cell death in proliferating cells (Seruga and Tannock, 2011). Recently, newer classes of drugs are emerging as potential treatments for CRCaP. Ixabepilone, a member of the epothilone class of drugs, has been shown to reduce PSA by greater than 50% in approximately 30% of CRCaP patients (Hussain *et al.*, 2005). Several other members of this drug class are in phase II clinical trials (Bhandari and Hussain, 2005).

1.2 Androgens

Androgens are essential in the formation of male phenotype from early embryogenesis to adulthood as well as the normal development and maintenance of the prostate epithelium (Gelmann, 2002). Testosterone, the major circulating androgen, is synthesised from cholesterol by the testes in response to LH stimulation (Gao *et al.*, 2005b). Figure 1.4 shows the basic structure of a steroid hormone, the structure of testosterone is illustrated in Figure 1.4b. Serum proteins, such as sex hormone binding globulin (SHBG) and albumin, sequester testosterone in the blood (Gao *et al.*, 2005b). Once testosterone enters the cell, it is converted by 5-α-reductase to DHT, which binds to and up-regulates transcriptional activity of the AR more potently than testosterone due to its higher binding affinity to the AR (Feldman and Feldman, 2001). It is suggested that the constant exposure of prostate cells to androgens is a driver in the development of prostate cancer as animal models of prostate cancer require the presence of functional testes or exogenous androgens (Gelmann, 2002). Deficiency in 5-α-reductase, as well as defects in the function of the AR, results in loss of prostate development (Griffin, 1992).

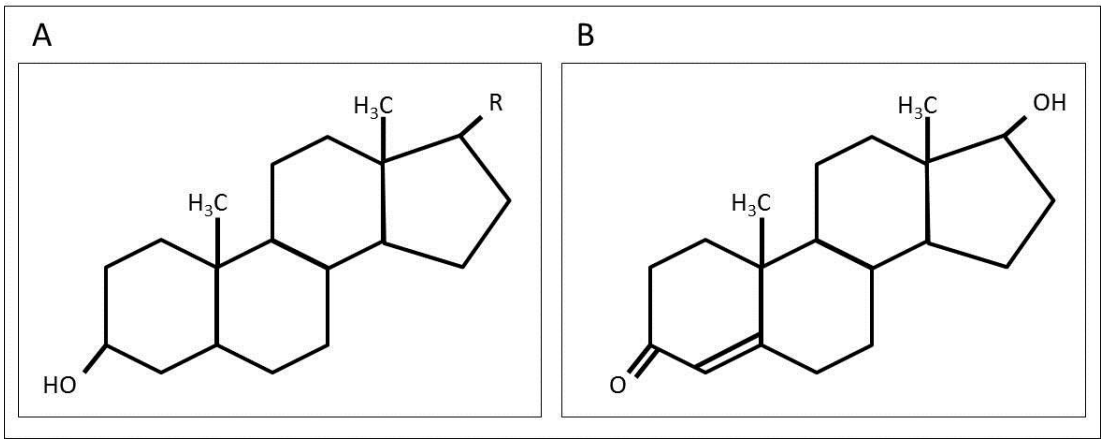

Figure 1.4. The structure of a steroid hormone.

A) The basic structure of a steroid hormone. B) The structure of testosterone. (Kicman, 2010)

1.2.1 The androgen receptor

The AR is a member of the steroid hormone receptor family of transcription factors. The *AR* gene, a non-essential but critical gene for male phenotype, is located at q11.2-q12 on the X chromosome and consists of 8 exons which encode the four domains of the AR (Figure 1.5) (Migeon *et al.*, 1981; Lubahn *et al.*, 1988; Gelmann, 2002).

1.2.1.1 Structure of the androgen receptor

1.2.1.1.1 The transactivation domain

Exon 1 of the *AR* gene encodes the N-terminal transactivation domain (NTD). This domain confers the majority of the ARs specific transcriptional activity and contains the ligand-independent activation function-1 (AF-1) (Gelmann, 2002). Deletion of the LBD region results in a constitutively active N-terminal fragment whose activity is similar to that of full length ligand bound AR (Simental *et al.*, 1991). The NTD also confers the primary interaction site of many co-activators of AR (Gelmann, 2002), such as the histone acetyltransferase SRC-1 (Bevan *et al.*, 1999), as well as components of the general transcription machinery, including TFIIH (Choudhry *et al.*, 2006).

The poly-glutamine (CAG) and poly-glycine (GGN) stretches are also located within the NTD. These regions of repetitive sequences are highly polymorphic due to slippage of DNA polymerase during replication (Gelmann, 2002) and thus vary in length naturally throughout the population (Sartor *et al.*, 1999). The poly-glutamine repeat, starting at codon 58, can range from 14-35 repeats (Sartor *et al.*, 1999) and its length is inversely proportional to AR activity (Buchanan *et al.*, 2004) hence shorter CAG repeats have been associated with increased risk of prostate cancer as well as more aggressive disease and a higher rate of recurrence (Giovannucci *et al.*, 1997).

1.2.1.1.2 The DNA-binding domain

The DNA-binding domain (DBD), which is encoded by exon 2 and the proximal region of exon 3, is the most conserved region between members of the steroid hormone receptor family (Gelmann, 2002). This 70 amino acid domain contains 8 conserved cysteine residues that form two zinc finger structures responsible for mediating discriminate contacts with the major groove of DNA (Chmelar *et al.*, 2007). The first zinc finger recognises and binds specific DNA sequences termed androgen response elements (AREs) that are primarily located in promoter and enhancer regions of AR

target genes whilst the second zinc finger facilitates stability of the DNA-bound AR by forming indiscriminate contacts with the phosphate-backbone of DNA (Schoenmakers *et al.*, 1999).

1.2.1.1.3 The hinge region

The hinge region, consisting of the C-terminal part of exon 3 and proximal part of exon 4, is a small linker domain between the DBD and ligand binding domain (LBD) (Gelmann, 2002). This region contains a bipartite nuclear localisation signal (NLS) deletion of which attenuates ligand-dependent nuclear translocation. As well as containing a NLS sequence, the hinge region is a major site of several post-translational modifications including phosphorylation, acetylation, methylation and ubiquitination and has also been implicated in receptor dimerisation and correct protein folding (Zhou *et al.*, 1994; Faus and Haendler, 2006; Chmelar *et al.*, 2007). The PEST sequence, rich in proline, glutamic acid, serine and threonine, is associated with turn-over of proteins and is suggested to be a signal for proteasomal degradation (Rogers *et al.*, 1986; Lin *et al.*, 2002a)

1.2.1.1.4 The ligand-binding domain

Finally, the distal part of exon 4 and exons 5 to 8 encode the C-terminal LBD (Gelmann, 2002). Members of the steroid hormone receptor family, including estrogen receptor, progesterone receptor and glucocorticoid receptor, display structurally conserved LBDs although they have very little sequence similarity. Unlike other family members, however, whose LBD is composed of 12 α-helices (named 1-12), the LBD of AR consists of 11 α-helices (Helix 2 is absent, although the numbering of the helices is consistent with other family members, (1-12)) (Dehm and Tindall, 2007). For the AR (and other steroid hormone receptors), helices 1-11 fold into a barrel-like globular structure that forms the ligand-binding pocket into which the DHT binds. Upon androgen binding, the flexible helix 12 folds back over the ligand binding pocket to form a lid-like structure, in doing so exposing the ligand-dependent activation function (AF-2) domain contained within helix 12 (Matias *et al.*, 2000; Gelmann, 2002). The functional AF-2 provides a binding surface for various co-regulator proteins, as well as mediating the interaction with the FXXLL and WXXLL motifs within the NTD (Alen *et al.*, 1999; Bevan *et al.*, 1999).

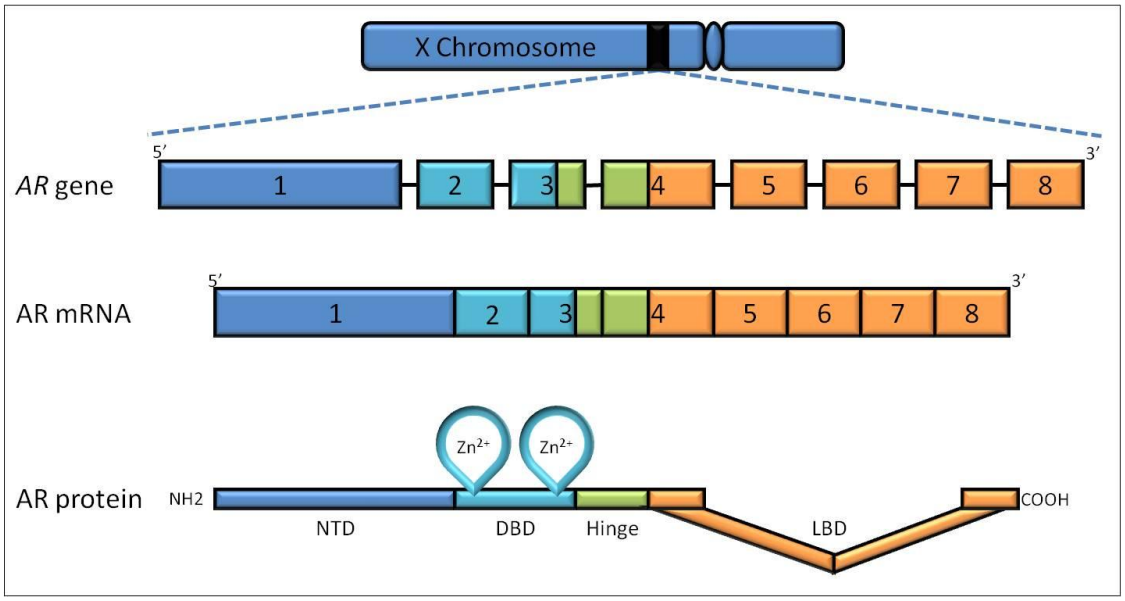

Figure 1.5. The structure of the androgen receptor.

The AR gene is located on the X chromosome and consists of 8 exons encoding the four domains of the AR; the N-terminal domain (NTD), the DNA binding domain (DBD), the hinge and the ligand binding domain (LBD). Adapted from (Gelmann, 2002).

1.2.1.2 Mechanism of AR action

In its inactive state, AR is bound by a complex of chaperone proteins, including heat-shock proteins (HSP) in the cytoplasm (Gelmann, 2002). It is postulated that these chaperones hold AR in a stable but partially unfolded conformation ready for ligand binding (Bohen *et al.*, 1995). The binding of androgen initiates dissociation of the chaperone proteins and permits allosteric re-organisation of the AR resulting in receptor dimerisation, intra-molecular N-C-terminal interaction between individual AR monomers and exposure of the NLS which potentiates nuclear import (Gelmann, 2002).

Once inside the nucleus, the AR binds to AREs within the *cis*-regulatory elements of target genes, such as PSA (Khorasanizadeh and Rastinejad, 2001), and recruits components of the basal transcription machinery, and a host of co-regulatory proteins to drive target gene expression by RNA polymerase (Shang *et al.*, 2002; Wang *et al.*, 2005; Chmelar *et al.*, 2007).

Once AR-mediated transcription is complete the chaperone complex facilitates cyclic disassembly of the AR transcription complex and aids recycling of the AR by returning it to a primed state ready for reactivation by further ligand binding (Freeman and Yamamoto, 2001).

1.2.1.3 Mechanisms of androgen-independence in prostate cancer

Many mechanisms of androgen independence have been described in CaP. These mechanisms include: (1) increased sensitivity of AR to low levels of androgens; (2) promiscuity of the AR towards other ligands; (3) ligand-independent activation by kinase pathways; (4) bypass of AR signalling by use of other pro-proliferative and anti-apoptotic pathways and (5) the cancer stem cell theory of tumour re-population by androgen-independent stem-like cells. All lead to increased AR activity following AAT resulting in the promotion of proliferation and survival in CaP cells (Feldman and Feldman, 2001).

1.2.1.3.1 Androgen receptor amplification

It is reported that 30% of CRCaP possess AR gene amplification (Linja *et al.*, 2001). AR amplification leads to over-expression of functional AR and therefore permits receptor activation in the presence of much lower concentrations of androgen. It is suggested

that cells containing AR amplification are selected for during AAT due to their proliferative advantage (Taplin *et al.*, 1999). Interestingly patients with AR amplification were found to have better survival as their tumours remained better differentiated, more closely resembling normal cells, and therefore more likely to respond to second line AAT than other CaPs (Palmberg *et al.*, 2000). Similarly, an increase in local DHT production by increased activity of 5-α-reductase can lead to hypersensitivity of the AR. AAT can reduce circulating testosterone levels by up to 95%, although DHT can remain at 40% of normal levels (Labrie *et al.*, 1986). Some males of African ethnicity have a polymorphism in 5-α-reductase, in which valine at position 89 is substituted to a leucine, resulting in a higher rate of conversion of testosterone to DHT (Makridakis *et al.*, 1997).

1.2.1.3.2 Androgen receptor point mutations

Many point mutations in AR have been identified in CaP. One of the first identified and most commonly reported is that of the threonine to alanine substitution at position 887 in the LBD (T877A) (Veldscholte *et al.*, 1992; Gaddipati *et al.*, 1994). This mutation is present in the endogenously expressed AR in the LNCaP CaP cell line and allows the AR to become activated by a broader spectrum of ligands including other steroid hormones, such as estrogens and clinically relevant anti-androgens, including flutamide (Sack *et al.*, 2001). This mutation is also postulated to interfere with co-regulator interactions with the receptor (Chmelar *et al.*, 2007).

Substitution of leucine at position 701 to histidine (L701H), again within the AR LBD, enhances LBD binding to adrenal corticosteroids at the expense of low level DHT during ATT that subsequently activates the receptor (Zhao *et al.*, 1999; Zhao *et al.*, 2000). Akin to this mode of aberrant AR activation, substitution of histidine 874 to tryrosine (H874Y) allows adrenal androgens, non-androgenic steroid and anti-androgens to activate the receptor (McDonald *et al.*, 2000).

1.2.1.3.3 Non-steroidal mechanisms of androgen receptor activation

The outlaw pathway describes a means by which AR can become activated independent of its ligand through activation by growth factors such as insulin-like growth factor (IGF) and epidermal growth factor (EGF) (Culig *et al.*, 1994). IGF-1 has been shown to induce PSA secretion in LNCaP cells by 5-fold (Culig *et al.*, 1994) . It has been suggested that these growth factors indirectly induce proliferation by activating

complex tyrosine kinase signalling cascades however it has been observed that treating cells with casodex can block the effects of growth factors implicating that AR LBD is important in this mechanism (Culig *et al.*, 1994). CRCaP cells lines generated from xenograft models in mice have been found to commonly over-express Her2/neu (Craft *et al.*, 1999). Interestingly, over-expression of this gene in androgen dependent cell lines is found to convert them to an androgen independent state (Craft *et al.*, 1999; Yeh *et al.*, 1999). Unlike the other growth factors however LBD binding is not thought to be involved as this mechanism as casodex treatment does not block the effects of HER2/neu over-expression (Yeh *et al.*, 1999).

Bypassing AR signalling completely and relying on other cellular pathways to promote proliferation is another mechanism of androgen-independence. Although not essential for CRCaP progression and not expressed in the normal prostate, the Bcl-2 pathway is frequently expressed in PIN and castrate resistant CaP (McDonnell *et al.*, 1992; Colombel *et al.*, 1993) and is associated with evasion of apoptosis (Colombel *et al.*, 1993).

1.2.1.3.4 The cancer stem cell theory

The cancer stem cell theory suggests that a cancer stem cell, described as a somatic cells that have acquired stem-like properties, reside within the basal layer of the tumour (Maitland and Collins, 2008) and are essentially androgen-independent. It is postulated that these cells remain after AAT due to their therapeutic resistance and are able to re-populate the tumour during hormonal therapy. The resulting progeny are differentiated tumour cells that are now androgen independent (Maitland and Collins, 2008).

1.3 Ubiquitination

Ubiquitination is one of the most important and evolutionarily conserved cellular processes involving a series of highly regulated enzymatic reactions that modifies a target lysine within substrates with the small protein ubiquitin either as a singular moiety or as a complex chain of multiple molecules (Ravid and Hochstrasser, 2008).

1.3.1 Structure of ubiquitin

Ubiquitin, encoded by four genes in humans, is transcribed and translated as a pro-protein that is cleaved to form a 76 amino acid ubiquitin molecule (Kalderon, 1996). The *Ubb* and *Ubc* genes, located on chromosomes 17p12-11.2 and 12q24.3 respectively, encode four and nine tandem repeats of ubiquitin as a multimeric pro-protein (Kimura and Tanaka, 2010). These genes appear redundant, but mouse models have shown significant differences; *Ubb* knockouts are infertile, but otherwise normal, while *Ubc* knockout is embryonic lethal as a consequence of a 60% reduction in total ubiquitin levels in embryonic fibroblasts resulting in attenuated fetal liver proliferation (Ryu *et al.*, 2007). Infertility of the *Ubb* knockout mice is due to failure of meiosis in the germ cells and it is shown that lower levels of ubiquitin are present in the testis and oocytes of these mice (Ryu *et al.*, 2008). The *Uba52* and *Rps27a* genes, located on chromosomes 19p13.1-p12 and 2p16 respectively, encode a single copy of ubiquitin that is fused to the ribosomal proteins L40 and S27a (Finley *et al.*, 1989; Redman and Rechsteiner, 1989).

Ubiquitin is highly conserved throughout evolution due to strong selective pressure (Pickart and Eddins, 2004), with human ubiquitin differing from the yeast protein at only 3 positions (Pickart and Eddins, 2004). The structure of ubiquitin is also well conserved (Figure 1.6); the ubiquitin-fold structure is found in several members of the ubiquitin-like proteins, such as SUMO, Nedd8 and ISG15 (Walters *et al.*, 2002), and all of these family members also terminate with a C-terminal di-glycine sequence required for substrate conjugation (Jentsch and Pyrowolakis, 2000). Importantly, ubiquitin contains seven lysine residues at positions 6, 11, 27, 29, 33, 48 and 63 which allow a multitude of poly-ubiquitin chains to form and therefore a diverse range of signals and functions to occur (Kim *et al.*, 2007).

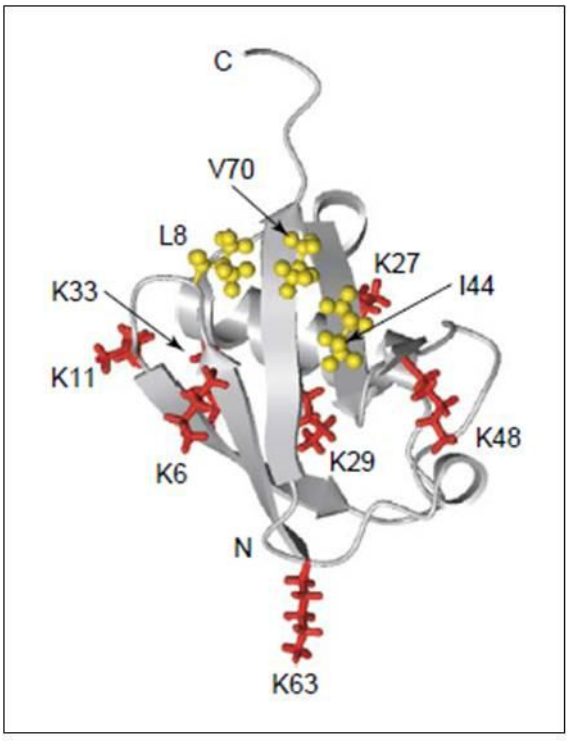

Figure 1.6. The structure of ubiquitin.

The seven lysine residues required for chain formation are indicated. Taken from (Pickart and Fushman, 2004).

1.3.2 Mechanism of ubiquitination

In order for ubiquitination to occur an ubiquitin molecule must first be activated and transferred via a series of enzymes onto the substrate. This three enzyme cascade involves the ubiquitin activating E1 enzymes, the ubiquitin conjugating E2 enzymes and the E3 ubiquitin ligases in a process highlighted in Figure 1.7.

1.3.2.1 Ubiquitin activating E1 enzyme

In order for ubiquitin to be covalently attached to a substrate it must first be activated by the ubiquitin activating E1 enzyme. This ATP-dependent reaction involves the formation of an adenylated ubiquitin intermediate, before a further reaction with the catalytic cysteine of the E1 enzymes forms a thiol ester bond with the ubiquitin (Pickart and Eddins, 2004). The E1 enzyme can bind two ubiquitin molecules at any one time, one as an adenylated intermediate and one via the thiol ester bond through its N-terminal and catalytic cysteine (Cys) domains (Pickart and Eddins, 2004). The final 100 amino acids of the enzyme form a ubiquitin fold structure and are required for interaction with the ubiquitin conjugating enzyme E2 (Jin *et al.*, 2007).

There are eight human E1 enzymes which activate the wide variety of ubiquitin-like proteins (Groettrup *et al.*, 2008). Until recently, only one ubiquitin-specific E1 enzyme, UBE1 had been identified (Ciechanover *et al.*, 1982), however the identification of UBA6 indicated that additional enzymes were responsible for activating this cascade. UBA6 was reported independently by three research groups as an E1 ubiquitin activating enzyme, sharing the structural components of UBE1 and 42% sequence identity (Chiu *et al.*, 2007; Jin *et al.*, 2007; Pelzer *et al.*, 2007). UBA6 was identified firstly as an E1 enzyme for ubiquitin-like FAT10 and the discovery of its ubiquitin E1 activity represented the first overlap in ubiquitin and ubiquitin-like protein activation (Chiu *et al.*, 2007).

1.3.2.2 E2 ubiquitin conjugating enzyme

The ubiquitin conjugating E2 enzyme receives activated ubiquitin from the E1 enzyme. There are 30 ubiquitin-specific E2 enzymes, divided into four classes, that all have a 150 amino acid globular catalytic domain which forms the conserved UBC-fold structure (van Wijk and Timmers, 2010). Class I enzymes are comprised only of the UBC-fold whereas classes II, III and IV have either N-terminal or C-terminal extensions or both (Winn *et al.*, 2004). These extended regions are thought to have diverse

functions; influencing stability as well as sites for E1 enzyme, E3 ligase and substrate interactions (van Wijk and Timmers, 2010).

The catalytic Cys residue is located within a catalytic groove in the UBC-fold surrounded by conserved amino acids that are involved in the formation of the thiol ester bond between the ubiquitin molecule and the E2 enzyme (Pickart and Eddins, 2004). Interestingly, the surfaces on which the E1 and E3 ligase enzymes interact with the E2 overlap whilst the ubiquitin binding surface is separate (Bencsath *et al.*, 2002). This suggests that during poly-ubiquitination the E2 must dissociate from the E3 ligase in order to receive further activated ubiquitin molecules (Wenzel *et al.*, 2011).

1.3.2.3 E3 ubiquitin ligase

There are a four major classes of E3 ubiquitin ligase; the homologous to E6-AP carboxyl terminus (HECT), really interesting gene (RING), plant homeogene (PHD) and UFD2 homology (U-box) E3 ligases (Pickart and Eddins, 2004). All of these ligases adjoin ubiquitin to the substrate via an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine residue within the substrate. Although approximately 650 E3 ubiquitin ligases have been identified, bioinformatics analysis of the human genome suggests that thousands of proteins potentially possess E3 ubiquitin ligase activity (Pickart and Eddins, 2004).

The HECT E3 ligases accept activated ubiquitin from the E2 enzyme, forming another thiol ester intermediate. A region of approximately 350 amino acids in the C terminus of E6-AP, the first discovered HECT, is found in all family members. These ligases then transfer the ubiquitin to a lysine residue within the target protein (Bernassola *et al.*, 2008). In contrast, the RING domain containing E3 enzymes act as a bridging factor between the substrate and E2 enzymes allowing transfer of activated ubiquitin directly (Hershko *et al.*, 1983; Pickart, 2001). These enzymes contain conserved cysteine and histidine rich motifs, which bind two zinc ions and are critical for function (Pickart and Eddins, 2004). The grouping of the PHD-containing E3 ligases into a separate sub-class is controversial as they demonstrate close similarity to the RING family, and are suggested to be within the same class (Aravind *et al.*, 2003).

Finally, the U-box enzymes were originally thought not be E3 ligases but E4 conjugation factors, acting to promote multi-ubiquitin chain extension of a previously

ubiquitinated target (Pickart and Eddins, 2004). However, evidence now suggests that these enzymes have both E3 and E4 functions. For example, C-terminus of Hsc70 interaction protein (CHIP) recognises and targets mis-folded proteins for degradation in addition to its E4 function (Imai *et al.*, 2002). Aravind and Koonin *et al*., postulated that U-box enzymes act as E3 ligases through a similar mechanism as the RING ligases; adopting a RING domain like structure but without the involvement of any metal ions (Aravind and Koonin, 2000).

Ubiquitination of each substrate protein is tightly regulated and recognition of targets is crucial. Substrate specificity is provided, in part, by both the E3 enzyme and the E2/E3 combination (Weissman, 2001), although bioinformatics sequence analyses have postulated that thousands of possible E3s exist, most of which are yet undiscovered (Pickart and Eddins, 2004), suggesting a vastly complex system capable of modifying and impacting on a multitude of cellular processes. Thus, specificity of action is of paramount importance for the ordered targeting of selected protein substrates. To this end, several E3 enzymes require chaperone proteins to bring the substrate to the enzyme (Rosser *et al.*, 2007), whilst others require further modification of their substrate before it can be recognised, such as phosphorylation of some receptors by ligand-binding correlates with their ubiquitination and degradation. Phosphorylation of some E3 ligases themselves has also been shown to be necessary for their activation providing an additional means of enzyme regulation (Haglund and Dikic, 2005).

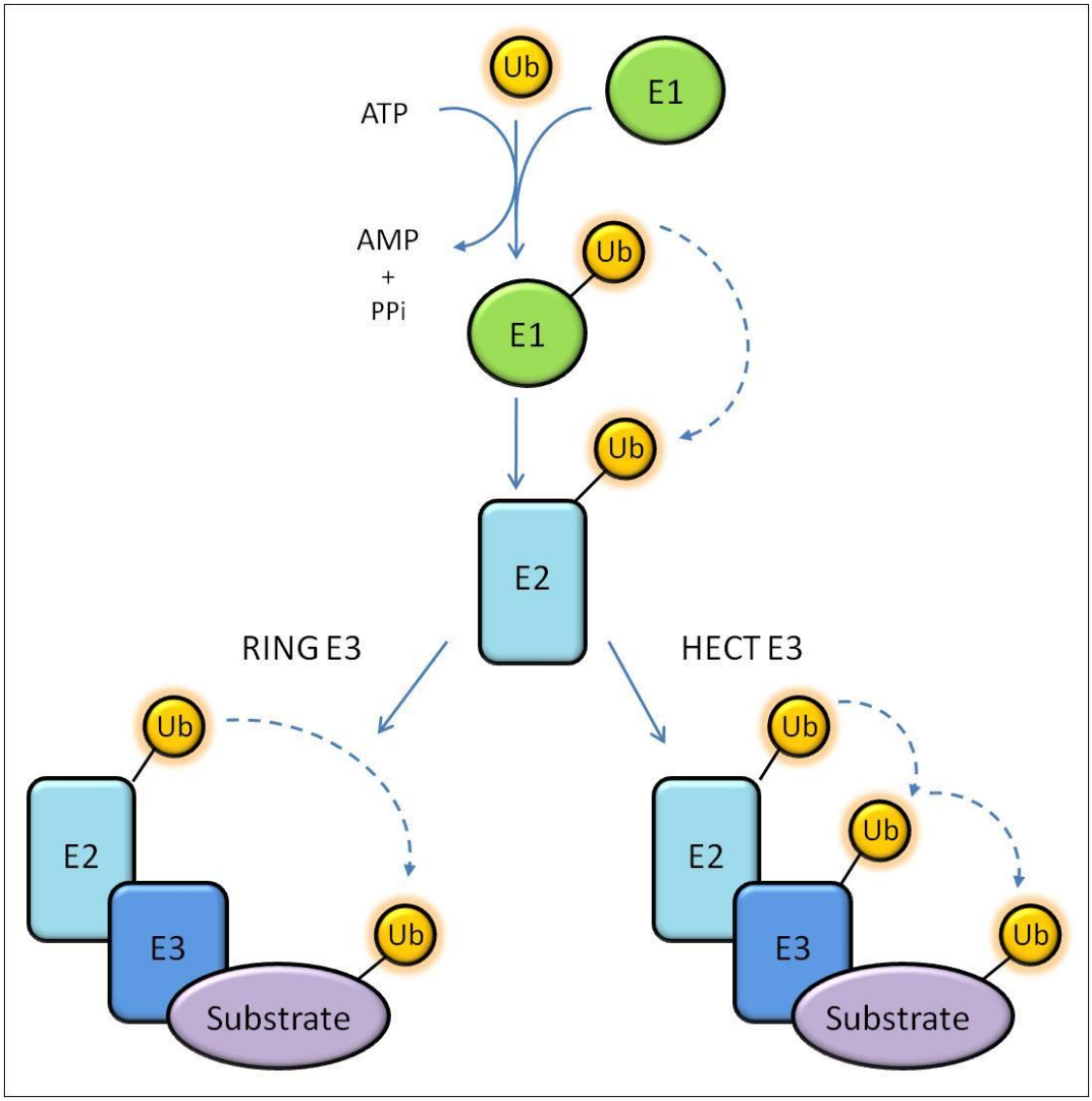

Figure 1.7 Conjugation of ubiquitin to its substrate.

E1 enzyme activates ubiquitin in an ATP dependent manner. The ubiquitin is passed to the E2 enzyme before being covalently attached to its substrate via either a RING- or HECT-domain containing E3 ligase.

1.3.3 Functions of ubiquitination

Ubiquitination has an effect on a diverse array of cellular function and its activity is largely dictated by the specific type of ubiquitin chain that is generated upon the target lysine residue of the substrate (Pickart and Fushman, 2004; Ikeda and Dikic, 2008). Some known functions of particular ubiquitin chains are illustrated in Figure 1.8.

1.3.3.1 Mono-ubiquitination

Mono-ubiquitination, or modification of a substrate with a single ubiquitin, and multiple mono-ubiquitination events have been shown to be involved in histone regulation, endocytosis, viral budding, DNA repair and nuclear export (Haglund *et al.*, 2003).

Histones H2A and H2B are approximately 10% and 1% ubiquitinated respectively in mammalian cells, whereas in yeast histone H2B is the major ubiquitinated histone (Hicke, 2001). Ubiquitination of histones is involved in regulation of transcriptional events; histone H2A ubiquitination has been shown to repress transcription while modification of histone H2B activates transcription. Furthermore, mutation of the histone H2B ubiquitination site or knockdown of the E2 enzyme Rad6 in yeast or of the Rad6 homologue Hr6b in mice results in defective meiosis (Roest *et al.*, 1996; Robzyk *et al.*, 2000).

Mono-ubiquitination of ion channels and receptors at the plasma membrane can lead to internalisation and lysosomal degradation (Hicke, 2001). It has been shown that the single ubiquitin molecule itself processes the internalisation signal. Fusion of mutant ubiquitin monomers, where the internal lysine residues have been substituted and therefore poly-ubiquitin chains cannot be formed, to receptors promotes internalisation (Shih *et al.*, 2000). Moreover, multi-mono-ubiquitination of some receptor tyrosine kinases, for example EGFR and PDGFR, has been shown to be sufficient for their endosomal trafficking in response to ligand activation (Strous and van Kerkhof, 2002; Huang *et al.*, 2006).

The E3 ligase MDM2 is known to regulate the function of p53 by poly-ubiquitination causing its proteasomal degradation (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). However, it was observed that low levels of MDM2 activity in unstressed cells promoted mono-ubiquitination of p53 by MDM2 and its subsequent nuclear

export (Boyd *et al.*, 2000; Geyer *et al.*, 2000). It is postulated that this is a regulatory mechanism to inhibit the transcriptional activities of p53 (Brooks and Gu, 2006).

1.3.3.2 Poly-ubiquitination

The seven lysine (K) residues within ubiquitin allow for distinct poly-ubiquitin chains to be formed, each with a specific function. Very little detail is known about the functions of K6, K27, K29 or K33-linked poly-ubiquitin chains and only recently has the function of K11-linked chains been elucidated, as described below (Pickart and Fushman, 2004; Behrends and Harper, 2011). Understanding of K48 and K63-linked poly-ubiquitination is much more advanced due to the significant nature of their biological functions (Hochstrasser, 1992; Yang *et al.*, 2010).

Perhaps the most well studied function of this system is degradation of mutated or damaged proteins via the proteasome. K48-linked ubiquitin chains, of four or more ubiquitin molecules, act as a recognition signal for the 26S proteasome (Hochstrasser, 1992). This multi-catalytic protease, consisting of a 20S catalytic subunit and a 19S regulatory particle, recognises the ubiquitinated proteins and allows them to enter the narrow channel of the catalytic subunit leading to proteolysis (Hochstrasser, 1992; Pickart and Fushman, 2004). K11 and K29-linked ubiquitin chains have also been implicated in proteasomal degradation (Baboshina and Haas, 1996; Koegl *et al.*, 1999; Lindsten *et al.*, 2002) although studies have uncovered specific roles of each modification type in cell cycle regulation and lysosomal degradation respectively (Chastagner *et al.*, 2006; Behrends and Harper, 2011).

K6-linked ubiquitin chains were recently discovered to be of importance in the BRCA1/BARD1-dependent localisation of conjugated ubiquitin to DNA damage foci (Wu-Baer *et al.*, 2003; Morris and Solomon, 2004). These chains are utilised in the auto-ubiquitination of BRCA1 and are recognised and deubiquitinated by the proteasome-associated deubiquitinase enzymes (DUBs) (Nishikawa *et al.*, 2004).

Ubiquitination of substrates with K63 poly-ubiquitin has been associated with DNA damage tolerance as poly-ubiquitination of PCNA has been shown to bypass DNA lesions (Hoege *et al.*, 2002). K63 linkages are also implicated in kinase activation, inflammatory responses, ribosomal synthesis and protein trafficking (Pickart and Fushman, 2004). Akt is poly-ubiquitinated by TRAF6, forming K63-linked ubiquitin

chains, which results in Akt recruitment to the plasma membrane and subsequent activation. Furthermore, K63-linked chains are thought to be involved in the interaction of adaptor proteins with Akt, facilitating activation (Yang *et al.*, 2009). TRAF6 is also known to ubiquitinate TAK1 in response to TGFβ stimulation, triggering TAK1 auto-phosphorylation and kinase activation (Sorrentino *et al.*, 2008). K63 poly-ubiquitination is on the most part non-proteolytic, although they can interact with the proteasome and when this occurs they are deubiquitinated more rapidly than K48 chains (Jacobson *et al.*, 2009).

Ubiquitination also has a role to play in immune signalling. The E3 ligase TRIM was found to mediate K27-linked poly-ubiquitination of NEMO causing IRF3 and NFκB activation and resulting in antiviral immune response (Arimoto *et al.*, 2010). Furthermore, T-cell receptor ζ was found to become K33 poly-ubiquitinated, affecting its protein associations and phosphorylation status; providing a mechanism of ubiquitin mediated T-cell signal transduction (Huang *et al.*, 2010).

Moreover, linear ubiquitin chains have been recently associated with immune signalling. Multimeric linear ubiquitin chains are produced as a result of transcription of the ubiquitin *Ubb* and *Ubc* genes and it was thought that this type of ubiquitin was produced exclusively in this manner and that it had no further functionality than to be processed into singular ubiquitin molecules ready for conjugation (Behrends and Harper, 2011). However, the discovery of the linear ubiquitin assembly complex (LUBAC) and that linear ubiquitination of NEMO results in NFκB activation revealed that this unusual chain type has biological significance (Haas *et al.*, 2009; Tokunaga *et al.*, 2009).

To add further complexity to the ubiquitin system, studies have shown that poly-ubiquitin chains can be formed utilising multiple lysine linkages within a single chain and there is evidence that multi-protein chains can be formed containing both ubiquitin and a member of the ubiquitin-like protein family such as SUMO or Nedd8. The significance and function of these more complex modifications is not known (Ikeda and Dikic, 2008).

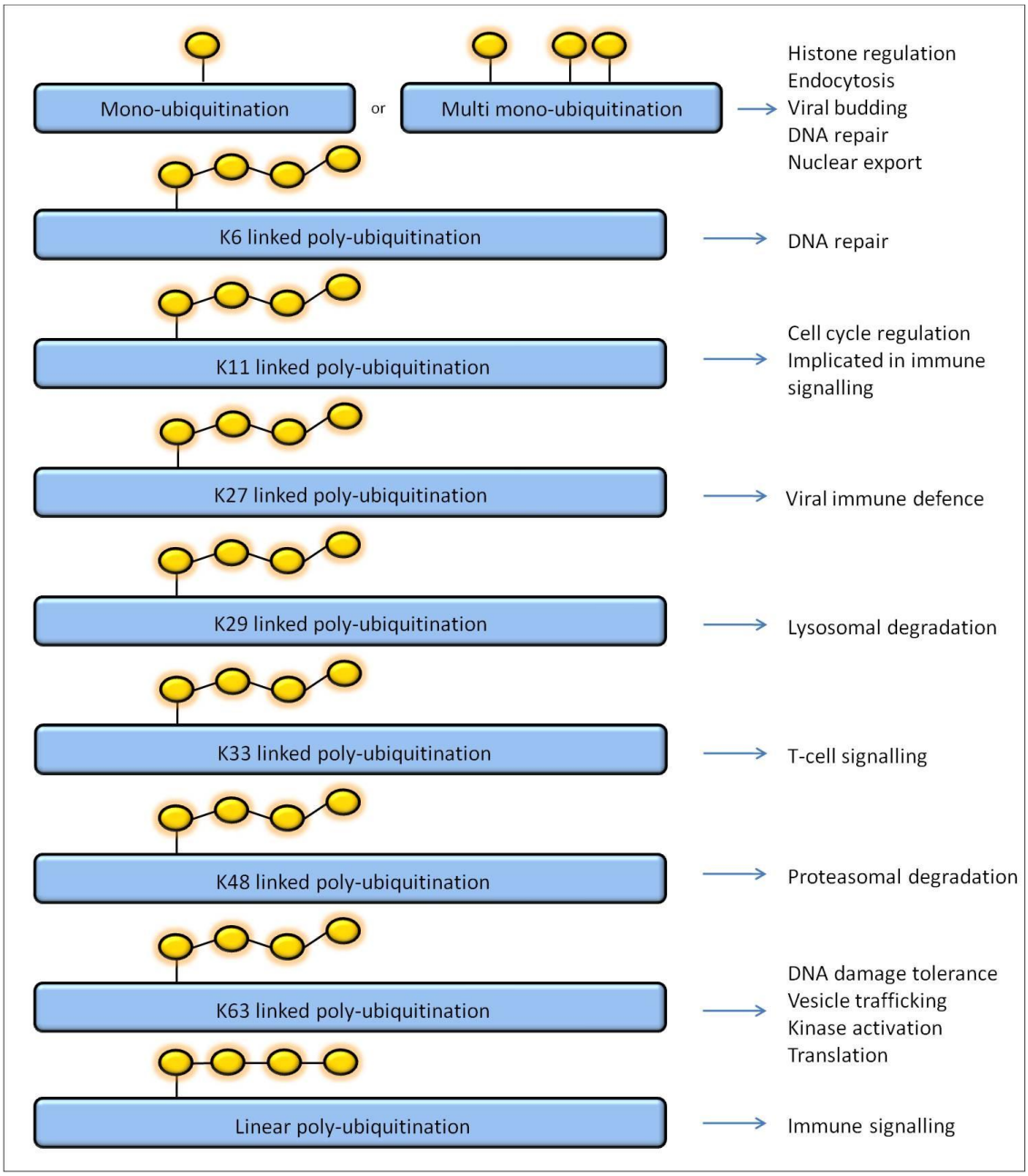

Figure 1.8. The known functions of mono-, multi-mono- and poly-ubiquitination.

Poly-ubiquitin chains can be linked through lysine residues K6, 11, 27,29,33,48 and 63.

1.4 Deubiquitinase enzymes and regulation of ubiquitination

Deubiquitinase enzymes (DUBs) provide an important regulatory role in the ubiquitin pathway by removing ubiquitin moieties from target proteins (Wilkinson, 1997). Among other functions, they regulate the rate of protein substrate turnover and maintain the pools of free ubiquitin (Amerik and Hochstrasser, 2004).

1.4.1 Structure and catalytic activity of deubiquitinase enzymes

Approximately 100 DUB enzymes have been identified within the human genome (Reyes-Turcu *et al.*, 2009), although some of them are yet to be characterised as catalytically active. These ubiquitin proteases have been classified in to five subfamilies based on sequence, structure and mechanistic properties. The first four subfamilies are cysteine (Cys) proteases; namely the ubiquitin-specific processing proteases (USP), the ubiquitin carboxyl-terminal hydrolases (UCH), the ovarian tumour related proteases (OTU-related) and Josephine domain DUBs (Amerik and Hochstrasser, 2004). The fifth subfamily consists of zinc-dependent metalloproteases containing the JAMM/MPN+ domain (Maytal-Kivity *et al.*, 2002). Examples of the structures of the five DUB sub-families are illustrated in Figure 1.9.

1.4.1.1 Ubiquitin-specific processing proteases (USPs)

The ubiquitin specific proteases or USP family is the largest and most diverse family of DUBs containing approximately 58 identified members (Ventii and Wilkinson, 2008). These enzymes contain the USP domain which consists of the conserved cysteine (Cys) and histidine (His) boxes that house the catalytically important cysteine, histidine and aspartate residues known as the catalytic triad residues (Nijman *et al.*, 2005b). The size of the USP domain can vary between enzymes, as some contain large sequence insertions which have been shown to affect enzyme activity as well as ubiquitin binding and cellular localisation (Nijman *et al.*, 2005b). However, it is important to note that although sequence similarity is low, the active site structure of the catalytic USP domain is super-imposable amongst all family members (Amerik and Hochstrasser, 2004).

Crystal structures have revealed that in general, the USP domain has a palm, thumb and fingers-like structure with the catalytic site sitting between the ‘palm’ and ‘thumb’ domains (Hu *et al.*, 2002) (Figure 1.9). The role of the ‘fingers’ is to interact with the distal most ubiquitin in the chain (Komander *et al.*, 2009a). Insights into the

mechanistic function of the USP catalytic reaction, and the other DUB Cys protease subfamilies, were provided by studies of the classical Cys protease papain (Storer and Menard, 1994). The catalytic triad of Cys, His and aspartic acid or asparagine (Asp or Asn) residues align allowing nucleophilic attack on the isopeptide bond between the C-terminal of ubiquitin and the lysine within the substrate with which it is conjugated. An acyl intermediate between the catalytic Cys and the carboxyl group of ubiquitin is formed which is further hydrolysed by a water molecule (Komander *et al.*, 2009a).

Analysis of apo-USP domains, those which are not bound to ubiquitin, has revealed that unlike classical cysteine proteases, they are in a non-catalytic configuration (Komander *et al.*, 2009a). Binding of substrate causes a conformational change either to align the catalytic triad residues or to remove structures that block access to the catalytic site (Amerik and Hochstrasser, 2004). However, this configuration is not true of all USP domains as the DUB enzyme CYLD has been shown to be ready for catalysis in an unbound form (Komander *et al.*, 2008).

1.4.1.2 Ubiquitin carboxy-terminal hydrolases (UCHs)

The ubiquitin carboxy-terminal hydrolases or UCHs were named due to their activity towards small amides and esters at the C-terminus of the ubiquitin molecule (Wilkinson, 1997). This family of DUBs again resembles the classical cysteine proteases containing a 230 amino acid catalytic core comprised of the catalytic triad residues and 3 dimensional structures similar to the USPs (Amerik and Hochstrasser, 2004). These enzymes also share a similar mechanism of catalysis to the USPs (Amerik and Hochstrasser, 2004). A predominant loop covers the active site of the UCHL3 enzyme which is several-fold smaller than loops of other un-folded DUBs meaning that it therefore dictates the substrates that UCHs can hydrolyse due to size constraints (Johnston *et al.*, 1997; Popp *et al.*, 2009). The substrate must be able to fit through the loop in order to access the catalytic residues therefore it is predicted that these proteases would only be able to act upon products of degradation, such as ubiquitin chains or on the ubiquitin precursor proteins to replenish cellular ubiquitin levels (Larsen *et al.*, 1998; Popp *et al.*, 2009). However, studies have now shown that larger, un-folded substrates can be accommodated (Johnston *et al.*, 1999).

1.4.1.3 Ovarian tumour related proteases (OTUs)

The ovarian tumour related (OTU-related) proteases were identified by bioinformatics analysis of genes that had homology to the ovarian tumour gene in drosophila melanogaster (Steinhauer *et al.*, 1989). There are approximately 14 human OTU proteins that have not all been assessed for DUB activity (Nijman *et al.*, 2005b). The OTU core domain consists of a 5 stranded β-sheet surrounded by helical domains that differ between family members (Reyes-Turcu *et al.*, 2009). The catalytic core resembles that of the USP and UCH DUBs, although variations in the catalytic triad residues have been identified that may impact on catalytic activity (Amerik and Hochstrasser, 2004; Nanao *et al.*, 2004) (Figure 1.9). For example, the drosophila OTU protein has a serine residue in place of the catalytic Cys, although it is unknown if this DUB is enzymatically active (Messick *et al.*, 2008; Reyes-Turcu *et al.*, 2009).

1.4.1.4 Josephin domain proteases

Ataxin-3 (ATXN3) is the best described Josephin domain protease whilst the other predicted family members remain relatively under-characterised (Nijman *et al.*, 2005b). The catalytic triad is again conserved and the catalytic core structure is similar to the USP and UCHs. However, ATXN3 has an extended helical arm that is proposed to regulate access to the active site (Mao *et al.*, 2005; Nicastro *et al.*, 2009). Furthermore, structural studies of ATXN3 bound to ubiquitin have indicated that a second ubiquitin molecule may bind to the back of the helix allowing ATXN-3 to bind 2 ubiquitin molecules simultaneously (Nicastro *et al.*, 2009).

1.4.1.5 JAMM/MPN+ proteases

The fifth subfamily to be described represent metalloproteases that bind two zinc ions in order to proteolytically cleave ubiquitin and are distinct from the Cys proteases USP, UCH, OTU and Josephin domain DUB enzymes (Komander *et al.*, 2009a). The first Zn^{2+} is involved intimately in catalysis by activating a water molecule to attack the isopeptide bond between the ubiquitin and its substrate, whilst the second Zn^{2+} stabilises the motif involved in recognising the distal ubiquitin (Maytal-Kivity *et al.*, 2002). JAMM/MPN+ DUBs have been found in association with the 26S proteasome, the COP9 signalosome and the ESCRT machinery implicating them in regulation of the ubiquitin-proteasome system as well as endosomal sorting (Cope *et al.*, 2002; Yao and Cohen, 2002; McCullough *et al.*, 2006).

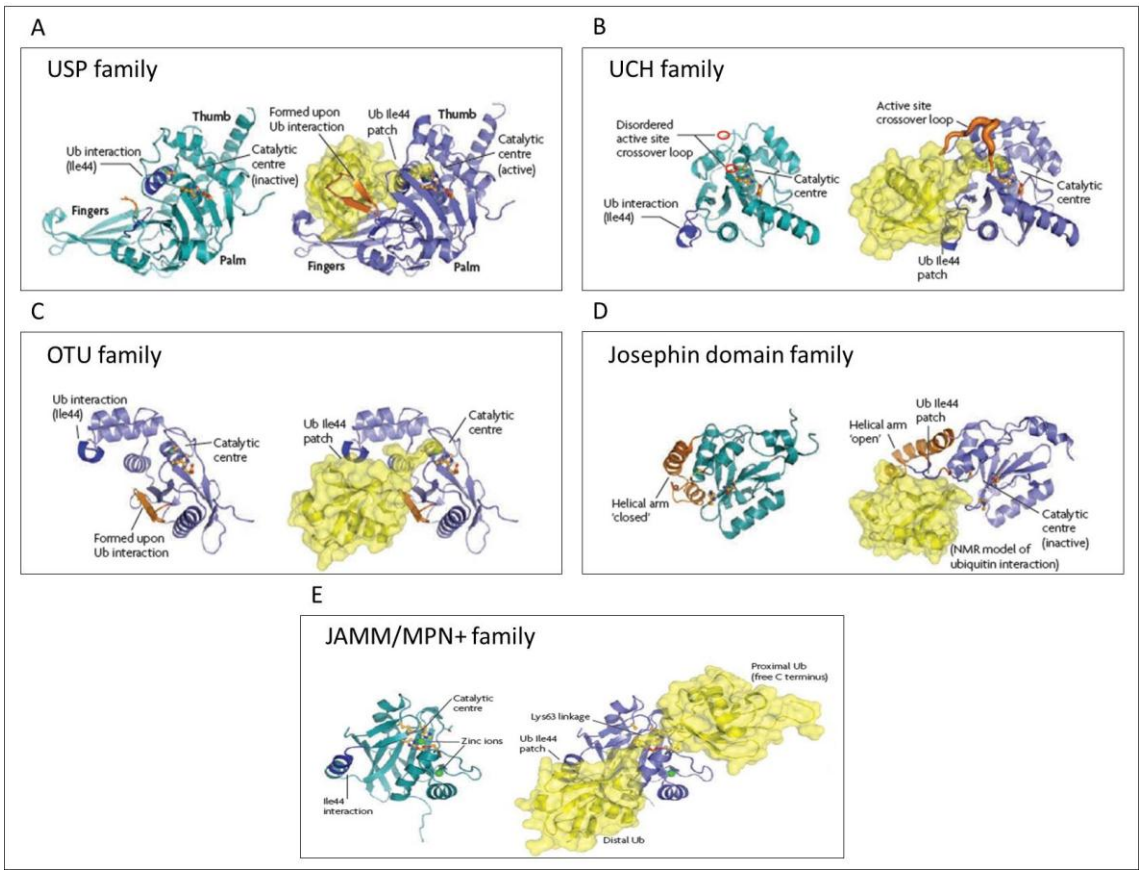

Figure 1.9. The structure of the catalytic domains of the deubiquitinase enzyme sub-families.

Examples of the inactive and active catalytic domain of the deubiquitinase enzymes (DUBs) are illustrated by the green and blue structures respectively. The interaction of the active domains with ubiquitin is also shown; ubiquitin is represented by the yellow structure. A) An example of the catalytic domain of a member of the USP family. B) An example of the catalytic domain of a member of the UCH family. C) An example of the catalytic domain of a member of the OTU family. D) An example of the catalytic domain of a member of the Josephin domain family. D) An example of the catalytic domain of a member of the JAMM/MPN+ family. Adapted from (Komander et al., 2009a)

1.4.2 Physiological functions of deubiquitinase enzymes

A selection of the biochemical functions of the DUB enzymes are illustrated in Figure 1.10. These functions allow DUBs to counteract and regulate the ubiquitination events described in Section 1.3.3. Like ubiquitination, DUB enzymes have been implicated in almost every cellular process.

1.4.2.1 Ubiquitin precursor processing

Ubiquitin is transcribed from the *Ubb* and *Ubc* genes as a multimeric 4 and 9 tandem repeat pro-protein and from the *Uba52* and *RPS27a* genes as a single moiety fused to ribosomal proteins (Kimura and Tanaka, 2010). In order for this newly transcribed ubiquitin to be used in the ubiquitin-proteasome system it must first be processed into single usable ubiquitin molecules (Wilkinson, 1997). Ubiquitin multimers and fusion proteins between ubiquitin and ribosomal subunits are cleaved at the α-amino group of the glycine residue at position 76 (Amerik *et al.*, 2000). Currently no DUB has been specifically identified for this exclusive function; enzymes that catalyse pro-protein cleavage are involved in many other ubiquitin-related processes (Wilkinson, 1997). However, multiple DUBs show activity towards linear ubiquitin chains and USP5 (also known as Isopeptidase T) has activity towards unanchored poly-ubiquitin chains (Dayal *et al.*, 2009). Pro-protein processing acts not only to regulate the levels of free ubiquitin but also as a quality control mechanism; if DUBs cannot process the precursors due to mutation or problem in folding then these molecule will not enter the ubiquitin system (Amerik and Hochstrasser, 2004).

1.4.2.2 Deubiquitination coupled to proteasomal degradation

Ubiquitin conjugates need to be removed from substrates targeted to the proteasome before proteolysis. This allows for recycling of free ubiquitin that acts to assist physiological rates of protein turnover by replenishing depleted pools of unconjugated ubiquitin protein (Song and Rape, 2008). Moreover, removing ubiquitin before substrate degradation avoids blockage of the narrow catalytic channel of the proteasome allowing efficient entry of targeted proteins (Verma *et al.*, 2002). Three DUBs have been closely linked to the proteasome, USP14, UCH37 and POH1. USP14 associates with the S2 subunit within the 19S regulatory particle of the 26S proteasome (Hu *et al.*, 2005; Hanna *et al.*, 2006), while in contrast, UCH37 and POH1, are integral subunits of the 19S particle itself (Hamazaki *et al.*, 2006; Qiu *et al.*, 2006;

Yao *et al.*, 2006). Together, these DUBs are responsible for the removal of poly-ubiquitin chains from proteins during degradation. UCH37 and USP14 are thought to be redundant as depletion of both is required to cause a build-up of free ubiquitin chains (Koulich *et al.*, 2008). However, USP14 is up-regulated upon depletion of the free pool of ubiquitin suggesting it is essential in the recycling process (Hu *et al.*, 2005).

Released poly-ubiquitin chains must undergo further disassembly, comparable to pro-protein processing, to remove potential competition with substrates for proteasome binding and degradation. In human cells, USP5 and USP13 are responsible for disassembling unanchored poly-ubiquitin chains down to the monomeric level (Reyes-Turcu *et al.*, 2008; Dayal *et al.*, 2009). These DUBs have a zinc-finger (ZnF)-UBP domain, which coordinates zinc ions to specifically recognise the di-glycine motif on the C-terminal tail of the ubiquitin chain (Reyes-Turcu *et al.*, 2006). USP5 specifically recognises K29, K48 and K63-linked poly-ubiquitin chains (Dayal *et al.*, 2009), whilst the specific chains targeted by USP13 are unknown.

Proteins may also be degraded via the lysosome, a cytoplasmic organelle containing acid hydrolase enzymes. In this case deubiquitination is not required for degradation but only as a means of maintaining levels of free ubiquitin (Komander *et al.*, 2009a). Mono-ubiquitination is important for the internalisation of surface proteins and further K63-linked poly-ubiquitination is thought to assist in this process (Hicke and Dunn, 2003). USP8, a homologue of yeast Doa4, is required for removal of ubiquitin chains in this scenario (Kato *et al.*, 2000; Mizuno *et al.*, 2005).

1.4.2.3 Ubiquitin chain removal and editing

DUBs are required for a level of ubiquitination 'proof-reading' within the cell. Proteins may be rescued, by chain shortening or chain removal, from the degradation pathway. For example, in response to DNA damage a K63-linked ubiquitin chain would result in cell cycle progression without DNA repair. However, editing of this chain to a mono-ubiquitinated mark would dramatically change the fate of the cell by triggering DNA damage repair (Hicke, 2001; Weissman, 2001). Removal of ubiquitin changes can also affect protein stability. There are many examples of DUBs that are intimately associated with E3 ligases that are known to regulate themselves through auto-ubiquitination (Li *et al.*, 2002b; Nijman *et al.*, 2005b). A well characterised example is that of the relationship between USP7 and MDM2. MDM2 regulates its own catalytic

activity towards p53, a prominent tumour suppressor gene, by auto-ubiquitination. MDM2-mediated ubiquitination of p53 leads to p53 proteasomal degradation and subsequent down-regulation of its downstream effects such as cellular senescence and apoptosis (Brooks and Gu, 2006). Auto-ubiquitination of MDM2 is reversed by USP7 which causes stabilisation of MDM2 and promotion of its activity towards p53. However, USP7 can also interact with and deubiquitinate p53, stabilising the protein and promoting p53 activity. Interestingly, both p53 and MDM2 are recognised by the N-terminal domain of USP7 which recognises the same two closely spaced motifs in both proteins (Meulmeester *et al.*, 2005; Sheng *et al.*, 2006).

Editing is also an important mechanism of specificity determination within the ubiquitin system. The DUB enzyme can only remove ubiquitin from chains and for this reason it is not unexpected that DUBs are often found in complexes with E3 ligases; regulating their activity and also their stability (Nijman *et al.*, 2005b). The DUB CYLD negatively regulates NFκB signalling by opposing the E3 ligase activity of TRAF2, TRAF6 and NEMO (Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003). Furthermore, A20, a member of the OUT sub-family of DUBs, also regulates NFκB signalling through chain editing. However, this enzyme not only processes the removal of ubiquitin but also uses its intrinsic E3 ligase activity to edit the K63-linked ubiquitination of adaptor proteins, such as RIP1, to K48-linked chains resulting in proteasomal degradation (Heyninck and Beyaert, 2005).

1.4.2.4 Control of transcription

Ubiquitination of histone H2A and H2B are important in transcriptional regulation. Histone H2A ubiquitination is transcriptionally repressive therefore deubiquitination allows for active transcription. USP16 is a histone H2A specific DUB and depletion of USP16 causes an increase in *Hox* gene expression. It was postulated that this DUB may regulate levels of ubiquitinated histone H2A at promoters and regulatory regions of these genes (Joo *et al.*, 2007; Reyes-Turcu *et al.*, 2009).

USP22 is a histone H2B specific DUB that is part of the SAGA complex (Zhang *et al.*, 2008). This complex regulates transcriptional activation and elongation by deubiquitination of histone H2B, allowing recruitment of RNA polymerase II kinase Ctk1. This recruitment in turn allows phosphorylation of RNA polymerase II at serine 2 and elongation to occur (Wyce *et al.*, 2004). Moreover, USP22 links deubiquitination to

mRNA export from the nucleus as the SAGA complex also binds to export factors which interact with the nuclear pore complex (Daniel and Grant, 2007). In yeast, deubiquitination of ubiquitinated histone H2B by Upb10 in rDNA and telomeric regions maintains gene silencing by association with histone deacetylase proteins (Gardner *et al.*, 2005).

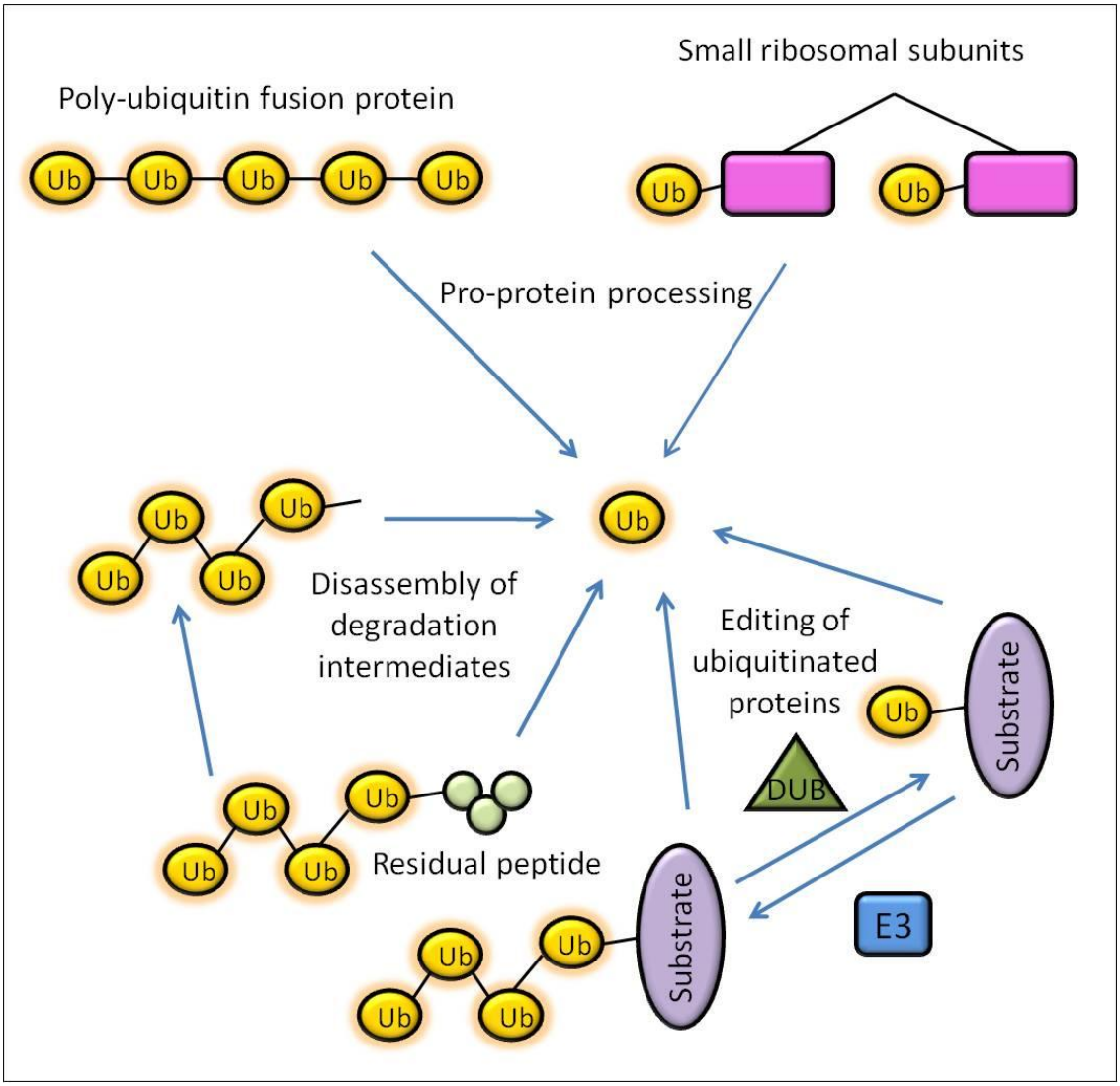

Figure 1.10. Some of the multiple functions of deubiquitinase enzymes.

DUBs are involved in processing and editing of ubiquitin chains allowing for negative regulation of the processes of ubiquitination.

1.4.3 Specificity of deubiquitinase enzymes

To conduct specific roles, DUBs must first be able to recognise and hydrolyse the correct substrates. Ubiquitin specific DUBs must firstly distinguish between ubiquitin and the closely related ubiquitin-like proteins, such as SUMO-1 and Nedd8 (Komander *et al.*, 2009a). This is achieved by extensive contacts between the DUB and the distal most ubiquitin; as much as 40% of the ubiquitin surface area is bound by the DUB (Zhu *et al.*, 2007b). Secondly, the ubiquitin C-terminus, which extends into the catalytic domain of the DUB, contains a unique amino acid sequence, Leu71-Arg72-Leu73-Arg74-Gly75-Gly76; it is Arg74 and Gly75 that are critical for recognition (Komander *et al.*, 2009a).

Furthermore, DUBs discriminately cleave ubiquitin chains and are capable of catalysing either the specific cleavage of the terminal ubiquitin in a chain or whole chains of ubiquitin. It is thought that certain lysine-linked ubiquitin chains have a 'closed' structure whilst others have a more 'open' accessible structure. USP14, a component of the proteasome, specifically recognises and catalyses K48-linked chains to facilitate ubiquitin recycling and substrate degradation (Hu *et al.*, 2005). The crystal structure of the JAMM/MPN+ sub-family member AMSH-LP in complex with a K63 di-ubiquitin chain has been solved, the only structure of its kind, and revealed that AMSH-LP contacts all the residues involved in the K63-linkage and has extensive interactions with the second ubiquitin molecule. Its recognises a motif containing glutamine 62 and glutamic acid 64 within the first ubiquitin molecule that is only accessible in K63-linked chains due to the closed conformation of other chains types (Sato *et al.*, 2008). Conversely, CYLD recognises both K63 and linear ubiquitin chains (Komander *et al.*, 2008). Interestingly, CYLD has an extended loop located near the enzymes active site that appears to recognise and select against K48-linked substrates (Komander *et al.*, 2009b). K63-linkages are very similar to linear chains of ubiquitin as they both have an extended open structure. However, these chains are hydrolysed in different ways; K63 chains, and all other lysine-linked ubiquitin chains, require isopeptide bond cleavage whilst linear ubiquitin chains are linked via a peptide bond. It has been found that most USP domain DUBs can cleave linear ubiquitin chains to a low efficiency so perhaps those that recognise K63-linked chains also have a function in linear and immature ubiquitin processing (Komander *et al.*, 2009a).

Cleavage of the final ubiquitin that is attached directly to the lysine of the substrate protein is performed by exo-DUBs, whilst internal linkage cleavage is performed by endo-DUBs. The structure of the catalytic domain can determine which type of activity a DUB has. For example, the finger domain of USP14 makes contacts with 40% of the surface area of the distal ubiquitin in a chain thereby blocking access of the DUB to internal linkage sites (Hu *et al.*, 2005). In contrast, CYLD lacks the finger domain and catalyses cleavage of internal isopeptide bonds (Komander *et al.*, 2008). Removal of mono-ubiquitin from a substrate, as a result of a ubiquitination event or cleavage of a ubiquitin chain by a DUB, requires substrate specific interactions as this isopeptide bond is the same in all cases i.e. it has no lysine specificity (Komander *et al.*, 2008). USP5 and USP13, as described previously, are ubiquitin recycling DUBs that mediate the breakdown of unanchored ubiquitin chains by recognition of a broad range of ubiquitin chains (Dayal *et al.*, 2009).

1.4.4 Regulation of deubiquitinase enzymes

In order to prevent inappropriate protease activity of DUBs towards cellular proteins they are tightly regulated by multiple mechanisms. Regulation of DUB enzyme transcription represents one mechanism of cellular control in which DUBs are expressed in the cell at specific times. For example, Dub-1 and Dub-2 are transcribed rapidly in mouse lymphocytes in response to interleukins due to the presence of a cytokine response element in their gene promoters and hence are only present during immune response (Zhu *et al.*, 1997; Jaster *et al.*, 1999).

Post-translational modification of DUBs is an additional control mechanism that regulates catalytic activity as well as DUB abundance in cells. For example, CYLD is phosphorylated in response to interaction with NEMO thereby inhibiting its ability to suppress NFκB signalling through deubiquitination of E3 ligase TRAF2 (Kovalenko *et al.*, 2003). Poly-ubiquitination of USP4 increases its activity towards its substrate Rho52, an E3 ligase which in turn ubiquitinates USP4 and down-regulates its activity (Wada and Kamitani, 2006). Lastly, SUMOylation of USP25, in which SUMO is conjugated to a target lysine in USP25, affects its binding affinity for ubiquitin therefore inhibiting its activity (Meulmeester *et al.*, 2008).

Conformational changes provide a structural method of regulation. Some DUBs, such as USP7, require binding of substrate to properly align the catalytic residues (Hu *et al.*,

2002). Allosteric re-organisation of internal structures that occlude the active site has also been shown to be important for activating some DUBs activity. For example, the unordered active site loop of UCHL3 is restructured into a α-helix upon substrate binding and interacts with the C-terminus of ubiquitin, therefore displacing it from the active site (Johnston *et al.*, 1997), while in contrast, the active site loop of yeast Otu1 is reorganised into a β-strand upon ubiquitin binding (Nanao *et al.*, 2004). In addition, the active site of USP8 is blocked by the finger structure of the USP domain; in order for a substrate to effectively access the catalytic residues it must first bind and displace this large domain (Avvakumov *et al.*, 2006).

Protein-protein interactions between DUBs and scaffolding or adaptor proteins not only regulate their activity, but also may assist in their specificity for substrates. The proteasome-associated DUBs, USP14, UCH37 and POH1 are found to have little activity until they are associated with the 26S proteasome as the interactions bring about conformational changes. USP14 has 300-fold greater activity when bound to the S2 subunits of the 19S regulatory particle (Leggett *et al.*, 2002).

1.4.5 Deubiquitinase enzymes in disease

Several DUBs were first identified through their relationship to disease phenotypes. Autosomal dominant mutations of CYLD were found in patients with familial cylindromatosis who develop skin tumours of the head and neck (Bignell *et al.*, 2000; Bowen *et al.*, 2005). CYLD functions to down-regulate NFκB signalling through removal of K63-linked poly-ubiquitin chains from TRAF2, TRAF6 and NEMO, important regulators of the NFκB signal cascade. This in turn stops the inhibitor of κB (IκB) from becoming degraded and releasing NFκB for gene activation function (Kovalenko *et al.*, 2003). In cells where CYLD is depleted, and hence NFκB displays aberrant activity, an increased resistance to apoptosis is observed providing a possible mechanism for tumour formation (Brummelkamp *et al.*, 2003).

USP6 was the first DUB to be identified as an oncogene after it was observed that over-expression in cells lead to a transformed phenotype (Onno *et al.*, 1993; Papa and Hochstrasser, 1993). The initial transcript was isolated from a Ewing's sarcoma and was found to be causative of aneurismal bone cysts. A translocation between chromosomes 16 and 17, t(16;17)(q22;p13) which results in the full length USP6 gene under the control of the osteoblast cadherin 11 promoter was identified in

approximately 66% of patients in a 52 patient study and results in up-regulation of USP6 expression (Oliveira *et al.*, 2005). Interestingly, catalytic activity of the DUB was found not to be important in the malignant transformation properties of USP6 as the first identified transcript was a splice variant containing no catalytic domain (Shen *et al.*, 2005).

Ataxin-3 is linked to the neurodegenerative disorder Machado-Joseph disease (Paulson *et al.*, 1997). Extension of ATXN3's poly-glutamine stretch confirms a toxic gain of function effect for this DUB due to altered protein associations and formation of toxic aggregates (Paulson, 1999; Perez *et al.*, 1999). UCHL1 is also linked to neuro-degeneration by mutations that confer both protection and susceptibility towards Parkinson's disease. The first mutation identified substituted isoleucine 93 to a methionine (I93M) and was found to diminish the catalytic activity of UCHL1 towards ubiquitin and also lower its E3 ligase activity (Leroy *et al.*, 1998). UCHL1 is also found in Lewy bodies, protein aggregates of normal and abnormal proteins (Singhal *et al.*, 2008). Conversely, a mutation of serine 18 to tyrosine (S18Y) is protective against Parkinson's disease and delays onset of the disease (Maraganore *et al.*, 1999). This is thought to be due to inhibition of UCHL1 E3 ligase activity by inhibiting dimer formation. UCHL1 is also linked to Alzheimer's disease where a low level of functional UCHL1 is inversely proportional to accumulation of amyloid protein aggregates (Ichihara *et al.*, 1995; Choi *et al.*, 2004). Additionally UCHL1 has been implicated in many malignancies including lung, breast and colon (Okochi-Takada *et al.*, 2006; Wang *et al.*, 2008; Fang *et al.*, 2010).

1.4.5.1 Deubiquitinase enzymes as drug targets

There are two potential methods for inhibiting DUBs; (1) inhibiting the enzymatic activity by targeting the active site; (2) interfering with the DUB-substrate interaction (Singhal *et al.*, 2008). Several DUB inhibitors are used in the context of research including ubiquitin-aldehyde (Ub-al) and ubiquitin vinyl sulfone (UbVS). Ub-al and UbVS are ubiquitin derivatives that cannot be hydrolysed resulting in the accumulation of poly-ubiquitinated products. Ub-al has been utilised in structural studies of DUBs bound to ubiquitin (Love *et al.*, 2007). These inhibitors, however, are broadly specific to the Cys proteases and are not thought to be good candidates for drug development programmes (Colland, 2010).

High-throughput screening of compounds against specific DUBs has identified several potential therapeutic compounds. Two small molecule inhibitors of UCHL1 have been developed that show up to 100-fold specificity for UCHL1 over UCHL3 and other DUBs (Liu *et al.*, 2003). Firstly, a series of istatin o-acyl oxime derivatives have been shown to counteract UCHL1 anti-proliferative effect in lung cancer and neuroblastoma cell lines. These inhibitors are directed towards the UCHL1 active site (Liu *et al.*, 2003). Secondly, a series of non-competitive, non-active site directed 3-amino-2-oxo-7H-theino[2,3-b]pyridine-6-one inhibitors are also in development (Mermerian *et al.*, 2007). A series of dihydro-pyrrole skeleton inhibitors have also been developed against UCHL3 which are thought to competitively bind to the enzymes active site (Hirayama *et al.*, 2007).

Hybrigenics, a pharmaceutical company, have also developed small molecule inhibitors towards USP7 and USP8. HBX41,108 reversibly inhibits USP7 deubiquitinase activity resulting in stabilisation and activation of p53 leading to inhibition of cell growth and promotion of apoptosis in p53 positive colon cancer cell lines (Colland *et al.*, 2009). HBX90,397 is a closely related compound to the USP7 inhibitor, although it has been shown to have anti-proliferative and pro-apoptotic effects due to its inhibition of USP8 (Colland, 2010). Currently, no DUB inhibitors are in clinical trial.

1.5 The role of ubiquitination and deubiquitination in prostate cancer

Defects in the ubiquitin system can have devastating effects. Loss of function mutations in enzymes involved in protein turn-over can lead to protein stabilisation whereas gain of function will lead to accelerated degradation or loss of 'healthy' proteins. Mutations that result in the loss of E1 enzyme function are lethal (Ciechanover, 2003). In cancer, stabilisation of oncogenes and growth factors along with degradation of tumour suppressors lead to aberrant growth and proliferation of cancer cells. BRCA1, a well-studied tumour suppressor gene, is a RING domain-containing E3 ligase; mutations of this catalytic domain is common for breast and ovarian cancer predisposition (Hashizume *et al.*, 2001). In CaP, several E3 ligases and DUBs act as AR co-regulators potentiating the notion that aberrant activity of these enzymes may drive deregulated AR activity and CaP development.

1.5.1 E3 ubiquitin ligases in prostate cancer

Firstly, proteasome function is suggested to be important for AR transcriptional activity. In a study by Lin *et al*., it was found that proteasomal inhibitor MG132 caused decreased AR expression and nuclear translocation as well as disrupting the interaction between AR and its co-regulators ARA70 and TIF2 (Lin *et al.*, 2002a). Contradictory to this, another study by Sheflin *et al*., reported an increase in AR expression after MG132 treatment (Sheflin *et al.*, 2000). The authors speculate that the proteasome may act directly as a co-regulator of AR due to enhanced activity upon over expression of a 20S proteasome subunit, PSMA7. They also suggest the PEST sequence of the hinge region as being important for the proteasomal recognition as it could be a target for ubiquitination (Sheflin *et al.*, 2000), although this remains unknown. Furthermore, a study by Godfrey *et al* reported that degradation of the AR by the proteasome was important for aiding stress-induced cell death by an AR-mediated mechanism (Godfrey *et al.*, 2010).

A number of E3 ligases interact with the AR including C-terminal HSP-interacting protein (CHIP), murine double minute (MDM2), p53-induced protein with a RING-H2 domain (PIRH2) and E6 associated protein (E6-AP). Rees *et al*., found that CHIP binds directly to AR after phosphorylation of the hinge region (Rees *et al.*, 2006). A further study reported over-expression of CHIP in LNCaP cells lead to decreased AR levels by acting as an inhibitor of AR folding (Cardozo *et al.*, 2003). MDM2 cooperates with

HDAC1 to co-repress AR leading to its degradation (Gaughan *et al.*, 2005). MDM2 promotes degradation of AR in a phosphorylation dependent manner; phosphorylation of both AR and MDM2 by AKT is required (Lin *et al.*, 2002a). MDM2 is well characterised as a negative regulator of p53 (Brooks and Gu, 2006) and interestingly knockdown of MDM2 in LNCaP cells restores sensitivity to AAT by increasing p53 expression (Mu *et al.*, 2008). Importantly, 30-40% of prostate tumours over-express MDM2 making this a potential therapeutic target (Mu *et al.*, 2008).

Conversely, PIRH2 and E6-AP act to increase AR activity. PIRH2 interacts with AR co-activator TIP60 leading to degradation of HDAC1 and therefore increased AR transcriptional activity (Logan *et al.*, 2006). Immuno-histochemical data correlates PIRH2 expression to grade of CaP, suggesting over-expression of PIRH2 may enhance AR activity in prostate malignancy (Logan *et al.*, 2006). E6-AP regulates a number of nuclear hormone receptors including AR (Ramamoorthy and Nawaz, 2008). Androgen treatment recruits E6-AP to the PSA promoter and enhances AR transcriptional activity (Khan *et al.*, 2006). However, contradictory data suggests that E6-AP degrades AR (Khan *et al.*, 2006). Interestingly, all the above mentioned enzymes also play a role in p53 ubiquitination (Traidej *et al.*, 2000; Brooks and Gu, 2006; Duan *et al.*, 2006; Tripathi *et al.*, 2007). TRIM68 is another E3 ligase that acts as a co-activator of AR transcriptional activity. TRIM68 was found to directly interact with AR and its co-activators TIP60 and p300 leading to increased AR activity. TRIM68 was found to be preferentially over-expressed in CaP cell lines and human CaP tissue was found to also over-express compared to normal prostate (Miyajima *et al.*, 2008).

Although ubiquitination of the AR is well studied, the specific sites and sites of ubiquitination have remained unclear. Recently, Xu *et al*., were the first to identify AR ubiquitination sites targeted by RNF6, a novel E3 ligase (Xu *et al.*, 2009). This study showed that RNF6-induced ubiquitination of AR is required for AR-mediated transcription via direct modification of two lysines within the LBD, namely K845 and K847. These ubiquitinated residues are then able to recruit the AR co-activator ARA54 to facilitate gene expression of androgen-responsive genes (Xu *et al.*, 2009). It is likely that each E3 ligase targeting the AR has specific target sites within the AR that are not limited to K845 and K847 of the LBD hence the study by Xu *et al*., represents the beginning of AR ubiquitination site identification not the end.

1.5.2 Deubiquitinase enzymes in prostate cancer

Members of the DUB enzyme family have also been implicated in AR regulation and prostate cancer. USP7, a regulator of both MDM2 and p53, has been found to be over-expressed in CaP and is associated with deubiquitination and reduced nuclear localisation of PTEN, a protein phosphatase of the AKT pathway that is frequently lost in CaP progression (Song *et al.*, 2008). UCHL1 has been identified as being expressed in CaP cell lines that have undergone neuroendocrine differentiation. Interestingly, only the AR-negative DU145 and PC-3 cell lines expressed UCHL1 (Leiblich *et al.*, 2007). USP2a protects CaP cells from apoptosis by promoting p53 degradation via MDM2 deubiquitination and stabilisation. USP2a was found to be over-expressed in CaP tumours and correlated with over-expression of MDM2. Tumours with low USP2a expression had a high level of p53-induced pro-apoptotic gene expression and better prognosis (Priolo *et al.*, 2006). USP2a is also involved in the stabilisation of fatty acid synthase (FAS), which is also over-expressed in CaP (Graner *et al.*, 2004).

Two DUBs have been reported as direct regulators of AR, USP10 and more recently USP26. USP10 was identified as an AR interacting protein in a proteomic study of PC-3 cell lines stably over-expressing ectopic AR (Faus *et al.*, 2005). Over-expression of USP10 was observed to stimulate AR activity by increasing its binding to both selective and non-selective AREs. The authors of this study hypothesise that this DUB is involved in the editing of ubiquitin chains from proteasome-targeting poly-ubiquitin chains to mono- or tri-ubiquitin chains which can be transcriptionally activating (Faus *et al.*, 2005). Additionally, USP10 has been identified to regulate the ubiquitination of histone H2A.Z thereby driving AR-mediated transcription of target genes. Depletion of USP10 results in retention of ubiquitinated histone H2A.Z at AR-target gene promoters and deubiquitination of this ubiquitinated histone by USP10 caused an increase in *PSA* and *KLK2* expression (Draker *et al.*, 2011).

USP26 was identified in a siRNA knockdown screen looking for DUBs that modulated AR transcriptional activity (Dirac and Bernards, 2010). Knockdown of USP26 in the HepG2 hepatocellular carcinoma cell line over-expressing ectopic AR caused an increase in AR activity, suggesting that the DUB is an AR co-repressor. However, over-expression of USP26 in the HEK293 kidney cell line confusingly caused an increase in AR activity. The authors suggest that this discrepancy is due to different ubiquitin

targets being hydrolysed in the different cell lines; mono-ubiquitination in HepG2 thereby inhibiting activation of AR activity and poly-ubiquitination in HEK293. Nevertheless, USP26 was found to interact directly with AR, co-localised with AR in the nucleus upon androgen stimulation and influence AR deubiquitination (Dirac and Bernards, 2010).

Given the complexity of the ubiquitination system in mammalian cells, and the evidence implicating various ubiquitination and deubiquitination pathways in AR regulation and CaP, new targets for CaP therapy may exist within these systems that must be identified, characterised and exploited for more effective treatments.

1.6 Aims

The AR is a known target for ubiquitination by several E3 ubiquitin ligase enzymes that can lead to either increased activity or degradation depending on the E3 ligase involved. Importantly, the ability of the AR to evade therapy in CRCaP suggests that deciphering the mechanisms that regulate AR stability in this disease state may provide extremely useful and novel therapies. A gap in the knowledge of the reversal of ubiquitination by deubiquitinase enzymes (DUBs) within AR signalling exists and the characterisation of these enzymes could be important in elucidating mechanisms for the aberrant AR function observed in CRCaP.

The aims of this study are:

- Identify DUBs involved in AR signalling by utilising an siRNA knockdown library screen in the androgen-sensitive LNCaP cell line
- To characterise the function of the identified DUBs within the AR signalling cascade and prostate cancer

Chapter 2: Material and Methods

2.1 General material and methods

The University guideline 'Safe Working with Micro-organisms' was observed when handling biological samples. All chemicals were handled in accordance with COSHH regulations.

2.1.1 Materials and reagents

Routinely used chemicals were analytical or molecular grade and purchased from Sigma-Aldrich or Fisher Scientific, unless otherwise stated. siRNA oligonucleotides and conventional and quantitative PCR (QPCR) primers were purchased from Sigma-Aldrich.

2.1.2 Plasmid vectors

pCDNA3-AR, pARE3-luc, pPSA-luc, pCMV-β-gal and pCMV5 empty vector have been described previously by (Brady *et al.*, 1999). pFLAG-AR was a kind gift from Ralf Janknecht (Oklahoma University, Rochester, MN) (Shin and Janknecht, 2007). pYFP-AR was a kind gift from Jan Trapman (Erasmus Medical Centre, The Netherlands). pCMV-HA-USP10 and pGEX-6P-1-USP10 were kind gifts from Bernard Haendler (TRG, Berlin, Germany) (Faus *et al.*, 2005). pDEST-FLAG-HA-USP12 (Sowa *et al.*, 2009), pRK5-HA-Ubiquitin (Lim *et al.*, 2005) and pCMV-Myc3-MDM2 (Zhang *et al.*, 2003) were purchased from Addgene.

2.2 Generation of p3XFLAG-CMV-10™-USP12 and p3XFLAG-CMV-10™-USP12$_{C48A}$

2.2.1 Agarose gel electrophoresis

All products of PCR, digestion, gel extraction and plasmid preparations were analysed by agarose gel electrophoresis on 1% agarose gels (1% agarose in Tris acetate EDTA (TAE) buffer). Samples were mixed with DNA sample buffer (30% sucrose, 100 mM EDTA pH8, 0.05% bromophenol blue) before loading alongside Hyperladder I DNA marker (Bioline). Samples were run at 100 V for approximately 40 minutes and gels were stained with GelRed (Biotium) and visualised using GelDoc™ DNA analysis equipment (Bio-Rad).

2.2.2 PCR

USP12 was amplified from pDEST-FLAG-HA-USP12 using the following primers; forward primer: TCT AGA ATG GAA ATC CTA ATG ACA; reverse primer: GGA TCC TCA GTC CCG

AGA CTG ATA. The PCR reaction contained 1 nM of each primer, 1 x reaction buffer (containing $MgCl_2$), 0.2 mM dNTPs, 100 ng pDEST-FLAG-HA-USP12 and 1.5 units of *Taq* polymerase (Bioline) in a total volume of 10 µl. PCR reactions were performed on PCR express thermal cycler HBX110 (Thermo Hybaid).

2.2.3 Diagnostic digestion and vector ligation

The resultant USP12 amplicon was cut from an agarose gel, DNA extracted using the QIAquick spin gel extraction kit (as described by the manufacturer, Qiagen) and ligated into the pCR2.1 vector using the TA cloning kit according to the manufacturer's recommendations (Invitrogen). Briefly, ligation reactions were set up containing 25 ng of vector and approximately 10 ng of USP12 amplicon with 4 units of T4 DNA ligase in a total volume of 10 µl. Ligation reactions were incubated at 14^{o}C overnight and were transformed into competent *E.coli* as described in Section 2.2.4. Transformants were selected by blue/white selection on ampicillin and bromo-chloro-indolyl-galactopyranoside (X-gal)-containing LB agar plates.

Small-scale plasmid preparations (minipreps) of colonies containing potentially recombinant pCR2.1-USP12 plasmids were performed using the GenElute plasmid miniprep kit (as described by the manufacturer, Sigma-Aldrich) and resultant plasmid DNA digested with the *Xba* I and *Bam* HI restriction enzymes (Fermentas) for 1 hour at 37^{o}C. Released fragments were size-verified by agarose gel electrophoresis prior to sub-cloning into the p3xFLAG-CMV-10™ vector (Sigma-Aldrich) following the same ligation protocol. Recombinant p3XFLAG-CMV-10™-USP12 vectors were identified by diagnostic *Xba* I and *Bam* HI restriction digests and subsequently subject to large-scale plasmid preparation (maxiprep) using an endotoxin-free plasmid maxiprep kit (Qiagen). Resultant plasmids were sequenced by Cogenics (UK).

2.2.4 Bacterial transformation

Transformation of NEB 5-alpha competent *E.coli* (New England Biolabs Inc.) was routinely conducted for all ligation reactions and recombinant plasmid DNA in accordance with the manufacturer's recommendations. Briefly, 1 µl of plasmid DNA was incubated with 25 µl of competent bacterial cell on ice for 25 minutes. The bacteria were then subject to heat shock by incubation at 42^{o}C for 90 seconds and chilled on ice for a further 5 minutes. 950 µl of SOC media was subsequently added to each sample and incubated at 37^{o}C for 45 minutes prior to plating on relevant

antibiotic-containing LB agar plates and incubating at 37^{o}C overnight. For blue/white selection 40 µl of 40 mg/ml X-gal and 40 µl of 100 mM isopropyl-D-1-thiogalactopyranoside (IPTG) were added to antibiotic-containing LB agar plates prior to plating of bacteria.

2.2.5 Site directed mutagenesis

Site directed mutagenesis of p3XFLAG-CMV-10™-USP12 was performed using the Quikchange site-directed mutagenesis kit (Stratagene) to substitute one of the critical catalytic residues of USP12 to generate an inactive mutant enzyme. In accordance with the manufacturers protocol, a PCR reaction utilising a forward primer: GTC AAT TTT GGG AAT ACC GCC TAC TGC AAT TCA GTT CTT CAA GCA; and reverse primer: TGC TTG AAG AAC TGA ATT GCA GTA GGC GGT ATT CCC AAA ATT GAC was used to substitute the catalytic cysteine at position 48 to an alanine. Reactions contained 125 ng of each primer, 1 µl of dNTPs, 1 x reaction buffer and 2.5 units of *Pfu turbo* DNA polymerase in a total volume of 50 µl. PCR reactions were performed on the PCR express thermal cycler (Thermo Hybaid) and resultant PCR products treated with *Dpn* I restriction enzyme (Stratagene) for 1 hour at 37^{o}C to remove template DNA. Subsequently, products were transformed into XL1-blue supercompetent *E.coli* (Stratagene) following the protocol highlighted in Section 2.2.4.

2.3 Tissue culture

Mammalian tissue culture experiments were undertaken in a class II tissue culture laminar flow hood, adhering to aseptic technique. Tissue culture plastic-ware (flasks, multi-well plates etc.) and sterile plastic pipettes were purchased from Corning Life Sciences. All tissue culture media and reagents were purchased from Sigma-Aldrich.

2.3.1 Cell lines

All cell lines were originally obtained from American Type Culture Collection (ATCC), unless otherwise stated. Cell lines were grown and maintained in RPMI-1640 media (with HEPES modification) supplemented with 10% foetal calf serum (FCS) and 1% L-glutamine (20mM) (termed serum-containing media) at 37^{o}C in a humidified atmosphere of 5% CO_2.

The androgen sensitive LNCaP cell line (lymph node carcinoma of the prostate) was used as an androgen sensitive model of prostate cancer. This cell line was originally

derived from a supraclavicular lymph node metastasis of a 50 year old Caucasian patient with prostate cancer in 1977 (Horoszewicz *et al.*, 1980). A sub-clone of this cell line with an integrated pPSA luciferase (luc) reporter, LNCaP-7B7, was utilised in luciferase assays investigating the effects of siRNA knockdown on AR transcriptional activity and was a kind gift from Jan Trapman (Erasmus Medical Centre, The Netherlands). The AR negative cell line, PC-3 was derived from a bone marrow metastasis of a Caucasian patient with an advanced grade, poorly differentiated prostate cancer after androgen ablation therapy (Kaighn *et al.*, 1979).

For transfection studies, COS-7 and U2OS cells were utilised. COS-7 is a kidney fibroblast-like cell line from the African Green Monkey, *Cercopithecus aethiops*. This cell line has been transformed by expression of the SV40 T antigen. U2OS are a human bone sarcoma cell line derived from a 15 year old female patient in 1964 (Ponten and Saksela, 1967).

Where media depleted of androgens was required, dextran-coated charcoal (DCC)-treated FCS was used. DCC treatment removes endogenous androgens from the FCS. This medium is termed steroid-depleted medium.

Cells were tested for mycoplasma routinely in 1 month intervals at the Northern Institute for Cancer Research using the Mycoalert mycoplasma detection kit (Cambrex).

2.3.2 Cell Passaging

Cells were grown on T175 cm^2 culture flasks to approximately 70% confluency and passaged every 2-3 days. Briefly, culture media was removed from cells before washing in pre-warmed PBS. Trypsin was applied to detach cells for approximately 5 minutes at 37^oC and then neutralised by the addition of serum-containing media. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes before resuspension in the appropriate volume of media. Cell numbers were calculated using a haemocytometer.

2.3.3 Cryopreservation of cell lines

In order to maintain cell lines routine cryopreservation was performed. Cells were passaged as described above. 2-5 x 10^6 cells were resuspended in 1 ml of freezing media (80% serum-containing, 10% FCS, 10% dimethylsulfoxide (DMSO)). Stocks were then stored at -80^oC in cryovials.

Cryopreserved cells were re-cultured by defrosting cells at 37^{o}C and transferring them into 9 ml pre-warmed serum-containing media. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes before being resuspended in appropriate volume of serum-containing media to remove excess DMSO. Cells were then plated onto T25 cm^2 or T75 cm^2 flasks. After 24 hours, culture media was replaced in order to remove any dead cells.

2.4 Transient siRNA knockdown of mammalian cells

LNCaP and LNCaP-7B7 cells were subject to RNA interference using small interfering RNA (siRNA) by reverse transfection using Lipofectamine™ RNAiMax (Invitrogen) in accordance with the manufacturer's protocol. All siRNAs were used at a final concentration of 25 nM (Table 2.1). As a negative control an siRNA which does not affect gene expression within the cells was used, this was termed scrambled (SCR) siRNA (catalogue number 1022076, Qiagen).

Briefly, SCR, AR siRNA and siRNA against each DUB target were transferred into 500 µl of basal media (RPMI-1640 supplemented with 10% L-glutamine) and mixed with Lipofectamine™ RNAiMax prior to plating onto 6-well plates for 30 minutes equilibration. 1.5 x 10^5 cells were then seeded onto each well of the plates containing the transfection mixtures in 1.5 ml steroid-depleted media. Non-transfected (NT) controls were included containing only basal media and Lipofectamine™ RNAiMax. For cell cycle or apoptosis analyses, cells were subject to knockdown using the same protocol, but cells were seeded in serum-containing media.

For proliferation assays (Section 2.12), 2 x 10^3 LNCaP cells per well were subject to siRNA knockdown on a 96-well plate format. A final concentration of 25 nM siRNA was transfected in 20 µl basal media using 0.2 µl Lipofectamine™ RNAiMax. Cells were seeded in serum-containing media.

Target	siRNA sequence
SCR	UUCUCCGAACGUGUCACGUdTdT
AR	CCAUCUUUCUGAAUGUCCUdTdT
UCHL1 A	GGACAAGAAGUUAGUCCUAdTdT
UCHL1 B	GGCCAAUAAUCAAGACAAAdTdT
UCHL1 C	CAGACCAUUGGGAAUUCCUdTdT
USP10 B	GUUCUAAUGUGGAGGCGGAdTdT
USP12 A	GAAACUCUGUGCAGUGAAUdTdT
USP12 B	CAGAUCUCUUCCAUAGCAUdTdT
USP12 C	CAUCAGAUAUCUCAAAGAAdTdT
USP29 A	GAAAGAAGCUCUCAUUGAAdTdT
USP29 B	GAAUAACGAGCAAGUUUAUdTdT
USP29 C	GAAUUGCAGCUCUCCCUUAdTdT
USP50 A	CAUCGUAUGUUUAAAGUGUdTdT
USP50 B	GUAUAUGACCGCUCUGCAAdTdT
USP50 C	GAGAAGAUCAUUAUGAGAAAdTdT

Table 2.1. Sequence of siRNAs used to knockdown targets

2.5 Transient plasmid DNA transfection

For luciferase reporter assays, stated cell lines were seeded onto 24-well plates at a density of 8 x 10^3 cells per well. Cells were transfected with 50 ng pFLAG-AR, 100 ng pARE3-luc reporter, 100 ng pCMV-β-gal reporter and various amounts of p3XFLAG-CMV-10™-USP12 (25-100 ng), p3XFLAG-CMV-10™-USP12$_{C48A}$ (50 ng), pCMV-HA-USP10 (25-100 ng) and pCMV-Myc3-MDM2 (50-100 ng) as stated using Lipofectamine™ LTX (Invitrogen) or TransITR-LT1 (Mirus) in concordance with the manufacturers protocol. All transfections were balanced with empty pCMV5 vector.

Plasmids were aliquoted into 100 µl basal media and transfection reagent added at a ratio of 1µg DNA: 3 µl transfection reagent. Complexes were left to equilibrate at room temperature for 30 minutes and then added to cells in a total volume of 600 µl media (100 µl transfection mixture and 500 µl serum-containing media). Cells remained in transfection mix until media was changed to steroid-depleted media prior to DHT treatments.

For immunoprecipitation studies, 1 µg of pCDNA3-AR and 1 µg of p3XFLAG-CMV-10™-USP12 were transfected into 3 x 10^5 COS-7 cells on 90 mm dishes. Plasmids were aliquoted into 1 ml basal media and transfection reagent added in the ratio described above. Transfection mixtures were then added onto the cells in a total volume of 10 ml media (1 ml of transfection mixture and 9 ml serum-containing media).

2.6 Gene expression analysis

2.6.1 Isolation of RNA

All techniques were carried out under RNase-free conditions using filter tips (Axygen). Diethylpyrocarbonate (DEPC) treated water was made by adding 1 ml of DEPC to 1 litre of distilled water for 12 hours. The DEPC water was then autoclaved. All solutions for RNA work were made using DEPC water.

RNA was isolated from cells in a 6-well plate format using the EZ RNA extraction kit (Geneflow). Briefly, media was removed from cells and washed with PBS before 0.5 ml homogenisation solution was added. Following 5 minutes incubation at room temperature to ensure complete homogenisation, samples were transferred to eppendorf tubes and 0.5 ml extraction solution was added. Samples were vortexed and incubated at room temperature for 10 minutes before being centrifuged at 13000

rpm for 15 minutes at 4°C. The aqueous phase, containing the RNA, was collected into a separate eppendorf tube and precipitated by adding 0.5 ml propan-2-ol and incubating overnight at -20°C. The RNA pellet was collected by centrifugation at 7600 rpm for 8 minutes at 4°C. The pellet was washed in 75% ethanol before being dissolved in 20 µl DEPC water. RNA was stored at -80°C.

RNA concentration and purity was assessed using a Nanodrop ND-1000 UV-vis spectrophotometer (Thermo Scientific). The RNA integrity was assessed by the 260/280 ratio (the ratio of absorbance at 260 nm and 280 nm). Optimal 260/280 ratio for RNA is 2.0.

2.6.2 Reverse transcription

1 µg of isolated RNA was diluted in a total volume of 12.7 µl DEPC water and incubated at 65°C for 5 minutes before 2 minutes at 37°C to remove secondary structure. All reverse transcription reagents were purchased from Promega. An enzyme master mix containing 4 µl Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (RT) buffer, 2 µl 4mM dNTPs (dATP, dCTP, dGTP, dTTP), 1 µl 5µM oligo dT and 0.3 µl MMLV-RT enzyme per sample was prepared. 7.3 µl of master mix was added to each sample and incubated at 37°C for 1 hour. In order to inactivate the MMLV-RT enzyme, samples were placed at 95°C for 5 minutes. Resulting cDNA was stored at -20°C.

2.6.3 QRT-PCR

Expression of mRNA was analysed using quantitative real time PCR (QPCR). QPCR measures the accumulation of PCR products following each amplification cycle by detecting the amount of a fluorescent reporter. SyBr Green (Invitrogen) is a fluorescent reporter which intercalates with the DNA. Upon binding DNA the signal of the SyBr Green is enhanced and can be detected.

Real time PCR primers were designed using Primer Express 2.0 (Applied Biosystems; Table 2.2). These were used to analyse mRNA expression using the absolute quantification method on an ABI 7900 sequence detection system (Applied Biosystems). HPRT1 was used as a housekeeping gene to normalise expression levels and results were analysed using the SDS 2.2 software (Applied Biosystems).

	Forward primer sequence	Reverse primers sequence
HPRT1	TTGCTTTCCTTGGTCAGGCA	AGCTTGCGACCTTGACCATCT
UCHL1	CATACAGGCAGCCCATGATG	TCTACCCGACATTGGCCTTC
USP10	TTGAATATCCTGTGGACTTGGAAATTA	AACCCCTGGAGAAAGCAGTTC
USP12	ATGTACGACCTTGTTGCTGTTGTG	TCGATTGGGACCACTTCCAC
USP29	GTCAGCCATATCGGGAGCTC	CACATCGCTGATGTAATGGCC
USP50	AAAGGAAGCTGAGAACGGATATTC	TGAGGTCCAAGTTAGTGAGTGGGTA
AR	CATGTGGAAGCTGCAAGGTCT	TCTGTTTCCCTTCAGCGGC
PSA	TCGGCACAGCCTGTTTCAT	TGGCTGACCTGAAATACCTGG
KLK2	AGCATCGAACCAGAGGAGTTCT	TGGAGGCTCACACACTGAAGA
TMPRSS2	CTGCTGGATTTCCGGGTG	TTCTGAGGTCTTCCCTTTCTCCT
NKX3.1	AGCCAGAAAGGCACTTGGG	GGCGCCTGAAGTGTTTTCA
NDRG1	ACAACCCCCTCTTCAACTACG	GCCAATAATGCTTTTCAGCCCA
PSA ARE I (ChIP)	CCTAGATGAAGTCTCCATGAGCTACA	GGGAGGGAGAGCTAGCACTTG
PSA ARE III (ChIP)	GCCTGGATCTGAGAGAGATATCATC	ACACCTTTTTTTTCTGGATTG

Table 2.2. Sequence of QPCR primers used for mRNA expression analysis

2.7 Chromatin Immunoprecipitation (ChIP) analysis

ChIP was performed as described in Schmidt et al. Briefly, $2x10^6$ LNCaP cells were subject to transient transfection in steroid-depleted media using either SCR or USP12 siRNA for 72 hours prior to 10 nM DHT treatment for 120 minutes. Cells were fixed in 1% formaldehyde for 10 minutes and glycine treated for 5 minutes prior to cell scraping and centrifugation. Resultant pellets were washed in PBS and lysed in LB1 solution (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% Triton-X-100) for 10 minutes with gentle agitation at 4^oC. The lysate was centrifuged at 1500g for 5 minutes prior to lysis in 10 ml LB2 buffer (10 mM Tris-HCl, pH 8; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA) at 4^oC for 10 minutes with gentle agitation. Lysates were centrifuged as before and resultant pellets re-suspended in 0.5 ml LB3 buffer (10 mM Tris-HCl, pH 8; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% Na-Deoxycholate; 0.5% N-lauroylsarcosine). Samples were subject to a pre-optimised sonication programme using a Bioruptor™ (Diagenode) incorporating 12 cycles of 30 seconds on/30 seconds off at the 'Hi' setting and then centrifuged for 10 minutes at 13,000g. DNA concentration for each supernatant was measured using a Nanodrop spectrometer and 50 µg of chromatin was transferred into a fresh tube and diluted 5-fold in LB3 buffer containing 1% Triton-X-100. 10% of each solution was taken as input and frozen until the next day.

50 µl per sample of Dynabeads conjugated to Protein A (Invitrogen) was transferred into fresh tubes and blocked with 3 washes of PBS containing 0.5% bovine serum albumin (PBS-BSA) prior to incubation with 2 µg anti-AR or HA (control) antibodies overnight at 4^oC. Dynabeads were then washed twice with PBS-BSA to remove any unbound antibody and the 50 µg chromatin samples prepared the previous day were added to the appropriate Dynabead samples and mixed overnight at 4^oC. Dynabeads were subsequently washed 6 times in RIPA buffer (50 mM HEPES-KOH, pH 7.5; 500 mM LiCl; 1 mM EDTA; 1% NP-40; 0.7% Na-Deoxycholate) and once in Tris-buffered saline (20 mM Tris-HCl, pH 7.6; 150 mM NaCl) prior to incubation overnight in 200 µl elution buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA) at 65^oC to elute protein-DNA complexes from beads and to reverse formaldehyde-induced cross-links. In addition, input samples taken the previous day were defrosted and, like the immunoprecipitated samples, subject to elution/cross-link reversal. Samples were

diluted 1-fold in TE buffer and subject to proteolytic digestion using 4 µl 20 mg/ml proteinase K (Invitrogen) for 1 hour at 55^{o}C. DNA was purified using a GenElute™ genomic DNA miniprep kit (Sigma-Aldrich) and subject to quantitative PCR using primers specific to ARE I and ARE III regions of the *PSA* promoter/enhancer (shown in Table 2.2).

Data is presented as % Input using the following formula: % Input = 100 x 2^(CT Adjusted Input sample - CT immunoprecipitated sample) (CT refers to cycle threshold). Three individual repeats of the experiment were conducted and data combined to give an average % Input over the three experiments.

2.8 Protein expression analysis

2.8.1 SDS-polyacrylamide electrophoresis

Cell lysates were generated by adding sodium dodecyl sulphate (SDS) sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.005% bromophenol blue) to cells in culture which had been washed in PBS. Samples were then placed at 100^{o}C for 10 minutes to assist lysis and to denature the proteins.

All samples were separated on 10% gels, except for GST-tagged protein purification samples which were separated on 15% gels, prepared in Bio-Rad PROTEAN-III apparatus (Bio-Rad). Firstly a resolving gel was poured and allowed to polymerise before the stacking gel was overlaid and insertion of an appropriate sized well comb. Gels were made using the components shown in Table 2.3. Buffer A contained 375 nM Tris-HCL, pH8.8, 0.1% SDS whilst buffer B contained 125 nM Tris-HCL, pH6.8, 0.1% SDS. 15 µl of protein lysate was loaded per well and a molecular weight marker, Spectra™ Multicolour broad range protein ladder (Fermentas), was run alongside. Samples were electrophoresed at 180V for approximately 60 minutes in reservoir buffer (25 mM Tris-HCL, 190 mM glycine, 0.1% SDS).

Samples for Western blotting were transferred onto Hybond C nitrocellulose membrane (GE healthcare) between pieces of Whatman paper in a Bio-Rad mini-PROTEAN-III Western transfer cell. Transfer was performed overnight at 30 V at room temperature or for 1 hour at 100V at 4^{o}C, in transfer buffer (25 nM Tris-HCl, pH 8.3, 0.15 M glycine, 10% methanol).

Coomassie staining of polyacrylamide gels was performed for all GST protein induction and purification samples. Briefly, after electrophoresis the gels were washed in distilled water before being stained with colloidal Coomassie for approximately 1 hour. The gels were de-stained using distilled water for 2-3 hrs. Gels were visualised on the GelDoc™ system (Bio-Rad) before being dried onto Whatman paper using the model 583 gel dryer (Bio-Rad)

Gel		2 x Buffer A	2 x Buffer B	Water	30 % acrylamide	10% APS	TEMED
10%	Resolving	5 ml	X	1.67 ml	3.33 ml	100 µl	10 µl
	Stacking	X	5 ml	3.34 ml	1.66 ml	100 µl	10 µl
15%	Resolving	5 ml	X	X	5 ml	100 µl	10 µl
	Stacking	X	5 ml	2.5 ml	2.5 ml	100 µl	10 µl

Table 2.3. Contents of polyacrylamide gels used to separate cell lysates.

2.8.2 Western analysis

Western blotting was performed as previously described (Armstrong *et al.*, 2006). Briefly, after transfer, the membranes were placed in 5% blocking solution (5% milk (Marvel) or 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 20 mM Tris-HCL, 500 mM NaCl) for 1 hour. Membranes were then washed twice for 10 minutes in TBS-Tween (TTBS; TBS, 0.1% tween-20) and then incubated with primary antibody in an appropriate diluent (1% milk or 1% BSA in TTBS) for 1 hour at room temperature. Membranes were then washed twice for 5 minutes in TTBS and incubated with HRP-conjugated secondary antibodies (Dako) in diluents for a further 1 hour at room temperature. Unbound secondary antibody was removed by two washes in TTBS and one wash in TBS for 10 minutes each. Enhanced chemiluminescence (ECL; GE Healthcare) was used to detect HRP-conjugated secondary antibodies and signals were visualised by exposure to X-ray film (Kodak). Antibodies used within the study are described in Table 2.4.

Where densitometry analysis was required Western blots were scanned and analysed using the Quantity one program on the GelDoc™ system (Bio-Rad).

Antibody	Host	Ig Type	Manufacturer	Catalogue number	Western dilution
α-tubulin	Mouse	Mono	Sigma-Aldrich	T9026	1:2000
AR	Mouse	Mono	BD Biosciences	554225	1:1000
AR (C-19)	Rabbit	Poly	Santa Cruz	sc-815	1:500
Flag (M2)	Mouse	Mono	Sigma-Aldrich	F3165	1:2000
GST	Goat	Poly	GE Healthcare	27-4577-01	1:2000
PARP 1/2 (H-250)	Rabbit	Poly	Santa Cruz	sc-7150	1:2000
PARP, Cleaved (Asp214)	Rabbit	Poly	Cell Signalling	9541	1:1000
PSA (C-19)	Goat	Poly	Santa Cruz	sc-7638	1:500
Ubiquitin, K48-specific	Rabbit	Mono	Millipore	05-1307	1:500
Ubiquitin, K63-specific	Rabbit	Mono	Millipore	05-1308	1:500
USP10	Rabbit	Poly	Abcam	ab72486	1:500
USP12	Mouse	Poly	Abcam	ab89870	1:200
USP12	Rabbit	Poly	Sigma-Aldrich	SAB2103646	1:200
USP12 (T-12)	Rabbit	Poly	Santa Cruz	sc-82072	1:200
USP12 (Y-13)	Rabbit	Poly	Santa Cruz	sc-82073	1:200
Rabbit anti-mouse HRP	Rabbit	Poly	Dako	P0260	1:1000
Swine anti-rabbit HRP	Swine	Poly	Dako	P0217	1:1000
Rabbit anti-goat HRP	Rabbit	Poly	Dako	P0160	1:1000

Table 2.4. Details of antibodies used in Western blotting studies.

2.9 Immunoprecipitation (IP)

Immunoprecipitation was performed to examine interactions between proteins of interest in mammalian cells. Briefly, transfected COS-7 cells or LNCaP cells were grown to approximately 60% confluency on 90 mm dishes in serum-containing media prior to collection by scraping into PBS. Cells were pelleted by centrifugation and resuspended in 1.5 ml IP lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 1% NP-40 alternative, 1 mM Na_3VO_4, 1 Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche)). Cells were incubated at 4^{o}C with gentle agitation for 30 minutes.

Following lysis, samples were centrifuged for 3 minutes at 13000 rpm to pellet cell debris. Resultant supernatants were then separated into a 100 µl input, 1 ml sample and 400 µl extract. The input sample was stored at -20^{o}C until after the IP procedure was complete and was used to examine expression levels of proteins of interest in the whole cell extract (WCE). Samples and extracts were precleared by incubation with Protein G Sepharose (PGS; GE Healthcare) for 4 hours at 4^{o}C with gentle agitation and centrifuged for 3 minutes at 13000 rpm to remove PGS. 2 µg of specific antibody was added to each of the sample tubes, but excluded from the extracts, and incubated overnight at 4^{o}C with agitation. An additional antibody-only control containing lysis buffer and antibody was also incubated overnight at 4^{o}C. The antibodies used in the IP studies are shown in Table 2.5.

An appropriate amount of PGS was added to samples, extracts and the antibody only control, following incubation with antibody, for a further 1 hour at 4^{o}C. The PGS beads were then collected by centrifugation for 3 minutes at 13000 rpm and washed with IP wash buffer A (PBS, 350 mM NaCl, 0.2% Triton) followed by two washes with IP wash buffer B (PBS, 0.2% Triton). All inputs, samples, extracts and antibody-only control were resuspended in SDS sample buffer and analysed by SDS-PAGE and Western blotting.

Antibody	Host	Ig Type	Manufacturer	Catalogue number
AR (C-19)	Rabbit	Poly	Santa Cruz	sc-815
FLAG (M2)	Mouse	Mono	Sigma-Aldrich	F3165
USP10	Rabbit	Poly	Abcam	ab72486

Table 2.5. Details of antibodies used in Immunoprecipitation studies.

2.10 Cytoplasmic/nuclear extraction

Cytoplasmic/nuclear extracts were generated using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's recommendations. Briefly, cells were harvested by trypsinisation and pelleted by centrifugation at 2000 rpm for 5 minutes. Cell pellets were washed twice in PBS before being resuspended in 60 µl of cold CER I buffer. Resuspension was assisted by vortexing samples for 15 seconds and incubating on ice for 10 minutes. 3.3 µl cold CER II buffer was then added and samples were vortexed twice for 5 seconds, with a minute incubation on ice between vortexing. The cytoplasmic fraction was collected by centrifugation of samples at 13000 rpm for 5 minutes and transferring the cytoplasmic fraction (supernatant) into chilled eppendorf tubes. The insoluble nuclear pellet was resuspended in 30 µl of cold NER buffer, vortexed for 15 seconds and incubated on ice for a total of 40 minutes. The samples were vortexed every 10 minutes for 15 seconds during this incubation. Finally, nuclear fractions were collected by centrifugation of samples at 13000 rpm for 10 minutes and the nuclear supernatant transferred into chilled eppendorf tubes. Both cytoplasmic and nuclear samples were resuspended in SDS sample buffer and analysed by SDS-PAGE and Western blotting.

2.11 Luciferase reporter assays

Cells grown on 24-well plates and subject to siRNA knockdown or plasmid DNA transfection were washed twice in PBS and then lysed in 50 µl reporter lysis buffer (Promega) at 37^{o}C for 10 minutes.

Luciferase assays were performed using the Promega luciferase assay system (Promega). Briefly, 10 µl of cell lysate was transferred into wells of an opaque, flat-bottomed 96-well plate. 50 µl of luciferase assay reagent (Promega) was added and luminescence read immediately on the Micro Beta Plus liquid scintillation and luminescence counter (PerkinElmer).

β-galactosidase (β-gal) assays were performed in order to normalise the luciferase activity data to transfection efficiency in each sample. 10 µl of cell lysate was transferred to a 96-well plate before 10 µl of β-gal reagent (2 mM MgCl2, 100 mM β-mercaptoethanol, 1.33 mg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and 100 mM sodium phosphate buffer, pH 7.5) added and incubated at 37^{o}C for 10 minutes. The reaction, which results in the colorimetric conversion of ONPG from

colourless to a yellow colour, was terminated by addition of 50 µl $NaHCO_3$. Optical density, which is proportional to the activity of the β-gal enzyme, was assessed by reading plates on the Bio-Rad 680 microplate reader using the 415 nm filter.

For luciferase assays involving LNCaP-7B7 cells, a bicinchoninic acid (BCA) assay (Pierce) was used to normalise the luciferase activity readouts to total cellular protein, which is equivalent to a reading of approximate cell numbers. BCA solution A was added to BCA solution B at a ratio of 50:1 and 10 µl of the mixture was added to 10 µl of cell lysate on a 96-well plate. Samples were incubated for 30 minutes at 37^{o}C before reading on the plate reader using the 570 nm filter.

Luciferase assay results were normalised against either β-gal or BCA assay readouts. As each luciferase experiment was performed using triplicate samples the average and standard deviation of each triplicate was calculated. Three independent repeats of each experiment were performed and combined, unless otherwise stated, and standard error of the mean calculated for each data-set.

2.12 Sulphorhodamine B cell proliferation/viability assay

Cellular proliferation/viability was determined by sulphorhodamine B (SRB) assay based on a published protocol by Skehan *et al.* SRB dye stains cellular proteins and is indicative of the cell numbers. 2 x 10^3 LNCaP cells seeded onto 96-well plates were subject to siRNA knockdown by reverse transfection. 96 hours post-knockdown, cells were fixed with cold trichloroacetic acid (TCA) at a final concentration of 10% for 1 hour at 4^{o}C. Cells were washed with water five times to remove residual TCA and growth media. Plates were dried at 37^{o}C for 1 hour before addition of 100 µl SRB dye (0.4% SRB in 1% acetic acid) for 30 minutes at room temperature. Cells were washed five times in 1% acetic acid and air dried. Dye was then solubilised in 10 mM Tris, pH 10.5 for 30 minutes with gentle agitation. Optical density was measured using the plate reader and the 570 nm filter.

2.13 Flow cytometry

2.13.1 Cell cycle analysis

LNCaP cells were subject to knockdown in 6-well plates in serum-containing media for 48 or 96 hours. Non-adherent cells (potentially apoptotic) contained within the growth media were collected into tubes before the adherent cells were trypsinised, collected

and added to the non-adherent population. Cell pellets were collected by centrifugation at 2000 rpm for 5 minutes and washed twice in PBS. Cells were subsequently permeabilised using 5% Triton X-100, stained with 2.5 mg/ml propidium iodide (PI) and treated with 100 µg/ml RNase to ensure DNA staining rather than RNA staining. Samples were analysed on the FACScan (BD Biosciences) At least $1x10^4$ cells were evaluated for each sample.

2.13.2 Apoptosis detection: cleaved caspase-3

Apoptosis in response to siRNA knockdown was measured using the FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences). LNCaP cells were subject to knockdown in 6-well plates in serum-containing media and after 48 hours, cells were treated with 200 nM doxorubicin or vehicle control for a further 48 hours. Both adherent and non-adherent cells were harvested and pelleted. Cell pellets were washed twice in PBS before fixation with cytofix/cytoperm™ for 20 minutes on ice. Cells were the washed twice with perm/wash™ and stained with FITC-conjugated anti-activate caspase-3 antibody for 30 minutes in the dark. Cells were washed and resuspended in perm/wash™ before analysis on the FACScan. At least $1x10^4$ cells were evaluated for each sample. A cells only control without antibody staining was included as a negative control in this experiment.

2.13.3 Flow cytometry data analysis

CellQuest software was used for data acquisition and data analysis performed on the WINMDI and Cychlred software programs. Cells were gated on Forward Scatter vs. Side Scatter to exclude cell debris and doublet discrimination was carried out on the FL2-Width vs. FL2-Area dot-blot to ensure only single cells were examined for cell cycle.

Chapter 3: Identification and validation of deubiquitinase enzymes involved in androgen receptor regulation

3.1 Introduction

Androgen receptor (AR) is the key target of current prostate cancer (CaP) therapies. In response to DHT binding the cytoplasmic, inactive AR translocates to the nucleus where it stimulates the transcription of androgen-regulated genes to promote proliferation and survival (Gelmann, 2002). Use of androgen ablation therapy (AAT), which functions by blocking androgen production or competitive inhibition of AR, suppresses the action of the AR thereby reducing pro-proliferative effects on prostate cells (Petrylak, 2005a). Targeting the AR is initially successful and results in gross tumour shrinkage. Unfortunately, in the vast majority of cases, patients relapse after a median time period of 18 months after commencing neo-adjuvant therapy, and develop tumours that have become androgen-independent, termed castrate-resistant CaP (CRCaP) (Feldman and Feldman, 2001). Importantly, AR is expressed, is active and remains important to the survival and progression of these malignancies (Feldman and Feldman, 2001). Many pathways resulting in androgen-independence have been identified, including mutations to the AR itself that lead to aberrant activation of activity in minimal or absent circulating androgens (Feldman and Feldman, 2001). The T877A mutation of AR, a threonine to alanine substitution at position 877, is one of the most commonly identified mutations in patients (Veldscholte *et al.*, 1992). This mutation has been shown to alter the ligand binding pocket of AR, therefore allowing activity to be stimulated by other steroid hormones, including estrogens and progesterone. Furthermore, the anti-androgenic agent flutamide becomes an agonist of this variant AR (Sack *et al.*, 2001).

An additional mechanism of AR activation in CRCaP that has received substantial interest in the past decade is the process by which aberrant expression of co-regulatory proteins can facilitate receptor-mediated transcription and drive cancer progression. Indeed numerous studies have shown that AR co-regulators, including TIP60, p68 and AIB1 are up-regulated in high grade CaP, suggesting that they may drive AR function during progression to CRCaP (Culig *et al.*, 1994; Clark *et al.*, 2008). The p53 induced protein with a RING H2 domain (PIRH2), is an E3 ligase which also acts as a co-activator of the AR. PIRH2 interacts with TIP60 leading to the degradation of histone deacetylase 1 (HDAC1) that in turn increases AR transcriptional activity (Logan *et al.*,

2006). There is a positive correlation between PIRH2 expression and grade of CaP tumour, with high grade disease expressing more PIRH2 protein (Logan *et al.*, 2006).

Ubiquitination is the covalent modification of a substrate protein through attachment of a 76 amino acid protein called ubiquitin via a multi-step cascade (Pickart and Eddins, 2004). The first step in the cascade is activation of the ubiquitin molecule by the E1 activating enzymes UBE1 and UBE6 (Groettrup *et al.*, 2008), followed by transfer of the ubiquitin to the E2 conjugating enzyme, of which there are approximately 30 members of this family (van Wijk and Timmers, 2010). Finally, ubiquitin is transferred by an E3 ubiquitin ligase enzyme to the target lysine within the substrate via the formation of an isopeptide bind between the C-terminal glycine residue of the ubiquitin protein and the ε-amino group of the substrate lysine, as described in Chapter 1.3 (Pickart and Eddins, 2004). Ubiquitination is a tightly regulated process in the normal cell which has been shown to be disrupted in many disease states including cancer. For example, mutations of the E3 ligase BRCA1 that disrupt catalytic activity have been associated with loss of BRCA1 tumour suppressor function (Ruffner *et al.*, 2001) and predispose to breast, ovarian and prostate cancers (Futreal *et al.*, 1994; Kirchhoff *et al.*, 2004; Turner *et al.*, 2007).

In addition to PIRH2, a number of other E3 ligases interact with the AR including C-terminal HSP-interacting protein (CHIP), murine double minute (MDM2) and E6 associated protein (E6-AP). MDM2, a negative regulator of p53, is over-expressed in a large proportion of prostate tumours and has been found to restore sensitivity of the LNCaP cell line to AAT (Mu *et al.*, 2008). MDM2 ubiquitinates AR after it becomes phosphorylated by AKT resulting in the proteasomal degradation of AR (Lin *et al.*, 2002b). Subsequently, Gaughan *et al*., found that MDM2 cooperates with HDAC1 to co-repress AR activity and drive AR degradation (Gaughan *et al.*, 2005). CHIP binds AR after phosphorylation of the hinge region, inhibiting AR folding and leading to degradation of AR (Cardozo *et al.*, 2003; Rees *et al.*, 2006). Conversely, PIRH2 and E6-AP act as co-activators of AR activity. E6-AP is recruited to the active PSA promoter and enhances AR-mediated transcription (Khan *et al.*, 2006). Interestingly, AR co-activation appears to be coupled with AR turnover as the same report demonstrated that E6-AP depletion enhances AR protein levels (Khan *et al.*, 2006).

Deubiquitinase enzymes (DUBs) negatively regulate ubiquitination by removing mono-ubiquitin and ubiquitin chains from substrates. Amongst other functions, DUBs allow for the normal rate of protein turnover and maintain the pools of available ubiquitin by processing ubiquitin precursors and recycling ubiquitin from proteins targeted for proteasomal degradation (Amerik and Hochstrasser, 2004). Five subfamilies of enzymes have been identified based on sequence homology and catalytic mechanism (Reyes-Turcu *et al.*, 2009). The first four classes are cysteine hydrolases called ubiquitin-specific processing proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour related proteases (OTU-related) and Josephine domain DUBs (Amerik and Hochstrasser, 2004). These four subfamilies differ in sequence and structure but do share mechanistic similarities. They contain similar cysteine and histidine rich motifs which are important for active site function (Wilkinson, 1997). Interestingly, the Josephin domain DUB family contains only one characterised member, ataxin-3 which was initially identified as a DUB by it binding to ubiquitin aldehyde, a DUB inhibitor (Burnett *et al.*, 2003). The fifth subfamily is the zinc-dependent metalloproteases containing the MPN+ domain. The MPN+ domain contains two histidine residues and an aspartate residue that bind a zinc ion, important for function (Maytal-Kivity *et al.*, 2002).

A number of DUBs have been reported to influence AR activity and prostate cancer. Prostate tumours expressing high levels of USP2a also express high levels of MDM2, while, reciprocally, low USP2a expression correlates with high expression of p53 target genes and pro-apoptotic genes (Priolo *et al.*, 2006). USP2a therefore has a role in protecting CaP cells from apoptosis by increasing p53 degradation through MDM2 stabilisation (Priolo *et al.*, 2006). Another DUB, USP10, was identified in purified nuclear extracts of AR-null PC-3 prostate cancer cells in complex with ectopically expressed AR bound to DNA. Over-expression of USP10 stimulates ectopically expressed AR activity by increasing its binding to both selective and non-selective response elements. The authors of this study hypothesise that this DUB edits ubiquitin chains modifying them from proteasome-targeting poly ubiquitin chains to mono or tri-ubiquitin chains that can be transcriptionally activating (Faus *et al.*, 2005). Additionally, USP10 deubiquitinates histone H2A.Z allowing AR regulated genes to be

transcribed (Draker *et al.*, 2011) However, as a whole this class of enzymes are not widely studied in AR signalling.

3.2 Aims

The interplay between DUB enzymes and AR signalling has not been fully explored. Only recently a small number of DUB enzymes have been identified as playing a role in this signalling pathway. To investigate the full impact of this class of enzymes on AR function, an unbiased siRNA library screening approach was utilised.

The specific aims of this Chapter are:-

- To identify DUB enzymes involved in regulating AR transcriptional activity
- To validate these potential 'hits' for both level of knockdown at the mRNA and protein level
- To validate the effect of target knockdown on *PSA* mRNA and protein expression
- To investigate the role of the identified DUBs on additional AR-target genes

3.3 Results

3.3.1 Identification of deubiquitinase enzymes that modulate AR function in LNCaP prostate cancer cells

Currently, USP10 and USP26 are the only reported DUB enzymes that have been directly linked to the AR signalling cascade (Faus *et al.*, 2005; Dirac and Bernards, 2010). In order to address whether any additional DUB enzymes are involved in AR regulation, an unbiased siRNA library screen was utilised targeting 72 DUB enzymes in the androgen-dependent LNCaP CaP cell line. This covers 75% of the currently known DUBs, those without catalytic activity or with only partial sequences were excluded from the investigation. Secretion of the AR regulated protein prostate specific antigen (PSA), using a commercially available PSA ELISA was used as a primary read-out for AR transcriptional activity. A schematic of the experimental design of the whole screening process is illustrated in Figure 3.1.

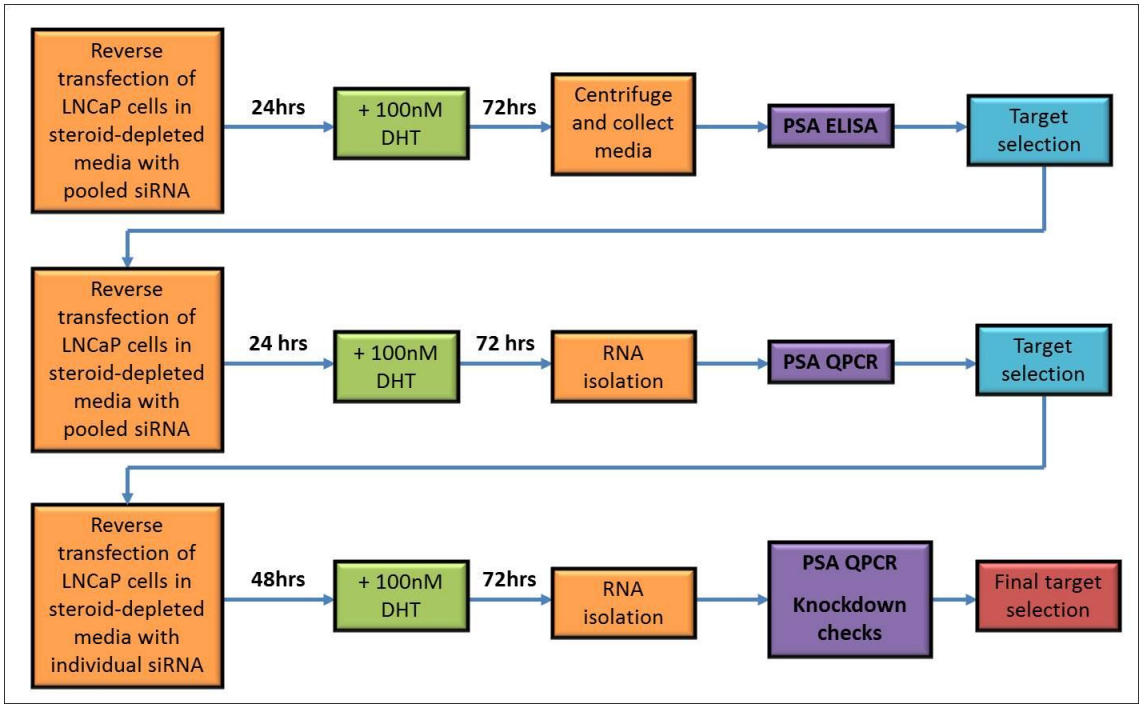

Figure 3.1. Schematic of experimental design of the siRNA screen.

LNCaP cells were subject to knockdown of DUB targets before 100 nM DHT treatment. ELISA measuring prostate specific antigen (PSA) secretion was the primary screen output. Targets selected from the ELISA were further examined for PSA expression. Any targets which consistently affected PSA secretion and expression were further validated before final target selection.

Each DUB was targeted with a pool of three individual siRNA oligonucleotides designed against different regions of target mRNA using the Rosetta Algorithm (Sigma-Aldrich) to ensure robust target knockdown and reduced off-target effects. Messenger RNAs of each target gene were knocked down in LNCaP cells in steroid-depleted media on a 96 well plate format. After 24 hours knockdown, the cells were treated with 100 nM DHT for a further 72 hours. An siRNA oligonucleotide specifically designed not to cause knockdown of any cellular target, known as scrambled (SCR), was used as a negative control. Given PSA was the main read-out for this experiment, siRNA targeting *AR* and the *PSA* mRNA were used as positive controls as both would attenuate *PSA* expression.

Figure 3.2 shows the results of four independent PSA ELISA experiments. The data is represented as fold changed compared to the SCR control which has been set to a value of 1. As expected, there was a wide variety of effects on *PSA* expression; knockdown of some targets resulted in increased PSA secretion whilst others decreased PSA secretion. Potential targets for the next round of screening were selected based on 50% PSA modulation, i.e. those with 1.5-fold up-regulation or 50% down-regulation in PSA secretion. The red stars indicate those targets which were taken forward for QPCR analysis. This work was performed by Dr Steven Darby, Northern Institute for Cancer Research, Newcastle University.

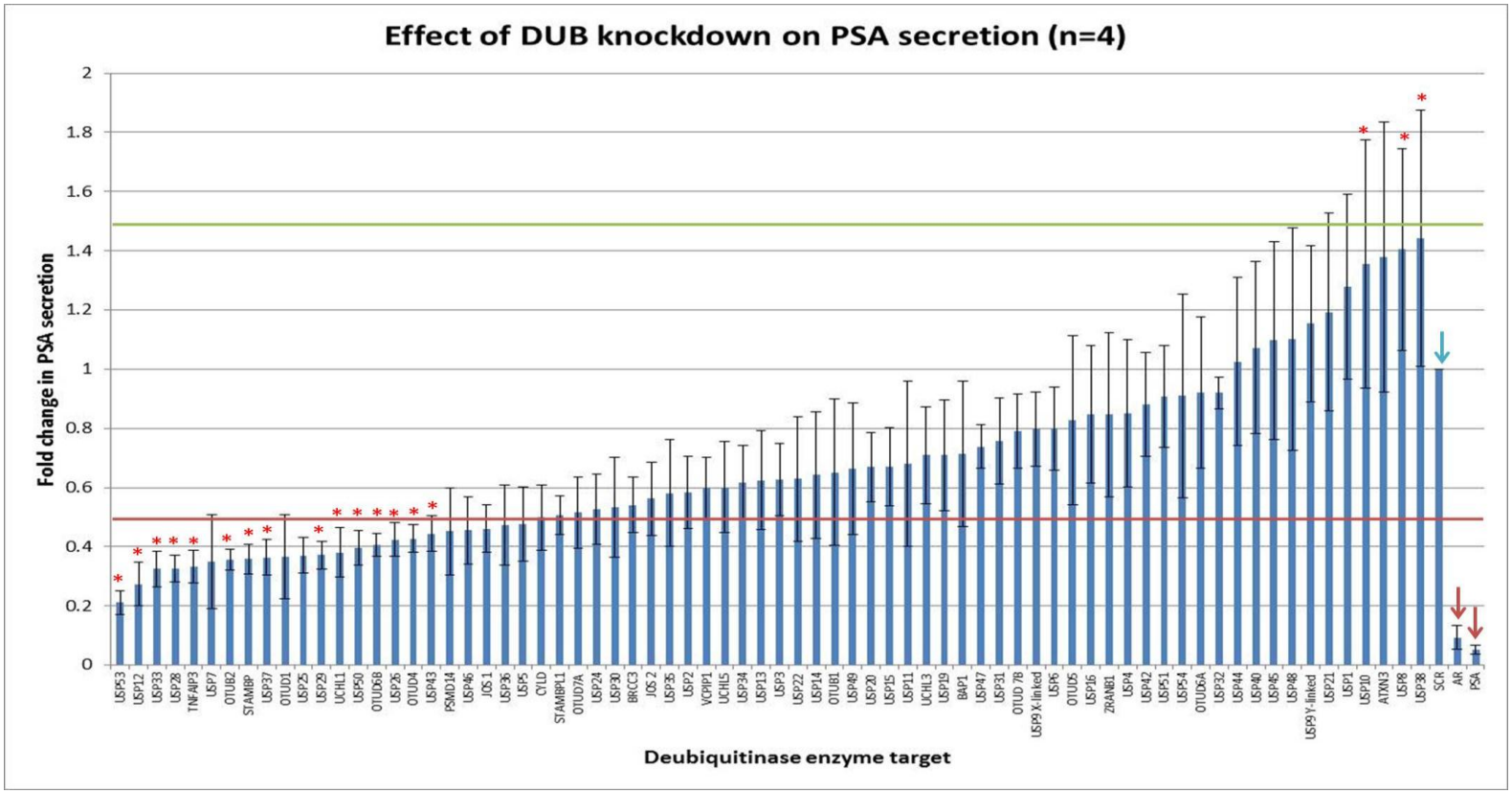

Figure 3.2. Effect of DUB knockdown on PSA secretion as measured by ELISA.

Deubiquitinase enzyme (DUB) targets were depleted in LNCaP cells for 24 hours before DHT was applied to cells for a further 72 hours. Secretion of prostate specific antigen (PSA) protein was measured by ELISA. Data is shown as fold change compared to the scrambled siRNA (SCR) control, represented by the blue arrow. SCR siRNA which does not affect gene expression was used as a negative control. Androgen receptor (AR) and PSA siRNA knockdown were used as positive controls, represented by red arrows. The red and green line represent a level of PSA secretion -/+ 50% of SCR respectively. Targets selected for further investigation are indicated by red stars. Data represents the mean of four independent repeats and error bars show the standard error. This work was performed by Dr Steven Darby.

To elucidate whether the effect of DUB knockdown was a result of a reduction in *PSA* transcription or due to an effect on PSA secretion, *PSA* expression was evaluated by QPCR. Each DUB selected from the ELISA was depleted as described for the initial screen. Figure 3.3 shows the results of four independent QPCR analyses of *PSA* mRNA expression for the eighteen selected targets from the PSA ELISA. Data is again represented as fold changed compared to the SCR control.

To aid selection of targets for further validation, ELISA and QPCR data for each DUB knockdown were compared. Some targets, for example, showed a reduction in PSA secretion at the protein level but did not show reduction in *PSA* mRNA expression, suggesting that these DUBs have a role in the secretion of PSA only. Targets which affected both PSA secretion and expression were thought to represent DUBs that potentially affected AR regulated transcriptional events and therefore only these targets were selected. The results of the PSA ELISA and QPCR experiments are tabulated in Table 3.1. The eight targets that consistently influenced PSA in both the ELISA and QPCR were selected for further validation (represented by highlighted targets in Table 3.1). This work was performed by Dr Steven Darby.

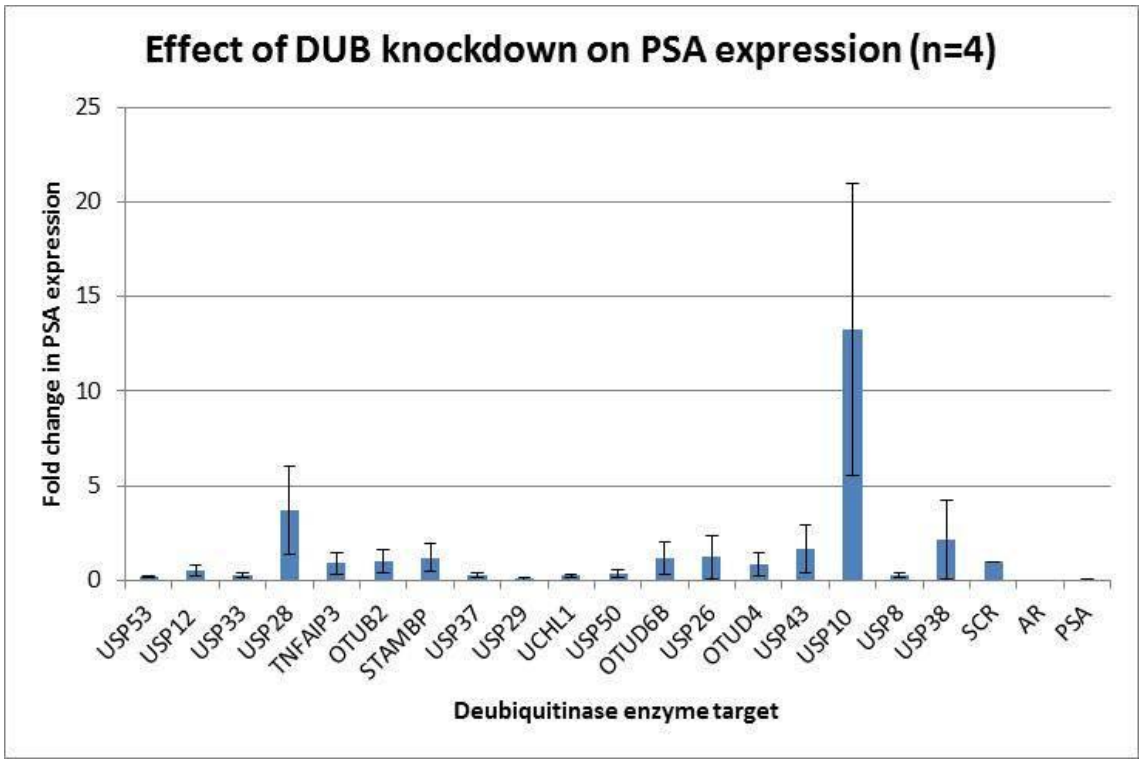

Figure 3.3. Effect of DUB knockdown on PSA mRNA expression.

Deubiquitinase enzyme (DUB) targets were depleted in LNCaP cells for 24 hours before DHT was applied to cells for a further 72 hours. RNA was collected for expression analysis. Data is shown as fold change compared to the scrambled siRNA (SCR) and is the mean of four independent repeats. SCR siRNA which does not affect gene expression was used as a negative control. Androgen receptor (AR) and prostate specific antigen (PSA) siRNA were used as positive controls. Error bars show standard error. This work was performed by Dr Steven Darby.

	Fold expression of PSA	
Targets	ELISA	QPCR
SCR	1.00 ± 0.00	1.00 ± 0.00
AR	0.09 ± 0.04	0.01 ± 0.00
PSA	0.05 ± 0.01	0.03 ± 0.02
USP53	0.21 ± 0.04	0.21 ± 0.06
USP12	0.27 ± 0.07	0.53 ± 0.26
USP33	0.32 ± 0.06	0.28 ± 0.14
USP28	0.33 ± 0.04	3.71 ± 2.32
TNFAIP3	0.33 ± 0.06	0.94 ± 0.57
OTUB2	0.36 ± 0.04	1.00 ± 0.63
STAMBP	0.36 ± 0.05	1.22 ± 0.75
USP37	0.36 ± 0.06	0.28 ± 0.09
USP29	0.37 ± 0.05	0.09 ± 0.06
UCHL1	0.38 ± 0.08	0.25 ± 0.11
USP50	0.39 ± 0.06	0.37 ± 0.22
OTUD6B	0.41 ± 0.04	1.16 ± 0.86
USP26	0.42 ± 0.06	1.23 ± 1.15
OTUD4	0.43 ± 0.05	0.86 ± 0.63
USP43	0.44 ± 0.06	1.71 ± 1.27
USP10	1.35 ± 0.42	13.25 ± 7.69
USP8	1.40 ± 0.34	0.29 ± 0.11
USP38	1.44 ± 0.43	2.17 ± 2.09

Table 3.1. Comparison of the results of DUB knockdown on PSA secretion and expression from the PSA ELISA and QPCR experiments.

All results are shown as fold change compared to the scrambled siRNA (SCR). Data represents and the mean of four independent repeats ± standard error. Targets highlighted in red are those which were selected for further validation.

3.3.2 Validation of potential targets

The first two rounds of screening identified eight targets which modulate *PSA* expression by at least 50%. Four of these targets were validated by Dr Steven Darby, USP10, USP33, USP37 and USP53, while I validated the remaining four targets which were UCHL1, USP12, USP29 and USP50.

In the initial screening, each DUB target was knocked down with a pool of three individual siRNA oligos and investigated only for the effect on *PSA* expression and secretion. Importantly, knockdown of each DUB had not been verified in these early experiments and thus it was unknown whether the effects of the siRNAs on *PSA* expression were genuinely via DUB enzyme depletion or an off-target phenomenon. In addition, use of a pool of multiple siRNAs directed against one target has been reported to increase the likelihood of knockdown, although it has also been suggested that the effects of a less efficient siRNA may attenuate the effects of more potent oligonucleotides and vice versa (Koller *et al.*, 2006; Dharmacon, 2011). Therefore, to validate depletion of the selected targets, individual and pooled siRNAs for each DUB were assessed for knockdown and investigated that their effects correlated with changes to *PSA* expression.

An additional control was also introduced into these validation experiments. Previous experiments used only the SCR siRNA as a base-line for effects on *PSA*. It is not known whether introduction of our control SCR siRNA itself may have had an effect on PSA secretion and/or expression. In order to address this, a non-transfected (NT) control was introduced which comprised of cells that had been subjected to the same procedures as the SCR control but without the addition of siRNA. As with previous experiments, *AR* knockdown was used as a positive control. Figure 3.4 shows the effect of the NT and SCR controls alongside *AR* knockdown on both *AR* and *PSA* mRNA expression. Firstly, it is shown that the NT and SCR control arms show no significant difference in either *AR* or *PSA* expression (Figure 3.4). As expected, transfection of AR siRNA caused almost complete attenuation of *AR* mRNA expression (Figure 3.4a) and reduced *PSA* expression by approximately 60-fold (Figure 3.4b).

For the subsequent validation experiments, targets were knocked-down in LNCaP cells, with each individual siRNA oligo or the pooled siRNA, in steroid depleted media on a 6-well plate format. 48 hours post-knockdown, cells were then treated with DHT for a

further 48 hours. QPCR analysis was performed to check target knockdown and the effect on *PSA* expression. Results are from a minimum of three independent repeats and are represented as fold change compared to the SCR control.

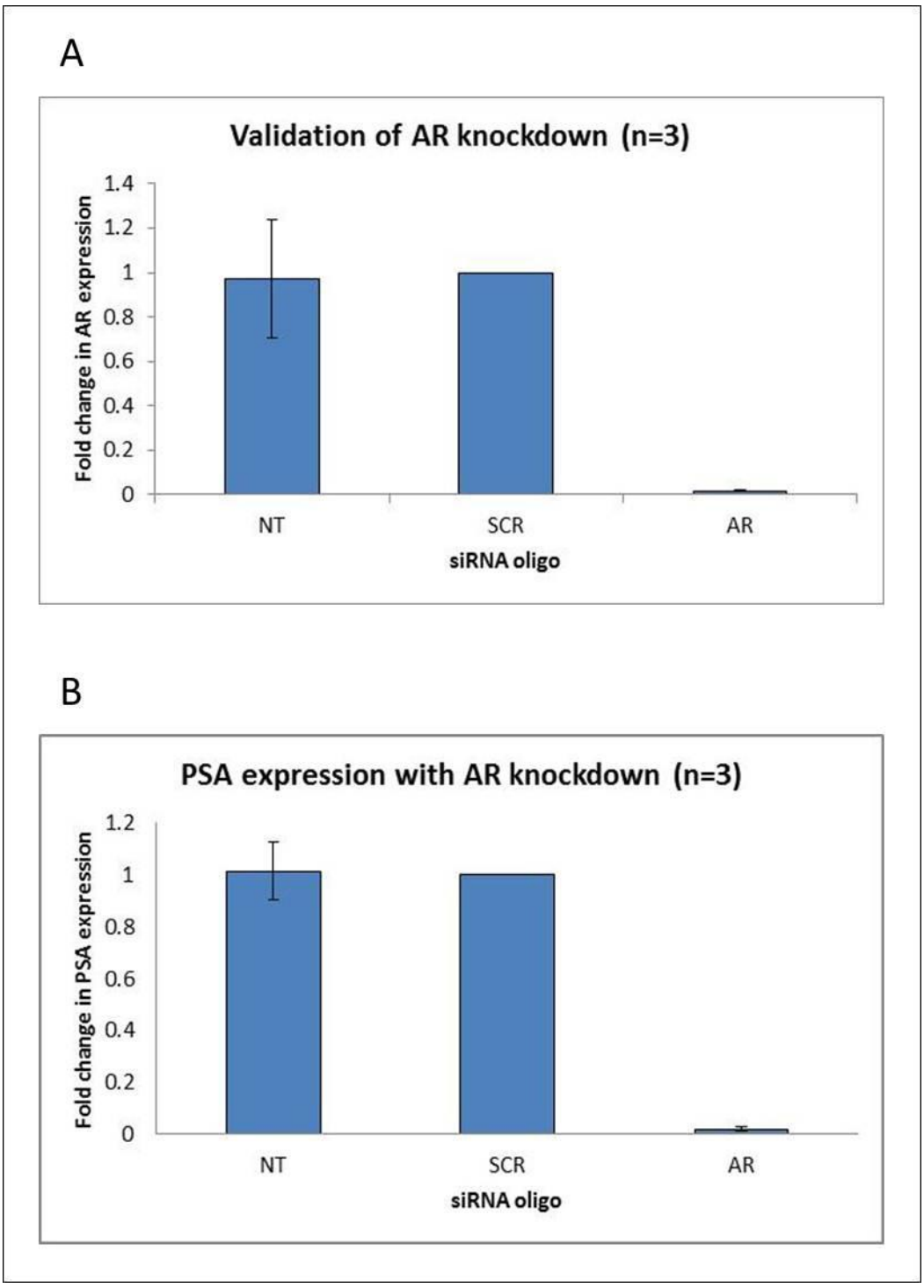

Figure 3.4. Effect of knockdown on AR and PSA mRNA expression.

Androgen receptor (AR) was depleted in LNCaP cells for 48 hours before DHT was applied to cells for a further 48 hours. RNA was collected for expression analysis. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Data is presented as fold change compared to the SCR control. Data is representative of the mean of three repeats and error bars show the standard error. A) The effect of AR knockdown on AR expression. No significant difference was observed between NT and SCR samples. B) The effect of AR knockdown on Prostate specific antigen (PSA) expression. No significant difference was observed between NT and SCR samples.

3.3.2.1 UCHL1

Figure 3.5 shows the results for both the knockdown and *PSA* expression analysis for *UCHL1*. In the PSA ELISA and initial QPCR analysis UCHL1 showed 60% and 70% reduction in PSA secretion and expression, respectively (Figure 3.2; Figure 3.3). Firstly, it is important to note that no significant difference is seen in the expression of *UCHL1* or *PSA* between the NT and SCR controls (Figure 3.5a and b). siRNA oligo A knocked-down *UCHL1* by around 2-fold (Figure 3.5a), but increased *PSA* expression by 1.8-fold (Figure 3.5b). siRNA B had no effect on expression of *UCHL1*, giving comparable levels to the SCR control (Figure 3.5a), but resulted in a 1.3-fold reduction in *PSA* (Figure 3.5b). siRNA oligo C and the pooled siRNA both caused an increase in *UCHL1* expression (3 and 1.5-fold, respectively; Figure 3.5a). However, oligo C caused a 1.3-fold increase in *PSA* while unexpectedly, the pooled siRNA reduced expression by 1.3-fold (Figure 3.5b). The level of *PSA* mRNA reduction seen in these validations with the pooled siRNA is much less than in the ELISA and initial QPCR. However, it is fair to conclude that any effect seen on PSA in the previous screening steps was not a result of *UCHL1* knockdown but likely an off-target effect.

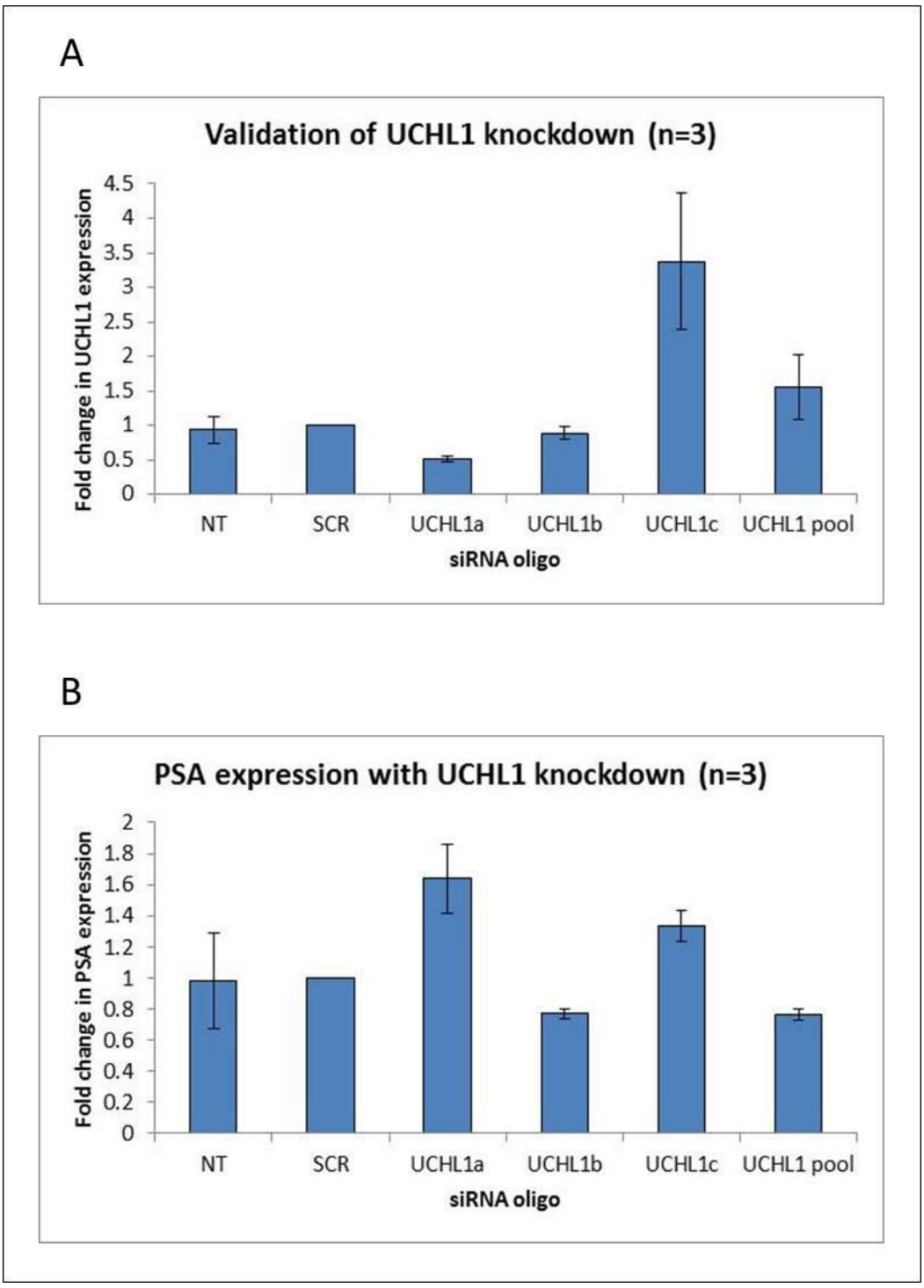

Figure 3.5. Validation of UCHL1 knockdown on PSA expression.

UCHL1 was depleted in LNCaP cells for 48 hours prior to DHT stimulation for a further 48 hours. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Data is represented as fold change compared to the SCR control. Data is representative of the mean of three independent repeats and error bars show the standard error. A) The effect of individual UCHL1 siRNA oligos and pooled siRNA on UCHL1 mRNA expression. B) The effect of individual UCHL1 siRNA oligos and pooled siRNA for UCHL1 on prostate specific antigen (PSA) mRNA expression.

3.3.2.2 USP29

USP29 showed the most promise in the initial screening reducing PSA secretion and mRNA expression by 60% and 90%, respectively (Figure 3.2; Figure 3.3). However, further analysis indicated that similarly to *UCHL1*, each individual siRNA and the pooled siRNA had differential effects on target knockdown (Figure 3.6). Oligonucleotides A and B showed induction of *USP29* expression (9 and 1.4-fold, respectively), oligo C showed 5-fold knockdown and the pooled siRNA showed no effect when compared to the SCR control (Figure 3.6a). It is important to note that a large amount of variability was observed between repeat knockdown validations and therefore the error bars on Figure 3.6a, particularly for the oligo A and B data-sets, were substantial. *USP29* showed very low expression in LNCaP cells resulting in 30-35 cycles of PCR being required to detect mRNA and therefore a high threshold cycle (Ct) value resulted. High Ct values are usually thought to be less accurate and therefore potentially unreliable. Interestingly, all siRNA oligos and the pooled siRNA showed reduction of *PSA* expression, to a maximum of 2.8-fold (Figure 3.6b). From these results it was concluded that the initial selection of USP29 may be potentially due to an off-target effect of siRNA causing reduction in *PSA* levels and that it could be a false positive.

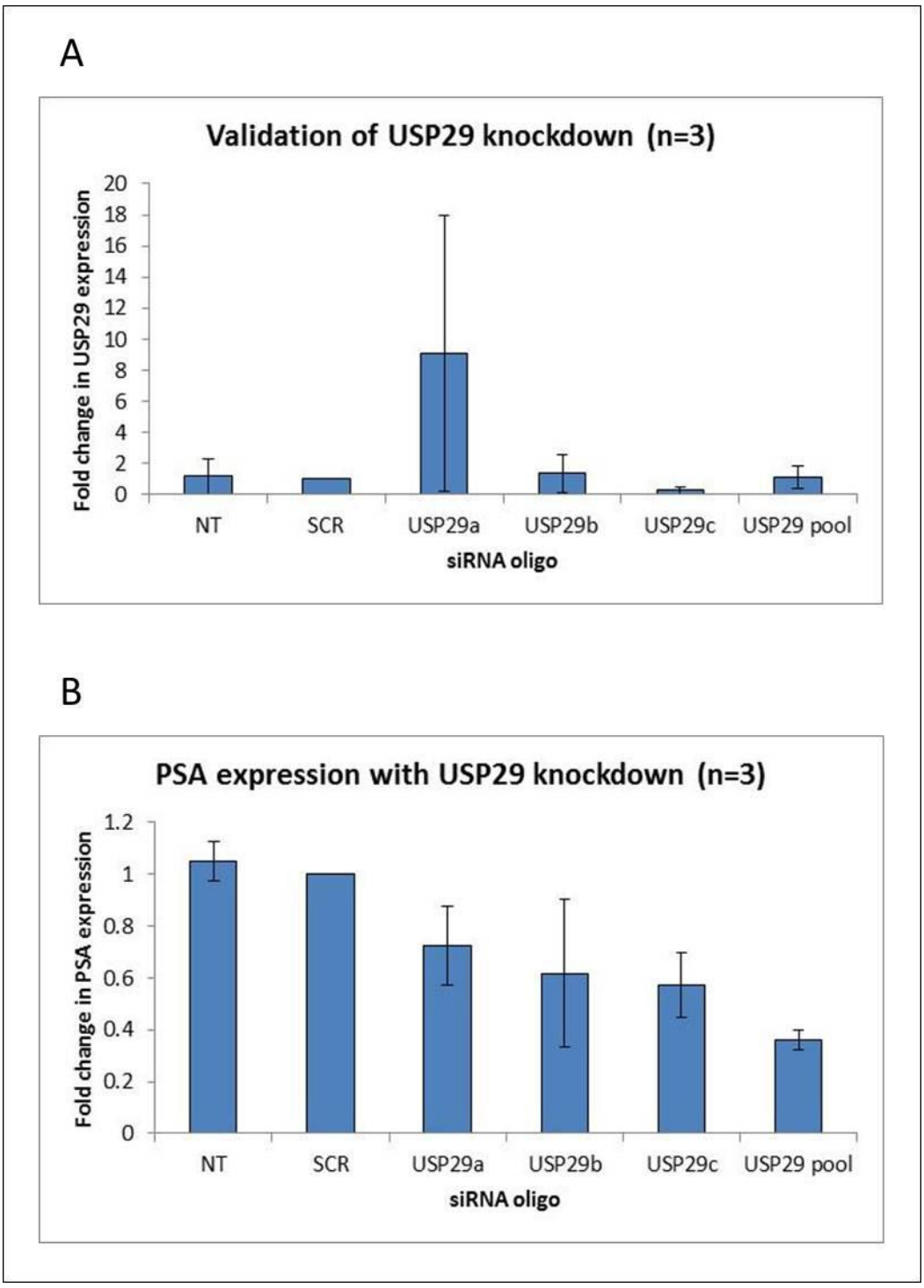

Figure 3.6. Validation of USP29 knockdown on PSA expression.

USP29 was depleted in LNCaP cells for 48 hours prior to DHT stimulation for a further 48 hours. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Data is represented as fold change compared to the SCR control. Data is representative of the mean of three independent repeats and error bars show the standard error. A) The effect of individual USP29 siRNA oligos and pooled siRNA on USP29 mRNA expression. B) The effect of individual USP29 siRNA oligos and pooled siRNA for USP29 on prostate specific antigen (PSA) mRNA expression.

3.3.2.3 USP50

Similar problems were encountered in the analysis of USP50. This target showed 60% decrease in PSA in both the ELISA and QPCR data (Figure 3.2; Figure 3.3). Expression of this target was also very low; represented by very high Ct values high in the QPCR analysis akin to USP29. siRNA oligonucleotides A and B caused induction of *USP50* expression (3 and 6-fold increase, respectively), whereas oligo C and the pooled siRNA showed no difference when compared to the SCR control (Figure 3.7a). Individual oligo knockdown showed no effect (oligo B, C and pooled) or an increase in *PSA* expression (2-fold maximum with siRNA oligo A). Interestingly, the pooled siRNA showed no effect on *PSA* expression which when compared to the initial QPCR data is disappointing (Figure 3.7b; Table 3.2). USP50 was therefore deemed as a potential false positive and the study of this enzyme was stopped.

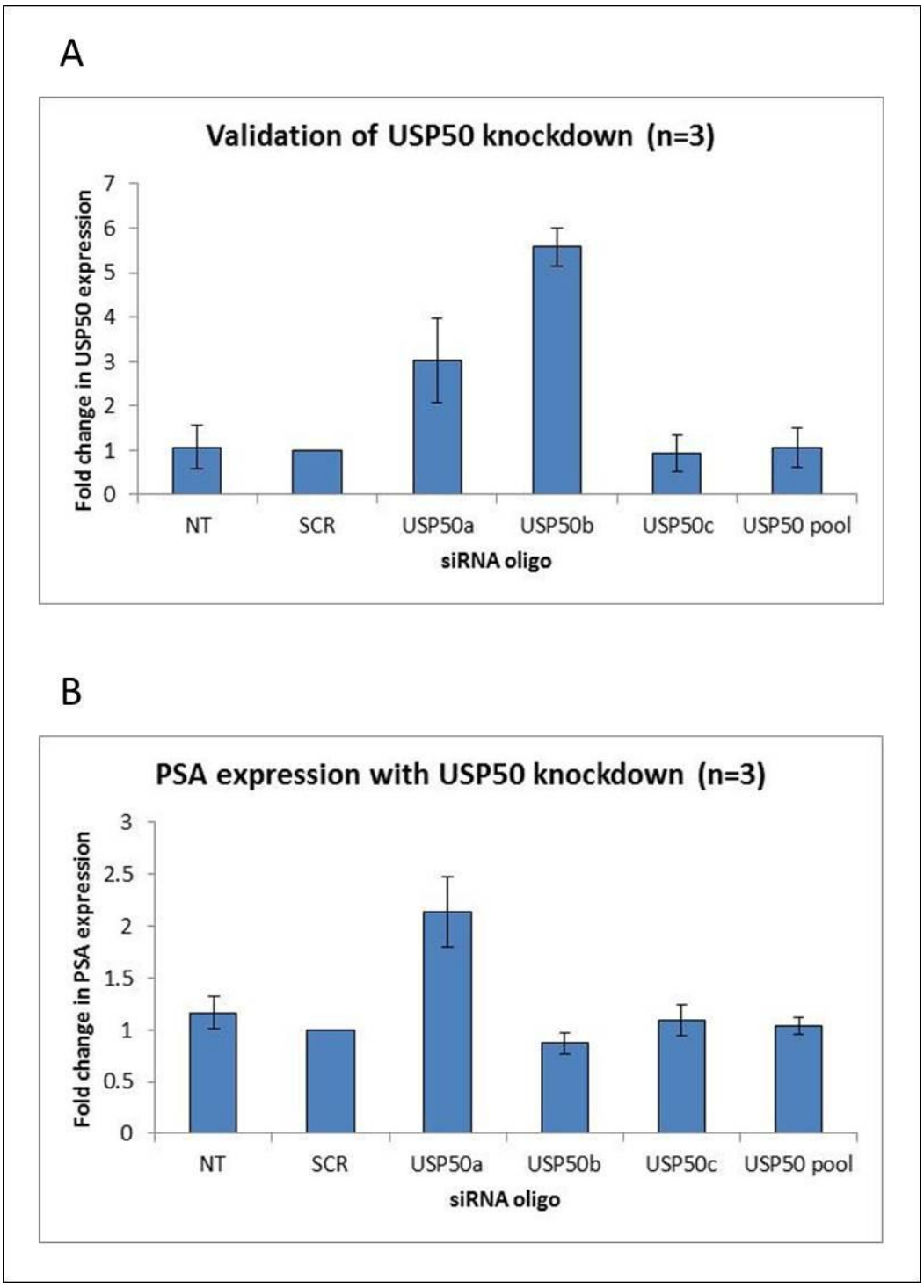

Figure 3.7. Validation of USP50 knockdown on PSA expression.

USP50 was depleted in LNCaP cells for 48 hours prior to DHT stimulation for a further 48 hours. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Data is represented as fold change compared to the SCR control. Data is representative of the mean of three independent repeats and error bars show the standard error. A) The effect of individual USP50 siRNA oligos and pooled siRNA on USP50 mRNA expression. B) The effect of individual USP50 siRNA oligos and pooled siRNA for USP50 on prostate specific antigen (PSA) mRNA expression.

	USP50 knockdown
Experiment	Fold change in PSA expression
PSA ELISA	0.39 ± 0.06
PSA QPCR	0.37 ± 0.22
Validation PSA QPCR	1.04 ± 0.08

Table 3.2. Comparison of effects on PSA with knockdown of USP50.

Knockdown of USP50 had 0.39-fold prostate specific antigen (PSA) secretion and 0.37-fold PSA expression compared to the scrambled siRNA (SCR). Data represented the mean of three independent repeats ± standard error in the PSA ELISA and initial PSA QPCR. Validation of USP50 knockdown showed no effect on PSA expression compared to the SCR control.

3.3.2.4 USP12

Unlike the other targets tested thus far, *USP12* levels were markedly depleted by each of the individual and pooled siRNAs, ranging from a minimum knockdown of 3-fold with oligo A to a maximum knockdown of 9-fold with oligo B (Figure 3.8a). However, it is important to note that a slight increase in *USP12* expression was seen upon treatment of cells with the SCR siRNA compared to the NT control (Figure 3.8a). Importantly, depletion of USP12 by each siRNA caused a reduction in *PSA* expression; individual oligos showed an approximate 2-fold decrease in *PSA* expression while the pooled siRNA showed a 3.5-fold decrease (Figure 3.8b). This was in accordance with the initial screening data that demonstrated a respective 70% and 50% reduction in PSA secretion by ELISA and expression by QPCR (Figure 3.2; Figure 3.3; Table 3.3).

Given these findings, USP12 was taken through to the next round of investigation, as described below.

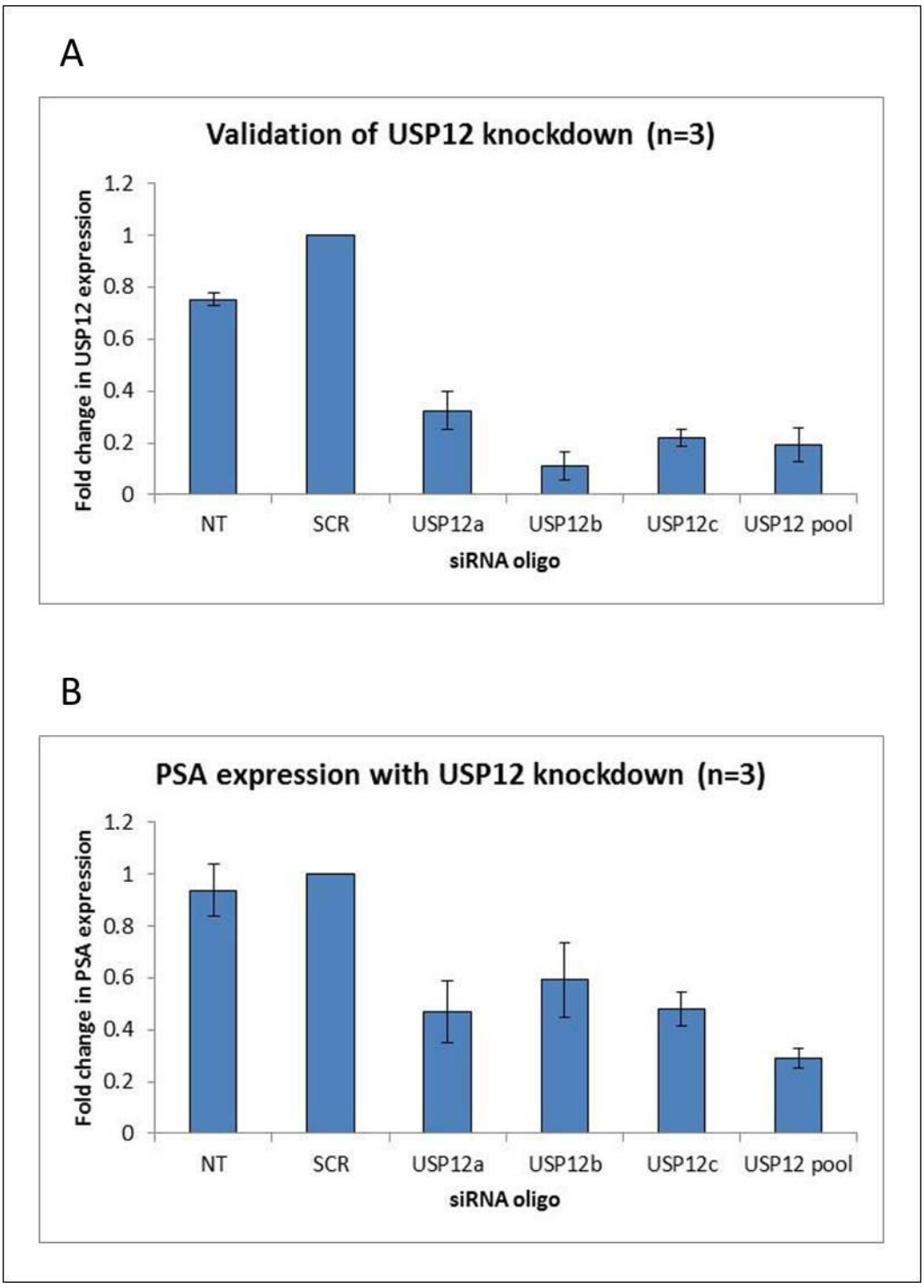

Figure 3.8. Validation of USP12 knockdown on PSA expression.

USP12 was depleted in LNCaP cells for 48 hours prior to DHT stimulation for a further 48 hours. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Data is represented as fold change compared to the SCR control. Data is representative of the mean of three independent repeats and error bars show the standard error. A) The effect of individual USP12 siRNA oligos and pooled siRNA on USP12 mRNA expression. B) The effect of individual USP12 siRNA oligos and pooled siRNA for USP12 on prostate specific antigen (PSA) mRNA expression.

	USP12 knockdown
Experiment	Fold change in PSA expression
PSA ELISA	0.27 ± 0.07
PSA QPCR	0.53 ± 0.26
Validation PSA QPCR	0.29 ± 0.04

Table 3.3. Comparison of effects on PSA with knockdown of USP12.

Knockdown of USP12 had 0.3-fold prostate specific antigen (PSA) secretion and 0.5-fold PSA expression compared to the scrambled siRNA (SCR) control. Data represents the mean of three independent repeats ± standard error in the PSA ELISA and initial PSA QPCR. Validation of USP12 knockdown showed 0.3-fold PSA expression compared to the SCR control.

3.3.3 Effect of USP12 knockdown on the AR-regulated PSA expression over 72 hours DHT stimulation

The next stage of validating USP12 as a *bona fide* AR co-regulator was to investigate the effect of *USP12* knockdown on *PSA* expression over an extended time course of 72 hours DHT treatment. This was to validate that the effect of *USP12* knockdown on *PSA* expression was directly as a result of modulating AR transcriptional activity rather than a secondary effect.

USP12 was depleted in LNCaP cells on a 6-well plate format for 48 hours prior to 100 nm DHT treatment for up to 72 hours. Cells were harvested at 0, 6, 12, 24, 48 and 72 hours post-treatment and both *PSA* and *USP12* levels measured by QPCR. For each experimental replicate SCR siRNA was used as a control and results from at least three independent repeats are shown as fold change compared to SCR at the 0 hour time-point.

As in the previous validations (Section 3.3.2), the effect of siRNA oligonucleotide transfection on *PSA* expression compared to a non-transfected control was assessed to provide evidence of toxicity or off-target effects of the transfection procedure. Figure 3.9a and b show the expression of *PSA* and *AR* in the NT and SCR controls over the duration of 72 hours DHT stimulation. As expected, *PSA* mRNA levels were elevated in response to androgen in the NT control which was mirrored in the SCR samples indicating minimal effect of the SCR siRNA transfection procedure on AR-mediated transcription (Figure 3.9a). Unexpectedly, expression of the AR was slightly reduced in the SCR control compared to the NT control (Figure 3.9b). Therefore, although the results of target knockdown in the subsequent time-course experiments were compared to the SCR control, the NT control was included to allow any effects of the SCR siRNA on mRNA expression to be observed.

Consistent with the previous validation experiments described in Section 3.3.2, and to confirm that *PSA* expression was mediated by the AR, depletion of the receptor was included as an additional control to the above experiment. As shown in Figure 3.9a, knockdown of *AR* showed almost complete ablation of *PSA* expression at all time-points and this correlated with depleted *AR* mRNA levels (Figure 3.9b). Interestingly, the levels of *AR* in the SCR control were seen to decrease over the 72 hour time-course

(Figure 3.9b), this is a trend that has been observed previously in a study investigating the effect of EGF and ErbB2 on *AR* expression (Cai *et al.*, 2009).

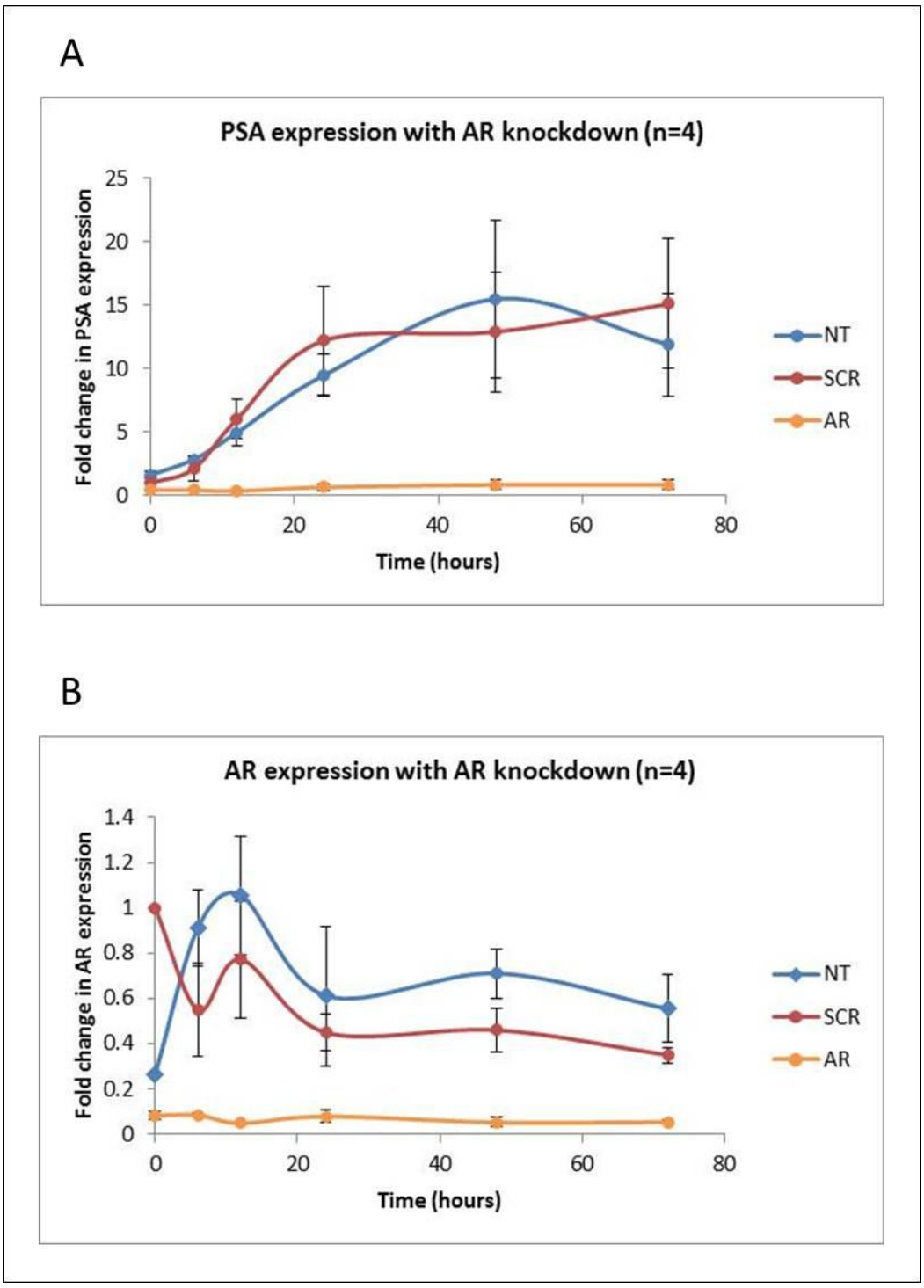

Figure 3.9. Effect of AR knockdown on PSA expression over 72 hours DHT stimulation.

Androgen receptor (AR) was depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Results are representative of the mean of four independent repeats and are shown as fold change compared to the 0 hours SCR control. Error bars show the standard error. A) The effect of AR knockdown on prostate specific antigen (PSA) expression over 72 hours DHT treatment. B) The effect of AR knockdown on AR expression over 72 hours DHT treatment.

To maximise USP12 depletion, *USP12* oligonucleotide B was used in the subsequent studies as this demonstrated the most robust knockdown of the deubiquitinase enzyme by the individual siRNAs. Importantly, knockdown of *USP12* showed reduced *PSA* expression at each time-point post 6 hour DHT treatment (Figure 3.10a). At 6 hours, knockdown showed little or no difference in *PSA* expression when compared to SCR control, but from this point onwards *PSA* expression was decreased dramatically with upwards of 2-fold reduction observed at 72 hours (Figure 3.10a). Profiling *USP12* mRNA levels over the duration of the experiment showed little difference between the NT and SCR controls and approximately 70% knockdown was achieved with *USP12* siRNA at each time-point indicating that the effects seen on *PSA* levels is a result of USP12 depletion (Figure 3.10b).

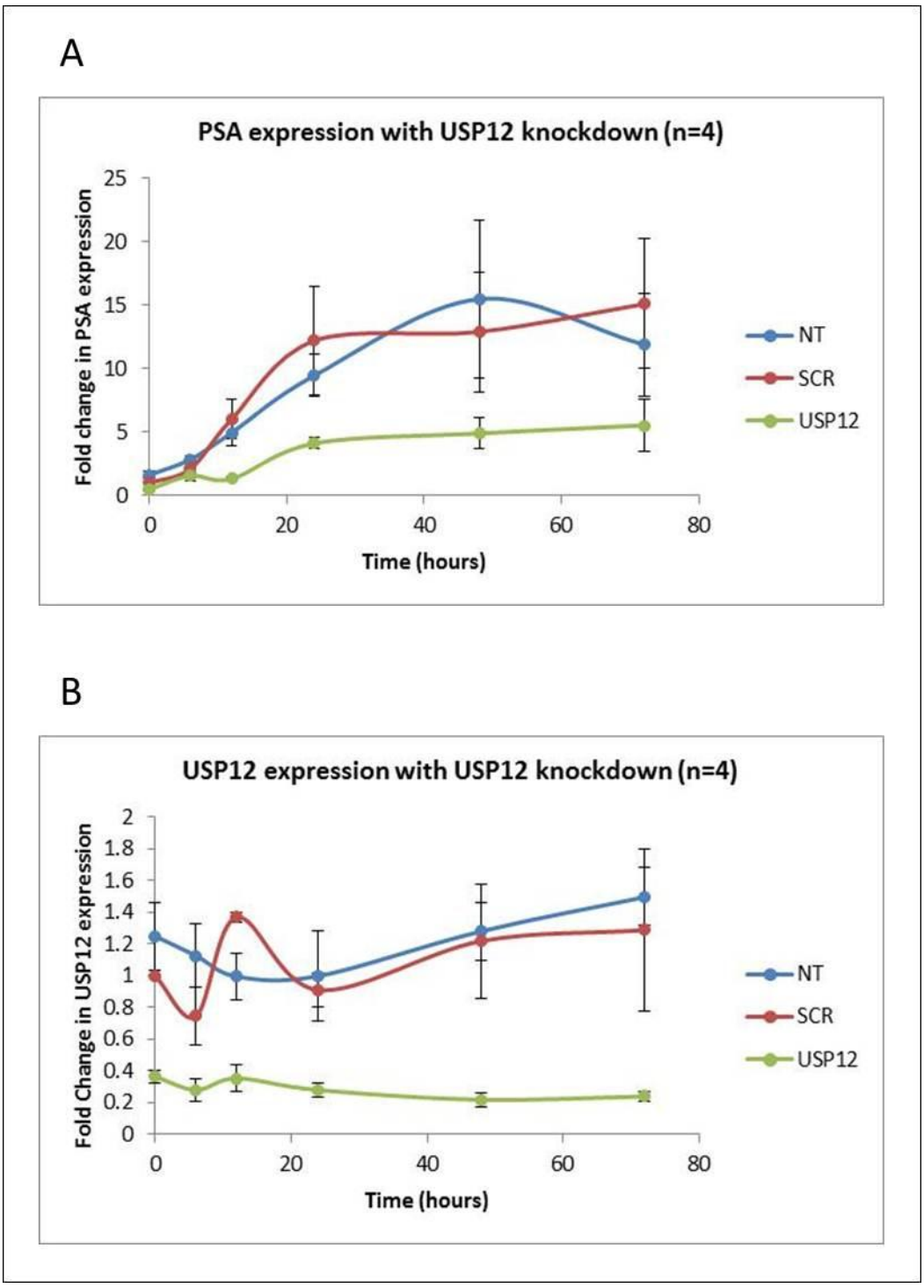

Figure 3.10. Effect of USP12 knockdown on PSA expression over 72 hours DHT stimulation.

USP12 was depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Results are representative of the mean of four independent repeats and are shown as fold change compared to the 0 hours SCR control. Error bars represent the standard error. A) The effect of USP12 knockdown on prostate specific antigen (PSA) expression over 72 hours DHT treatment. B) The effect of USP12 knockdown on USP12 expression over 72 hours DHT treatment.

To elucidate whether *USP12* was itself an AR-regulated gene, *USP12* expression was investigated in cells subject to AR depletion. No significant difference in *USP12* expression was apparent between *AR* knockdown and the NT and SCR controls suggesting that *USP12* is not an AR-responsive gene. Furthermore, *AR* expression was investigated in cells subject to *USP12* knockdown to see if USP12 had any effect on the transcription of the *AR* gene. Again, no significant difference in *AR* expression was observed between *USP12* knockdown and the NT and SCR controls suggesting that USP12 does not regulate the transcription of *AR*.

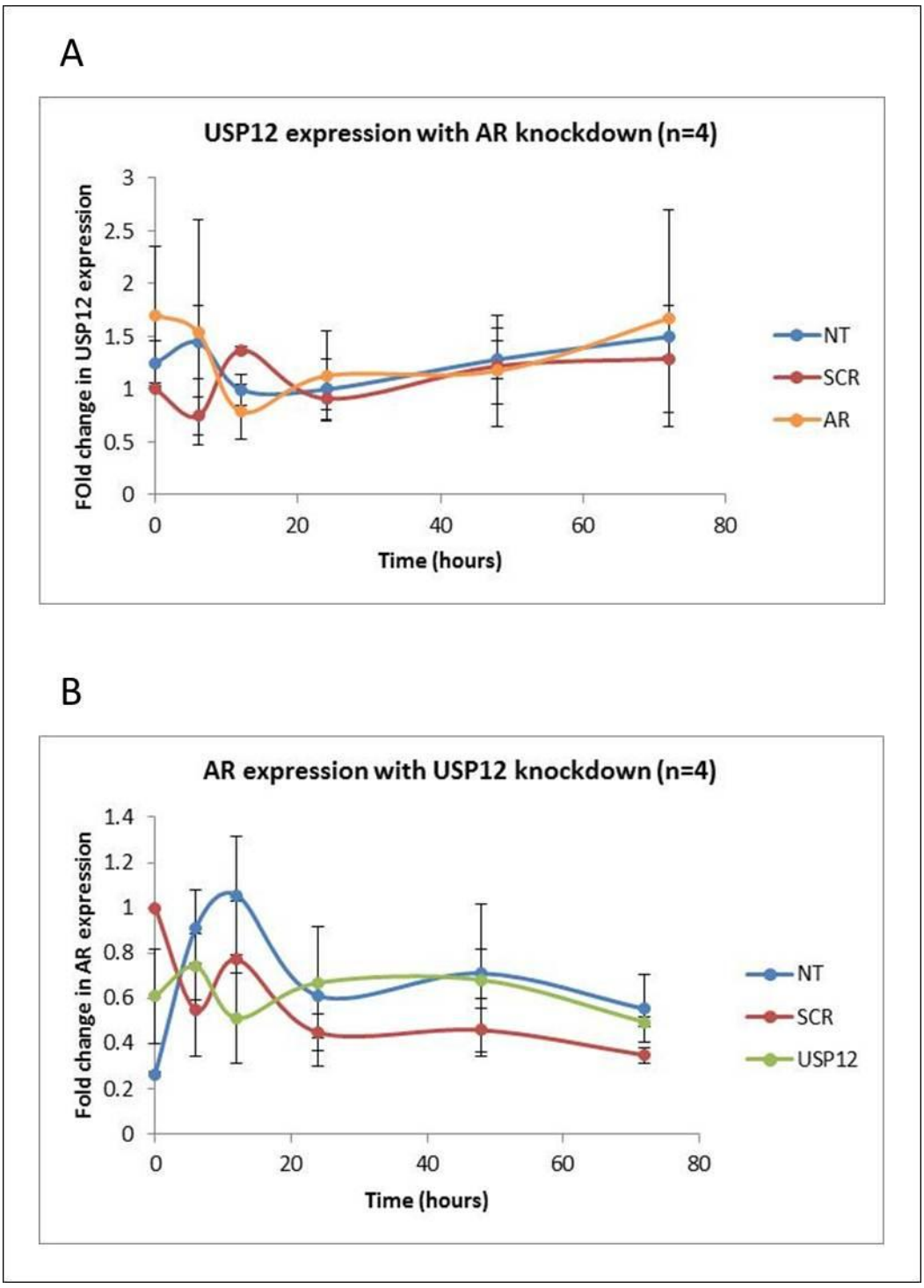

Figure 3.11. Effect of AR and USP12 knockdown on USP12 and AR expression over 72 hours DHT stimulation.

Androgen receptor (AR) and USP12 were depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Results are representative of the mean of four independent repeats and are shown as fold change compared to the 0 hour SCR control. Error bars represent the standard error. A) The effect of AR knockdown on USP12 expression over 72 hours DHT treatment. B) The effect of USP12 knockdown on AR expression over 72 hours DHT treatment.

To investigate whether the observed effect of USP12 knockdown on mRNA expression impacted on PSA protein expression in LNCaP cells, PSA protein levels were assessed by Western analysis. Using the same time-course experiment as described above, with DHT treatment from 0-72 hours, cell lysates were collected and subject to SDS-PAGE and Western blotting for PSA and AR. α-tubulin was used as a loading control. A representative series of Western blots from one of three independent experimental repeats is show in Figure 3.12. Figure 3.13 shows the densitometry analysis of the three experimental repeats and is presented as the mean relative protein expression normalised to the respective α-tubulin loading control. Data was analysed for statistical significance using 2-way ANOVA, significant data is indicated on the figure (Figure 3.13).

In concordance with the QPCR data, NT and SCR samples were comparable and showed a marked increase in PSA protein at 24 hours DHT stimulation that increased further at 48 and 72 hour time-points (Figure 3.9; Figure 3.12a and b; Figure 3.13a). In contrast, knockdown of *USP12* reduced PSA protein at each time-point tested to undetectable levels and was comparable to the effect of *AR* knockdown on PSA levels (Figure 3.12c and d; Figure 3.13a) indicating a robust effect on AR function upon USP12 depletion. Importantly, these findings are consistent with the QPCR data (Figure 3.9; Figure 3.10). Interestingly, knockdown of *USP12* reduced the total amount of protein in each sample, indicated by lower levels of α-tubulin (Figure 3.12d), suggesting that it may be involved in regulating the proliferation of LNCaP cells (this point is addressed in Section 4.3.5.1).

Assessment of AR levels in the NT and SCR controls demonstrated an expected increase in AR levels in response to DHT stimulation after 6 hours treatment that was maintained until 24 hours after treatment; AR levels then returned to basal levels (Figure 3.12a and b; Figure 3.13b). AR protein was not detectable in *AR* knockdown samples (Figure 3.12c; Figure 3.13b). Taking into account the reduced α-tubulin levels in *USP12* knockdown cells, there was minimal effect of USP12 depletion on AR protein levels and a similar profile to both NT and SCR controls was observed (Figure 3.12d; Figure 3.13). Unfortunately, due to a lack of appropriate antibodies the USP12 protein levels could not be assessed (refer to Section 4.3.1.2).

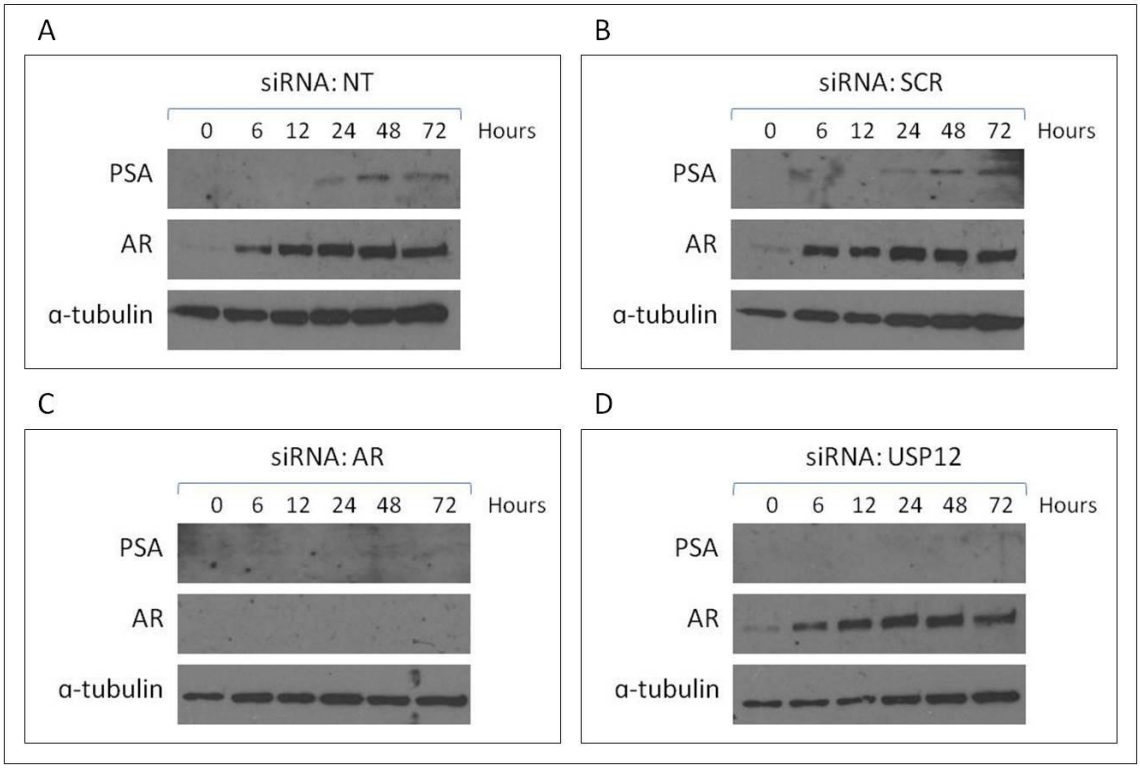

Figure 3.12. Effect of USP12 knockdown on PSA and AR protein levels.

LNCaP cells were subject to siRNA knockdown for 48 hours prior to 72 hours 100 nM DHT stimulation. Cell lysates were collected at 0, 6, 12, 24, 48 and 72 hours post-treatment. Western blotting for prostate specific antigen (PSA) and androgen receptor (AR) were performed. α -tubulin was used as a loading control. Non-transfected (NT), scrambled siRNA (SCR) and AR knockdown were used as negative and positive controls. Western blots are representative of three independent experimental repeats. A) The effect of the NT control on PSA and AR protein. B) The effect of the SCR control on PSA and AR protein. C) The effect of AR knockdown on PSA and AR protein. AR Western blot confirmed AR was knocked down throughout the time course. D) The effect of USP12 knockdown on PSA and AR protein.

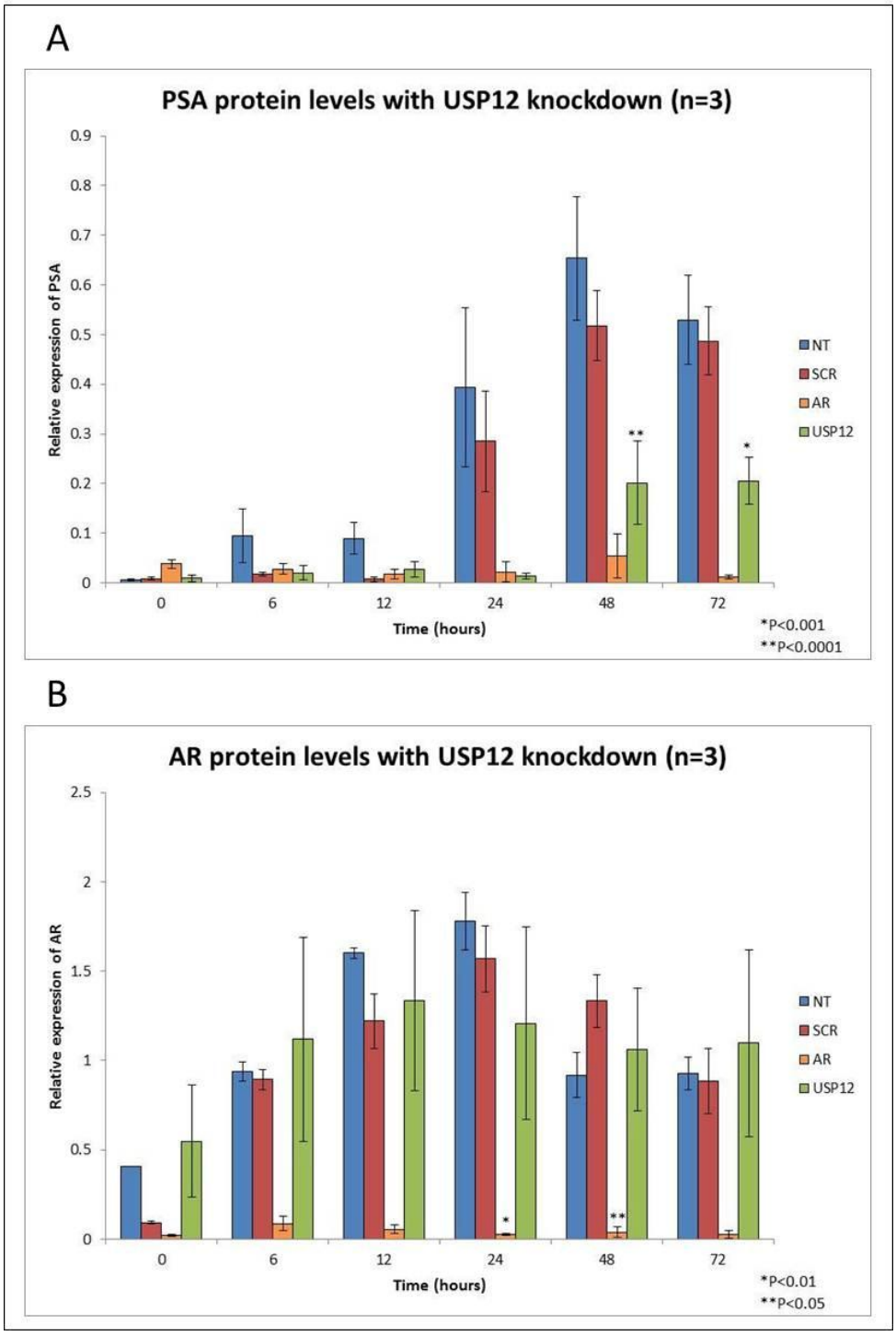

Figure 3.13. Densitometry analysis of the effect of USP12 knockdown on PSA and AR protein levels.

Western blot data from the time-course experiments was analysed using the Quantity one programme on the GelDoc™ system. Data for prostate specific antigen (PSA) and androgen receptor (AR) protein levels were normalised to the respective α-tubulin loading control data. Data is presented as the mean relative expression of three independent experimental repeats. Error bars represent the standard error. Data was analysed for statistical significance using 2-way ANOVA. Each knockdown was compared to the SCR control at each time point, significant data is indicated by an asterisk. A) The effect of siRNA knockdown on PSA protein levels over 72 hours DHT treatment. B) The effect of siRNA knockdown on AR protein levels over 72 hours DHT treatment.

3.3.4 Effect of USP12 knockdown on other AR-regulated gene expression

To assess whether the effect of *USP12* knockdown was limited exclusively to the *PSA* gene, the same sample set was analysed for expression of a number of other AR regulated genes; *KLK2*, a member of the same kallikrein family as *PSA*, *TMPRSS2*, *NKX3.1* and *NDRG1*.

All of the AR-regulated genes examined showed characteristic induction with DHT treatment in the NT and SCR controls (Figure 3.14) (Ngan *et al.*, 2009).*KLK2* showed a similar expression pattern to *PSA* in the NT and SCR control (Figure 3.14a), while *TMPRSS2*, *NKX3.1* and *NDRG1* demonstrated an increase in expression at approximately 20 hours DHT treatment before returning to basal levels at 72 hours (Figure 3.14b, c and d). Large error bars were observed due to variation between sample sets and taking these into account little difference was observed in mRNA expression of the AR-regulated between the NT and SCR controls (Figure 3.14). As expected, almost complete loss of androgen-responsiveness was observed for all genes upon *AR* knockdown (Figure 3.14), although the difference in expression was minimal at 72 hours for *TMPRSS2*, *NKX3.1* and *NDRG1* due to mRNA levels returning to basal levels in the SCR controls (Figure 3.14b, c and d).

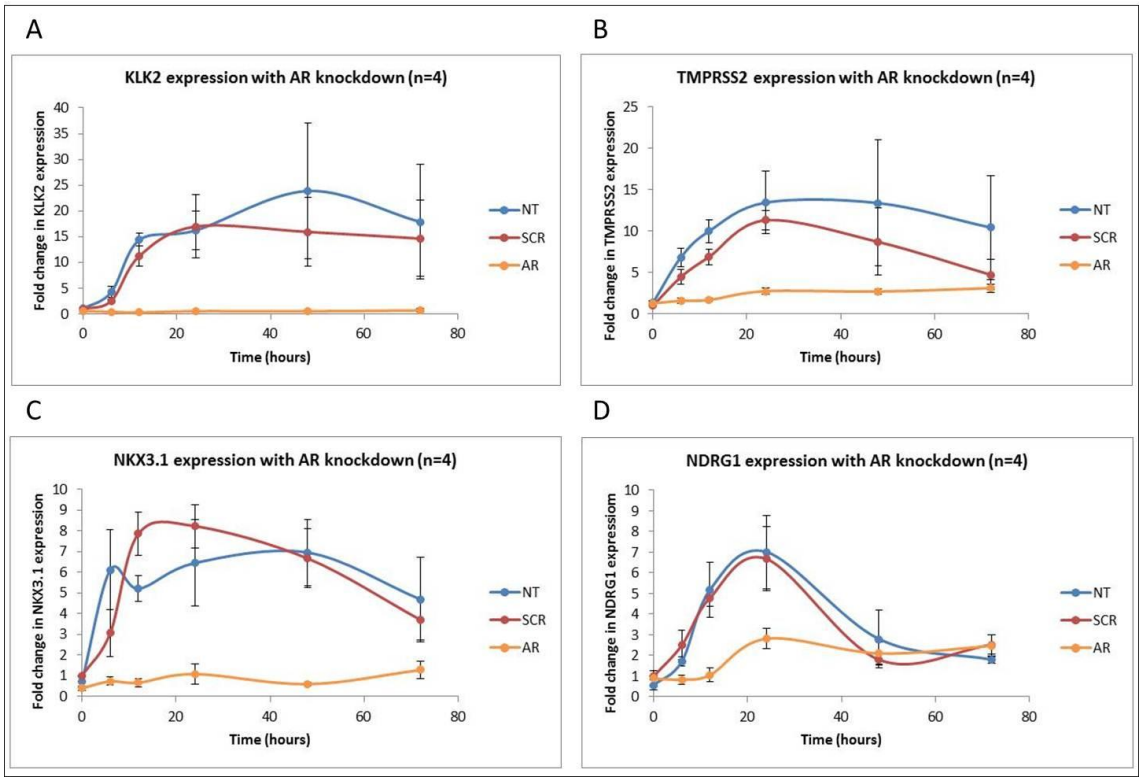

Figure 3.14. Effect of AR knockdown on other androgen receptor regulated gene expression over 72 hours DHT stimulation.

Androgen receptor (AR) was depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Results are representative of the mean of four independent repeats and are shown as fold change compared to the 0 hours SCR control. Error bars show the standard error. A) The effect of AR knockdown on KLK2 expression over 72 hours DHT treatment. B) The effect of AR knockdown on TMPRSS2 expression over 72 hours DHT treatment. C) The effect of AR knockdown on NKX3.1 expression over 72 hours DHT treatment. D) The effect of AR knockdown on NDRG1 expression over 72 hours DHT treatment.

The effect of *USP12* knockdown on *KLK2* expression was consistent with that observed for *PSA*; a decrease in expression throughout the 72 hours, with a maximum of 10-fold decrease observed at 24 hours DHT stimulation (Figure 3.15a). *TMPRSS2* expression was reduced by approximately 3-fold when USP12 was depleted, however similarly to *AR* knockdown, the difference in expression at 72 hours post-treatment was minimal (Figure 3.15b). Likewise, *NKX3.1* and *NDRG1* expression were both reduced following *USP12* knockdown with between 2 to 5-fold decreases observed over the course of the 72 hours DHT time-course (Figure 3.15c and d). Consistent, with the *TMPRSS2* data-set, the difference in expression between the *USP12* knockdown and SCR samples for both *NKX3.1* and *NDRG1* was not significant at the 72 hour time-point (Figure 3.15c and d).

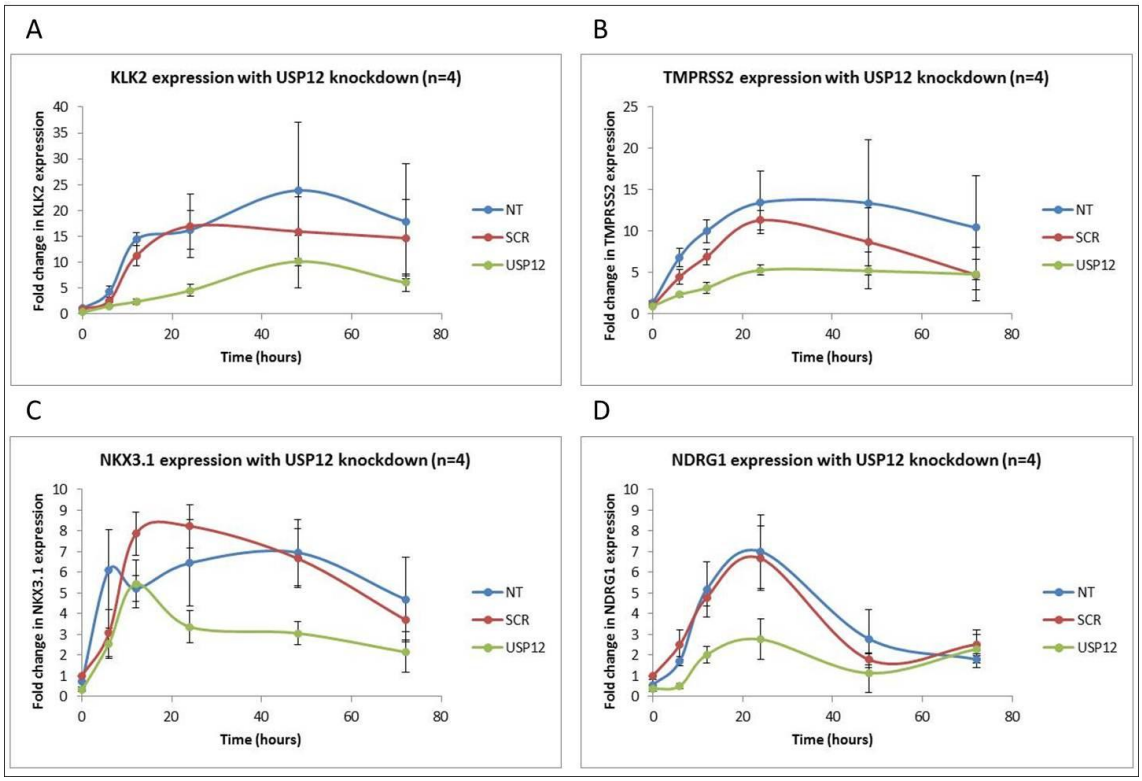

Figure 3.15. Effect of USP12 knockdown on other androgen receptor regulated gene expression over 72 hours DHT stimulation.

USP12 was depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Non-transfected cells (NT) and scrambled siRNA (SCR) were used as negative controls. Results are representative of the mean of four independent repeats and are shown as fold change compared to the 0 hours SCR control. Error bars show the standard error. A) The effect of USP12 knockdown on KLK2 expression over 72 hours DHT treatment. B) The effect of USP12 knockdown on TMPRSS2 expression over 72 hours DHT treatment. C) The effect of USP12 knockdown on NKX3.1 expression over 72 hours DHT treatment. D) The effect of USP12 knockdown on NDRG1 expression over 72 hours DHT treatment.

3.4 Discussion

Ubiquitination of AR acts to regulate both AR activity and stability. The E3 ubiquitin ligase PIRH2 acts as a co-activator of AR activity, potentially ubiquitinating AR or other components of the AR protein complex, whilst other E3 ligases, such as MDM2, ubiquitinates AR promoting its proteasomal degradation (Lin *et al.*, 2002b; Logan *et al.*, 2006). DUBs provide a means of negatively regulating these ubiquitination signals. The aim of this study was to identify DUBs that significantly affect the transcriptional activity of AR resulting in altered levels of *PSA* expression in LNCaP cells. These DUBs may prove to be important in the development and progression of prostate cancer and hence provide potential targets for therapy.

Only approximately 80 DUBs had been identified at the planning stage of the siRNA screen and of these, several were eliminated based on their lack of catalytic activity or incomplete sequence hence 72 DUBs were investigated. siRNA was utilised to individually knockdown each DUB in the LNCaP cell line prior to DHT treatment. The primary experimental readout was PSA secretion by PSA ELISA. As *PSA* is an AR-regulated gene, any effect on AR activity may have a downstream effect on PSA expression and secretion. Targets found to modulate PSA secretion by 50% were selected for further analysis of *PSA* expression by QPCR. *PSA* expression analysis allowed discrimination between DUBs that potentially effected AR transcriptional activity and those that may have had a role in the secretion of PSA.

The initial siRNA knockdown data-set from the PSA ELISA revealed that 18 of the 72 DUB enzymes screened influenced PSA secretion by 50% above or below the SCR control value. Subsequent profiling of *PSA* mRNA levels after knockdown of each of these 18 targets by QPCR showed 8 consistently altered *PSA* expression; these were taken forward for further validation. Validation of four of these targets, namely UCHL1, USP12, USP29 and USP50 was performed in this Chapter.

It is a possibility that some of the 54 targets ruled out of the QPCR analysis may have had an effect on *PSA* expression. DUB targets that modulated PSA secretion by 50% were selected. However, none of the DUBs that increased PSA secretion reached the threshold and the three closest targets were selected for further investigation; one of these targets, USP10, was validated (this is discussed in Chapter 5). This flexibility in

target selection for those that increased PSA secretion was not applied in those that reduced secretion meaning other potential hits may have been missed.

Little is known about the function of USP29, USP50 or USP12, especially within CaP. UCHL1 is an exception with a previous study reporting that it is expressed in the AR-negative, androgen-independent PC-3 and DU145 cell lines but not in the androgen-dependent LNCaP (Leiblich *et al.*, 2007). UCHL1 is a widely studied neuronal protein with implications in neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Setsuie and Wada, 2007). Therefore, the authors of the CaP study suggest that cell lines expressing UCHL1 may be derived from neuronal lineages (Leiblich *et al.*, 2007). Interestingly, UCHL1 was found to be expressed in LNCaP cells at the mRNA level and at easily detectable quantities (Figure 3.5). Leiblich *et al.,* used PCR to confirm *UCHL1* expression in PC-3 and DU145 cells, although they used conventional gel electrophoresis instead of a quantitative method and this may account for not detecting UCHL1 in LNCaP cells. Increasingly, UCHL1 has been associated with a wide variety of cancers including lymphoma (Hussain *et al.*, 2010), breast (Wang *et al.*, 2008), colorectal (Okochi-Takada *et al.*, 2006) and small cell lung carcinoma (Kim *et al.*, 2009). In a head and neck squamous cell carcinoma cell line study performed by Tokumaru *et al.,* the authors postulated that UCHL1 is a tumour suppressor gene as it is methylated during tumourigenesis (Tokumaru *et al.*, 2008). Contradictory to this notion, however, primary tumour samples within this study were found to over-express the protein potentiating the conclusion that UCHL1 acts as a tumour suppressor gene in normal tissue but is over-expressed in cancer due to oncogenic signals (Tokumaru *et al.*, 2008). Li *et al*., also found that UCHL1 acts as a tumour suppressor through its interaction with and promotion of p53 activity, and the same study found that UCHL1 was frequently methylated in nasopharyngeal carcinoma (Li *et al.*, 2010). More recently, conflicting reports have suggested that UCHL1 actually acts as an oncogene (Kim *et al.*, 2009; Hussain *et al.*, 2010). Hussain *et al.,* reported that UCHL1 drives the development of lymphoma through down regulation of apoptosis and promotion of proliferation through the AKT pathway (Hussain *et al.*, 2010). Similarly, UCHL1 was found to stimulate tumour invasion in a lung cancer cell line through activation of the Akt pathway (Kim *et al.*, 2009). Disappointingly, UCHL1 could not be validated in the context of our screen. PSA ELISA data suggested knockdown of

UCHL1 caused a decrease in PSA secretion which was confirmed by QPCR analysis (Figure 3.2, Figure 3.3). However, further validation revealed that the pool of three siRNA oligonucleotides failed to knockdown *UCHL1* and that the decrease in *PSA* may have been an off target effect of the siRNA (Figure 3.5). Importantly, knockdown of *UCHL1* was only successful using oligonucleotide A, and in this case, *PSA* mRNA expression was increased (Figure 3.5), suggesting this may be the genuine effect of *UCHL1* knockdown on AR activity. In order to resolve this conflicting result and elucidate the true effect of UCHL1 knockdown on *PSA* expression new siRNA oligonucleotides would need to be designed.

Complete validation of both USP29 and USP50 was also unsuccessful due to both targets being expressed at very low levels in LNCaP cells that made determination of knockdown difficult. 30-35 cycles of PCR were required to detect *USP29* and *USP50*. Ct values of this level would not normally be considered reliable and accurate and could account for some of the variability seen between experimental repeats particularly for USP29 (Figure 3.6a). Therefore, to determine whether these targets would be worth further follow up, the study is limited to analysis of *PSA* expression only as no informed decision on the robustness of USP29 and USP50 knockdown can be made from the expression data of each of the DUBs (Figure 3.6a and Figure 3.7a). Importantly, *USP29* knockdown showed consistent reduction in *PSA* expression with each individual siRNA oligo and the greatest effect was observed with the pooled siRNA (Figure 3.6b). Therefore, it is possible that knockdown of the already low levels of the enzyme reduces AR activity, suggesting an involvement of USP29 in the AR signalling cascade. One possible method of validating *USP29* knockdown would be at the protein level by Western blotting. Several USP29 antibodies are available commercially, however, no USP29 antibodies have been successfully used in the literature and verification would have to be performed. With hindsight, USP29 could have been brought forward to the next steps of validation; even though knockdown could not be accurately determined by QPCR, the effect on *PSA* expression was consistent. However, without thorough validation of *USP29* knockdown levels, the function of the enzyme in AR regulation remains an intriguing possibility.

Interestingly, *USP50* knockdown did not show reduction in *PSA* expression with the pooled siRNA or individual oligos (Figure 3.7b), while both the ELISA and initial QPCR

experiments at the on-set of the screening programme demonstrated robust reduction in PSA by utilising the same pool of siRNA (Table 3.1). This condition effectively acts as an internal control for these experiments and should give similar results to the ELISA. The data sets theoretically should not be different as the cell line used was the same, although there were differences in passage numbers. As the ELISA and first *PSA* QPCR were performed without knockdown checks there may have been some large scale off-target effect, although the knockdown validations proved difficult.

Of the four targets chosen for additional validation, USP12 was the only target that was successfully taken forward and shown to be a regulator of AR gene expression. All individual siRNA oligos and the pooled siRNA showed robust knockdown of *USP12* that markedly reduced *PSA* expression (Figure 3.8) with maximal effect seen with the pooled oligonucleotides (approximately 70% reduction). For subsequent DHT time-course validation experiments, siRNA oligo B was used alone to knockdown *USP12* as this provided up to 90% depletion of *USP12* mRNA and was a simpler and cheaper way of achieving robust knockdown of the target and decreases in AR target gene expression. Over 72 hours DHT stimulation, *USP12* knockdown was shown to reduce the expression of all the AR-regulated genes investigated. No effect was seen on AR protein levels and so it can be postulated that USP12 regulates the transcriptional functions of AR rather than AR protein stability.

RNA interference screens have been utilised by a number of laboratories to identify DUB enzymes that are important in a wide variety of pathways. Many endpoint assays have been used throughout the literature. USP39 and USP44 were identified in screens performed in looking for defects in mitosis (Stegmeier *et al.*, 2007; van Leuken *et al.*, 2008). These screens, performed in human osteosarcoma and cervical cancer cell lines respectively, used a visual endpoint to select candidate DUBs for further validation by observing defects in cells under the microscope. Western blot analysis of downstream targets of TGF β signalling was used to identify USP9x as an important regulator in TGF-β signalling (Dupont *et al.*, 2009). Other screens such as Brummelkamp *et al*., and Kayagaki *et al*., used luciferase reporter assay-based approaches to look for the effect of DUB knockdown on transcriptional activity and identified CYLD as a regulator of NFκB, and DUBA as important in type I interferon production, respectively (Brummelkamp *et al.*, 2003; Kayagaki *et al.*, 2007). With such a wide selection of

endpoint assays, the question remains as to which is the most appropriate for such a screen. At first glance, it may appear a protein based-assay would be most favourable as ubiquitination is a post-translational modification, and has been implicated in protein turnover. However, ubiquitination events themselves have such a broad spectrum of consequences that both protein stability and transcriptional activity can be affected (Pickart and Eddins, 2004). The study herein aimed to identify DUBs that effected AR transcriptional activity by monitoring production of the AR-responsive PSA protein in an ELISA. One potential problem with this protein-based read-out approach was that DUBs influencing PSA protein stability may have been inadvertently identified. It was a necessity, therefore, to include an additional QPCR screening step to look directly at PSA mRNA expression.

A luciferase-based reporter approach may have simplified the identification of DUBs involved in AR transcriptional activity. This system would utilise the luciferase reporter gene downstream of an AR-responsive promoter, such as the *PSA* promoter. Upon androgen stimulation, the AR binds to the promoter and drives transcription of the luciferase gene. Addition of luciferin, a substrate of the luciferase enzyme, then results in a measurable emission of light that is proportional to luciferase levels and hence reflects AR activity. An LNCaP cell line with a stably transfected androgen-responsive luciferase reporter would be a useful tool cell line for this type of experiment, while other non-AR-responsive lines have also been engineered to assess mechanisms of receptor regulation (Faus *et al.*, 2005; Caliper Life Sciences, 2011). For example, Dirac *et al.,* identified USP26 as an important regulator of AR signalling using the hepatocellular carcinoma cell line HepG2 (Dirac and Bernards, 2010). This cell line does not endogenously express AR but has been shown to become androgen-responsive upon over-expression of ectopic AR. Additionally, the plasmid-based luciferase gene downstream of an androgen response element (ARE) was transiently introduced into this cell line and subsequent luciferase analysis demonstrated USP26 knockdown increases AR activity. However, over-expression of USP26 also showed increased AR activity in HEK293 cells upon the same reporter; the authors suggesting that this discrepancy is due to differences in AR ubiquitination status between cell lines (Dirac and Bernards, 2010). Importantly, both the HepG2 and HEK293 cell lines used in this study do not endogenously express the AR and are therefore not reliant on this

signalling pathway. It is also probable that other important components of the AR signalling pathway are missing that may be required for the effect of USP26 on AR in a cell line that does endogenously express AR. The data described in this Chapter have utilised the LNCaP cell line which both expresses and is dependent upon AR, suggesting that any effect seen with knockdown of a DUB on AR signalling is likely to be more representative of the true cellular state. As shown in Figure 3.1 and Table 3.1, USP26 was identified as a DUB that reduced PSA secretion in the ELISA by 60%. Unfortunately, analysis of PSA mRNA levels was not consistent with the results of the PSA ELISA; demonstrating an increase in *PSA* expression by 20% over the SCR control. Therefore this DUB was not brought forward for further validation.

Early DUB knockdown screens such as Brummelkamp *et al*., and Nijman *et al*., targeted 50 proteins postulated to contain the conserved DUB catalytic domain (Brummelkamp *et al.*, 2003; Nijman *et al.*, 2005a). When our study was initially designed, 79 functional DUB enzymes had been identified from previous literature and bioinformatics searches (Nicholson *et al.*, 2007). To date, approximately 100 DUB enzymes have been identified (Frappier and Verrijzer, 2011), and this highlights how quickly the field of DUB research is expanding. We targeted 72 of the 90 known DUBs based on those which were thought to have intrinsic DUB activity. However, the recent demonstration that the E3 ubiquitin ligase Siah2 is repressed by the DUB enzyme USP13 independently of deubiquitinase activity indicates that DUB enzymes have additional mechanisms of action that are independent of their catalytic activity (Scortegagna *et al.*, 2011). Therefore, analysis of the DUBs that lacked the catalytic domain and were excluded from the initial screen may provide additional enzymes that regulate AR activity or stability.

siRNA screens have a number of limitations. siRNA by nature is unstable and the effects of knockdown can be transient. Some studies have used vector driven short hairpin RNA (shRNA) based approaches to alleviate these issues (Nijman *et al.*, 2005a; Stegmeier *et al.*, 2007). Luckily, USP12 knockdown proved to be very stable, lasting at least 120 hours with siRNA in LNCaP cells. Another problem with siRNA is the possibility of off-target effects due to partial homology of the sense strand with other cellular mRNA. Newer siRNA technologies have been developed to minimise this by chemical modification of the sense strand to prevent its interaction with the RISC

complex such as Dharmacon ON-TARGET *plus* siRNA oligonucleotides. Additionally, chemical modification of the anti-sense strand has been shown to increase specificity (Dharmacon, 2011).

USP12 was the only DUB of the four investigated in this Chapter to be fully validated. USP12 was first identified close to chromosomal translocation breakpoints in leukaemia (Hansen-Hagge *et al.*, 1998). More recently, USP12 was implicated in the development of *Xenopus laevis*, by regulating histone H2A and histone H2B ubiquitination that controls transcription of developmentally important genes (Joo *et al.*, 2011). To date USP12 has not been associated directly with any disease state. USP12 is investigated and discussed in more detail in Chapter 4.

In conclusion, USP12 has been identified and validated as a DUB that effects AR transcriptional activity. Knockdown of *USP12* causes a reduction in *PSA* mRNA expression at almost all time-points over the course of 72 hours DHT stimulation. Levels of USP12 knockdown are substantial with depleted expression being maintained at 30% throughout the 120 hour duration of this experiment. The effect of *USP12* knockdown on AR activity reduces both *PSA* mRNA and protein levels. Likewise, USP12 depletion has effects on expression of AR-regulated genes outside of the kallikrein family, as similar effects were seen in all the other genes analysed. Validation of USP29 and USP50 proved problematic but further validation experiments, at the PSA expression level, could be used to elucidate whether these are true regulators of AR activity.

Chapter 4: Characterisation of USP12 as a deubiquitinase involved in androgen receptor regulation

4.1 Introduction

Deubiquitinase enzymes (DUBs) fall into five specific sub-families based on structure and catalytic mechanism (Amerik and Hochstrasser, 2004). The largest and most diverse of these families are the ubiquitin-specific processing proteases (USPs) with 58 USPs having been identified to date (Ventii and Wilkinson, 2008). The USP family are defined as cysteine proteases and consist of two conserved domains, the cysteine (Cys) box and the histidine (His) box, that contain conserved Cys, His and aspartic acid (asp) residues, referred to as the triad residues, and are critical for enzymatic activity (Nijman *et al.*, 2005b). Unlike other proteases, structural analysis has revealed that the USP enzymes are non-catalytic in the free form, needing ubiquitin binding to induce conformational changes to align the triad residues or to remove bulky loops and domains that occlude the active site (Amerik and Hochstrasser, 2004). UCHL3 has an active site loop that blocks the entry of substrates into the active site, but upon recognition of ubiquitin, this loop is restructured into an α-helix and displaced from the active site allowing catalysis (Johnston *et al.*, 1997). This is an important regulatory mechanism that prevents non-appropriate substrate interactions (Amerik and Hochstrasser, 2004).

4.1.1 USP12

USP12 is a relatively uncharacterised member of the USP family. Also known as ubiquitin hydrolysing enzyme 1 (UBH1), it was first identified by Hansen-Hagge *et al.,* whilst sequencing the breakpoints of the t(5;14)(q33-q34;q11) translocation in a subset of leukaemia patients (Hansen-Hagge *et al.*, 1998). The predicted primary sequence of *USP12* was found to be similar to products of the *Caenorhabditis elegans* gene *R10E11.3* and the *Sacchormyces cerevisiae Ubp9* and *Ubp13* genes that are active DUB enzymes (Hansen-Hagge *et al.*, 1998), suggesting that USP12 is an additional DUB protein. Interestingly, regions of sequence homology between the proteins were limited to within the catalytic Cys and His boxes consistent with USP12 being potentially catalytically active; although at just 370 amino acids it is the smallest member of the USP family of enzymes (Komander *et al.*, 2009a). USP12 is postulated to be a nuclear protein due to the presence of two nuclear localisation signals between amino acids 74-77 and 96-112 but this has not been confirmed (Hansen-Hagge *et al.*, 1998). USP12 is evolutionarily conserved within vertebrates; mouse Usp12 shows

98.3% amino acid homology to human USP12 (Baek *et al.*, 2002). Interestingly, USP12 is highly homologous to the 366 amino acid DUB enzyme, USP46 (Cohn *et al.*, 2009). Due to the high degree of homology and comparable size of the two proteins, distinguishing between them at the protein level is highly difficult, with antibodies generated detecting both DUBs (Cohn *et al.*, 2009).

4.1.1.1 USP12-interacting proteins

In vitro deubiquitination assays utilising turn-over of the fluorogenic ubiquitin conjugated 7-amido-4-methyl-coumarin (AMC) revealed that USP12 possesses very little DUB activity (Cohn *et al.*, 2009). Studies have shown that interaction with various WD40-repeat containing proteins, such as UAF1 and WDR20, facilitate and enhance USP12 enzymatic activity (Cohn *et al.*, 2009; Kee *et al.*, 2010). Cohn *et al.,* firstly reported the presence of USP12 in complex with the deubiquitinase regulatory protein UAF1, a protein that contains eight WD40-repeats, and showed that USP12 activity towards ubiquitin-AMC was markedly increased upon addition of UAF1 in *in vitro* deubiquitination assays. Importantly, loss of any one of the WD40-repeats was sufficient to compromise the UAF1-USP12 interaction, implicating the dependency of these domains for complex formation (Cohn *et al.*, 2009). Furthermore, addition of WDR20, an uncharacterised five WD40-repeat containing protein, was shown to further enhance the DUB activity of USP12 in a UAF1-dependent manner; interacting with both USP12 and UAF1, but requiring UAF1 to further enhance USP12 activity. In contrast to UAF1, however, only the 2nd WD40-repeat of WDR20 was shown to be indispensable for USP12 enzymatic activity (Kee *et al.*, 2010). The most recent USP12 study identified DMWD, WDR26 and WDR77 as low abundance interacting proteins, in addition to confirming the interactions with UAF1 and WDR20 (Joo *et al.*, 2011). Reciprocal immunoprecipitation (IP) and mass spectrometry analysis identified Akt phosphatases PHLPP and PHLPPL as interacting partners of USP12 (Sowa *et al.*, 2009), although, Joo *et al*., did not identify PHLPP or PHLPPL in their study (Sowa *et al.*, 2009; Joo *et al.*, 2011).

4.1.1.2 USP12 and regulation of ubiquitination

Cohn *et al.,* and Kee *et al.,* both investigated USP12 in the context of Fanconi anaemia (FA) (Cohn *et al.*, 2009; Kee *et al.*, 2010). FA is an autosomal recessive disorder characterised by defects in DNA repair and chromosome instability leading to

hypersensitivity to DNA cross-linking agents and a predisposition to cancer (Jacquemont and Taniguchi, 2007). Approximately 13 proteins co-operate in the FA DNA repair pathway and mutation or loss of any can lead to FA (Jacquemont and Taniguchi, 2007). FANCD2, a DNA repair protein important in the FA pathway, is regulated by mono-ubiquitination. Deubiquitination of FANCD2 by the USP1-UAF1 complex is essential for its DNA repair function. Loss of USP1 leads to an accumulation of mono-ubiquitinated FANCD2 and a hypersensitivity of cells to DNA cross-linking agents (Jacquemont and Taniguchi, 2007; Cohn *et al.*, 2009). Stable knockdown of USP1 and UAF1 in HeLa cells showed an accumulation of mono-ubiquitinated FANCD2 whilst knockdown of USP12 had no effect (Cohn *et al.*, 2009). As WDR20 specifically regulates the activity of USP12-UAF1, Kee *et al*., hypothesised that depletion of WDR20 would also have no effect on FANCD2 mono-ubiquitination status. Transient siRNA knockdown of *WDR20* in HeLa cells had no effect on FANCD2 ubiquitination confirming the author's hypothesis (Kee *et al.*, 2010).

Joo *et al.,* provided the first insight into the functional role of USP12 in early embryonic development of *Xenopus Laevis*. Ubiquitination of histones H2A and H2B are markers of transcriptional silencing and activation, respectively, although more recent reports have suggested that this is context specific and both marks facilitate both outcomes (Weake and Workman, 2008; Joo *et al.*, 2011). In this study, USP12 was shown to deubiquitinate histones H2A and H2B at certain developmentally important gene promoters to regulate expression of these genes. Specifically, upon USP12 depletion, ubiquitinated histone H2A levels were decreased whilst ubiquitinated histone H2B levels were increased at the promoter region of the mesodermal fate marker gene *Xbra*, resulting in reduced expression of *Xbra*, suggesting USP12 regulates mesodermal cell fate determination in the early *Xenopus* embryo. The *Hox* genes, encoding transcription factors which control the positional transcription of genes involved in determination of the structure and orientation of an organism, are expressed during early development and are regulated by histone modifications including ubiquitination of histones H2A and H2B (Wang *et al.*, 2004; Zhu *et al.*, 2005; Barber and Rastegar, 2010). Although USP12 affected histone ubiquitination at the *Xbra* promoter, the authors found no effect of USP12 on *Hox* gene expression (Joo *et al.*, 2011). Finally, both knockdown and over-expression of USP12 caused major physical defects in the

Xenopus embryo including an inability of the embryo to undergo complete gastrulation or formation of the three germ layers.

4.1.1.3 USP12 as a potential AR co-regulator

USP12 was identified using a siRNA library screen in LNCaP cells as discussed in Chapter 3. *USP12* knockdown consistently reduced *PSA* mRNA and protein expression in addition to down-regulating expression of all other AR regulated genes studied (Figure 3.10 and Figure 3.15). *USP12* was shown not to be an AR target gene, as androgen-treatment and receptor-depletion failed to affect *USP12* gene expression (Figure 3.11). Importantly, *AR* mRNA and protein levels were unaffected by *USP12* knockdown in LNCaP cells (Figure 3.11 and Figure 3.12), strongly suggesting that USP12 is a co-activator of AR transcriptional activity.

4.2 Aims

The cellular function of USP12 remains poorly characterised and it is currently unknown if the enzyme functions as a co-regulator of transcription factor function, including the AR. In addition, there is a major knowledge gap in our understanding of the potential role of USP12 in prostate cancer (CaP). The data highlighted in Chapter 3 suggested that this DUB enzyme is an important regulator of AR activity in CaP cells and this needs to be further investigated.

The specific aims of this Chapter are:

- To further characterise the effect of USP12 on AR transcriptional activity including determining whether the deubiquitinase activity of USP12 is required for AR co-regulation
- To investigate whether *USP12* knockdown impedes AR movement into the nucleus upon DHT stimulation and therefore affecting recruitment of AR to target gene promoters
- To elucidate whether USP12 exerts its effect through a direct interaction with AR
- To investigate whether *USP12* knockdown has any phenotypic effects on LNCaP cells

4.3 Results

4.3.1 USP12 up-regulates the activity of the AR in a deubiquitinase-dependent manner

4.3.1.1 Effect of USP12 knockdown on AR transcriptional activity

Analysis of gene expression in Chapter 3 (Figure 3.10 and Figure 3.15) showed that knockdown of USP12 had a negative impact on all AR regulated genes studied. To confirm that this effect was due to a change in AR transcriptional activity, we exploited the LNCaP-7B7 pPSA cell line that contains a stably integrated pPSA luc reporter which, like endogenous AR regulated genes, is driven by the active receptor.

LNCaP-7B7 pPSA cells were subject to siRNA knockdown for 48 hours in steroid-depleted media in a 24 well plate format, including both SCR and AR siRNAs as negative and positive controls, respectively. Cells were treated with DHT for an additional 48 hours before they were harvested and luciferase reporter activity assayed. Total protein levels, as measured by bicinchoninic acid (BCA) assay, were used to normalise the luciferase activity data. Data is represented as mean fold change compared to the untreated SCR control of three independent experiments.

In the SCR control, DHT stimulation caused a dramatic increase in reporter activity (94.4-fold, P=0.0034) indicating potent AR activity in this cell line (Figure 4.1, compare lanes 1 and 2). As expected, knockdown of *AR* significantly decreased AR activity in DHT-treated, but not un-stimulated LNCaP cells, compared to the SCR control (P<0.001). Importantly, knockdown of *USP12* also caused a reduction in luciferase activity in both DHT-treated and untreated cells. Basal, un-stimulated AR activity was reduced to 0.4-fold compared to the SCR control (Figure 4.1, compare lanes 1 and 5), and upon addition of DHT, luciferase expression was 45% less than the androgen-treated SCR control (Figure 4.1, compare lanes 2 and 6). This result was not statistically significant (P=0.057) but does support the previous data from Chapter 3.

In addition, supporting evidence for the effect of *USP12* knockdown resulting in reduced LNCaP cell proliferation, as represented by reduced α-tubulin levels presented in Figure 3.13, the *USP12* knockdown reduced the BCA measurements by approximately 20% compared to the SCR control in the absence of DHT (Table 4.1). As observed in Figure 3.12, α-tubulin levels increased with addition of androgen even

with *USP12* knockdown, and this is also observed in the BCA measurements as, although still reduced, protein levels increased to approximately 10% below the SCR (-DHT) control (Table 4.1). AR knockdown also reduced BCA readout in both the presence and absence of DHT by approximately 20%, supporting its pro-proliferative function and the reduced α-tubulin levels also observed with AR knockdown in Figure 3.12 (Table 4.1).

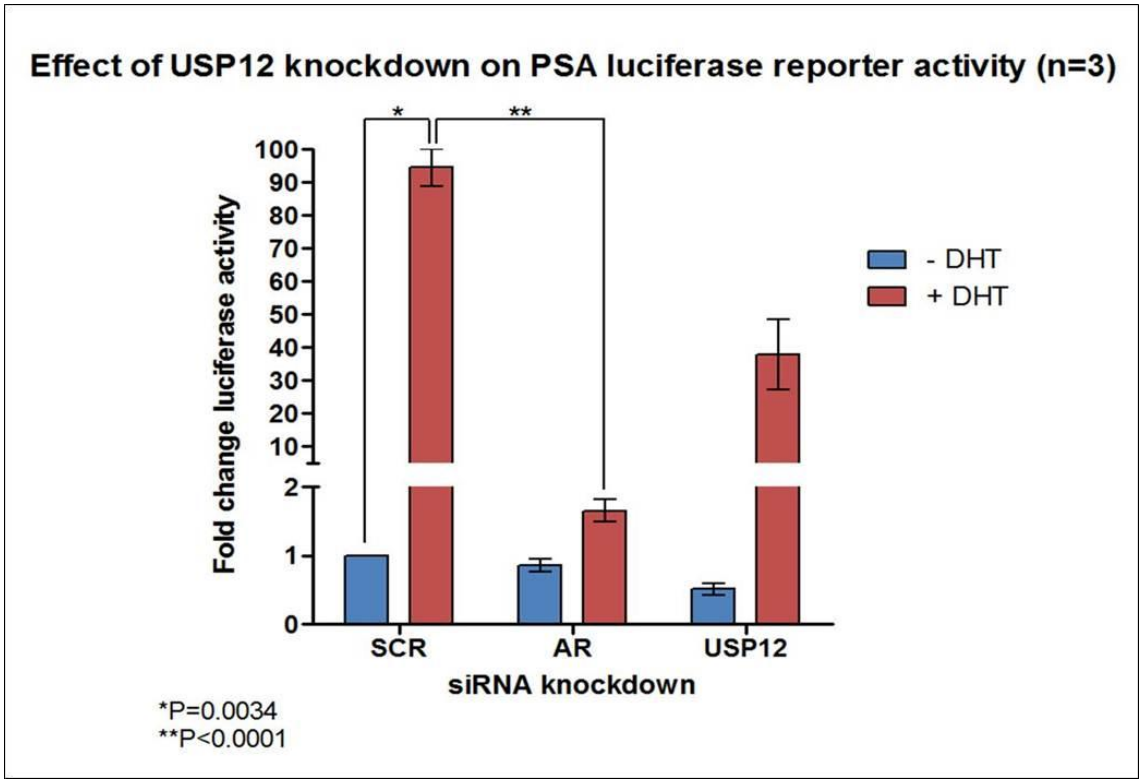

Figure 4.1. Effect of USP12 knockdown on luciferase reporter activity in the LNCaP-7B7 pPSA cell line.

LNCaP-7B7 pPSA cells were subject to knockdown in steroid-depleted media for 48 hours prior to 48 hour treatment with 100 nM DHT. Scrambled (SCR) and androgen receptor (AR) siRNA were used as negative and positive controls, respectively. Data is normalised to the total protein as measured by bicinchoninic acid (BCA) assay and is represented as fold change in luciferase reporter activity compared to the untreated SCR control. Data is the mean of three independent repeats and error bars show the standard error between the experimental replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk.

	Fold change in BCA readout	
siRNA knockdown	- DHT	+ DHT
SCR	1.0 ± 0.00	1.16 ± 0.01
AR	0.82 ± 0.05	0.83 ± 0.06
USP12	0.76 ± 0.08	0.89 ± 0.03

Table 4.1. Effect of USP12 knockdown on total protein levels in the LNCaP-7B7 pPSA cell line.

Total protein levels were measured by bicinchoninic acid (BCA) assay to allow for normalisation of the luciferase reporter assays. Data is the mean of three independent experiments and is represented as fold change compared to the scrambled (SCR) – DHT control. Data represents the mean of three independent repeats ± standard error.

4.3.1.2 Generation of p3xFLAG-CMV-10™-USP12 mammalian expression vector

The above result suggested that in the normal cellular context USP12 may be acting as a co-activator of AR transcription. In order to confirm this, additional luciferase assays using ectopic *USP12* expression were undertaken. For these experiments, a USP12 mammalian expression vector was required to permit production of the enzyme in cell line models utilised for luciferase assays, including the osteosarcoma cell line, U2OS and the monkey kidney cell line, COS-7. In addition, given that USP12 antibodies are unable to detect both the endogenous and ectopic enzyme (see below); a tagged version of the enzyme was required to permit immunoprecipitation and Western analysis using tag-specific antibodies.

Initially, the pDEST-FLAG-HA-USP12 vector was purchased from Addgene (Plasmid 22567) (Sowa *et al.*, 2009), and sequence analysis confirmed that the full *USP12* protein coding sequence was present. However, upon transfection into COS-7 cells, Western analysis using both HA and FLAG-specific antibodies was unable to detect HA- (Figure 4.2a, lane 3) or FLAG-tagged USP12 protein (predicted size of tagged USP12 is 52 kDa) (Figure 4.2b, lane 3) suggesting that the purchased vector was possibly out of frame. Importantly, HA-tagged ubiquitin and FLAG-tagged AR were specifically detected by the respective HA and FLAG antibodies (Figure 4.2a and b, lanes 1 and 2). Therefore, a FLAG-tagged USP12 vector was generated using the p3xFLAG-CMV-10™ vector backbone (Sigma-Aldrich).

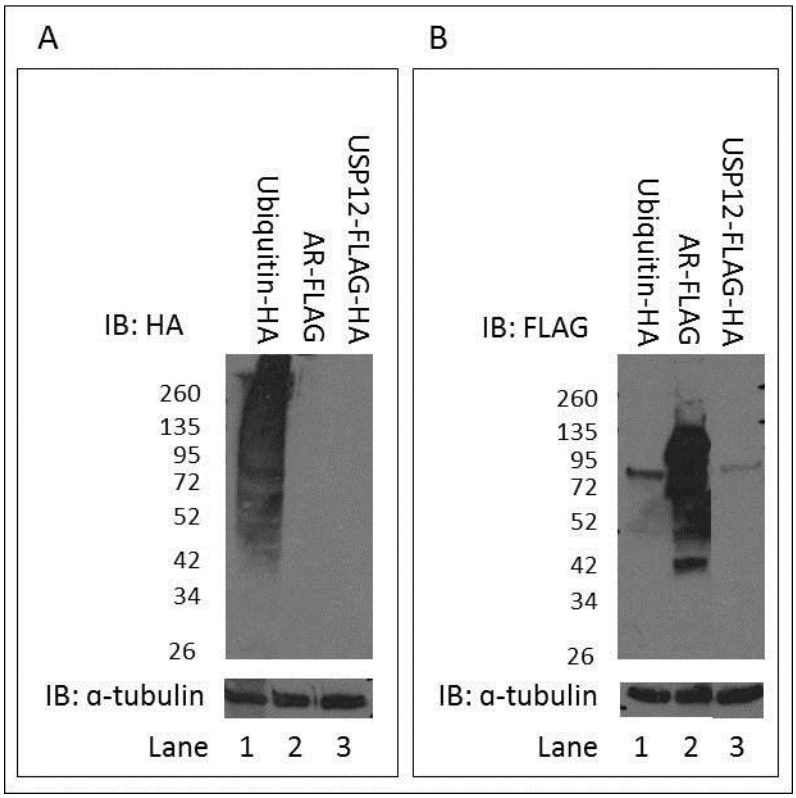

Figure 4.2. Anti-HA and anti-FLAG antibodies do not detect ectopically expressed USP12 in COS-7 cells.

Lysates from Lysates from pDEST-FLAG-HA-USP12-transfected COS-7 cells were analysed by Western blotting with anti-HA and anti-FLAG antibodies. HA-tagged ubiquitin and FLAG-tagged androgen receptor (AR) were used as positive controls for the HA and FLAG antibodies, respectively. α-tubulin was used as a loading control.

Specific forward and reverse primers were designed to amplify USP12 from the pDEST-FLAG-HA-USP12 vector by PCR, and contained sites for *Xba* I and *Bam* HI restriction enzymes cleavage. USP12 cDNA was successfully amplified by PCR and sub-cloned into the pCR2.1 vector (Invitrogen) (Figure 4.3a and b). *Xba* I and *Bam* HI restriction enzymes were used to excise the USP12 cDNA from the pCR2.1 vector that was then sub-cloned into the p3xFLAG-CMV-10™ vector. Diagnostic digestion of the recombinant p3xFLAG-CMV-10™-USP12 vectors was achieved using the *Xba* I and *Bam* HI enzymes (Figure 4.3c). Finally, the recombinant vector was sequenced to ensure no point mutations had been introduced during the cloning process. Restriction maps and sequencing details of the vectors generated can be seen in the appendix.

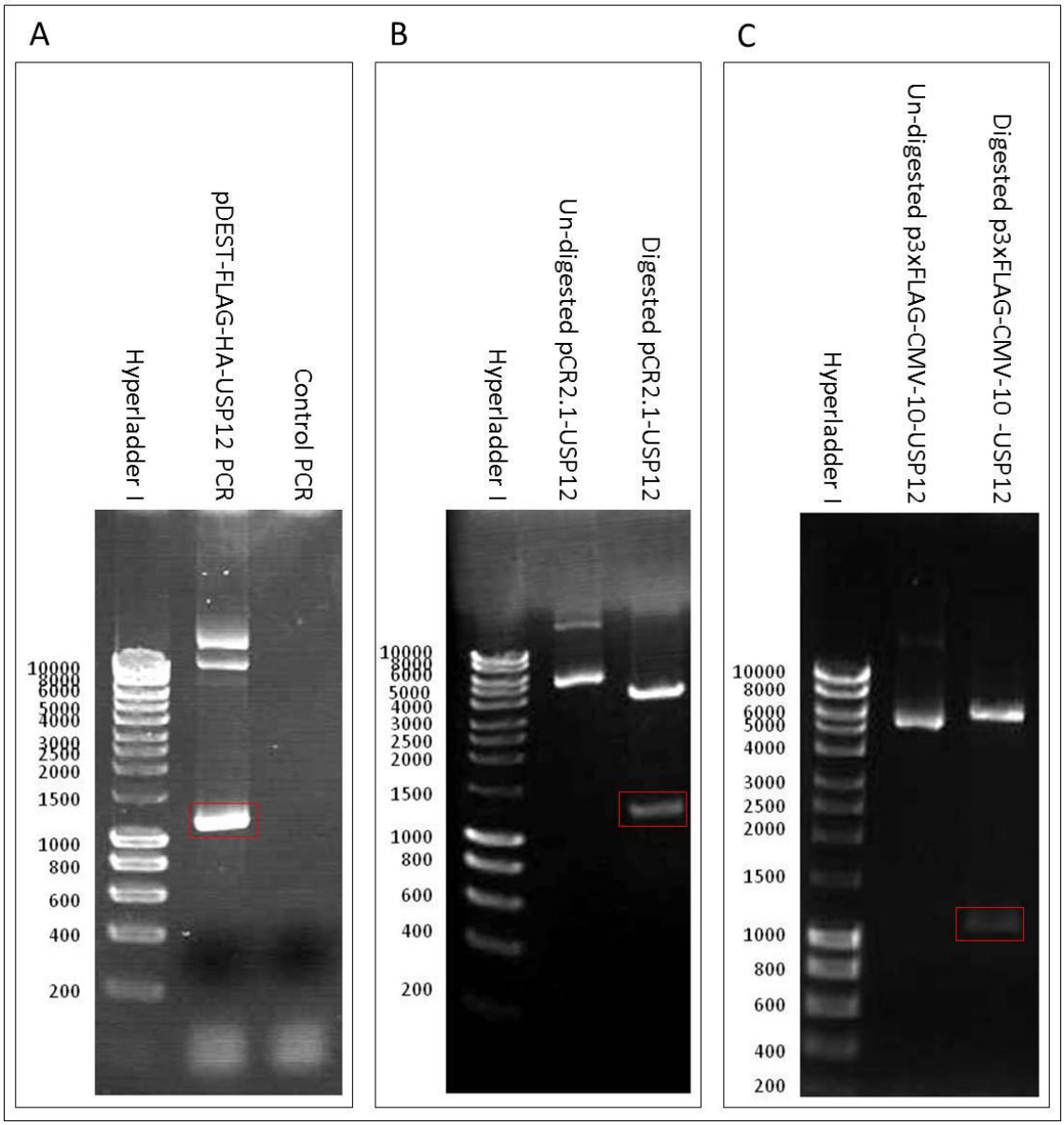

Figure 4.3. Cloning of p3xFLAG-CMV-10™-USP12.

A) USP12 was amplified by PCR from the pDEST FLAG HA USP12 vector (indicated by the product at .10, 000 bp). The PCR product was of the correct predicted size of 1100 bp (indicated by the red box). B) The USP12 PCR product was ligated into the pCR2.1 vector. Positive transformants were digested with Xba I and Bam HI to release the USP12 sequence (indicated by the red box). C) The restriction enzyme digested fragment of USP12 was ligated into the p3xFLAG-CMV-10™ vector via Xba I and Bam HI restriction sites. Recombinant vectors were assessed by digestion with Xba I and Bam HI (indicated by the red box).

To test that p3xFLAG-CMV-10™-USP12 construct expressed the FLAG tagged USP12 protein, it was transiently transfected into COS-7 cells for 48 hours prior to Western blot and QPCR analyses to detect the fusion protein product at the protein and RNA level, respectively.

Figure 4.4a shows detection of the 52 kDa FLAG-tagged USP12 by an anti-FLAG antibody in the p3xFLAG-CMV-10™-USP12 transfected cells, but not in the mock-transfected experimental arm (Figure 4.4a, lane 2). Additionally, QPCR analysis of *USP12* expression showed that 2200-fold more USP12 mRNA was present in the over-expression sample compared to the non-transfected (NT) control (Figure 4.4b), confirming that over-expression of the DUB enzyme is achievable in transient transfection experiments that can be verified by Western and QPCR analyses. In order to verify protein expression, a range of commercially available anti-USP12 antibodies were tested. Unfortunately, none of the antibodies tested detected either endogenous or ectopic USP12 (Figure 4.5), hence Western blotting incorporating the anti-FLAG antibody was used in all subsequent experiments to detect USP12 over-expression.

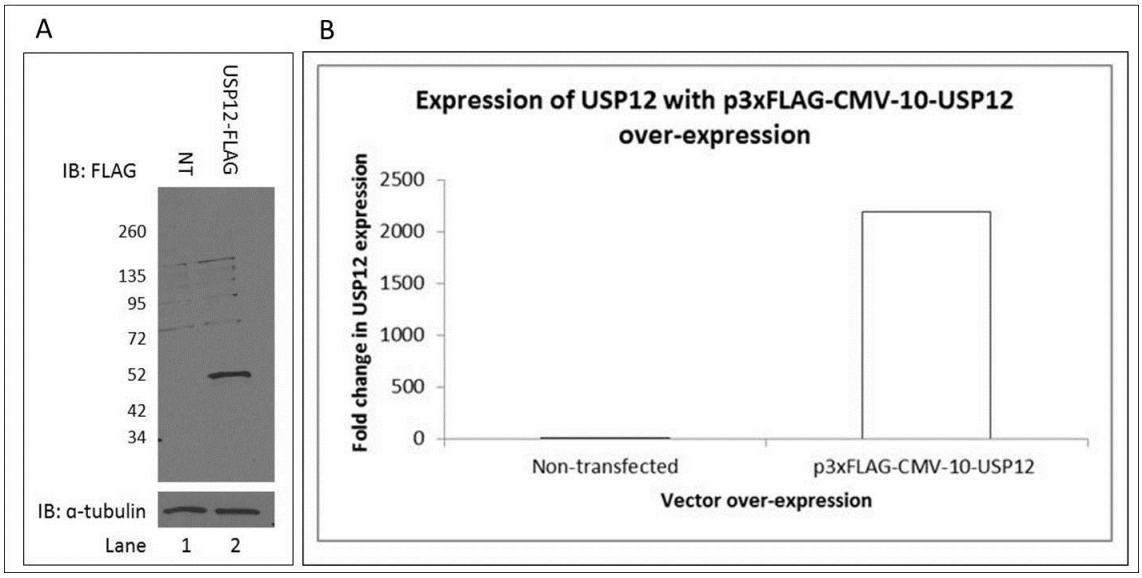

Figure 4.4. Validation of p3xFLAG-CMV-10^{TM}-USP12 over-expression in COS-7 cells.

COS-7 cells were either non-transfected or transfected with either 1 µg p3xFLAG-CMV-10^{TM}-USP12 for 48 hours in serum-containing media. A) Cell lysates were analysed by Western blotting for expression of FLAG-tagged USP12 with anti-FLAG antibodies. α-tubulin was used as a loading control. B) RNA was collected and QPCR analysis for USP12 expression performed.

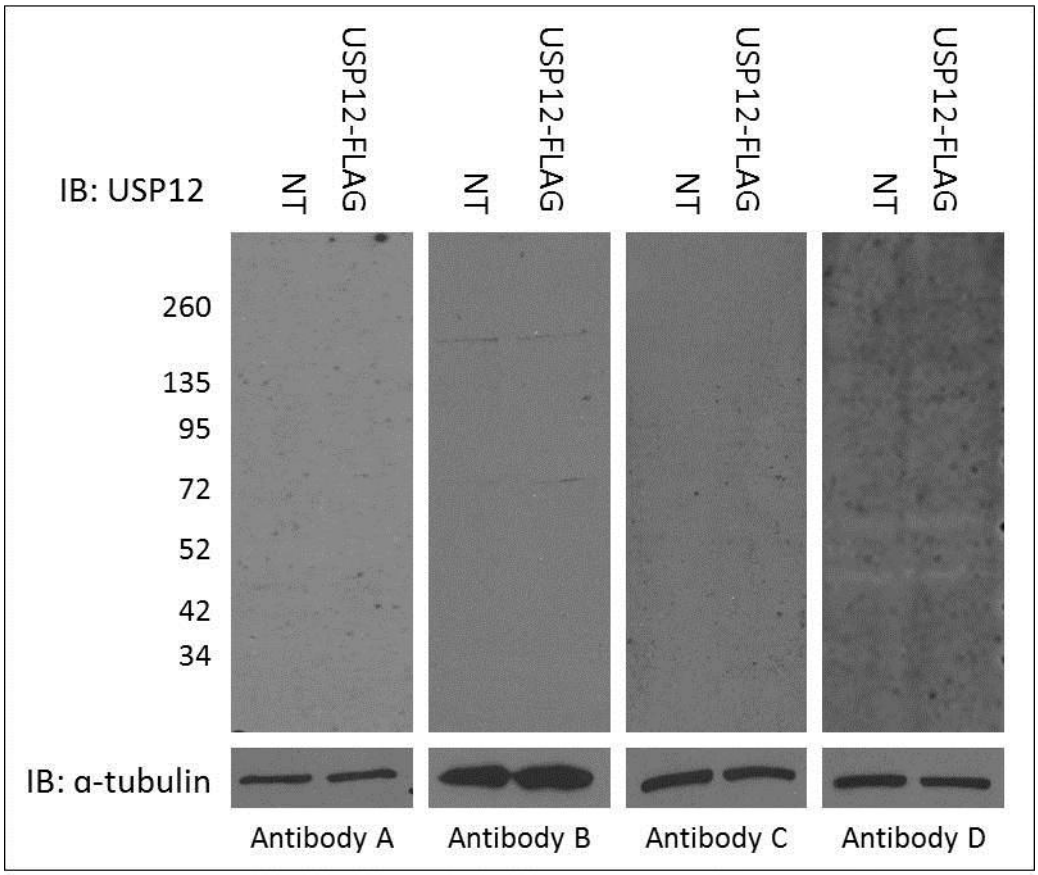

Figure 4.5. Anti-USP12 antibodies do not detect endogenous or ectopically-expressed USP12 protein in COS-7 cells.

Lysates from mock or p3xFLAG-CMV-10™-USP12 transfected COS-7 cells were analysed by Western blotting with four commercially available anti-USP12 antibodies. Antibodies A and B corresponds to ab89870 (Abcam) and SAB1408437 (Sigma-Aldrich), while antibodies C and D are both from Santa Cruz (sc-82072 and sc-82073, respectively). α-tubulin was used as a loading control.

4.3.1.3 Effect of USP12 over-expression on AR transcriptional activity

Given that USP12 knockdown reduces PSA protein levels in LNCaP cells as measured by ELISA, and reduces both PSA mRNA in LNCaP cells and luciferase expression in the LNCaP-7B7 pPSA cells, it was important to assess whether USP12 acted as a co-activator of AR transcriptional activity in luciferase-based reporter experiments. To this end, mammalian expression vectors for AR and USP12, together with an androgen-responsive luciferase reporter, ARE3, were transiently transfected into COS-7 cells, and 72 hours later, luciferase activity was assessed. The ARE3 reporter contains a triplicate repeat of a consensus androgen response element (ARE) upstream of the luciferase gene. Therefore, consistent with the PSA reporter, upon DHT stimulation, the AR binds to the synthetic ARE and drives transcription of the luciferase gene. A constitutively expressed β-galactosidase (β-gal) reporter was also included in the transfection to act as an internal experimental control to measure transfection efficiency. USP12 was transfected at increasing quantities between 25-100 ng, represented as +, ++ and +++, respectively (Figure 4.6a).

COS-7 cells were transfected with 100ng of both ARE3 and β-gal and 50ng of AR in serum-containing media. FLAG-tagged USP was transfected at increasing quantities as described above. Media was replaced 24 hours post-transfection with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for an additional 48 hours. Luciferase activity was assayed and normalised against β-gal activity. Data shows the mean of 3 independent experimental repeats and is represented as fold change compared to AR only control without DHT treatment (Figure 4.6a). Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

As expected, stimulation of AR with DHT caused a 5.3-fold increase in luciferase activity (P=0.032), which is similar to that reported by other members of the Solid Tumour Target Discovery Group (Gaughan *et al.*, 2011) (Figure 4.6a, compare lanes 1 and 2). Importantly, co-transfection of USP12 increased AR-mediated transcription to 7.2-fold and 10.2-fold with 25 ng and 50 ng respectively; although a further increase in the amount of USP12 to 100 ng did not follow this trend (Figure 4.6a). Unfortunately, none of the changes observed with increasing amounts of USP12 were statistically significant.

Western blot analysis revealed that ectopic AR was expressed in each of the samples (Figure 4.6b, upper panel). FLAG-tagged USP12 was not present in the AR only samples as expected (Figure 4.6b, middle panel, lanes 1 and 2) but was detected at increasing levels with increasing amounts of FLAG-tagged USP12 (Figure 4.6b, middle panel, lanes 3–8).

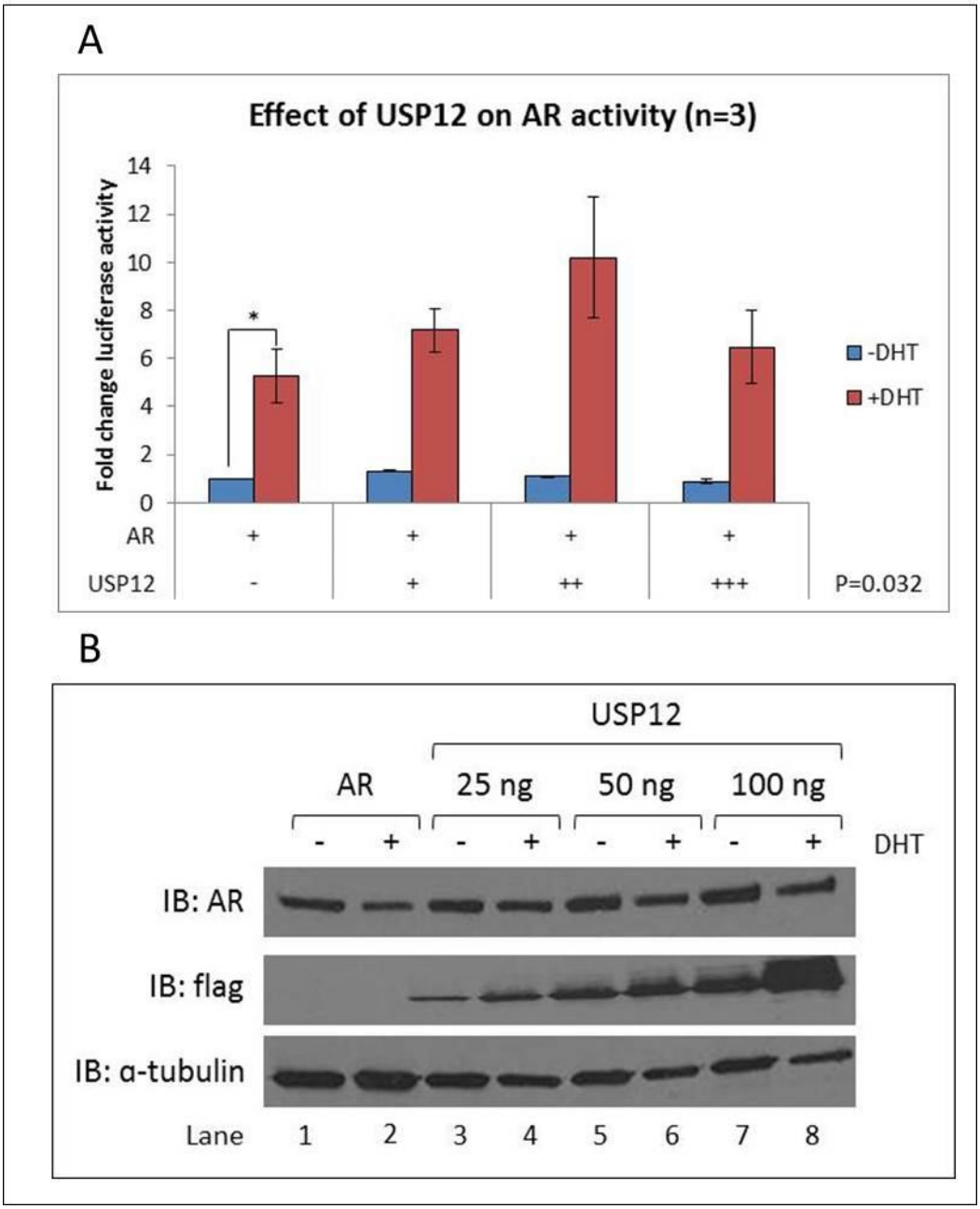

Figure 4.6. Effect of USP12 over-expression on AR transcriptional activity.

COS-7 cells were transiently transfected with 50 ng androgen receptor (AR), 100 ng of an AR-responsive ARE3 luciferase reporter and 100ng of a β-gal reporter. Increasing amounts of p3xFLAG-CMV-10™-USP12 were also introduced; 25 ng (+), 50ng (++) and 100ng (+++). 24 hours after transfection cells were grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is a mean of three independent repeats and error bars represent the standard error of the mean of the three replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk. B) Luciferase assay samples were analysed by Western blotting with anti-AR and -FLAG antibodies to ensure over-expression of ectopically expressed proteins. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats.

4.3.1.4 Effect of USP12$_{C48A}$ mutant on AR transcriptional activity

An important knowledge gap that remains to be addressed is whether the deubiquitinase activity of USP12 is required for AR co-activation. To address this, site-directed mutagenesis of the p3xFLAG-CMV-10™-USP12 vector was employed to convert the catalytic cysteine (Cys) residue at position 48 to an alanine residue C48A mutation) using the primers and methodology described in Table 2.2. Cys48 was reported previously to be important for the deubiquitinase function of USP12 and that mutation of this residue resulted in loss of catalytic activity (Cohn *et al.*, 2009). Sequencing of the vector confirmed that the C48A mutation had been introduced, generating the vector p3xFLAG-CMV-10™-USP12$_{C48A}$.

To assess whether the deubiquitinase activity was required for co-activation of AR transcriptional activity, wild-type and mutant USP12 vectors were transiently transfected into COS-7 cells as above with an AR mammalian expression vector and both ARE3 and β-gal reporters. 50 ng of either wild-type or mutant USP12 vectors were used in this experiment as AR co-activation by the wild-type DUB enzyme is maximal at this amount (Figure 4.6), hence potential differences between co-activation ability of the wild-type versus deubiquitinase-dead mutant would be more pronounced. Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

In keeping with experiments in Figure 4.6, AR activity was induced by addition of DHT, although the absolute fold induction (13.1-fold) was markedly larger than the previous experiment which may be due to a lower total amount of transfected DNA into the cells (Figure 4.7, compare lanes 1 and 2). Androgen-dependent AR activation was further increased, albeit non-significantly, to 20.7-fold by co-transfection of wild-type USP12, confirming the previous data (Figure 4.7, lane 4). Interestingly, ectopic expression of the USP12$_{C48A}$ mutant potentiated a 15.1-fold increase in AR activity over that of AR alone, but this increase was not as great as with wild type USP12. The difference between AR co-activation by wild type USP12 and USP12$_{C48A}$ was not significant, however it does suggest that USP12 deubiquitinase activity is at least in part required for AR co-activation (Figure 4.7, compare lanes 4 and 6).

Western blot analysis revealed that ectopic AR was again expressed in each of the samples (Figure 3.7b, upper panel). FLAG-tagged USP12 was detected only in the

samples with FLAG-tagged wild-type or mutant USP12 over-expression although at a lower level than previously observed (Figure 4.7b).

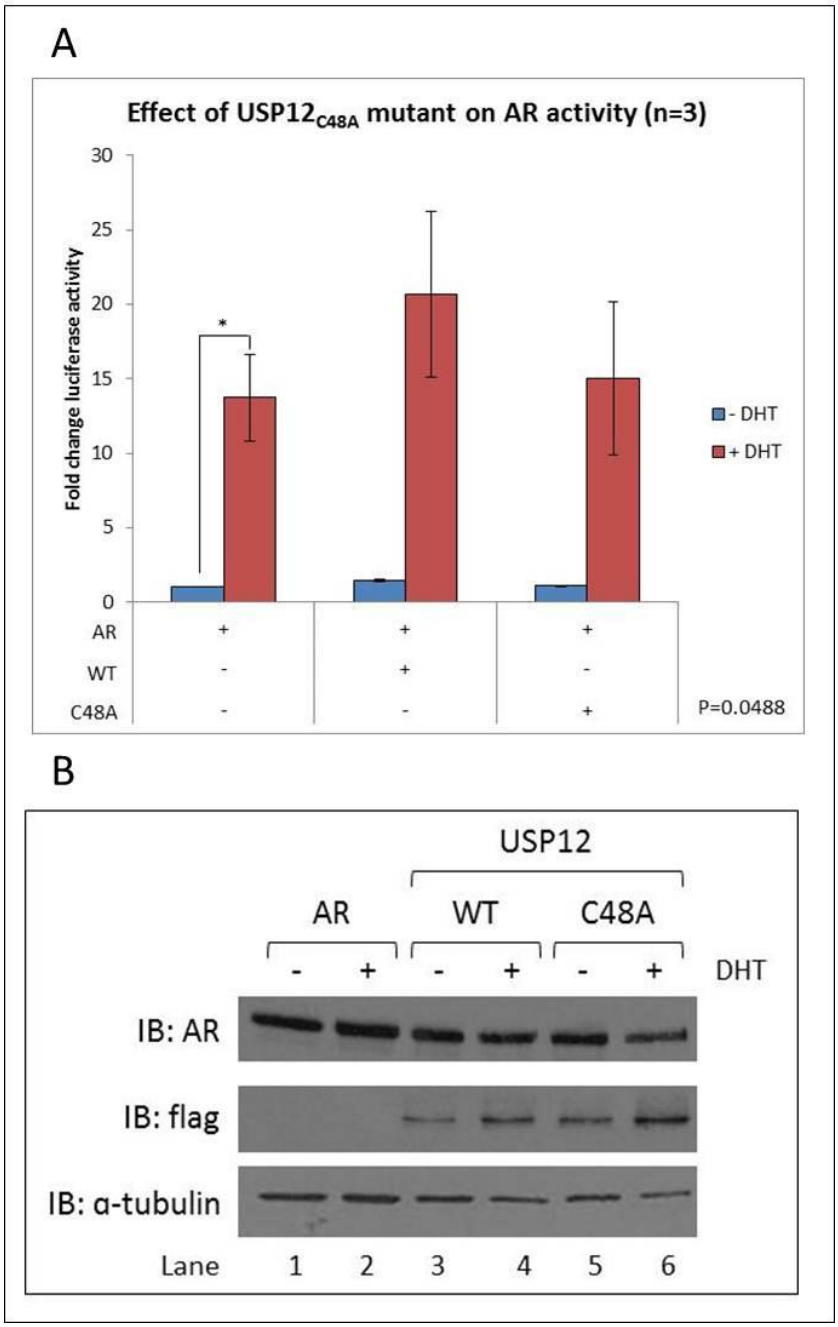

Figure 4.7. Effect of the USP12$_{C48A}$ mutant on AR transcriptional activity.

COS-7 cells were transfected with 50 ng androgen receptor (AR), 100 ng of both ARE3 luciferase and β-gal reporters. 50ng of wild type (WT) or mutant (C48A) USP12 expression vectors were also introduced. 24 hours after transfection, media was replaced with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is a mean of three independent repeats and error bars represent the standard error of the mean of the three replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk. B) Luciferase assay samples were analysed by Western blotting with anti-AR and -FLAG antibodies to ensure over-expression of ectopically expressed proteins. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats.

4.3.2 The effect of USP12 knockdown on AR nuclear translocation

One potential mechanism for USP12-mediated AR co-activation is enhancement of nuclear import of the receptor. To address whether USP12 facilitates ligand-induced AR nuclear shuttling, cytoplasmic-nuclear extractions were performed in LNCaP cells depleted of USP12 by siRNA and AR cellular distribution analysed by Western blotting. LNCaP cells were transiently transfected with either SCR or USP12 siRNA for 48 hours in steroid-depleted media prior to 100 nM DHT stimulation over a time-course of 0-8 hours. Cells were harvested, cytoplasmic-nuclear extractions performed and both fractions analysed by Western blotting using antibodies raised against AR, and both PARP1 and α-tubulin as nuclear and cytoplasmic controls, respectively. Figure 4.8 shows representative Western blots from two individual experimental repeats. Figure 3.9 shows the densitometry analysis of the two experimental repeats and is presented as the mean relative protein expression normalised to the respective loading control. Data was analysed for statistical significance using 2-way ANOVA, however no data points were found to be significant.

As expected, in the SCR control LNCaP cells, nuclear translocation of the AR occurred within 30 minutes of DHT stimulation and increased markedly by 8 hour ligand treatment, while cytoplasmic levels of receptor were unchanged (Figure 4.8a and b, upper panel). The dramatic fluctuation of AR levels in the nuclear fraction of the SCR control between 1 and 2 hours DHT treatment can be accounted for, in part, by unequal loading shown by the PARP-1 loading control (Figure 4.8b, lower panel, compare lane 3 with lanes 4 and 5), further repeats of this experiment and more equal loading of the Western blots would rectify this.

The reduction in cytoplasmic AR in the USP12-depleted LNCaP cells over the duration of the DHT time-course, can be accounted for by reduced total protein loading as evidenced by lower α-tubulin levels, particularly at the 4 and 8 hour time-points (Figure 4.8c, upper and middle panels). Comparing nuclear AR levels between SCR and USP12 knockdown samples indicated an increased level of the receptor in un-stimulated USP12-depleted cells that increased further after 30 minutes androgen treatment (Figure 4.8b and d, upper panels, lane 1 and 2). In addition, in contrast to the control, nuclear AR levels after 8 hour DHT treatment in the USP12-depleted cells was markedly lower, suggesting a potential role of the deubiquitinase in nuclear export

of the receptor, or stabilising active AR in the nucleus (Figure 4.8d, upper panel, lane 6). Importantly, no cytoplasmic contamination was observed in the nuclear fraction nor was any nuclear contamination observed in the cytoplasmic fraction (Figure 4.8, middle and lower panels).

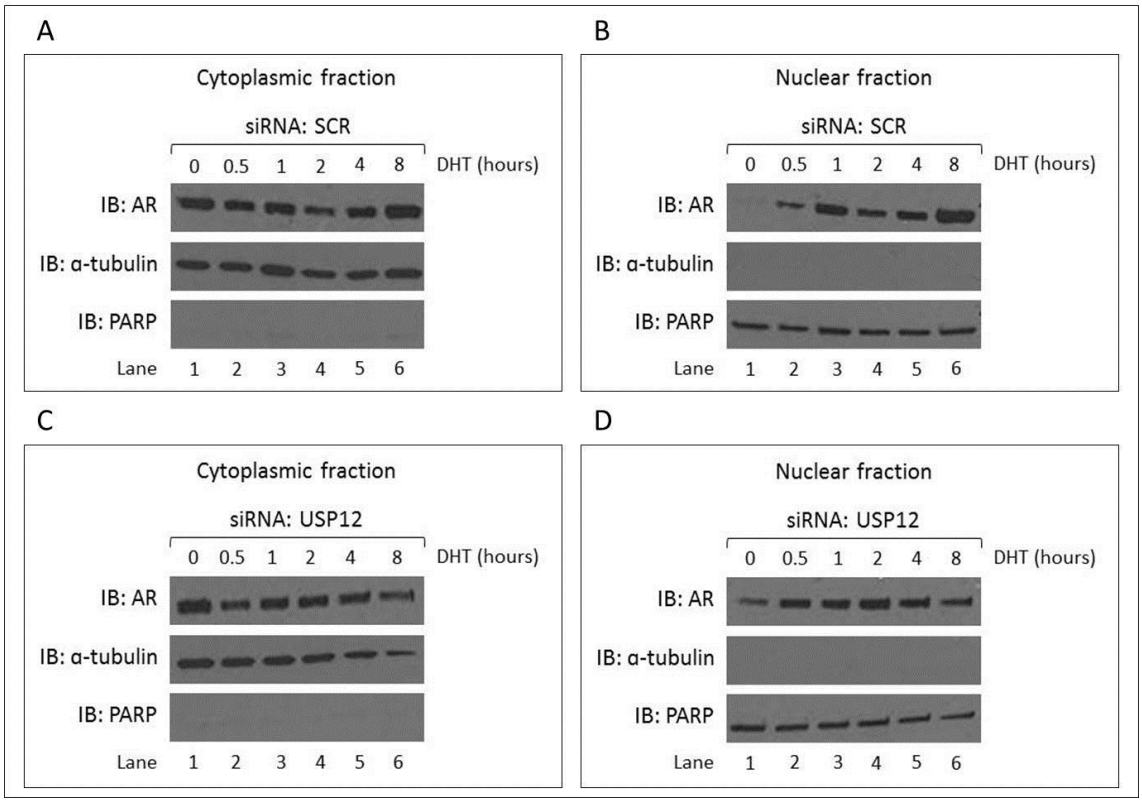

Figure 4.8 Effect of USP12 knockdown on AR translocation to the nucleus upon DHT stimulation.

LNCaP cells were subject to knockdown of USP12 for 48 hours in steroid-depleted media. Scrambled (SCR) siRNA was used as a negative control.100 nM DHT was applied to cells for up to 8 hours. Cells were harvested and cytoplasmic and nuclear extractions performed. AR movement was analysed by Western blotting with anti-AR antibodies. α-tubulin and PARP-1 were used as cytoplasmic and nuclear controls. Western blots are representative of two independent experimental repeats. A) The effect of SCR siRNA on cytoplasmic AR protein levels over 8 hours DHT treatment. B) The effect of SCR siRNA on nuclear AR protein levels over 8 hours DHT treatment. C) The effect of USP12 knockdown on cytoplasmic AR protein levels over 8 hours DHT treatment. D) The effect of USP12 knockdown on nuclear AR protein levels over 8 hours DHT treatment.

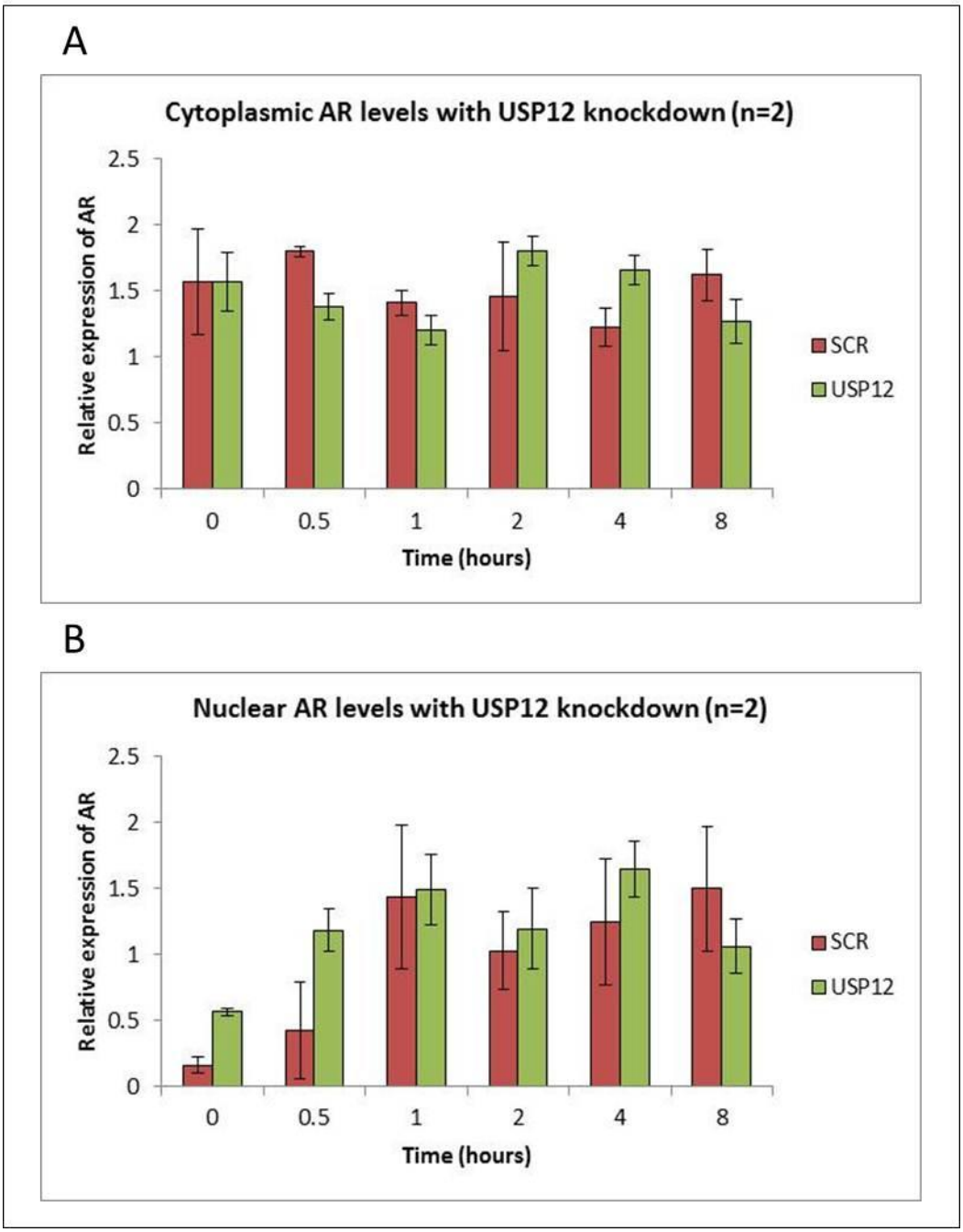

Figure 4.9. Densitometry analysis of the effect of USP12 knockdown on AR translocation to the nucleus upon DHT stimulation.

Western blot data from the cytoplasmic and nuclear extraction experiments was analysed using the Quantity one programme on the GelDoc™ system. Data for androgen receptor (AR) protein levels were normalised to the respective loading control data, α-tubulin in the cytoplasmic samples and PARP1 in the nuclear samples. Data is presented as the mean relative expression of two independent experimental repeats. Error bars represent the standard error. A) The effect of USP12 knockdown on cytoplasmic AR protein levels over 8 hours DHT treatment. B) The effect of USP12 knockdown on nuclear AR protein levels over 8 hours DHT treatment.

4.3.3 USP12 knockdown reduces AR recruitment to target gene promoters

USP12 has been linked directly to transcription by its function in histone deubiquitination. Transcription of developmentally important genes in *Xenopus laevis* was affected by USP12 deubiquitination of histones H2A and H2B (Joo *et al.*, 2011). Furthermore, given that many AR co-regulators, such as SET9, p300 and TIP60, also affect histone post-translational modifications and regulate AR association at target gene promoters (Gaughan *et al.*, 2002; Gaughan *et al.*, 2005; Chmelar *et al.*, 2007; Gaughan *et al.*, 2011), it was hypothesised that given that USP12 depletion reduced AR-target gene expression, USP12 may facilitate AR association with its target gene promoter elements. To assess whether *USP12* knockdown affects AR recruitment to androgen-responsive genes, chromatin immunoprecipitation (ChIP) was performed using an anti-AR antibody in LNCaP cells subject to SCR or *USP12* knockdown for 72 hours in steroid depleted media prior to 120 minute DHT stimulation. SCR siRNA was used as a negative control. Cells were fixed, lysed and subjected to ChIP as described in Chapter 2.7. Resultant DNA was subject to QPCR using primers specific to the AREs within the proximal promoter (ARE I) and enhancer (ARE III) of the *PSA cis*-regulatory elements. Results are the mean of three independent repeats of the entire ChIP procedure, including triplicate PCR reactions per sample, per ChIP, and are represented as percentage of input. Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure. This work was performed by Dr Luke Gaughan.

Figure 4.10a displays the result for AR recruitment to ARE I of the proximal *PSA* promoter. As expected, AR was absent at ARE I without DHT treatment in the SCR control, and in response to hormone, showed an approximate 5-fold increase in promoter association (Figure 4.10a, compare lanes 1 and 2), although this was not statistically significant possibly due to intra-experimental variation. Upon USP12 depletion, there was a slight increase in AR association to the inactive *PSA* promoter (Figure 4.10a, compare lanes 1 and 3), but this is not statistically significant, while DHT treatment failed to facilitate AR promoter binding to ARE I, although, again this was not statistically significant, suggesting USP12 may not be important for regulating receptor recruitment to the proximal PSA promoter (Figure 4.10a, compare lanes 2 and 4).

Figure 4.10b shows the recruitment of AR to ARE III of the *PSA* promoter. As reported previously DHT-stimulated AR recruitment to the enhancer was greater than observed at the proximal ARE I region (Shang *et al.*, 2002; Gaughan *et al.*, 2011), with an approximate 6.5-fold increase over IgG background levels compared to 2.5-fold, respectively (compare Figure 4.10a lane2 with Figure 4.10b lane 2). AR association at the ARE III element was 5.9-fold greater in the androgen-stimulated SCR control than those without stimulation, which was statistically significant (P=0.0495) (Figure 4.10b, compare lanes 1 and 2). Importantly, consistent with data from ARE I, *USP12* knockdown reduced androgen-stimulated AR recruitment to ARE III by approximately 60%, indicating that USP12 may potentiate AR-promoter association at androgen-responsive genes, although this decrease was not significant (Figure 4.10b, compare lanes 2 and 4). Given that total AR levels do not change in response to *USP12* knockdown, the effect of depletion of the deubiquitinase on receptor-chromatin association is not a consequence of reduced AR protein levels. However, to provide a more robust validation for the effects of *USP12* knockdown in AR-target gene association, it would be necessary to assess AR recruitment to additional androgen-responsive genes, such as *KLK2* and *TMPRSS2*.

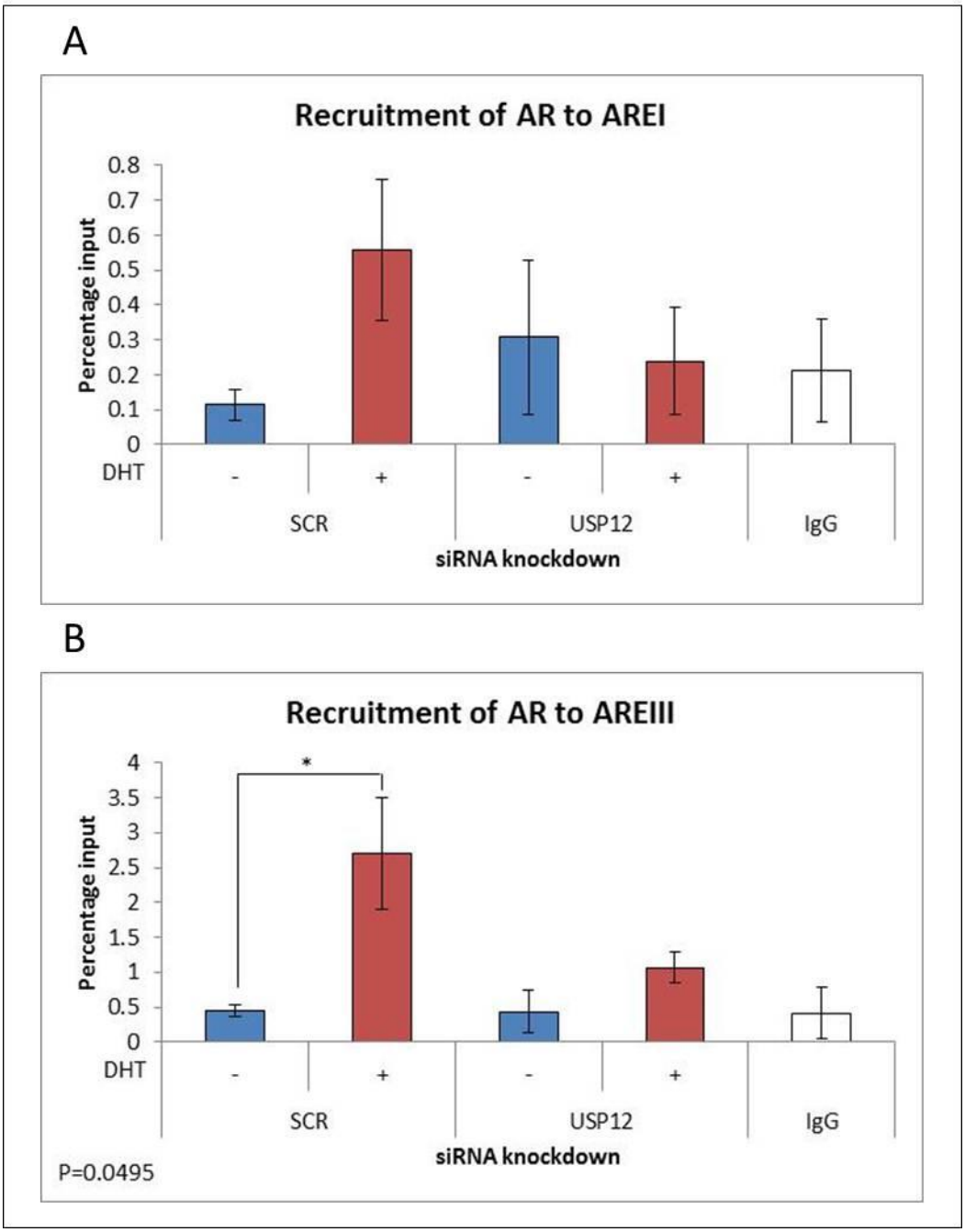

Figure 4.10. Effect of USP12 knockdown on AR recruitment to androgen-response elements upstream of the PSA gene.

LNCaP cells were subjected to USP12 knockdown for 72 hours in steroid-depleted media. 10 nM DHT was applied for 120 minutes before cells were harvested and ChIP for AR or control IgG performed. Scrambled (SCR) siRNA was used as a negative control. Data is represented as percentage of input and is the mean of three independent experimental repeats. Error bars show the standard error of the mean of the three replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk. A) The effect of USP12 knockdown on recruitment of AR to the AREI of the PSA promoter. B) The effect of USP12 knockdown on recruitment of AR to the AREIII of the PSA enhancer. This work was performed by Dr Luke Gaughan.

4.3.4 USP12 interacts with the AR

The above data suggest a role for USP12 in AR gene transcription as a receptor co-activator. It is intriguing to speculate that USP12 and the AR interact in CaP cells, given that USP12 impacts on AR-mediated transcription and chromatin association. Presently, there is no evidence to suggest that USP12 interacts with any members of the steroid hormone receptor family thus providing a novel angle of study that may strengthen our hypothesis that USP12 is a genuine co-regulator of the AR.

To address this, FLAG-tagged USP12 and AR were over-expressed in COS-7 cells in serum-containing media for 48 hours prior to lysis and immunoprecipitation with either AR or FLAG antibodies. 10% of the total cell lysate was taken as an input to verify that the ectopically expressed proteins were indeed over-expressed. The 'sample' underwent the entire IP procedure and was incubated with anti-AR antibodies whilst the 'extract' acted as a control for non-specific interactions between proteins in the cell lysate and the protein-G sepharose, and thus did not receive antibody. Resultant IP samples were analysed by SDS-PAGE and Western blotting using anti-AR and anti-FLAG antibodies.

As shown in Figure 4.11, the input sample confirmed over-expression of both AR and FLAG-tagged USP12, as detected by AR and FLAG antibodies, respectively (Figure 4.11, upper and lower panels, lane 1). IP using the anti-AR antibody successfully immuno-precipitated the receptor (Figure 4.11, lower panel, lane 2) and immuno-blotting with an anti-FLAG antibody revealed the presence of FLAG-tagged USP12 exclusively in the AR immuno-complex, indicating that the receptor and USP12 interact (Figure 4.11, upper panel, lane 2). Importantly, no non-specific protein or antibody binding was observed in the extract or antibody only controls, respectively (Figure 4.11, upper and lower panels, lanes 3 and 4). To confirm this finding in a more physiological setting, it would be beneficial to repeat this experiment using endogenously expressed AR and USP12. However, due to difficulties with the currently available USP12 antibodies for Western analysis, as described in Figure 4.5, this is currently unachievable.

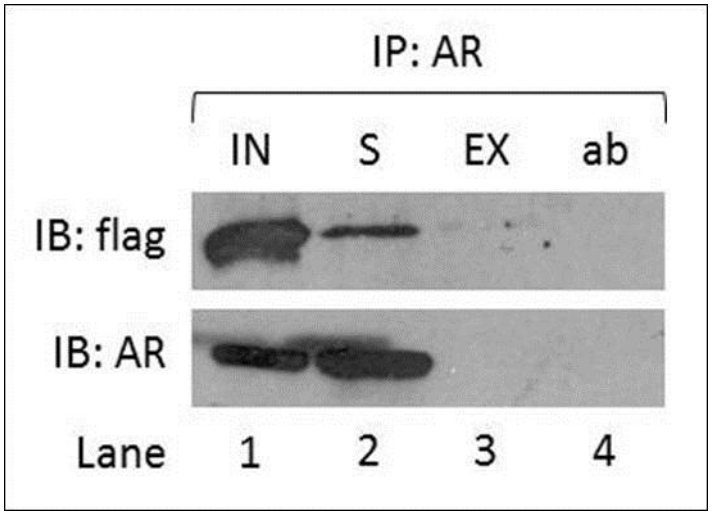

Figure 4.11. USP12 interacts with AR in COS-7 cells.

COS-7 cells were transfected with androgen receptor (AR) and FLAG-tagged USP12 mammalian expression vectors for 48 hours in serum-containing media. Cells were harvested and immunoprecipitation (IP) performed using an anti-AR antibody. Inputs (IN) were taken before IP to ensure that both proteins were over-expressed. Samples (S) were incubated with anti-AR antibodies before incubation with PGS, while extract (EX) samples were incubated with PGS only. The antibody only control (ab) contained only lysis buffer mock immuno-precipitated with anti-AR antibodies. Western blots of the resultant AR immuno-complexes and input samples using anti-AR and anti-FLAG antibodies are representative of three independent experimental repeats.

4.3.5 Phenotypic effects of USP12 knockdown in LNCaP cells

4.3.5.1 Effect of USP12 knockdown on LNCaP cell proliferation/viability

In Figure 3.12 a reduction in the total amount of protein present after *USP12* knockdown in LNCaP cells, as demonstrated by an α-tubulin Western blot was shown, suggesting that the proliferation and/or viability of LNCaP cells is potentially reduced upon USP12 depletion. Given that USP12 facilitates AR activity in LNCaP cells, as demonstrated in Figure 4.1, and that the AR is pro-proliferative, it was hypothesised that USP12 may have a role in driving CaP cell proliferation or protecting CaP cells from apoptosis. To investigate this, sulphorhodamine B (SRB) cell proliferation assays were employed in LNCaP cells previously subjected to SCR or *USP12* siRNA knockdown for 96 hours in serum-containing media prior to SRB staining. Additionally, *AR* siRNA knockdown was included as a positive control for effects on androgen-dependent cell proliferation/viability. Data presented in Figure 4.12 is the mean of three independent repeats and is represented as fold change compared to the SCR control. Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

As shown in Figure 4.12, *AR* knockdown significantly reduced LNCaP cell proliferation/viability by approximately 30% compared to SCR control (P=0.0011), and is consistent with previous studies (Yang *et al.*, 2005). *USP12* knockdown resulted in an approximate 40% reduction in LNCaP cells proliferation/viability compared to the SCR control, albeit failing to reach significance due to some variability in experimental repeats (Figure 4.12). Therefore, USP12, potentially via the AR signalling cascade, regulates the growth rate and/or the cell death of LNCaP cells. Further investigation of the effect of combined *AR* and *USP12* knockdown would elucidate whether the affect seen with USP12 knockdown are due to the direct effect on AR transcriptional activity. Furthermore, analysis of the effect of USP12 depletion on both AR positive and negative cell lines would strengthen the hypothesis that *USP12* knockdown causes a reduction in cell proliferation and viability due to its effect on AR signalling.

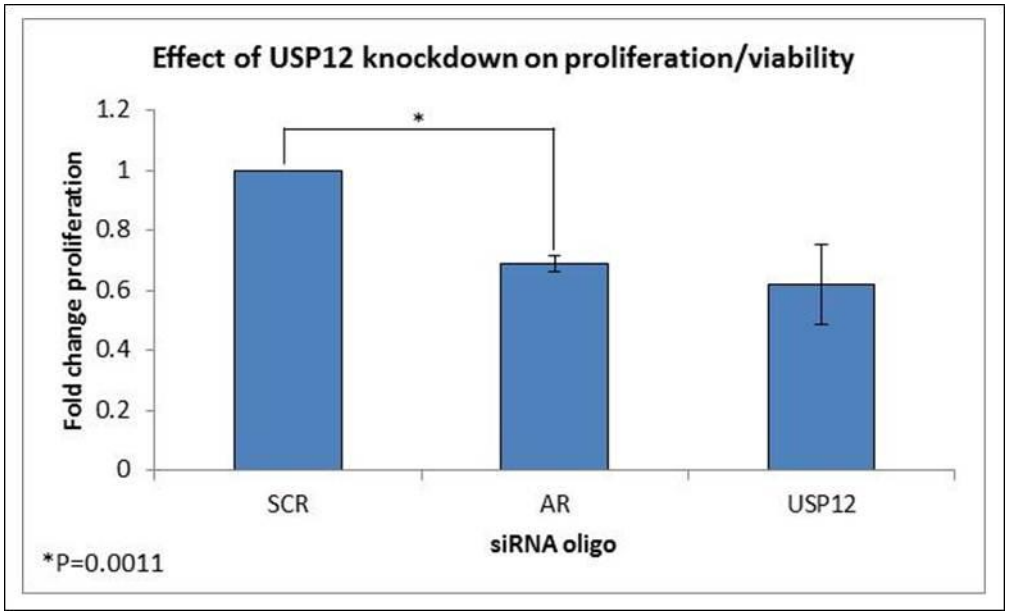

Figure 4.12. Effect of USP12 knockdown on LNCaP cell proliferation/viability.

LNCaP cells were subjected to USP12 and androgen receptor (AR) knockdown for a total of 96 hours in serum-containing media and cell proliferation/viability was compared to scrambled siRNA (SCR) control. Data is represented as mean fold change compared to the SCR control of three independent experimental repeats. Error bars represent the standard error between the three replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk.

4.3.5.2 Effect of USP12 knockdown on LNCaP cell cycle

Due to the effects of *USP12* knockdown observed in the SRB assays, the phenotypic effect of *USP12* knockdown on cell cycle profile was assessed. LNCaP cells were subject to USP12 depletion for 48 hours in serum-containing media prior to propidium iodide (PI) staining and flow cytometry analysis. Data represents the mean of three independent repeats and each cell cycle phase is expressed as a percentage of the total number of cells analysed. SCR siRNA was used as a negative control.

The cell cycle profiles for each siRNA are shown in Figure 4.13. A graphical overview of the cell cycle profiles and the effect of USP12 knockdown on the individual phases of the cell cycle are shown in Figure 4.14 and Figure 4.15 respectively. The percentage of cells in each phase of the cell cycle is summarised in Table 4.2. Data in Figure 4.15 was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

The SCR control, representing the 'normal' LNCaP cell cycle, demonstrated that the largest proportion of cells was in G1 with 70.43% of the population; 6.55% were in subG1; 10.36% were in S-phase and 12.66% were in G2/M phase of the cell cycle (Figure 4.13a; Table 4.2). As expected, *AR* knockdown caused significant G1 arrest (P=0.026) with reductions in both S and G2/M phases (P=0.0014, 0.0054, respectively), in-line with previously published data (Kokontis *et al.*, 1998; Feng *et al.*, 2011) (Figure 4.13b; Figure 4.15b, c and d, lane 2). *USP12* knockdown however, had no effect on G1 phase but did have a significant reductive effect on both S and G2/M phases similar to *AR* knockdown (P=0.0035, 0.0137 respectively), (Figure 4.13c; Figure 4.15c and d, lane 3), these data were consistent with the effects of USP12 depletion in the SRB assays (Figure 4.12). Interestingly, knockdown of the USP12 enzyme caused an accumulation of cells in the subG1 phase of the cell cycle, and although this affect was not significant it suggests that *USP12* knockdown causes some degree of apoptosis (Figure 4.15a, lane 3).

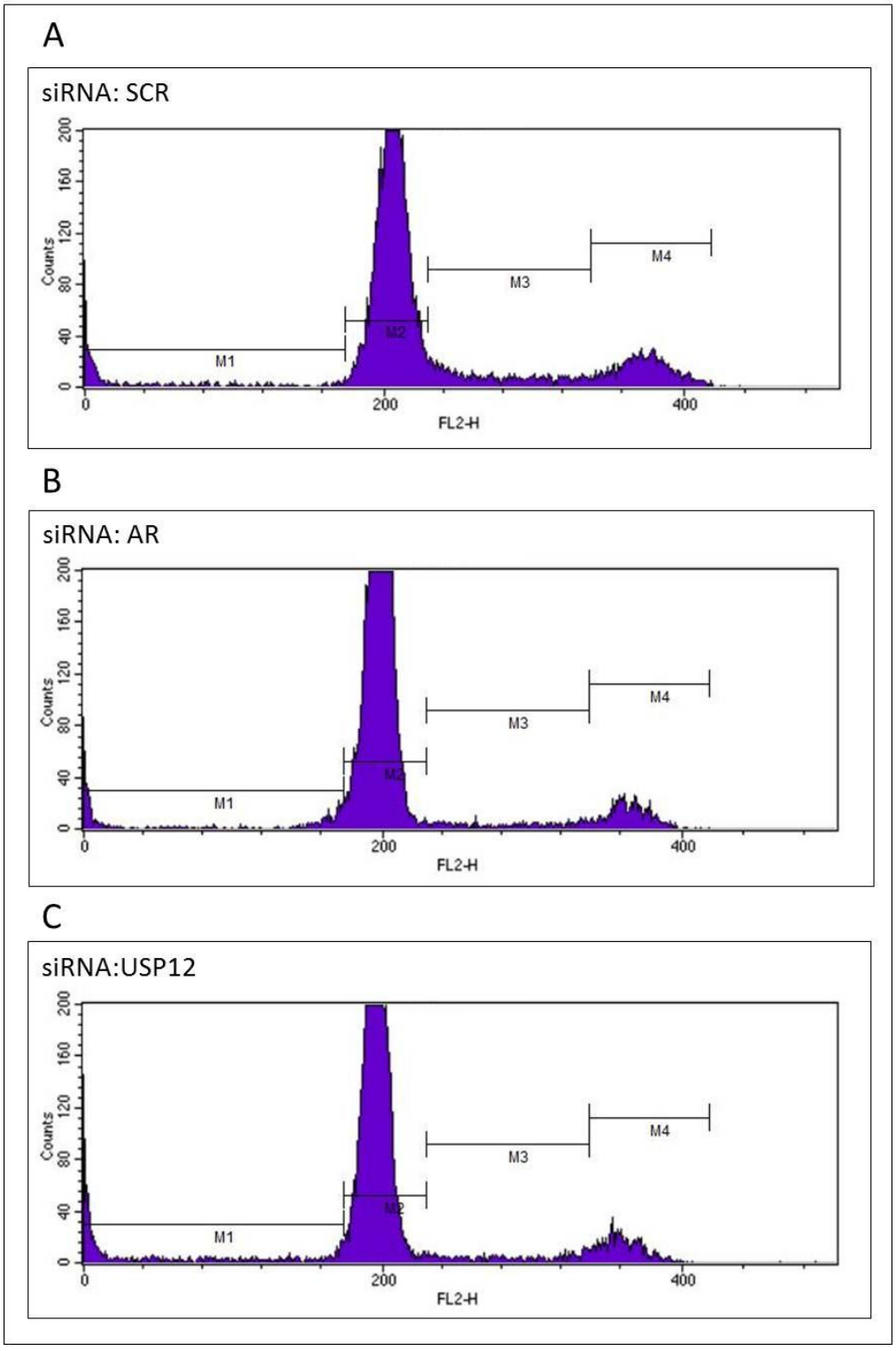

Figure 4.13. Effect of USP12 knockdown on the cell cycle in LNCaP cells.

LNCaP cells were subjected to USP12 depletion in serum-containing media for 48 hours. Scrambled (SCR) and androgen receptor (AR) siRNA were used as negative and positive controls respectively. Cells were harvested and stained with propidium iodide (PI) before flow cytometry analysis. Cell cycle profiles are representative of three independent experimental repeats. Markers indicate the individual phases of the cell cycle; subG1 (M1), G1 (M2), S (G3) and G2/M (M4).A) The effect of SCR siRNA on the cell cycle of LNCaP cells. B) The effect of AR siRNA on the cell cycle of LNCaP cells. C) The effect of USP12 siRNA on the cell cycle of LNCaP cells.

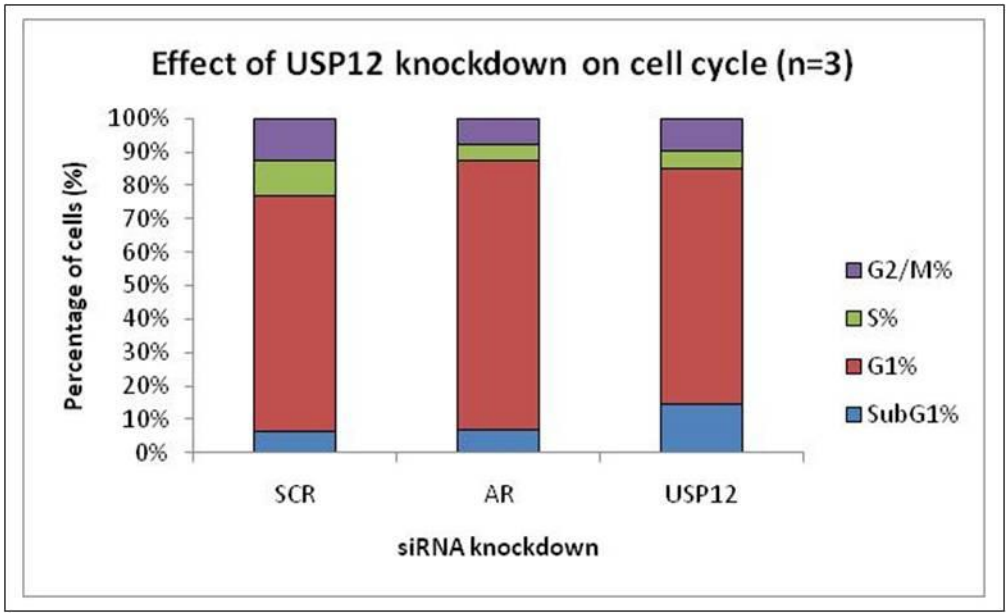

Figure 4.14. Overall profile of the effect of USP12 knockdown on the cell cycle of LNCaP cells.

Graphical representation of the cell cycle profiles from the flow cytometry analysis. Data represents the mean of three independent repeats and is expressed as percentage of cells in each cell cycle phase.

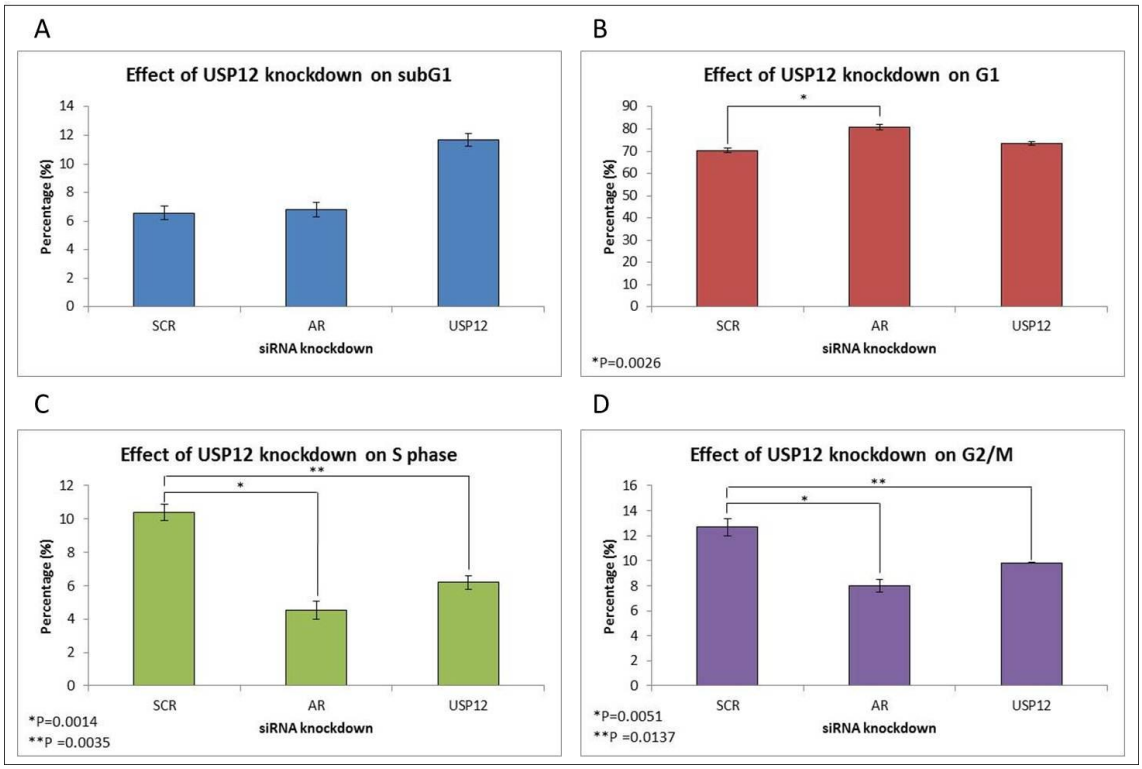

Figure 4.15. Effect of USP12 knockdown on individual phases of the cell cycle in LNCaP cells.

Data from the flow cytometry analysis was analysed as individual cell cycle phases. Data is from three independent repeats and is represented as percentage of cells in each phase. Error bars show the standard error between each experimental replicate. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk. A) The percentage of cells in the SubG1 population. B) The percentage of cells in the G1 population. C) The percentage of cells in the S population. D) The percentage of cells in the G2/M population.

	Cell cycle phase (%)			
siRNA knockdown	subG1	G1	S	G2/M
SCR	6.55 ± 0.48	70.43 ± 1.03	10.36 ± 0.48	12.66 ± 0.68
AR	6.77 ± 0.51	80.82 ± 1.17	4.53 ± 0.57	7.88 ± 0.49
USP12	14.73 ± 3.04	70.05 ± 3.41	5.70 ± 0.58	9.52 ± 0.31

Table 4.2 Effect of USP12 knockdown on the individual phases of the cell cycle phase in LNCaP cells.

Numerical representation of the percentages of cells in each cell cycle phase as determined by flow cytometry analysis. Data is presented as the mean of three independent repeats ± standard error.

4.3.5.3 Effect of USP12 knockdown on LNCaP cell apoptosis

The increase in subG1 of the LNCaP cell cycle with USP12 knockdown suggested an increase in cell death that prompted further investigation. To do this, a flow cytometry-based approach to assess the specific apoptosis marker cleaved caspase-3 was utilised.

LNCaP cells were subjected to 96 hours of *USP12* knockdown in serum-containing media. SCR siRNA was again used as a negative control. After 48 hours knockdown, cells were treated with 200 nM doxorubicin for a further 48 hours. At this doxorubicin concentration, up to 50% of the cell population are expected to enter apoptosis (Shaheen *et al.*, 2011). Therefore in cells subjected to SCR siRNA, treatment with doxorubicin acts as a positive control. Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

As shown in Figure 4.16, the SCR control population contained approximately 8.89% apoptotic cells which increased significantly upon treatment with doxorubicin to 47.93% (P=0.0019) (Figure 4.16, compare lanes 1 and 2). *AR* knockdown marginally increased the percentage of apoptotic cells to 11.79% which agrees with the cell cycle data (Figure 4.16, compare lanes 1 and 3). Following doxorubicin treatment, the percentage of apoptotic cells increased significantly increased in the *AR* knockdown cells to 34.47% (P=0.0051) (Figure 4.16, compare lanes 3 and 4). *AR* knockdown appeared to protect LNCaP cells from the effects of doxorubicin compared to the SCR control treated with doxorubicin, although this result was not significant (Figure 4.16, compare lanes 2 and 4).

Consistent with the subG1 cell cycle analysis, *USP12* knockdown, although not causing as dramatic an affect as the SCR + doxorubicin control, did significantly increase the apoptotic population to 17.28% (P=0.0408) (Figure 4.16, compare lanes 1 and 5). Interestingly, *USP12* knockdown combined with doxorubicin treatment had a dramatic effect on apoptosis increasing the percentage of cells to 82.43% (P<0.0001) (Figure 4.16, compare lanes 5 and 6). This increase was approximately 9-fold above the SCR control (P= 0.0005) (Figure 4.16, compare lanes 1 and 6) and nearly 2-fold above the SCR control treated with doxorubicin (Figure 4.16, compare lanes 2 and 6).

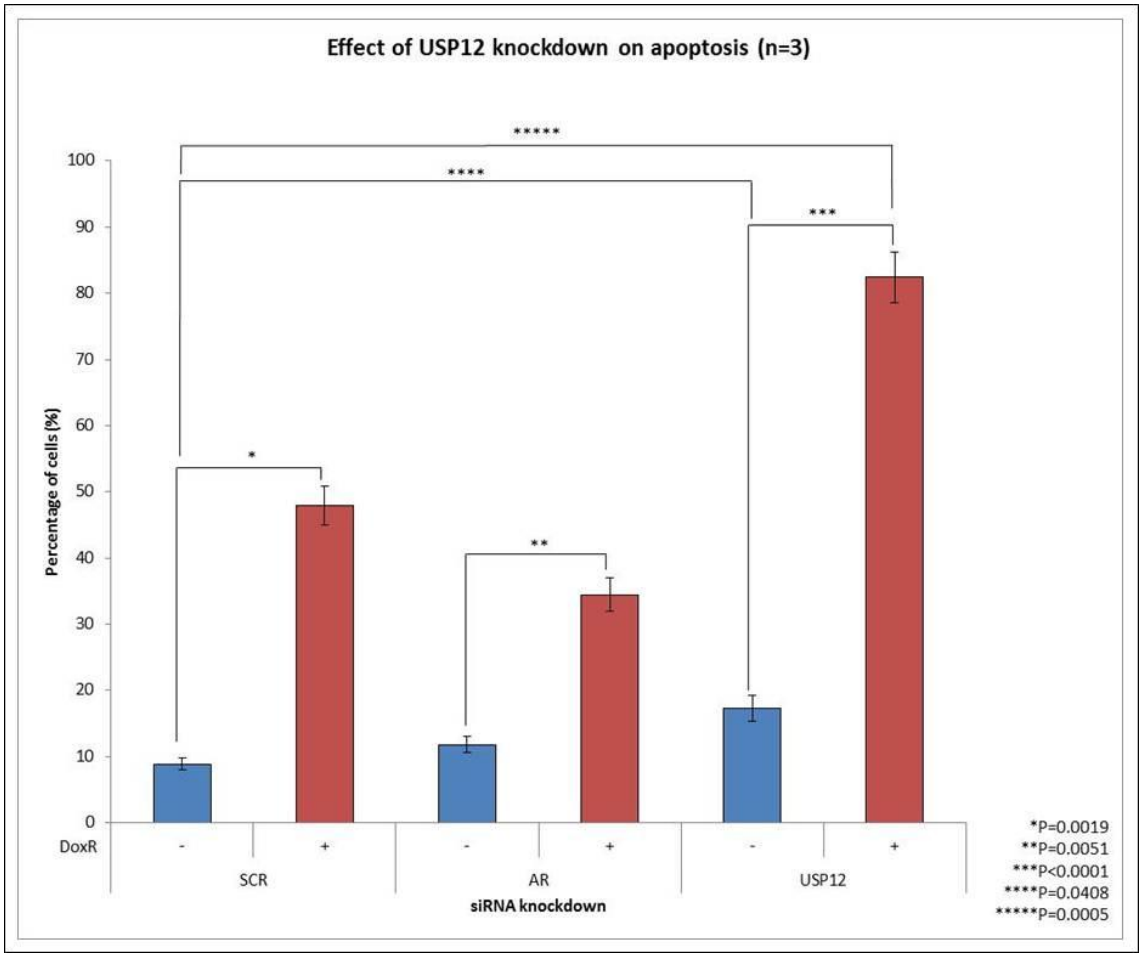

Figure 4.16. Effect of USP12 knockdown on apoptosis in LNCaP cells.

LNCaP cells were subjected to USP12 and androgen receptor (AR) depletion in serum-containing media for a total of 96 hours. Scrambled (SCR) siRNA was used as a negative control. Following 48 hours knockdown, cells were subjected to treatment with 200 nM doxorubicin (DoxR) for a further 48 hours. Cells were harvested and stained with fluorescent anti-cleaved caspase-3 antibodies before undergoing flow cytometry analysis. Data is the mean of three independent repeats and is represented as the percentage of total analysed cells with cleaved caspase 3. Error bars represent the standard error. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk.

Considering that the cell cycle data was obtained after 48 hours of *USP12* knockdown and the apoptosis data was collected after 96 hours knockdown, additional cell cycle analysis of LNCaP cells after 96 hours USP12 depletion was performed to make it consistent with the caspase-3 experiments. Unfortunately, due to time constraints, data is restricted to one experimental repeat and hence would need to be repeated in order to draw statistically relevant conclusions from the data. Table 4.3 shows a summary of the percentage of cells in each phase if the cell cycle.

Firstly, the data demonstrated that 96 hours knockdown increases the percentage of cells in subG1 in all experimental arms when compared to 48 hours knockdown (Figure 4.18 and Figure 4.14; Table 4.4). As shown in Figure 4.18, doxorubicin treatment for 48 hours increased the population of SCR cells in the subG1 phase of the cell cycle from 9.14% in untreated cells to 22.24% which agrees, in part with the caspase-3 data (Figure 4.18, compare lanes 1 and 2). Longer knockdown of *AR* increased the percentage of cells in subG1 from 6.77% after 48 hours to 14.77% post-96 hours AR depletion at the expense of cells in the G1 phase of the cell cycle (Figure 4.18, lane 3; Table 4.4). Treatment of these cells with doxorubicin again increased the subG1 population (Figure 4.18, compare lanes 3 and 4). Remarkably, *USP12* knockdown had the greatest effect on the subG1 cell population as evidenced by an increase from 9.14% in the SCR control to 54.26% (Figure 4.18, compare lanes 1 and 5) and this was increased further to 88.08% in *USP12* knockdown cells treated with doxorubicin (Figure 4.18, compare lane 5 and 6).

USP12 depletion caused the greatest increase in LNCaP apoptosis between the 48 and 96 hour experiments (Table 4.4). This large increase could be due to a prolonged effect of USP12 depletion on AR function or simply due to toxicity of prolonged depletion, although a total of 120 hours knockdown was utilised in the validation time-course experiments and was achieved with minimal toxic effects (Figure 3.10and Figure 3.15). Importantly, PI staining only provides an approximate representation of apoptotic cells as necrotic and senescent cells are also detected as part of the subG1 population, therefore an increase in these other populations may have caused the dis-proportional increase in cells detected. Caspase-3 flow cytometry analysis is a more robust and specific assay, allowing definite identification of apoptotic LNCaP cells. Therefore,

further repeats of the PI flow cytometry would assist in strengthening the evidence for an effect of *USP12* knockdown on LNCaP apoptosis.

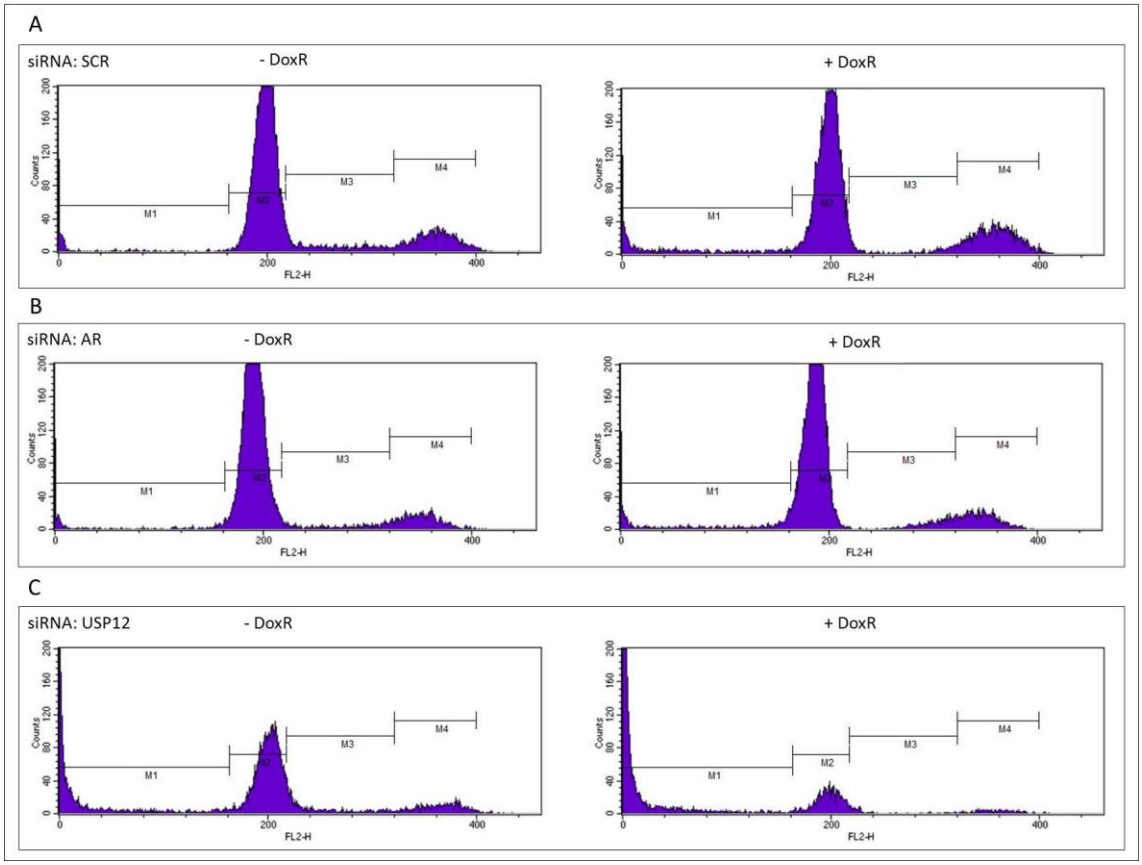

Figure 4.17. Effect of 96 hours USP12 knockdown on the cell cycle in LNCaP cells.

LNCaP cells were subjected to USP12 and androgen receptor (AR) depletion in serum-containing media for a total of 96 hours. Scrambled (SCR) siRNA was used as a negative control. Following 48 hours knockdown, cells were subjected to treatment with 200 nM doxorubicin (DoxR) for a further 48 hours. Cells were harvested and stained with PI before flow cytometry analysis. Cell cycle profiles are representative of three independent experimental repeats. Markers indicate the individual phases of the cell cycle; subG1 (M1), G1 (M2), S (G3) and G2/M (M4). A) The effect of SCR siRNA with (+DoxR) and without (-DoxR) doxorubicin treatment on the cell cycle of LNCaP cells. B) The effect of AR siRNA with (+DoxR) and without (-DoxR) doxorubicin treatment on the cell cycle of LNCaP cells. C) The effect of USP12 siRNA with (+DoxR) and without (-DoxR) doxorubicin treatment on the cell cycle of LNCaP cells.

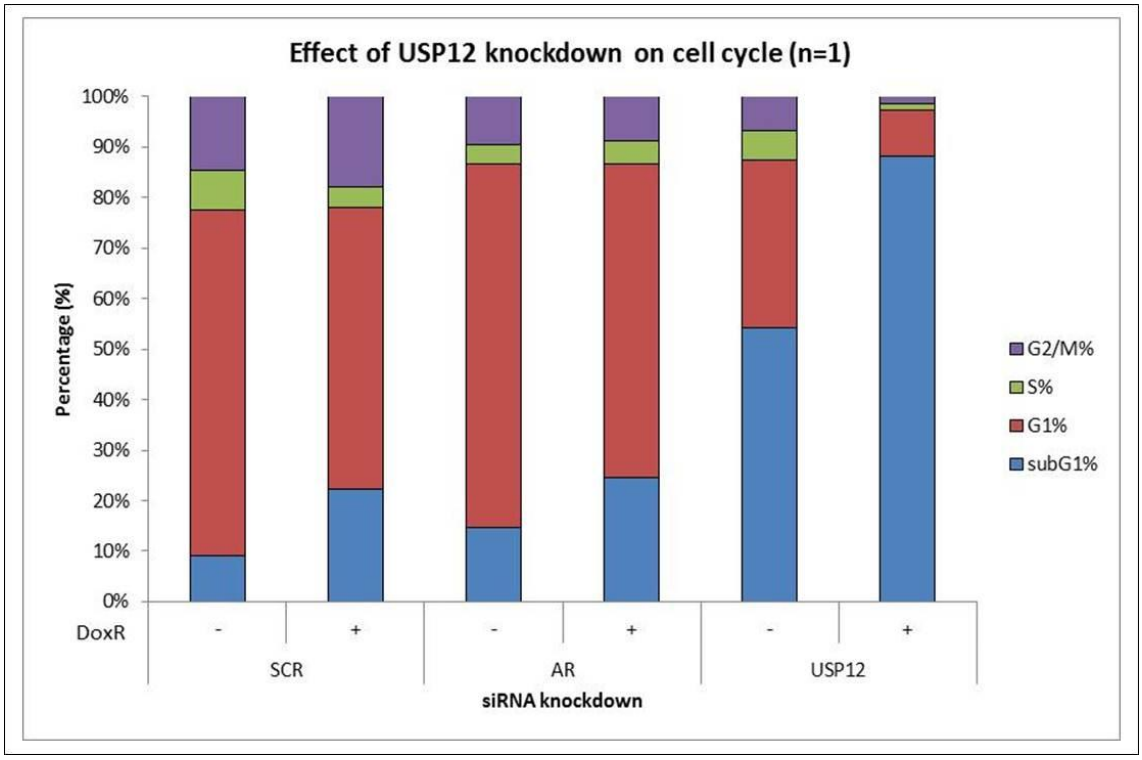

Figure 4.18. Overall profile of the effect of 96 hours USP12 knockdown on the cell cycle of LNCaP cells.

Graphical representation of the cell cycle profiles from the flow cytometry analysis. Data represents the mean of three independent repeats and is expressed as percentage of cells in each cell cycle phase.

		Cell cycle phase (%)			
DoxR treatment	siRNA knockdown	subG1	G1	S	G2/M
-	SCR	9.14	68.47	7.68	14.71
+	SCR	22.24	55.90	3.86	18.00
-	AR	14.77	71.86	3.85	9.39
+	AR	24.48	62.19	4.56	8.77
-	USP12	54.26	33.30	5.76	6.68
+	USP12	88.08	9.30	1.10	1.52

Table 4.3. Effect of 96 hours USP12 knockdown on the individual phases of the cell cycle phase in LNCaP cells.

Numerical representation of the percentages of cells in each cell cycle phase as determined by flow cytometry. Data is from one experimental repeat.

		SubG1 population (%)	
DoxR treatment	siRNA knockdown	48 hours	96 hours
-	SCR	6.55	9.14
+	SCR	-	22.24
-	AR	6.77	14.77
+	AR	-	24.48
-	USP12	14.73	54.26
+	USP12	-	88.08

Table 4.4. Comparison of the effect of48 and 96 hours USP12 knockdown on subG1 population in LNCaP cells.

The percentage of LNCaP cells in the subG1 population as measured by flow cytometry for both 48 hours and 96 hours siRNA knockdown experiments.

In an attempt to confirm these data, Western blot analysis of another marker of apoptosis, cleaved PARP-1, was performed on lysates of LNCaP cells treated as described above. Figure 4.19 shows the results a Western blot for cleaved PARP-1 and α-tubulin as a loading control. Due to time constraints this data is from one experimental repeat and would require further repeats.

A proportion of cleaved PARP-1 was present in the SCR control without doxorubicin treatment, consistent with the cleaved caspase-3 data (Figure 4.19, upper panel, lane 1). Cleaved PARP-1 levels only marginally increased upon doxorubicin treatment, although the level of α-tubulin was greatly diminished in this sample, suggesting a large proportion of the cells had undergone cell death (Figure 4.19, upper and lower panels, compare lane 1 and 2). In contrast to *AR* knockdown, that showed no change in cleaved PARP-1 levels (Figure 4.19, upper panel, compare lanes 1 and 3), USP12 depletion caused an increase in cleaved PARP-1 that is likely to be more enhanced with equalised protein loading (Figure 4.19, upper and lower panels, compare lanes 1 and 4). Unfortunately, *AR* and *USP12* knockdown samples treated with doxorubicin failed to yield a protein band within the loading control suggesting that a greater proportion of cells had undergone apoptosis than in the SCR + doxorubicin control (data not shown).

In conclusion taking all of the flow cytometry, caspase-3 and cleaved PARP-1 analyses together, *USP12* knockdown increases LNCaP cell apoptosis suggesting it may constitute a potential target for CaP therapy. Additionally, combination of *USP12* knockdown and chemotherapeutic agents may provide a further means of treatment in CaP although this requires more extensive investigation.

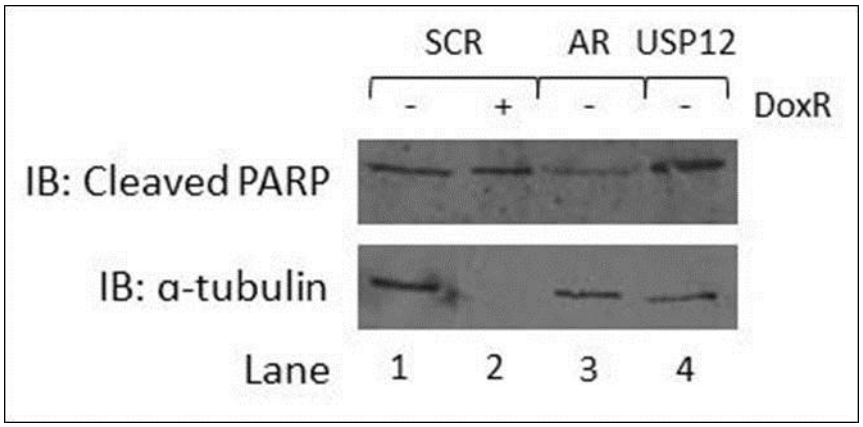

Figure 4.19. Effect of USP12 knockdown on levels of the apoptosis marker cleaved PARP-1 in LNCaP cells.

LNCaP cells were subjected to USP12 and androgen receptor (AR) depletion in serum-containing media for a total of 96 hours. Scrambled (SCR) siRNA was used as a negative control. Following 48 hours knockdown, cells were subjected to treatment with 200 nM doxorubicin (DoxR) for a further 48 hours. Cell lysates were collected and analysed by Western blot using anti-cleaved PARP-1 and α-tubulin antibodies. Data is from one experimental repeat.

4.4 Discussion

USP12 was identified by the Solid Tumour Target Discovery Group, Newcastle University, as a regulator of AR activity using an siRNA library screen to investigate the effect of DUB enzyme knockdown on AR function in LNCaP cells. Depletion of USP12 reduced the mRNA expression of all AR-regulated genes studied, including *PSA*, *TMPRSS2* and *KLK2* (Figure 3.10 and Figure 3.15), without affecting *AR* levels. It was therefore hypothesised that USP12 acts as a co-activator of AR transcriptional activity. To investigate this further, luciferase-based reporter assays in multiple cell lines was employed, including the LNCaP-7B7 pPSA luciferase cell line that contains an integrated AR-responsive PSA promoter upstream from the luciferase gene thus providing an informative read-out for receptor activity. Knockdown of *USP12* in the LNCaP-7B7 pPSA luc cell line confirmed that depletion of USP12 did indeed have a negative effect on AR transcriptional activity (Figure 4.1), consistent with the role of USP12 as an AR co-activator. In support of this notion, increased amounts of ectopically expressed USP12 enhanced AR function in COS-7 cells upon the androgen-responsive ARE3 luciferase reporter (Figure 4.6); although it was noted that at higher amounts of USP12 (100 ng) no AR co-activation was observed. This effect was possibly due to overloading of the system with ectopically expressed plasmids that may have caused transcriptional "squelching" or may have interfered with the normal stoichiometry of the USP12 or AR protein complexes.

Recently USP13 was reported to regulate the stability of the E3 ligase Siah2 though a non-catalytically-dependent mechanism (Scortegagna *et al.*, 2011). This is not the case for the majority of DUB enzymes, however, with most requiring the catalytic triad residues of histidine, cysteine and aspartic acid to carry out their function. USP2a, for example, requires its DUB activity to protect fatty acid synthase (FAS) from degradation. Similarly, conversion of cysteine 221 to alanine in USP21 to alanine generates an enzymatically dead DUB that no longer inhibits TNFα-mediated NFκB activation in luciferase reporter assays (Xu *et al.*, 2010). It was therefore important to assess if mutation of the catalytic cysteine at position 48 in USP12 was important for co-activation of the AR. Due to the conserved nature of the USP catalytic triad residues and their surrounding sequence, the catalytic Cys48 residue of USP12 at position 48 provided an ideal candidate for mutation to produce a deubiquitinase-dead USP12

enzyme. In addition, mutation of Cys48 had previously been shown to cause loss of USP12 deubiquitinase activity towards the synthetic ubiquitin-AMC substrate (Cohn *et al.*, 2009). Importantly, in luciferase-based AR reporter experiments, the $USP12_{C48A}$ mutant dampened the AR co-activation capacity of USP12 to near basal levels, although there was considerable variation in the data-set as evidenced by large error bars in each experimental arm (Figure 4.7). Additional repeats, and utilising other androgen-dependent reporters in similar experiments, will be important to confirm the requirement of the catalytic activity of USP12 in stimulating AR activity.

To further investigate the function of USP12 on AR-regulated transcription, it was important to assess whether USP12 facilitated movement of the receptor into the nucleus upon DHT stimulation and hence increase the transcriptional response. Overall, no dramatic effect of USP12 depletion on AR movement was observed other than the surprising observation that more AR appeared to be present in the nucleus before DHT stimulation, and that nuclear AR levels declined at 6 and 8 hours DHT treatment compared to SCR controls (Figure 4.8). Interestingly, MDM2-mediated mono-ubiquitination of p53 has been shown to cause nuclear export of p53 (Li *et al.*, 2003). An in-frame fusion of ubiquitin to p53 in H1299 p53-null lung carcinoma cells was shown to be sufficient to drive nuclear export and MDM2 was shown to be critical in this process (Li *et al.*, 2003). Similarly, mono-ubiquitination of the human DCN1-like protein (DCNL1) was also shown to be sufficient for its nuclear export by over-expression of a DCNL1-ubiquitin fusion protein (Wu *et al.*, 2011). The outcome of these studies could be extrapolated to suggest that depletion of USP12 from LNCaP cells could allow ubiquitination of AR leading to its nuclear export, although further studies would have to be performed in order to confirm this. Furthermore, mono-ubiquitination of AR has not been studied in cell lines and it would be interesting to generate an AR-ubiquitin fusion protein to observe the effects on cellular localisation and see if the effects of USP12 knockdown are replicated.

To address the question of whether AR-chromatin association is regulated by USP12, and hence may be a reason for enhanced AR-mediated transcription, ChIP experiments were performed in LNCaP cells after USP12 depletion (Figure 4.10). It was observed that knockdown of *USP12* reduced the recruitment of AR to both AREs within the proximal promoter (ARE I) and the distal enhancer (ARE III) of the *PSA* gene, with the

greatest effect being observed for ARE III. Other AR co-activators including Ku70 and Ku80 have been shown to be important for AR transcriptional activity with both proteins being recruited to AR responsive promoters but without affecting nuclear translocation of AR due to their nuclear functionality (Mayeur *et al.*, 2005). To support this, USP12 has also been postulated to be a nuclear protein due to the presence of nuclear localisation signals within its coding sequence (Hansen-Hagge *et al.*, 1998). Unfortunately, due to the lack of effective USP12 antibodies and failure to optimise the use of anti-FLAG antibodies for immuno-fluorescence this could not be confirmed, but remains an important point to be addressed in the future. Interestingly, the USP10 protein affects the function of p53 in the nucleus, although it was found to localise mainly to the cytoplasm with only a small fraction translocating to the nucleus upon DNA damage (Yuan *et al.*, 2010). This may also be the case for USP12, with certain stimuli such as androgen treatment causing movement. Equally, production of USP12 antibodies would help to elucidate if the enzyme itself is recruited to AR responsive promoters.

Interplay between multiple post-translational modifications on histones, including acetylation, methylation and ubiquitination, provides a dynamic code for gene transcription. Deubiquitination of histone H2A has an impact on hyper-acetylation of histones in nucleosomes as well as leading to phosphorylation of the linker histone H1. This phosphorylation event results in dissociation of histone H1, which is generally an indicator of transcriptional activation (Zhu *et al.*, 2007a). Interestingly, in a few cases H1 dissociation is involved in transcriptional repression and histone H2A deubiquitination also shares this dual role in transcriptional regulation (Zhu *et al.*, 2007a). Nakagawa *et al.,* reported that histone H2A deubiquitination by USP21 promoted di- and tri-methylation of histone H3 lysine 4 (H3K4), possibly through increasing substrate specificity of the involved methylases or access to the methyl target, leading to intiation of transcription (Nakagawa *et al.*, 2008). Conversely, ubiquitination of histone H2A K119 coupled with methylation of both K9 and K27 of histone H3, have been implicated in the silencing of early developmental genes and inactivation of the X chromosome (Shilatifard, 2006). Dover *et al.,* identified the ubiquitin conjugating E2 enzyme *Rad6* as a gene involved in the methylation of histone H3K4 by SET1 in *S. cerevisiae* (Dover *et al.*, 2002). Rad6 is involved in mono-

ubiquitination of histone H2B K123 mediated by the E3 ubiquitin ligase Bre1. Mutations affecting the interaction and transfer of ubiquitin between Rad6 and Bre1 resulted in loss of methylation of histone H3K4 (Wood *et al.*, 2003). Joo *et al.,* described USP12 as a histone H2A and H2B specific DUB and showed that knockdown of USP12 promoted the retention of ubiquitinated histone H2B, whilst histone H2A ubiquitination was lost, at the promoter region of the *Xbra* gene thereby reducing expression in *Xenopus* (Joo *et al.*, 2011). USP12 may also target histones in the LNCaP cell line; depletion of USP12 shows a reduction in AR-regulated gene expression that is similar to the effect of USP12 knockdown on *Xbra* expression. ChIP studies to investigate the levels of ubiquitinated histones H2A and H2B in the promoter and coding regions of AR-target genes such as *PSA*, *KLK2* and *TMPRSS2* using commercially available antibodies specific to ubiquitinated histone H2A and H2B would help to unravel this. Moreover, many histone modifiers have been shown to interact with and affect the function of the AR, independently of their histone-directed activity. This is true for the histone methyltransferase SET9 which is responsible for histone H3K4 mono-methylation as well as direct methylation of the AR at lysine 632 within the hinge region (Gaughan *et al.*, 2011). It would be interesting to investigate whether the effect of USP12 on AR could be accounted for by modification of an ubiquitinated histone or whether both histone modification and direct deubiquitination of AR is required.

IP data obtained from over-expression of FLAG-tagged USP12 and AR demonstrated an interaction between the two proteins, although the proportion of USP12 that was found to interact with the AR was relatively low when compared to the level of over-expression (Figure 4.11). Use of another experimental system, such as the mammalian two-hybrid assays could further confirm these data in the absence of a suitable USP12 antibody to detect endogenous proteins.

Analysis of the phenotypic effects of *USP12* knockdown revealed that under normal conditions, USP12 may act to promote LNCaP cell proliferation whilst inhibiting apoptosis (Figure 4.12; Figure 4.14; Figure 4.16). This is comparable to other AR co-regulators such as hPIRH2 and SET9; depletion of hPIRH2 by siRNA resulted in decreased LNCaP proliferation and knockdown of *SET9* resulted in both decreased proliferation and increased apoptosis in LNCaP cells (Logan *et al.*, 2006; Gaughan *et al.*,

2011). Many DUBs have been linked to apoptosis including USP14, USP7 and CYLD (Ramakrishna *et al.*, 2011; Vucic *et al.*, 2011). USP2a protects prostate cancer cells from apoptosis through stabilisation of FAS and interaction with the p53 pathway via MDM2 (Graner *et al.*, 2004). Although not conclusively proven, the Akt phosphatase PHLPP has been identified by mass spectrometry to interact with USP12; connecting it directly with a pathway involved in both cell proliferation and apoptosis (Sowa *et al.*, 2009). This phosphatase is responsible for de-phosphorylation of Akt resulting in suppression of growth and promotion of apoptosis (Gao *et al.*, 2005a). Investigation of this alleged relationship may reveal the mechanism by which USP12 protects cells from apoptosis. Pilot studies investigating the levels of phosphorylated Akt in LNCaP cells subjected to SCR or *USP12* knockdown for 48 hours in steroid-depleted media prior to treatment with insulin-like growth factor-1 (IGF-1), which is known to induce phosphorylation of Akt, were performed but unfortunately due to time constraints could not be fully optimised. Preliminary results, however, indicated that knockdown of USP12 led to a reduced level of serine 473 phosphorylated Akt whilst total Akt and α-tubulin levels remained unaffected (data not shown). Reduced Akt phosphorylation would theoretically result in an increase in apoptosis and a decrease in cell proliferation, therefore this result would conform promisingly to the LNCaP proliferation and flow cytometry data. Further experimental repeats would be required and investigation to confirm and characterise USP12 and PHLPP interaction may attach a further cellular role to USP12.

In conclusion, USP12 has been shown to consistently co-activate AR transcriptional activity. Acting in a potentially nuclear location, USP12 affects the recruitment of AR to AREs in target genes. Knockdown of USP12 not only dampens the expression of AR-regulated genes but also promotes apoptosis and growth arrest in prostate cancer cells. Therefore, USP12 could prove to be a prospective therapeutic target.

Chapter 5: Characterisation of USP10 as a deubiquitinase involved in androgen receptor regulation

5.1 Introduction

USP10 is a member of the ubiquitin-specific processing protease (USP) family of deubiquitinating enzymes that contains an ataxin-2C domain, a region of homology with the carboxyl-terminus of the ataxin-2 protein with no known function, and a USP domain, containing the catalytically important residues required for deubiquitinase activity (Komander *et al.*, 2009a). USP10 was first described as an interacting partner of the Ras-GTPase activating protein, G3BP and has subsequently been linked to regulating the function of many other proteins involved in an array of cellular processes, as described below.

5.1.1 USP10 and the endo-membrane system

One of the most characterised cellular functions of USP10 is in regulation of vesicular and endosomal transport. The endo-membrane system consists of the endoplasmic reticulum (ER), the Golgi apparatus and lysosomes. Vesicles allow transport of proteins between the organelles. Endosomes are a specific type of vesicle that forms at the plasma membrane to internalise membrane proteins such as cell surface receptors. These endosomes are then transported to the Golgi where the internalised proteins are either recycled back to the plasma membrane, by recycling endosomes, or targeted to the lysosome for degradation (Lee *et al.*, 2004; Hsu *et al.*, 2009). Furthermore, proteins destined for the cell membrane can be transported from the ER to the Golgi by vesicles (Lee *et al.*, 2004).

5.1.1.1 USP10 and Ras-GAP SH3 binding protein

The COPII complex is a coat protein which is incorporated into vesicles transported between the ER and the Golgi, allowing transport of membrane proteins which are assembled in the ER (Lee *et al.*, 2004). A study in yeast identified that Ubp3p, a DUB similar to USP10, deubiquitinated the COPII component Sec23p, protecting it from degradation. Furthermore, Bre5p was found to positively regulate the deubiquitinase activity of Ubp3p towards Sec23p (Cohen *et al.*, 2003) (Figure 5.1). Interestingly, a yeast two-hybrid study using a of human lymphocyte cDNA library identified Ras-GAP SH3 binding protein (G3BP), a homologue of Bre5p, as an interacting partner of USP10 suggesting that this interaction between Ubp3p and Bre5p is evolutionarily conserved and that USP10 may have a role in transport of proteins between the ER and the Golgi. However, unlike Bre5p, G3BP negatively regulates the deubiquitinase activity of USP10

towards substrates (Soncini *et al.*, 2001) (Figure 5.1). Further study of the Ubp3p-Bre3p complex identified that β'-COP, a component of the COPI vesicular coat protein, was also a substrate. USP10 was subsequently observed to interact with the mammalian homologue of β′-COP, implicating it further in vesicle transport (Cohen *et al.*, 2003) (Figure 5.1).

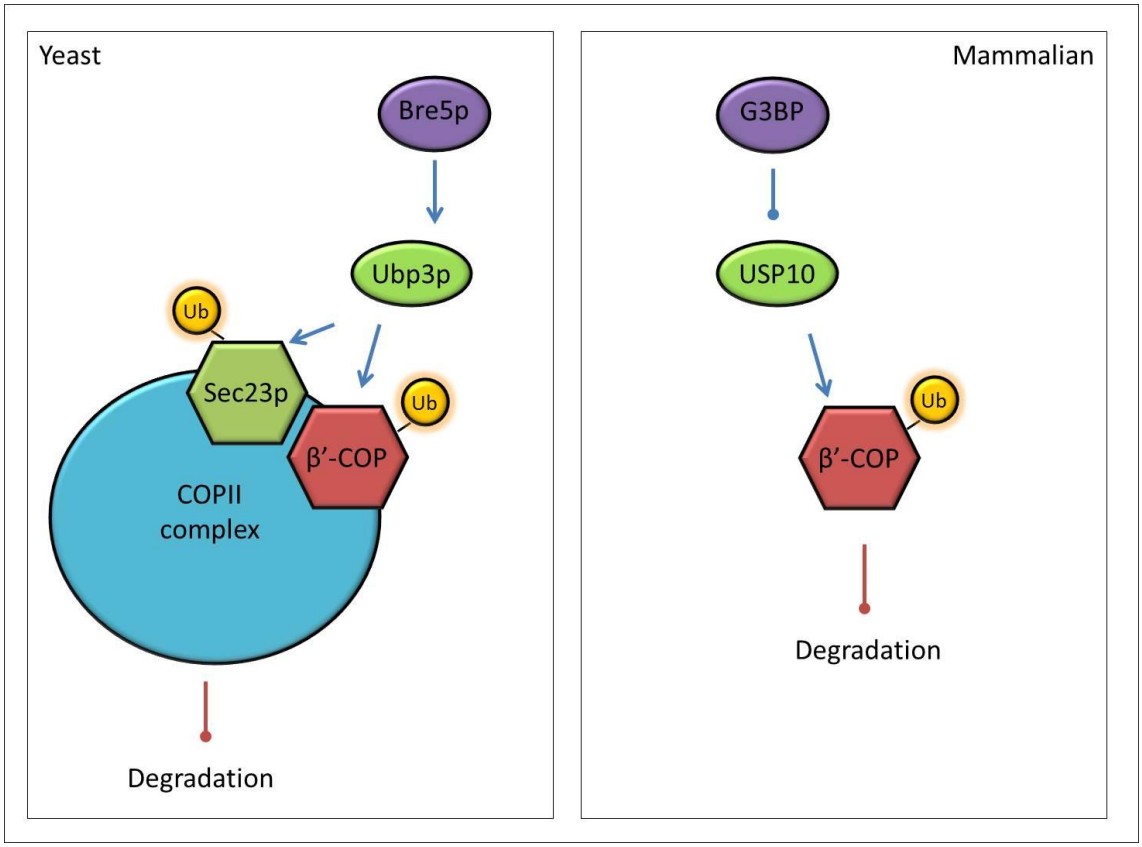

Figure 5.1. USP10 and Ras-GAP SH3 binding protein.

Ubp3p, a yeast deubiquitinase enzyme (DUB) of USP10,deubiquitinates the Sec23p and β'-COP components of the COPII complex leading stabilisation of the complex. Bre5p positively regulates Ubp3p. In mammalian cells, USP10 deubiquitinates β'-COP. Ras-GAP SH3 binding protein (G3PB), a mammalian homologue of Bre5p, negatively regulates USP10.

5.1.1.2 USP10 and CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic-AMP regulated chloride channel that is regulated by endocytosis, lysosomal degradation and endosomal recycling (Bomberger *et al.*, 2010). Mutations in CFTR cause cystic fibrosis, a genetic disease that is characterised by cysts of the pancreas as well as breathing difficulties caused by excess mucus in the lungs (Boucher, 2004).

CFTR that has been internalised into endosomes is deubiquitinated by USP10, promoting its recycling back to the plasma membrane and preventing its degradation by the lysosome (Bomberger *et al.*, 2009; Bomberger *et al.*, 2010) (Figure 5.2). A further study by Bomberger *et al.,* found that the bacterium *Pseudomonas aeruginosa* secretes a toxin which stabilises the inhibitory effect of G3BP upon USP10, therefore preventing CFTR deubiquitination by USP10 and causing increased lysosomal degradation of CFTR (Bomberger *et al.*, 2011) (Figure 5.2). *P. aeruginosa* is an opportunistic bacterial infection which affects patients with chronic lung diseases including cystic fibrosis. The above studies not only provided evidence of USP10s role in endosomal sorting of CFTR, but highlighted a specific insight for the role of USP10 in human disease.

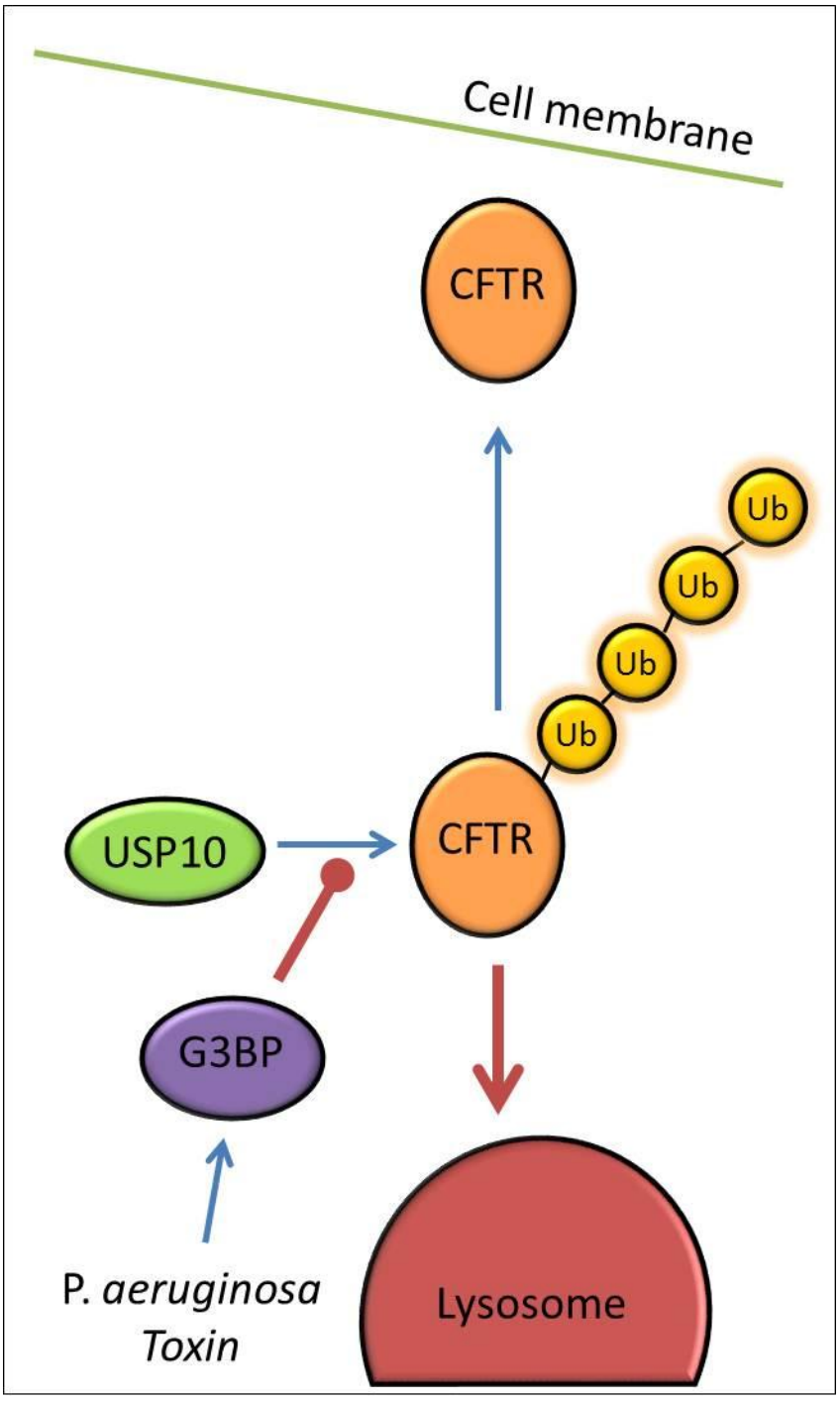

Figure 5.2. USP10 and CFTR.

USP10 deubiquitinates CFTR enabling recycling of the receptor to the cell membrane. Toxins secreted from P. aeruginosa during infection increase the negative effect of Ras-GAP SH3 binding protein (G3BP) towards USP10 causing ubiquitinated CFTR to be degraded by the lysosome.

5.1.1.3 USP10 and sorting nexin 3

USP10 gene expression is regulated by the anti-diuretic hormone vasopressin. Vasopressin regulates sodium homeostasis by promoting translocation of water and sodium channels to the plasma membrane of cells in the kidney. USP10 was found to increase the cell surface expression of epithelial sodium channels (ENaC) when co-transfected into 293T cells, although ENaC was not found to be a substrate for USP10 (Boulkroun *et al.*, 2008). The endocytic adaptor protein, sorting nexin 3 (SNX3), was identified in a yeast two-hybrid screen, as a direct substrate of USP10 and deubiquitination of SNX3 led to its stabilisation. Furthermore, over-expression of SNX3 increased the cell surface expression of ENaC in a USP10-dependent manner due to increased endocytic transport of the channel to the plasma membrane (Boulkroun *et al.*, 2008).

5.1.2 USP10 and p53-MDM2 network regulation

The tumour suppressor gene *p53* is often termed the 'guardian of the genome' as it regulates numerous cellular pathways involved in maintaining DNA integrity during cellular and genotoxic stress (Brooks and Gu, 2006). MDM2 triggers nuclear export and proteasomal degradation of p53 by direct ubiquitination (Li *et al.*, 2003). In unstressed cells, p53 activity is maintained at low levels by MDM2-mediated ubiquitination, triggering nuclear export and proteasomal degradation (Vucic *et al.*, 2011) (Figure 5.3). However, in cells experiencing stress, such as DNA damage, p53 is stabilised leading to increased transcription of p53-target genes that potentiate cell cycle arrest, repression of growth or apoptosis (Vucic *et al.*, 2011). Phosphorylation of p53, by kinases involved in cell cycle checkpoints or up-regulated by genotoxic stress, allows co-activator proteins such as p300 and PCAF to bind p53 and enhance transcriptional activity (Vucic *et al.*, 2011) (Figure 5.3). Moreover, this modification disrupts the ability of MDM2 to bind and ubiquitinate p53 leading to its enhanced stability (Vucic *et al.*, 2011). MDM2 is itself regulated by auto-ubiquitination and degradation (Brooks and Gu, 2006) (Figure 5.3).

With regards to a role of deubiquitinase enzymes in the regulation of this cascade, USP7 was shown to regulate the ubiquitination of both MDM2 and p53 (Figure 5.3). Li *et al.,* reported that USP7 was a novel interacting partner of p53, identified by mass spectrometry, that directly deubiquitinated and stabilise p53, resulting in p53-

dependent cell growth inhibition and apoptosis. Conversely, over-expression of deubiquitinase-dead USP7 resulted in accumulation of ubiquitinated p53 (Li *et al.*, 2002a). In contrast to this study, Cummins *et al.,* reported that USP7 depletion stabilised p53 due to an increase in ubiquitination and degradation of MDM2 (Cummins and Vogelstein, 2004). Importantly, cellular and genotoxic stress regulates the substrate specificity of USP7 (Vucic *et al.*, 2011). In unstressed cells, USP7 deubiquitinates MDM2 leading to increased p53 ubiquitination, whilst preferential deubiquitination and stabilisation of p53 in response to DNA damage increases p53 transcriptional activity and assists p53-mediated DNA repair, cell cycle arrest and apoptosis (Li *et al.*, 2002a; Meulmeester *et al.*, 2005; Vucic *et al.*, 2011). Furthermore, Ataxia telangiectasia mutated (ATM), a kinase activated during cellular response to DNA damage, was found to lower the affinity of USP7 for MDM2, thereby leading to its increased degradation (Meulmeester *et al.*, 2005).

Importantly, and in keeping with the focus of this Chapter, USP10 has been found to play a role in the p53-MDM2 network by counteracting MDM2-mediated ubiquitination of p53 and hence facilitating p53 activity in response to genotoxic stress. USP10, a mainly cytoplasmic protein, was found to interact directly with p53 by immunoprecipitation. Depletion of USP10 resulted in a reduction of p53 protein, but did not affect mRNA expression suggesting USP10 had a role in the stability of p53. Subsequent use of the proteasomal inhibitor MG132 confirmed that USP10 regulated p53 stability in a proteasome-dependent manner. MDM2-mediated ubiquitination and nuclear export of p53 was rescued by USP10 with mutation of the catalytic cysteine residue of USP10 abrogating its ability to reverse this ubiquitination *in vitro.* Although MDM2 expression was decreased in response to USP10 depletion in p53-proficient cells, this effect was due to a decrease in p53 protein levels as knockdown of USP10 in p53-deficient cells did not yield the same result. Furthermore, unlike USP7, USP10 did not directly interact with MDM2. Reversal of MDM2-mediated ubiquitination of p53 by USP10 resulted in increased p53 transcriptional activity leading to increased expression of p53-target genes *p21* and *Bax* and increased p53-induced apoptosis (Yuan *et al.*, 2010) (Figure 5.3).

As p53 activity is up-regulated in response to genotoxic stress, Yuan *et al* hypothesised that USP10 may play a key role in p53 regulation during DNA damage response.

Expression of USP10 was stimulated and protein levels in the nucleus increased in response to ionising radiation. Phosphorylation events, at threonine 42 and serine 337, mediated by ATM were found to be important for translocation of USP10 into the nucleus where it deubiquitinated and stabilised p53. Inhibition of ATM blocked nuclear translocation of USP10 and mutation of Thr42 and Ser337 led to defective p53 stabilisation and reduced p53-target gene expression (Yuan *et al.*, 2010) (Figure 5.3).

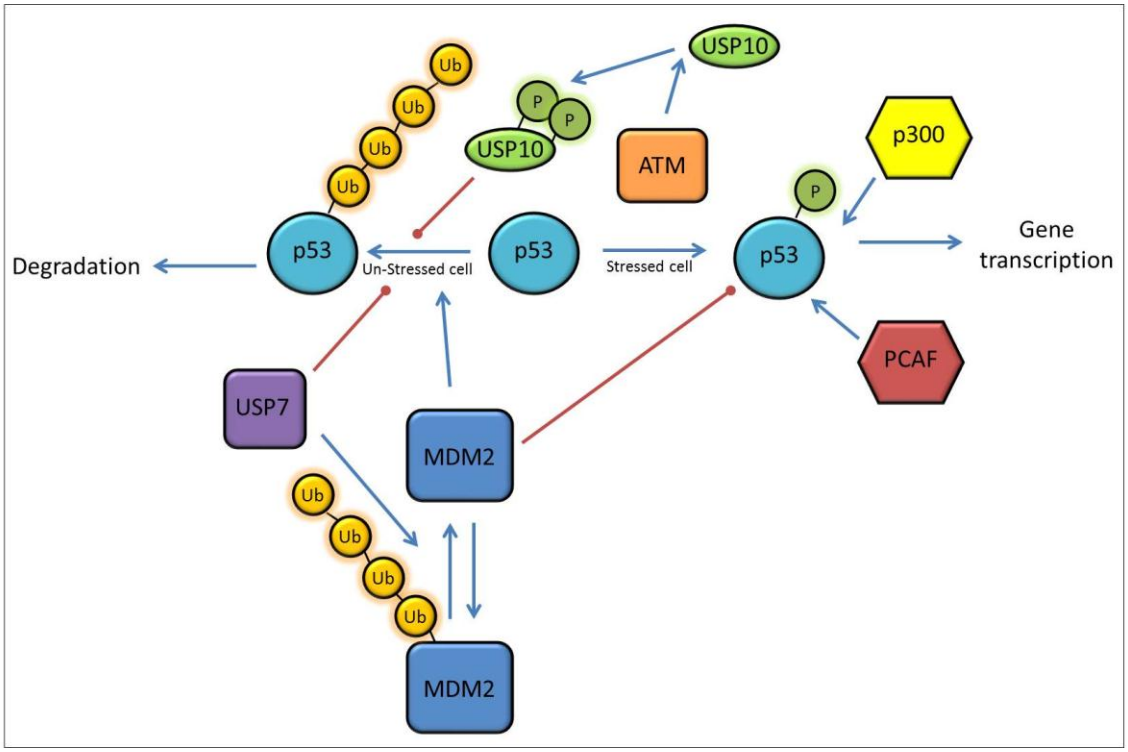

Figure 5.3. USP10 and p53-MDM2 network regulation.

The activity of p53 is maintained at low levels by MDM2 under normal cellular conditions. MDM2-mediated ubiquitination of p53 leads to degradation. In cells experiencing stress p53 is stabilised by deubiquitination via USP7 and phosphorylation. USP10 deubiquitinates p53 in an ATM-mediated phosphorylation-dependent manner. Stabilised p53 interacts with co-activator proteins leading to increased transcriptional activity.MDM2 is regulated by auto-ubiquitination which is counter-acted by USP7.

5.1.3 USP10, MDM2 and AR regulation

Both USP10 and MDM2 have been shown to function as AR co-regulators. USP10 was identified in a study by Faus *et al.,* to be in complex with DNA-bound AR in nuclear extracts of a PC-3, AR-null, cell line stably over-expressing AR. This result was confirmed using radio-labelled *in vitro* translated AR and recombinant GST-tagged USP10 in GST pull-down experiments, where AR was only observed in USP10 containing samples compared to GST-tag only controls. The cellular localisation of USP10 was found to be both cytoplasmic and nuclear in the PC-3/AR cells and USP10 increased AR transcriptional activity upon two luciferase reporters, containing four copies of either the ARE-1 or ARE-2 of the murine androgen-responsive *Pem* gene. Moreover, the effect of USP10 on AR activity was found to be deubiquitinase-dependent as mutation of the catalytic cysteine of USP10, Cys424, showed almost basal levels of AR activity towards the ARE-1 reporter. Similarly, depletion of USP10 with siRNA in PC-3/AR cells stably expressing a MMTV luciferase reporter construct showed basal levels of AR activity (Faus *et al.*, 2005).

Most recently, Draker *et al*., have linked USP10 to AR transcriptional activity through association with and regulation of mono-ubiquitinated histone H2A.Z. Cell-free *in vitro* deubiquitination assays and the observation that levels of ubiquitinated histone H2A.Z decreased in 293T cells over-expressing USP10 suggested that USP10 directly deubiquitinated histone H2A.Z. Furthermore, depletion of USP10 by stable shRNA expression in LNCaP cells increased the levels of ubiquitinated histone H2A.Z at the promoter regions of both the *PSA* and *KLK2* genes suggesting that this mark is retained upon knockdown and providing additional evidence that USP10 is responsible for deubiquitination of this histone variant. Deubiquitination of histone H2A.Z increased expression of both *PSA* and *KLK2* genes in LNCaP cells and PC-3/AR luciferase experiments using an ARE3 luciferase reporter, similar to that used in the current study, confirming USP10 is a potential co-activator of AR transcriptional activity (Draker *et al.*, 2011). Interestingly, immuno-fluorescence and cytoplasmic-nuclear extraction in LNCaP cell confirmed that USP10 resides in both the nuclear and cytoplasmic compartments in concordance with Faus *et al*.

Consistent with the role of MDM2 as an E3 ubiquitin ligase, the enzyme has been shown to repress AR transcriptional activity by causing ubiquitin-dependent

degradation of the receptor (Lin *et al.*, 2002b; Gaughan *et al.*, 2005). The initial demonstration that MDM2 was an E3 ligase of the AR came on the back of some earlier observations that the Akt pathway actively reduced receptor protein levels (Lin *et al.*, 2001). Akt was found to directly phosphorylate serine residues 210 and 790 of the receptor, as well as key residues in MDM2, that was required to drive interaction between AR and the E3 ligase. Upon binding, MDM2 catalysed poly-ubiquitination of the AR that facilitated degradation of the AR; a process blocked by the proteasomal inhibitor MG132, implicating a role for the proteasome in receptor turnover (Lin *et al.*, 2002b). Interestingly, MDM2 is found in complex with AR at the PSA promoter suggesting that transcription and degradation of the receptor is coupled allowing tight regulation of AR transcriptional activity (Gaughan *et al.*, 2005).

5.1.4 USP10 and disease

A quantitative real-time gene array study conducted In the aggressive brain tumour glioblastoma multiforme identified USP10, in conjunction with thymidylate synthetase and survivin, as being significantly over-expressed in patients who die of the disease (DOD) compared to those with long term survival (LTS) over 36 months post-surgery (Grunda *et al.*, 2006). This study, however, analysed only a small number of tissue samples, 8 LTS and 13 DOD patients, and the 96 genes included on the array were selected based on their known involvement in cancer-related pathways such as apoptosis, tumour progression and patient survival. Immuno-histochemical staining of paraffin-embedded glioblastoma tissue for USP10 confirmed over-expression of the protein in DOD tissue compared to LTS tissue. Unfortunately, this study did not elucidate the molecular role for USP10 in glioblastoma but the authors suggested that further study into proteins involved in the ubiquitin-proteasome system may provide further prognostic indicators of survival in this disease (Grunda *et al.*, 2006).

Intriguingly, expression profiling of 6 infiltrating ductal breast carcinoma samples, to identify proteins associated with the ubiquitin proteasomal pathway and ubiquitination, identified USP10 as one of ten ubiquitin pathway associated genes, including four USPs, to be over-expressed by more than three-fold in carcinoma compared to normal breast tissue (Deng *et al.*, 2007). 2D gel electrophoresis and mass spectrometry analysis did not detect USP10 as an over-expressed protein in the breast cancer samples, as a threshold of 4-fold over-expression compared to normal breast

tissue was applied; therefore USP10 was not investigated by immuno-histochemistry. The authors speculate that the four over-expressed USPs identified, may aberrantly regulate the rate of protein turn-over thereby preventing protein degradation in cancer and that these, along with the five proteasome subunits and E3 ubiquitin ligase E6-AP, potentially provide therapeutic targets in infiltrating ductal carcinoma (Deng *et al.*, 2007).

In a study investigating the role of USP10 as a regulator of MDM2-mediated ubiquitination and degradation of p53, USP10 deubiquitination of p53 increased p53 function and inhibited cell proliferation of the p53-wild type colorectal carcinoma cell HCT116 (Yuan *et al.*, 2010). The authors suggested that USP10 may act as a tumour-suppressor and in renal cell carcinoma (RCC), a cancer that rarely harbours p53 mutations; down-regulation of USP10 may act as a mechanism for down-regulating p53. USP10 was confirmed to be down-regulated in p53-wild type RCC cell lines and the level of p53 protein was also found to be lower. 90% of clear cell carcinoma, 50% of chromophobe and 20% of papillary RCC tissues on tissue microarrays containing a total of approximately 230 RCC cases showed negative staining for both USP10 and p53. Interestingly, all cell lines and samples with p53 mutation showed up-regulation of both USP10 and p53 protein. Furthermore, reconstitution of USP10 expression in the USP10 down-regulated RCC cell lines Caki-1 and Caki-2 restored p53 protein levels, increased expression of p53-target genes p21 and Bax and inhibited cell proliferation and colony forming efficiency (Yuan *et al.*, 2010).

5.2 Aims

USP10 was identified as a potential AR co-regulator by the Solid Tumour Target Discovery Group, using a siRNA library screen containing 72 DUB targets in LNCaP cells. USP10 knockdown was shown to consistently up-regulate PSA secretion, as measured by ELISA, and *PSA* mRNA expression that is in contrast to recently published work describing the DUB as a co-activator of the AR (Faus *et al.*, 2005; Draker *et al.*, 2011).

The specific aims of this Chapter are:

- To validate the effect of *USP10* knockdown on *PSA* mRNA and protein expression
- To further characterise the effect of USP10 on AR transcriptional activity
- To elucidate if USP10 and AR interact directly

5.3 Results

5.3.1 Effect of USP10 knockdown on AR-regulated PSA gene expression over 72 hours DHT stimulation

USP10 was initially identified in the Solid Tumour Target Discovery Laboratory, Newcastle University, as a potential AR co-regulator using an in-house DUB siRNA library in androgen-dependent LNCaP cells to address the effect of DUB depletion on AR activity (Figure 3.2). Individual DUB enzymes were depleted in LNCaP cells for 24 hours in steroid-depleted media prior to 100 nM DHT treatment for 72 hours and PSA secretion analysed by ELISA. As described in Chapter 3.3.1, the criteria for target selection was based on 50% modulation of PSA secretion in response to knockdown, and although none of the targets caused an increase in PSA secretion over the 50% threshold, USP10, USP8 and USP38 all increased PSA secretion by 40% above the SCR control and hence were selected for further validation experiments.

Knockdown of these targets was then investigated for effect on *PSA* mRNA expression. USP10 was the only target to consistently increase *PSA* mRNA levels upon knockdown and was therefore selected for further validation (Figure 3.3).

As described in Chapter 3.3.2 each target was initially depleted using a pool of three siRNA oligonucleotides and none of the knockdowns were confirmed during the initial QPCR analysis to validate the PSA ELISA data. Therefore, to demonstrate that the effect of USP10 depletion on *PSA* expression was a consequence of DUB knockdown, USP10 levels were analysed using individual *USP10* oligonucleotides. LNCaP cells were subjected to USP10 depletion by each siRNA oligo individually and SCR siRNA was used as a negative control. This work was performed by Dr Steven Darby.

Figure 5.4 shows the mean of three experimental repeats for *USP10* expression and indicates that siRNA oligo B gave the best knockdown; this was subsequently used as the sole siRNA in the further validation steps (Figure 5.4). Unfortunately, no *PSA* expression data was obtained for this validation experiment and the pooled siRNA was not included as a comparison.

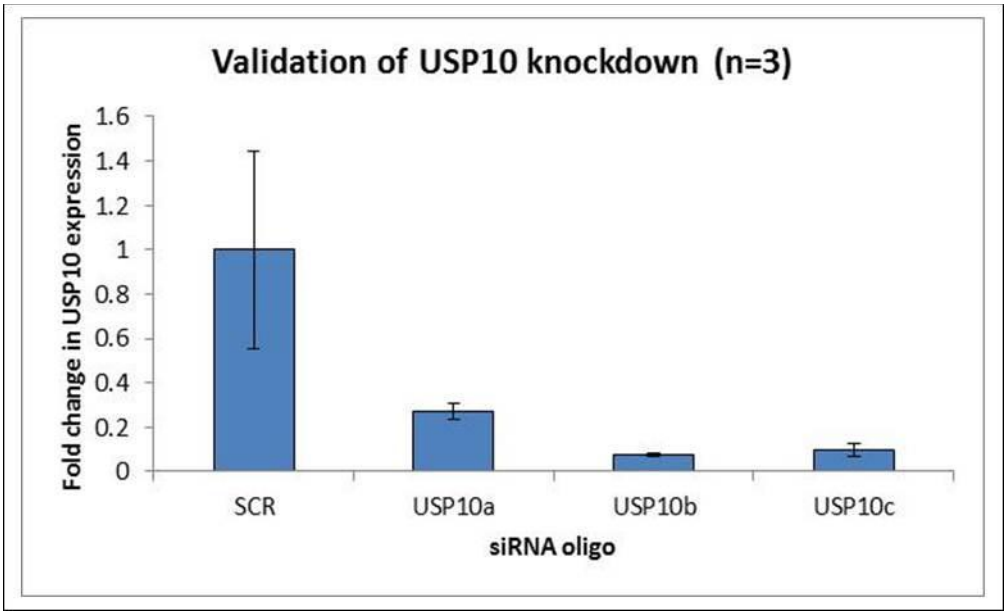

Figure 5.4. Validation of USP10 knockdown in LNCaP cells.

USP10 was depleted in LNCaP cells for 48 hours prior to 100 nM DHT stimulation for a further 48 hours. Scrambled siRNA (SCR) was used as a negative control. Data shows the effect of individual USP10 siRNA oligos on USP10 mRNA expression. Data is represented as fold change compared to the SCR control. Data is representative of the mean of three independent repeats and error bars show the standard error. This work was performed by Dr Steven Darby.

As with the initial validation experiments in Chapter 3.3.3, the next step of the investigation was to study the effect of *USP10* knockdown on *PSA* mRNA expression over a time course of 72 hours DHT treatment. *USP10* was depleted in LNCaP cells for 48 hours before 100 nM DHT was applied and cells harvested for RNA extraction at 0, 12, 48 and 72 hours post-DHT treatment. QPCR was used to analyse both *PSA* and *USP10* knockdown and scrambled (SCR) siRNA was used as a control. Given that this experiment was performed at the very early stages of target validation, the work was performed by Dr Steven Darby who had performed the initial PSA ELISA screen. Figure 5.5 shows a representative data-set of three independent repeats, and the data is presented as relative expression compared to *HPRT1* house-keeping gene expression.

As shown in Figure 5.5a, knockdown of USP10 showed an increase in *PSA* expression at all time-points in the absence of DHT stimulation, *PSA* expression was 5.9-fold above the SCR control, suggesting a role for USP10 in regulating the basal activity of the AR. As expected, ligand treatment enhanced *PSA* expression in the SCR control by approximately 10-fold at 10 hours that remained at this level for the duration of the experiment. Remarkably, USP10 depletion steadily enhanced *PSA* expression in the presence of hormone to a maximum level of 48.7-fold above SCR control at 72 hours. Interestingly, this is a much greater level than observed at the same time point for the initial PSA QPCR (Figure 3.3) and may be attributed to better knockdown achieved with a single siRNA. *USP10* expression was reduced by at least 80% in response to knockdown throughout the 72 hour time-course experiment (Figure 5.5b, compare the purple and red lines).

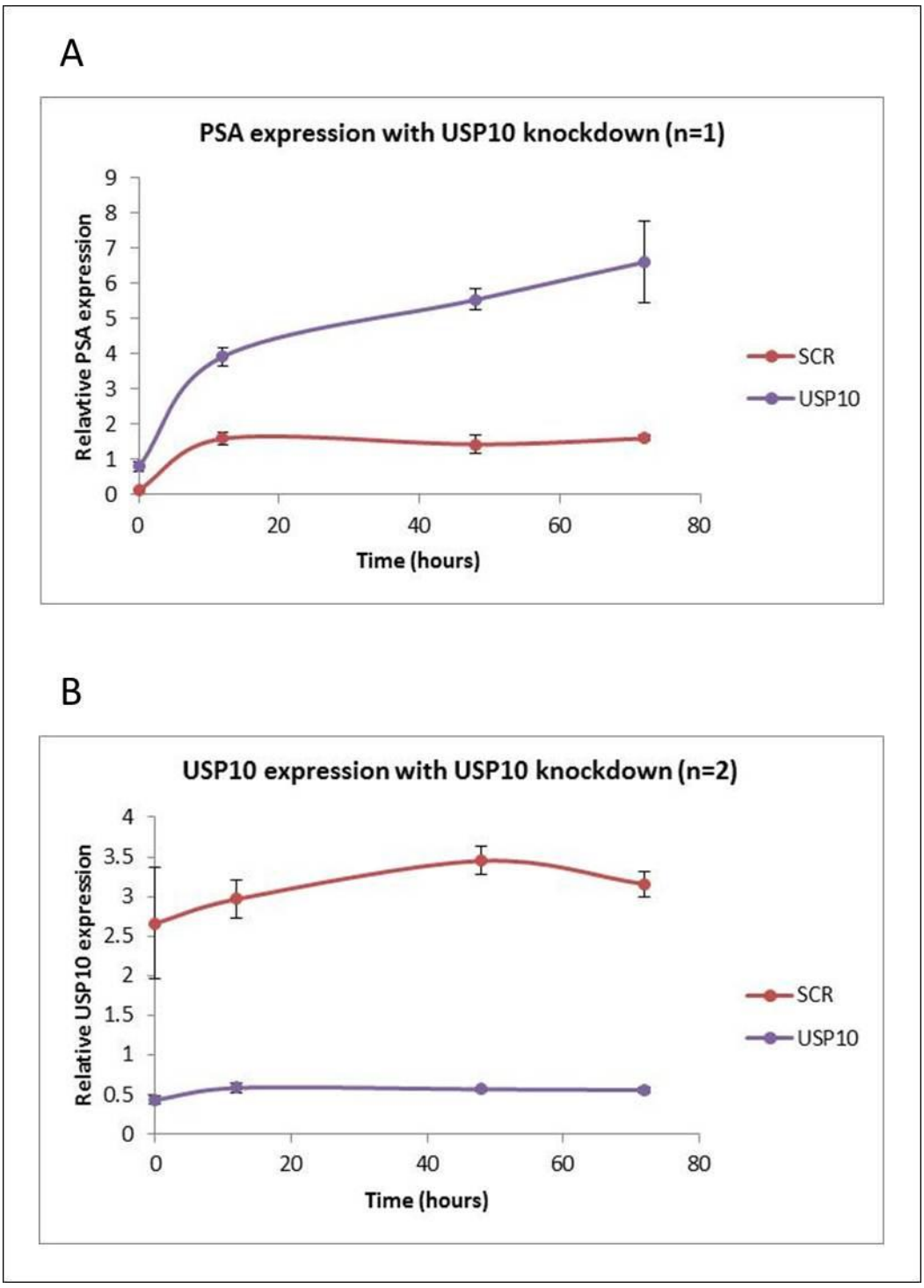

Figure 5.5. Effect of USP10 knockdown on PSA expression over 72 hours DHT stimulation.

USP10 was depleted by siRNA in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 12, 48 and 72 hours post treatment. Scrambled siRNA (SCR) was used as a negative control. Results are from one repeat and are representative of three independent repeats. Data is shown as relative expression compared to the HPRT1 house-keeping gene and error bars show the standard deviation between experimental triplicates. A) The effect of USP10 knockdown on prostate specific antigen (PSA) expression over 72 hours DHT treatment. B) The effect of USP10 knockdown on USP10 expression over 72 hours DHT treatment. This work was performed by Dr Steven Darby.

To confirm that the effect on *PSA* was also apparent at the protein level, Western blot analysis of PSA was performed. Furthermore, to address whether the effect of USP10 depletion on PSA expression could be attributed to a change in AR protein, receptor levels were measured by Western analysis. A time-course experiment was set up as described above and cell lysates were collected at each time point. SDS-PAGE and Western blotting for PSA, AR and USP10 to confirm protein knockdown was performed. α-tubulin was used as a loading control. Figure 5.7 shows the densitometry analysis of the three experimental repeats and is presented as the mean relative protein expression normalised to the respective α-tubulin loading control. Data was analysed for statistical significance using 2-way ANOVA, significant data is indicated on the figure (Figure 5.7).

Consistent with Chapter 3.3.3, no significant difference was observed between the non-transfected (NT) and SCR controls for each protein analysed indicating that there are no discernible off-target or transfection-related affects during each of the experiments. As expected, PSA and AR protein levels both increased in response to DHT stimulation in the NT and SCR controls, while USP10 levels remain largely unchanged over the course of the experiment (Figure 5.6a and b). Consistent with *PSA* being an androgen-responsive gene, *AR* knockdown, as confirmed by anti-AR Western blotting, reduced PSA expression but had no effect on USP10 levels (Figure 5.6c).

As shown in Figure 5.6d, knockdown of *USP10* showed a similar effect on PSA protein levels to the SCR control within the first 24 hours DHT treatment in that there was a small increase in PSA protein compared to the un-treated control. However, at 48 and 72 hour time-points, PSA protein levels were massively elevated in the *USP10* depleted samples compared to the SCR controls suggesting a role for USP10 in AR-mediated transcription. This notion was further strengthened by demonstrating that AR levels in the *USP10* depleted samples mimic those in the NT and SCR controls suggesting changes to PSA expression is not a result of increased AR protein (Figure 5.6d). USP10 Western analysis confirmed depletion of the DUB enzyme (Figure 5.6d).

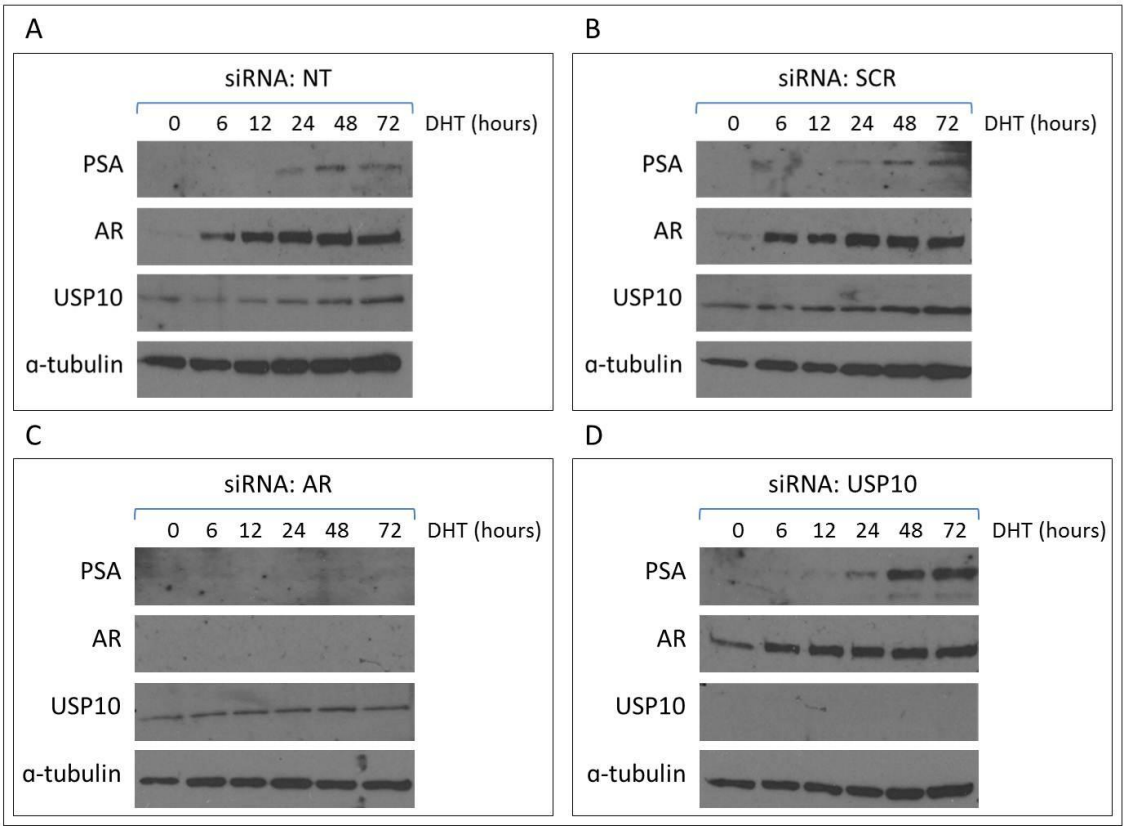

Figure 5.6. Effect of USP10 knockdown on PSA and AR protein levels.

LNCaP cells were subject to siRNA knockdown for 48 hours prior to 72 hours 100 nM DHT stimulation. Cell lysates were collected at 0, 6, 12, 24, 48 and 72 hours post-treatment. Western blotting for prostate specific antigen (PSA), androgen receptor (AR) and USP10 were performed. α -tubulin was used as a loading control. Non-transfected (NT), scrambled siRNA (SCR) and AR knockdown were used as negative and positive controls. Western blots are representative of three independent experimental repeats. A) The effect of the NT control on PSA and AR protein levels. B) The effect of the SCR control on PSA and AR protein levels. C) The effect of AR knockdown on PSA and AR protein levels. AR Western blot confirmed AR was depleted throughout the time course. D) The effect of USP10 knockdown on PSA and AR protein levels. Western blot for USP10 confirmed USP10 was depleted throughout the time course.

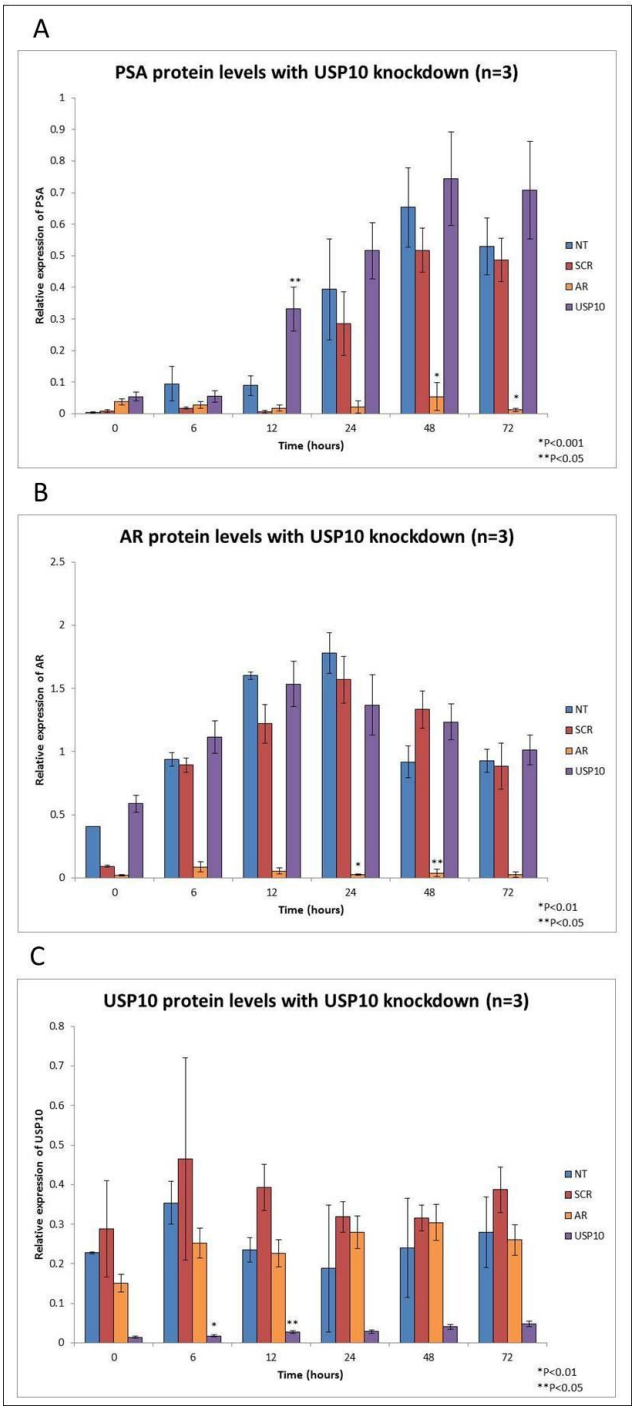

Figure 5.7. Densitometry analysis of the effect of USP10 knockdown on PSA and AR protein levels.

Western blot data from the time-course experiments was analysed using the Quantity one programme on the GelDoc™ system. Data for prostate specific antigen (PSA) and androgen receptor (AR) protein levels were normalised to the respective α-tubulin loading control data. Data is presented as the mean relative expression of three independent experimental repeats. Error bars represent the standard error. Data was analysed for statistical significance using 2-way ANOVA. Each knockdown was compared to the SCR control at each time point, significant data is indicated by an asterisk. A) The effect of siRNA knockdown on PSA protein levels over 72 hours DHT treatment. B) The effect of siRNA knockdown on AR protein levels over 72 hours DHT treatment. C) The effect of USP10 knockdown on USP10 protein levels over 72 hours DHT treatment.

5.3.2 Effect of USP10 knockdown on other AR-regulated gene expression

To assess whether *USP10* knockdown also had a similar effect on four other AR-regulated genes studied in Chapter 3.3.4, *KLK2*, *TMPRSS2*, *NKX3.1* and *NDRG1* mRNA expression was analysed using the same sample set as above (Figure 5.5). Figure 5.8 shows a representative data-set of three independent repeats, and the data is presented as relative expression compared to *HPRT1* house-keeping gene expression. This work was performed by Dr Steven Darby.

As expected, all of the androgen-responsive genes showed characteristic induction in expression in response to DHT treatment (Figure 5.8, red line) (Ngan *et al.*, 2009). *KLK2*, *TMPRSS2* and *NDRG1* showed similar expression profiles to those observed previously (Figure 3.14a, b and c, red line), however in all cases, the peak of expression was delayed, for example, *KLK2* and *TMPRSS2* showed maximal expression at approximately 55 hours DHT treatment (Figure 5.8a and b, red line) whereas previous experiments showed expression to peak at approximately 20 hours post-androgen treatment (Figure 3.14a and b, red line). Unlike the other androgen-responsive genes, the expression profile of *NKX3.1* was markedly different to that observed previously, as expression did not reach a maximum level before returning to basal levels (Compare Figure 3.14c and Figure 5.8c, red line).

Unlike *USP12* knockdown, that reduced transcription of each of the AR-target genes, depletion of USP10 did not have the same effect on all of the tested AR-regulated genes. *KLK2* and *TMPRSS2* expression were enhanced with *USP10* knockdown and were consistent with *PSA* expression (Figure 5.8a and b, purple line). *NKX3.1* expression, on the other hand, was reduced in cells depleted of *USP10* compared to the SCR control during the first 24 hour of the time-course. Expression of this AR target gene then increased to higher levels than the SCR control at 48 hours post-DHT treatment which then decreased and returned to the same level of expression as the SCR control at 72 hours (Figure 5.8c, compare red and purple lines). *USP10* knockdown appeared to delay the expression of *NDRG1* in response to DHT, with an increase in expression observed after approximately 24 hours DHT treatment compared with approximately 12 hours in the SCR control (Figure 5.8d, compare red and purple lines). Overall the expression of *NDRG1* was lower in response to USP10 depletion compared

to the SCR control, however, unlike the SCR control where expression returned to basal levels, *NDRG1* expression continued to increase (Figure 5.8d, purple line).

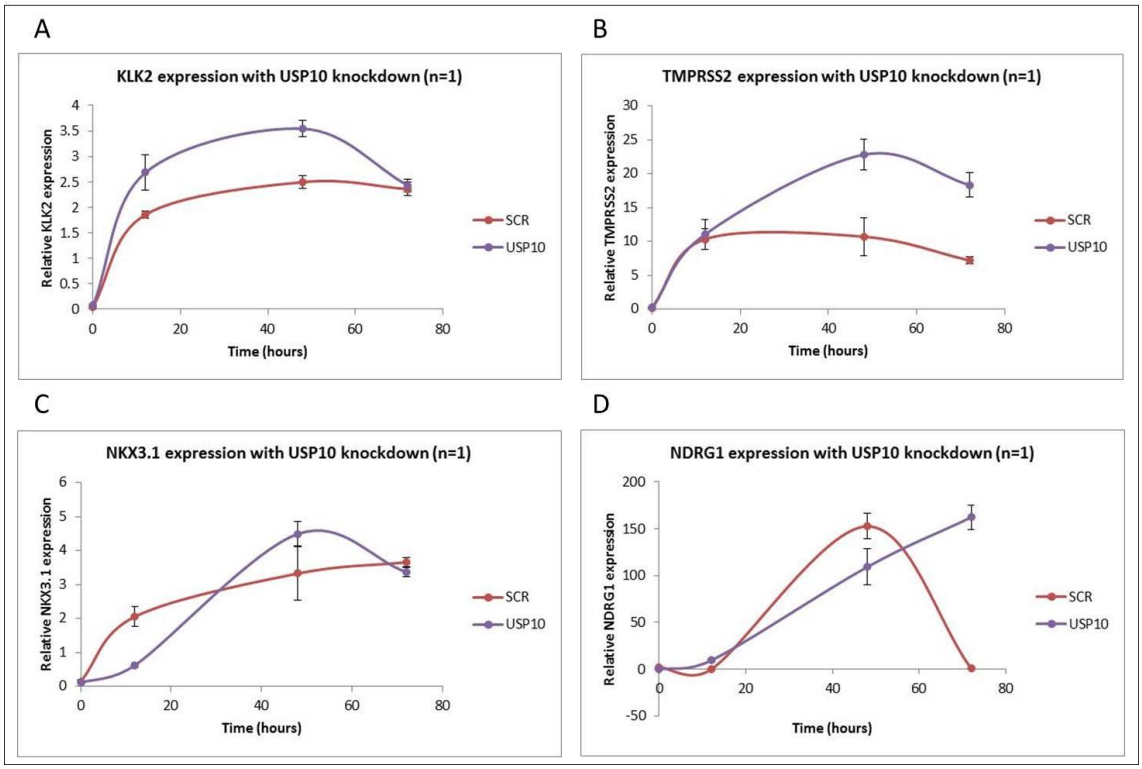

Figure 5.8. Effect of USP10 depletion on AR regulated gene expression over 72 hours DHT stimulation.

USP10 was depleted in LNCaP cells for 48 hours before 100 nM DHT treatment was applied for a further 72 hours. RNA was collected at 0, 6, 12, 24, 48 and 72 hours post treatment. Scrambled siRNA (SCR) was used as a negative control. Results are from one repeat and are representative of three independent repeats. Data is shown as relative expression compared to the HPRT1 house-keeping gene and error bars show the standard deviation between experimental triplicates. A) The effect of USP10 knockdown on KLK2 expression over 72 hours DHT treatment. B) The effect of USP10 knockdown on TMPRSS2 expression over 72 hours DHT treatment. C) The effect of USP10 knockdown on NKX3.1 expression over 72 hours DHT treatment. D) The effect of USP10 knockdown on NDRG1 expression over 72 hours DHT treatment. This work was performed by Dr Steven Darby.

5.3.3 USP10 down-regulates the transcriptional activity of the AR

The above data showed that, at least for a subset of AR regulated genes, *USP10* knockdown caused an up regulation of expression. This suggested, therefore, that USP10 may be acting as a transcriptional co-repressor of the AR in LNCaP cells. To investigate this further, an androgen-responsive luciferase reporter system was utilised to interrogate whether USP10 acts as a repressor of AR transcriptional activity and whether any effect could be amplified with increasing amounts of USP10.

To this end, COS-7 cells were transfected with 100ng of both ARE3 and β-gal and 50ng of AR in serum-containing media. USP10 was transfected at increasing quantities between 25-100 ng (represented as +, + and +++). Media was replaced 24 hours post-transfection with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for an additional 48 hours. The average and standard error of the mean was calculated for each triplicate. Only assays that showed greater than 3-fold AR induction with DHT stimulation were deemed to have been successful; this was applied to all luciferase reporter assays. Data shown is from three independent repeats and is represented as fold change compared to the un-stimulated AR only control. Data was analysed for statistical significance using the Student's t-test, significant data are indicated on the figure.

As expected, AR activity was significantly increased by approximately 6-fold upon treatment with DHT (P=0.0017) (Figure 5.9, compare lanes 1 and 2). Co-transfection of 25 ng of USP10 (+) had no effect on DHT-stimulated AR activity, while 50 ng (++) and 100 ng (+++) of USP10 slightly reduced AR-mediated luciferase activity (Figure 5.9, compare lanes 6 and 8 with lane 2), although this effect was not statistically significant (P=0.4745 and P=0.266, respectively).

Western blot analysis revealed that ectopic AR was again expressed in each of the samples (Figure 5.9b, upper panel). Interestingly, there appeared to be increased AR in the samples containing 50 ng and 100 ng USP10 which were not treated with DHT (Figure 5.9b, Upper panel, lanes 5 and 7). USP10 was detected in each of the samples with ectopic expression (Figure 5.9b, middle panel, lanes 3-8). Low levels of endogenous USP10 were observed in the AR only controls (Figure 5.9b, middle panel, lanes 1 and 2).

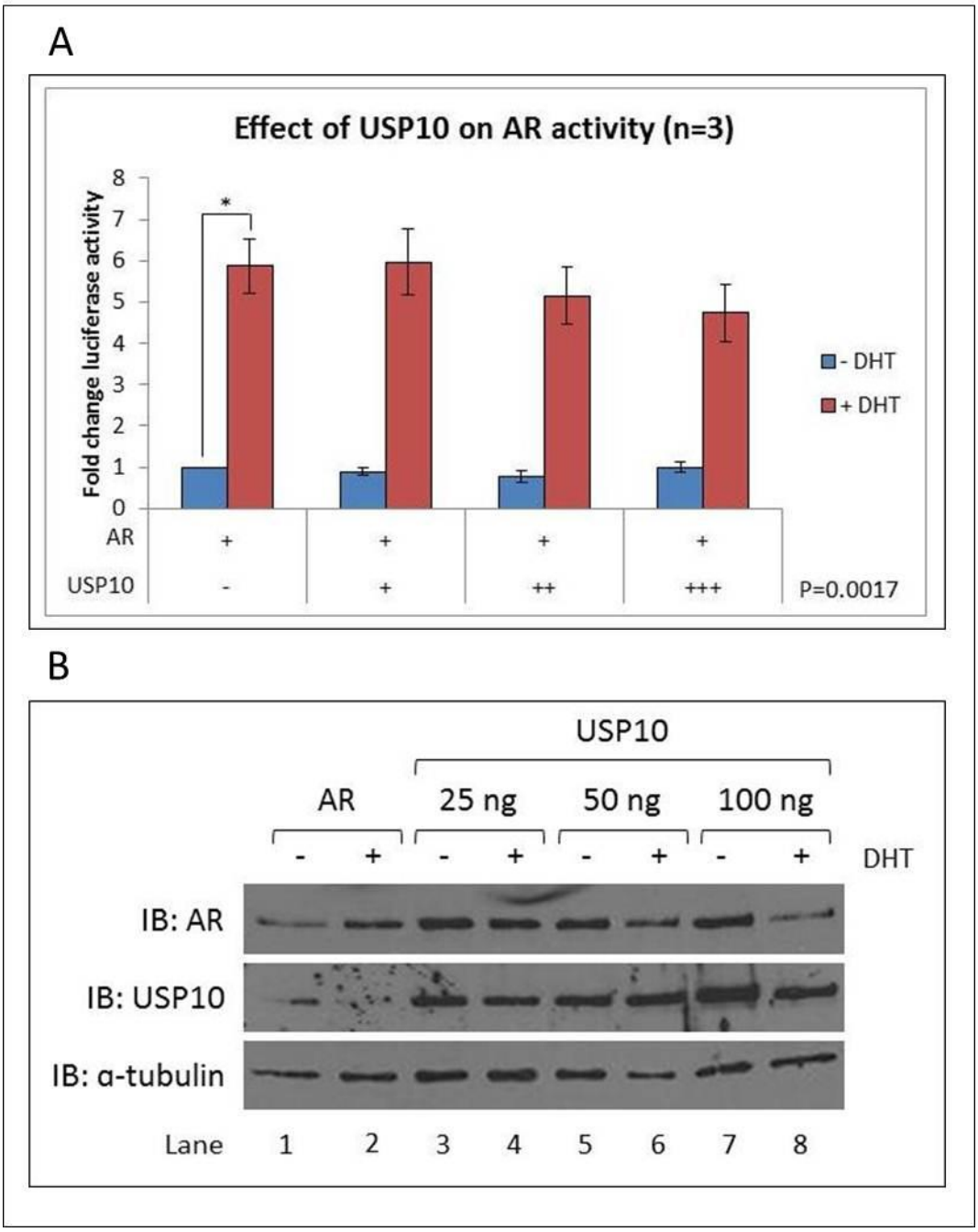

Figure 5.9. Effect of USP10 on AR transcriptional activity in COS-7 cells.

COS-7 cells were transfected with 50 ng androgen receptor (AR), 100 ng of both an AR responsive ARE3 luciferase reporter and a β-gal reporter and increasing amounts of USP10; 25 ng (+), 50ng (++) and 100ng (+++). 24 hours after transfection, cells were grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is a mean of three independent repeats and error bars represent the standard error of the mean of the three replicates. Data was analysed for statistical significance using the Student's t-test, significant data is indicated by an asterisk. B) Luciferase assay samples were analysed by Western blotting with anti-AR and -FLAG antibodies to ensure over-expression of ectopically expressed proteins. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats..

In contrast to our findings above, the study conducted by Faus *et al.*, demonstrated that exogenous over-expression of USP10 (75 ng) stimulated AR transcriptional activity in luciferase reporter-based assays utilising two reporter constructs containing four copies of either the ARE-1 or ARE-2 of the androgen responsive murine *Pem* gene promoter. These findings were confirmed by knockdown of *USP10* in a PC-3 cell line stably over-expressing both AR and an MMTV luciferase construct; depletion of USP10 resulted in decreased luciferase activity. Furthermore, Draker *et al.,* confirmed that both knockdown of *USP10* increased AR transcriptional activity in experiments utilising an ARE3 luciferase reporter, supporting the notion that USP10 is a transcriptional co-activator of the AR. However, these experiments were performed in the AR null PC-3 cell lines that had been stably transfected with AR, suggesting the observed disparity between data-sets may be a consequence of variability in the cell background. It was therefore pertinent to ask whether transiently expressing AR and USP10 in PC-3 cells would reproduce the results described in the Faus *et al*., and Draker *et al*., studies (Faus *et al.*, 2005; Draker *et al.*, 2011).

PC-3 cells were transfected with 100ng of both ARE3 and β-gal and 50ng of AR in serum-containing media. USP10 was transfected at increasing quantities between 25-100 ng (represented as +, + and +++). Media was replaced 24 hours post-transfection with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for an additional 48 hours. As the data shown in Figure 5.10 is from only two repeats it should be further repeated to draw firm conclusions, particularly when considering the variability between the experimental replicates. Data was analysed for statistical significance using the Student's t-test, however no data points were found to be significant.

AR activity was induced 15.5-fold upon DHT stimulation that was markedly higher than that observed in COS-7 cells (Figure 5.9 and Figure 5.10). Increasing amounts of USP10 showed a marginal decrease in AR activity; 25 ng of USP10 had little effect compared to the AR only control reducing induction to approximately 15-fold whilst 50 ng of USP10 reduced induction to approximately 11-fold (Figure 5.10, compare lanes 4 and 6 with lane 2). 100 ng USP10 did not have any additional effect over 50 ng with induction remaining at 11-fold (Figure 5.10, compare lane 8 with lane 6). Although none of these results reached statistical significance these results support our previous findings and

are contradictory to both the Faus *et al*., and Draker *et al*., reports. Further experimental repeats would be necessary in order to obtain statistically relevant results. AR and USP10 over-expression was confirmed by Western blot analysis (Figure 5.10b).

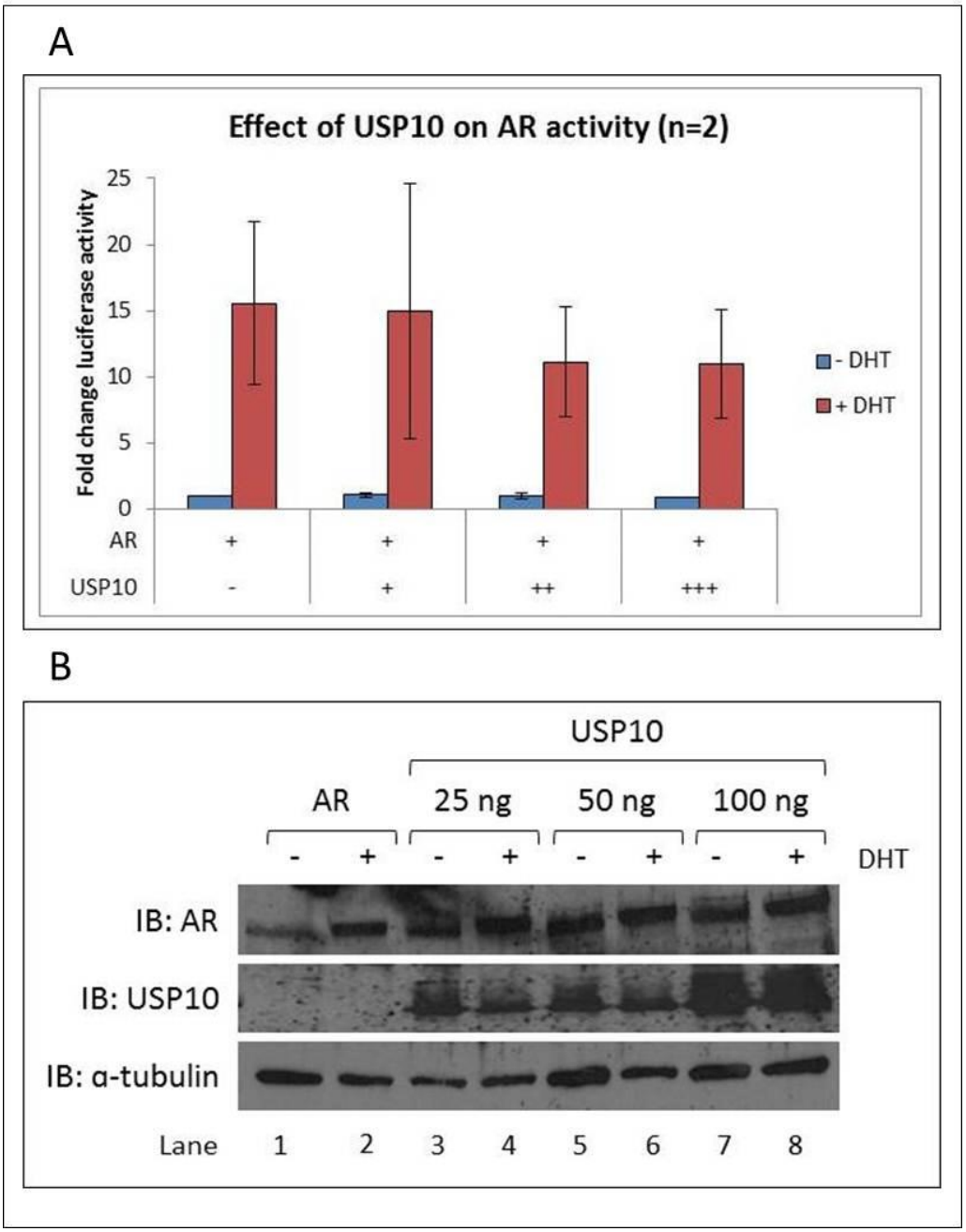

Figure 5.10. Effect of USP10 on AR transcriptional activity in PC-3 cells.

PC-3 cells were transfected with 50 ng androgen receptor (AR), 100 ng of both ARE3 luciferase and β-gal reporters and increasing amounts of USP10; 25 ng (+), 50ng (++) and 100ng (+++). 24 hours after transfection, cells were grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is a mean of two independent repeats and error bars represent the standard error of the mean of the two replicates. Data was analysed for statistical significance using the Student's t-test, however no data points were significant. B) Luciferase assay samples were analysed by Western blotting with anti-AR and -USP10 antibodies to ensure over-expression of ectopically expressed proteins. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats.

5.3.4 USP10 does not directly interact with the AR in LNCaP cells

The data thus far has revealed that USP10 acts to repress endogenous AR activity in the LNCaP cell line, and does not act as a receptor co-activator in luciferase-based reporter experiments in COS-7 and PC-3 cells as previously documented (Faus *et al.*, 2005; Draker *et al.*, 2011). Faus *et al.,* also provided evidence that there is a direct association between both proteins in PC-3 cells ectopically expressing AR and USP10. USP10 was identified firstly as an AR interacting protein in DNA response element bait experiments. Nuclear extracts of the PC-3/AR cell line treated with synthetic androgen R1881 were incubated with a biotin-tagged ARE-2 of the androgen responsive gene *Pem.* The interacting proteins were then purified by streptavidin affinity chromatography, separated by 2D gel electrophoresis and identified by mass spectrometry (Faus *et al.*, 2005). Furthermore, radio-labelled AR generated using the rabbit reticulocyte lysate transcription/translation system was found to interact with GST-tagged USP10 *in vitro* (Faus *et al.*, 2005). These experiments however were performed in the stably transfected PC-3/AR cell line or in a cell free environment providing no evidence of an endogenous interaction between USP10 and AR in an AR-positive cell line. As USP10 depletion in LNCaP cells showed an effect on AR transcriptional activity it suggested that USP10 may exert its effects on the endogenous receptor through a direct interaction therefore immunoprecipitation studies were performed to elucidate this.

LNCaP cells were cultured in serum-containing media for 48 hours prior to immunoprecipitation using both anti-AR or USP10 antibodies and Western analysis using the same immuno-globulins. Extract and antibody only controls were also included as described in Chapter 2.9.

Firstly, input samples, that represent a sample of the whole cell extract, confirmed expression of both AR and USP10 in LNCaP cells (Figure 5.11a and b, lane 1). However, although anti-AR and USP10 antibodies were able to immuno-precipitate AR and USP10, respectively (Figure 5.11a and b, lower panels lane 2), probing the immuno-precipitates with reciprocal antibodies failed to detect an association between USP10 and the receptor (Figure 5.11a and b, upper panels lane 2). These results, therefore, suggest that endogenous AR and USP10 do not interact within LNCaP cells however studies of the interaction between the endogenous proteins in other cell lines would

be required to draw any firm conclusions. In addition, ectopic expression of both AR and USP10 in PC-3 cells would provide a logical next step in the investigation as although the luciferase reporter data in PC-3 cells contradicted the previous studies (Faus *et al.*, 2005; Draker *et al.*, 2011), the fact that USP10 impacted on AR activity may suggest an interaction between the two proteins.

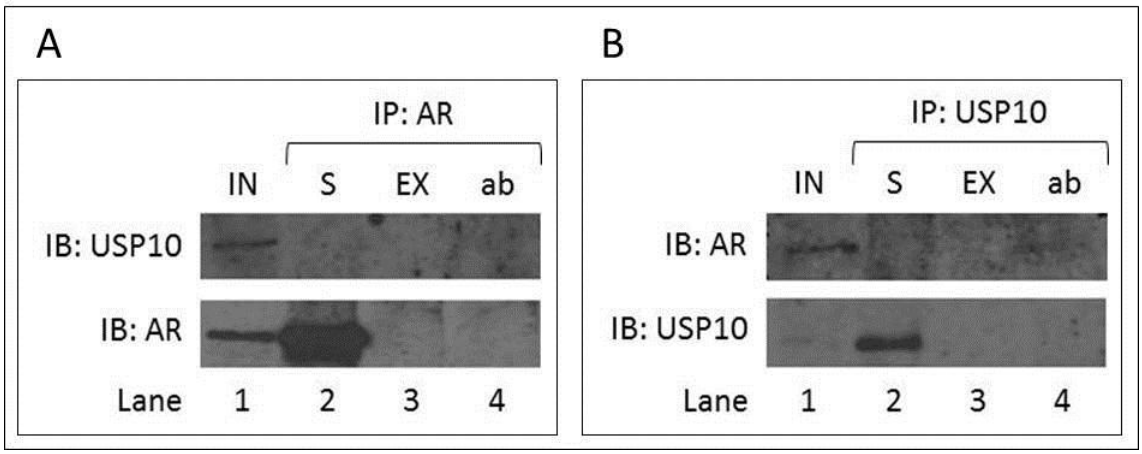

Figure 5.11. AR and USP10 do not interact in LNCaP cells.

LNCaP cells grown in serum-containing media were subject to immunoprecipitation. Inputs (IN) were taken before IP to ensure endogenous expression of both androgen receptor (AR) and USP10. Samples (S) were incubated with anti-AR or anti-USP10 antibodies before incubation with Protein G sepharose (PGS). Extract (EX) samples were incubated with PGS but where not exposed to antibodies. The antibody only control (ab) contained only lysis buffer mock immuno-precipitated with anti-AR or anti-USP10 antibodies. Western blots are representative of three independent experimental repeats. A) Representative Western blots for AR IP, using anti-USP10 (upper panel) and anti-AR (lower panel) antibodies. B) Representative Western blots for USP10 IP using anti-AR (upper panel) and anti-USP10 (lower panel) antibodies.

5.3.5 Effect of USP10 and MDM2 on AR transcriptional activity

5.3.5.1 Effect of MDM2 on AR transcriptional activity

As described above, a number of E3 ubiquitin ligases involved in ubiquitination of p53 are also linked to the regulation and ubiquitination of the AR, including MDM2 (Lin *et al.*, 2002b; Gaughan *et al.*, 2005). MDM2 promotes proteasomal degradation of the AR, potentially via catalysis of lysine-48 linked poly-ubiquitin chains, ultimately reducing activity of the receptor (Lin *et al.*, 2002b; Gaughan *et al.*, 2005). Furthermore, a recent study associated USP10 with both MDM2 and p53 showing that MDM2-mediated nuclear export and subsequent degradation of p53 could be reversed by USP10 (Yuan *et al.*, 2010). Considering that USP10 acts to reduce AR activity in LNCaP cells (Figure 5.5 and Figure 5.6) coupled to what is already known about the role of MDM2 in receptor regulation, it was hypothesised that unlike p53, USP10 may cooperate with MDM2 to enhance repression of AR activity.

To this end, luciferase assays were performed to firstly confirm that MDM2 has a repressive effect on the AR as reported previously (Lin *et al.*, 2002b; Gaughan *et al.*, 2005). COS-7 cells were transfected with 100ng of both ARE3 luciferase and β-gal reporters together with 50 ng of AR and either 50 ng or 100ng of MDM2 (represented as + and ++ respectively) for 24 hours in serum-containing media prior to replacement with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. Luciferase activity was assayed and normalised against β-gal activity. Data was analysed for statistical significance using the Student's t-test, however no data points were found to be significant.

As expected and consistent with data in Figure 5.9, AR activity was increased approximately 6-fold in response to DHT treatment (Figure 5.12, compare lanes 1 and 2). However, unlike the literature, co-transfection of cells with MDM2 caused no change in androgen-dependent AR activity compared to the control; 6.7-fold and 5.9-fold above the un-treated AR control with 50 ng and 100 ng MDM2, respectively (Figure 5.12, compare lanes 4 and 6 with lane 2). Inclusion of MDM2 overall caused no significant change in AR activity compared to the AR only control with DHT treatment (P=0.7979 and P=9899 for 50 ng and 100 ng MDM2, respectively). Unfortunately, a large amount of variation between individual repeats resulted in large error bars; however, it is important to note that no singular repeat resulted in MDM2-mediated

reduction of AR activity compared to the AR only control but a variable amount of AR induction with androgen stimulation was observed.

Western blot analysis showed over-expression of AR in each of the samples (Figure 5.12b, upper panel). Unfortunately, due to problems with the anti-MDM2 antibodies analysis of MDM2 over-expression was not possible.

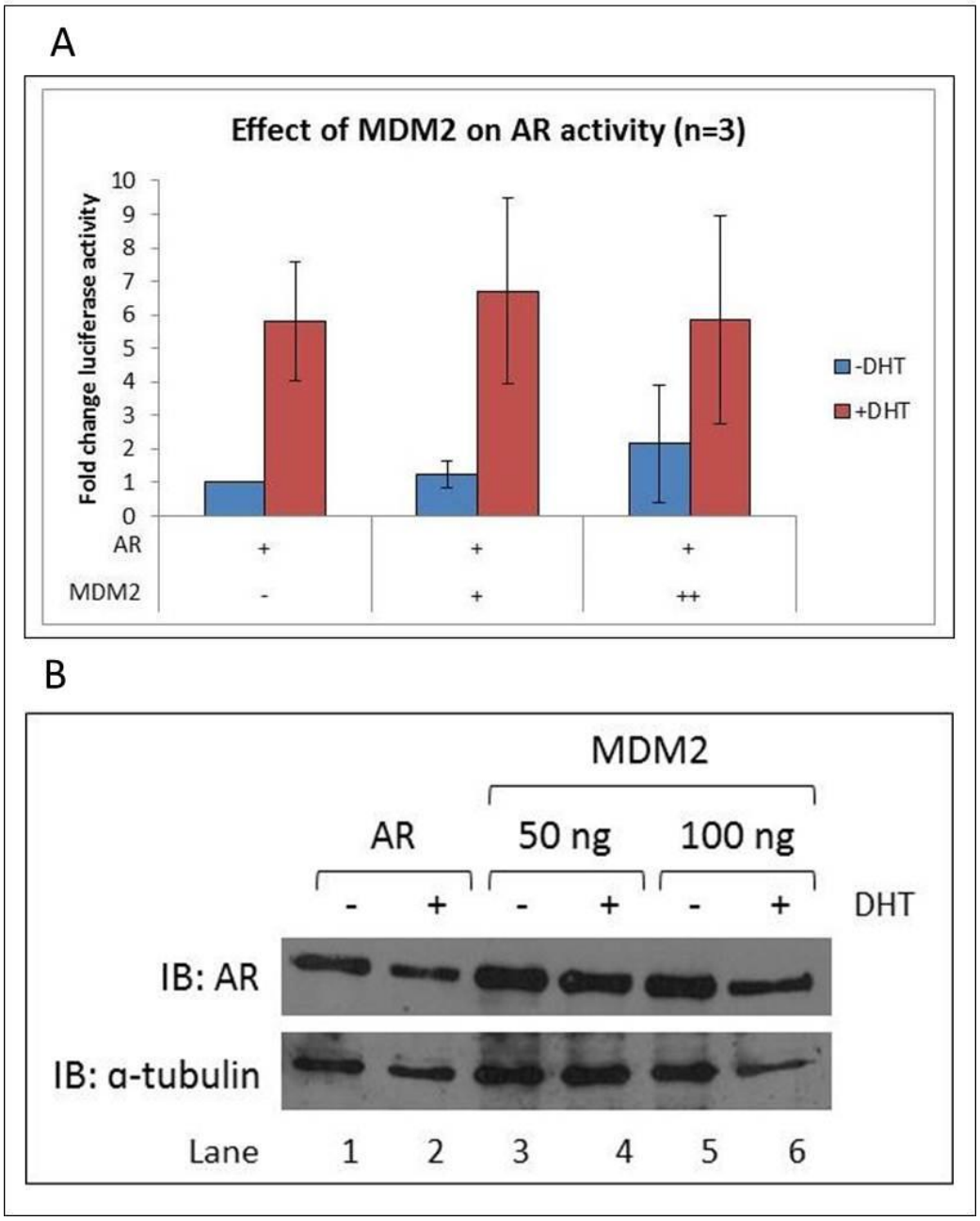

Figure 5.12. Effect of MDM2 on AR transcriptional activity in COS-7 cells.

COS-7 cells were transfected with 100 ng of both ARE3 luciferase and β-gal reporters together with 50 ng androgen receptor (AR) and increasing amounts of MDM2 expression vectors; 50 ng (+) and 100ng (++). 24 hours after transfection, media was replaced with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT and incubated for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is the mean of three independent repeats and error bars represent the standard error of the three replicates. Data was analysed for statistical significance using the Student's t-test, however no data points were significant. B) Luciferase assay samples were analysed by Western blotting with an anti-AR antibody to ensure over-expression of ectopically expressed AR. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats..

The intriguing result obtained in Figure 5.12 may have been due to number of differences between this experimental design and that of the previous Gaughan *et al.,* study including the use of a different luciferase reporter and a different AR construct, pcDNA3-AR (Gaughan *et al.*, 2005).

In an attempt to overcome these differences, the same androgen-responsive luciferase reporter containing approximately 700 base pairs of the *PSA* promoter upstream of the luciferase gene, pPSA-luc, was used. COS-7 cells were transfected with 100ng of both pPSA-luc and β-gal reporters together with 50 ng of AR and either 50 ng or 100 ng of MDM2 (represented as + and ++ respectively) for 24 hours in serum-containing media prior to replacement with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. Luciferase activity was assayed and normalised against β-gal activity. Figure 5.13 shows a representative experiment of three independent repeats.

Employing the pPSA-luc reporter in COS-7 cells was unsuccessful as there was no androgenic stimulation of the receptor in cells expressing the AR treated with 100 nM DHT (Figure 5.13, compare lanes 1 and 2). AR over-expression was confirmed by Western blotting as shown in Figure 5.13b.

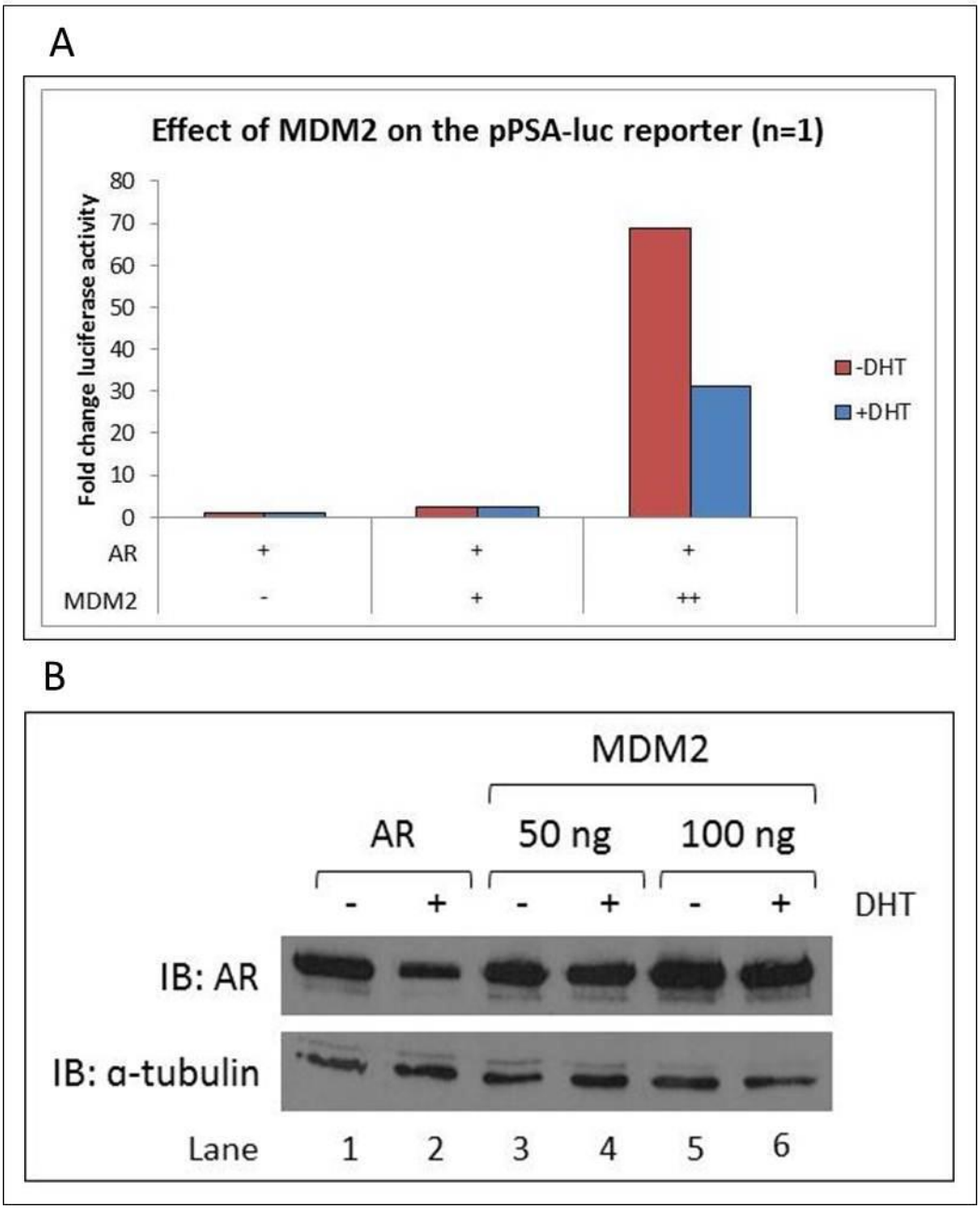

Figure 5.13. Effect of DHT treatment and MDM2 on the pPSA-luc reporter in COS-7 cells.

COS-7 cells were transfected with 100 ng of both pPSA-luc and β-gal reporters together with 50 ng androgen receptor (AR) and increasing amounts of MDM2 expression vectors; 50 ng (+) and 100ng (++). 24 hours after transfection, media was replaced with steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT and incubated for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is from one repeat and is representative of three independent repeats. B) Luciferase assay samples were analysed by Western blotting with an anti-AR antibody to ensure over-expression of ectopically expressed AR. α-tubulin was used as a loading control.

To further address this issue, and to provide evidence that MDM2 reduces AR-mediated transcription, the ARE3 luciferase reporter experiments were repeated in the human osteosarcoma cell line U2OS as this cell line is relatively easy to transfect with plasmid DNA, and has been used to interrogate the activity of numerous co-regulators on ectopically-expressed AR (Gaughan *et al.*, 2011).

U2OS cells were transfected as described above for 24 hours in serum-containing media prior to growth in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. Results are collated from three independent repeats and are represented as the mean fold change compared to the AR control without DHT treatment. Data was analysed for statistical significance using the Student's t-test, however no data points were found to be significant.

Similarly to COS-7 cells using the ARE3 reporter, AR transcriptional activity was induced upon DHT treatment in U2OS cells approximately 4-fold (Figure 5.14, compare lanes 1 and 2). However, in contrast to the COS-7 cells, 50 ng (+) and 100 ng (++) MDM2 repressed AR transcriptional activity upon DHT stimulation by 50% (Figure 5.14, compare lanes 4 and 6 with lane 1). AR over-expression was again confirmed by Western blot analysis (Figure 5.14b, upper panel).

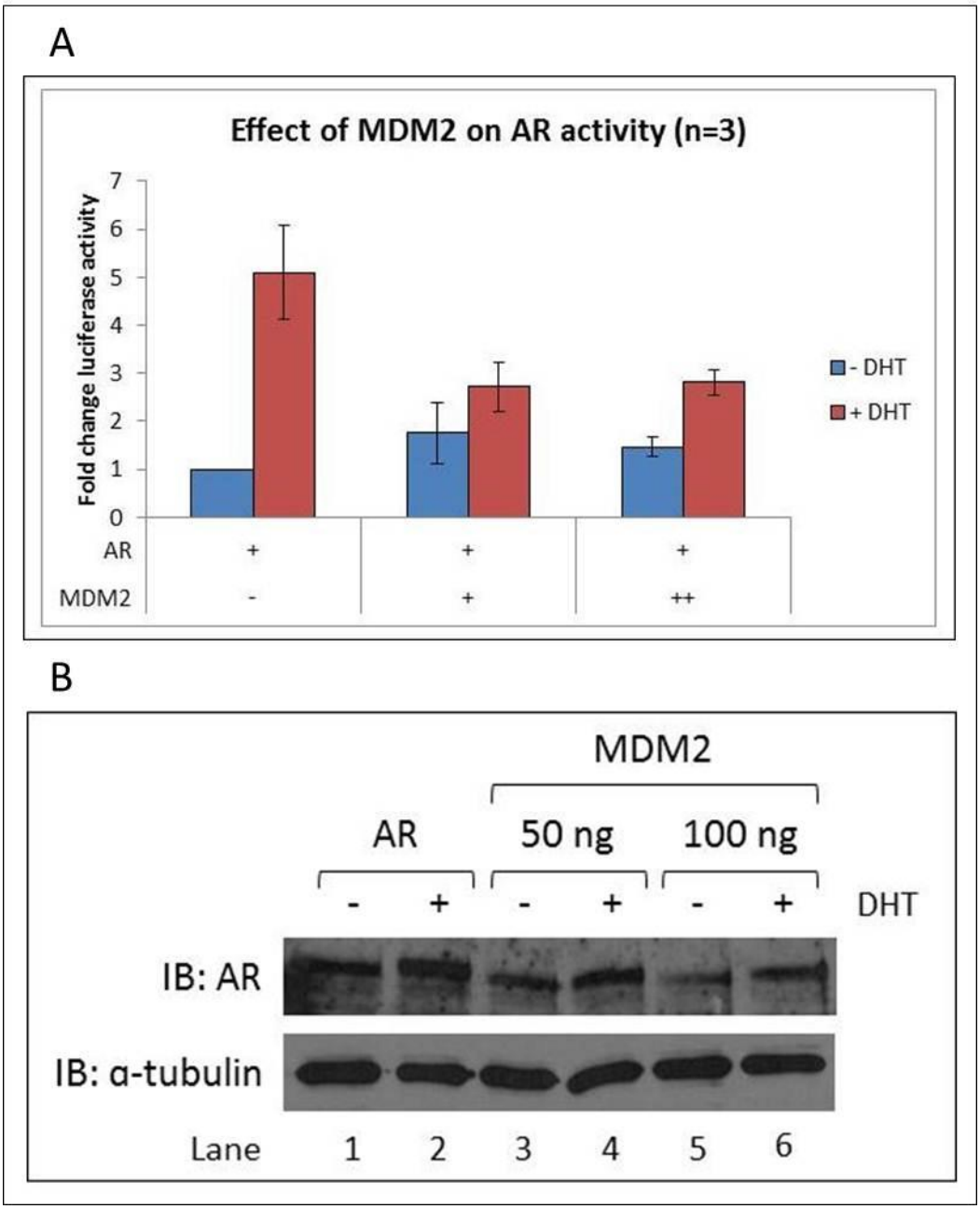

Figure 5.14. Effect of MDM2 on AR transcriptional activity in U2OS cells.

U2OS cells were transfected with 100 ng of both ARE3 luciferase and β-gal reporters together with 50 ng androgen receptor (AR) and increasing amounts of MDM2 expression vectors; 50 ng (+)and 100ng (++). 24 hours after transfection, cells grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is the mean of three independent repeats and error bars represent the standard error of the three replicates. Data was analysed for statistical significance using the Student's t-test, however no data points were significant. B) Luciferase assay samples were analysed by Western blotting with an anti-AR antibody to ensure over-expression of ectopically expressed AR. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats.

Given that USP10 only marginally reduced AR activity in COS-7 cells (Figure 5.9), but MDM2 repressed AR transcriptional activity robustly in the U2OS cell line, it was important to investigate the effect of USP10 on AR-mediated transcription in U2OS cells.

To this end, U2OS cells were transfected as described in Figure 5.9 and results are derived from three independent experimental repeats and are again represented as the mean fold change compared to the AR only control without DHT treatment. Data was analysed for statistical significance using the Student's t-test, however no data points were found to be significant.

As expected, AR activity in U2OS cells was induced upon DHT stimulation by approximately 5.5-fold (Figure 5.15, compare lanes 1 and 2). Importantly, USP10 over-expression had a much greater repressive effect on AR activity in U2OS cells compared to COS-7, with a maximal 50% reduction in AR induction observed with 25 ng of USP10 (Figure 5.15, compare lanes 2 and 4) that steadily declined with increasing amounts of the DUB enzyme (Figure 5.15, compare lanes 6 and 8 with lane 4). This result, in conjunction with the USP10 depletion data in LNCaP cells and luciferase reporter data in COS-7 and PC-3 cells suggests a role for USP10 as co-repressor of the AR. Once again Western blot analysis confirmed over-expression of both AR and USP10 (Figure 5.15b).

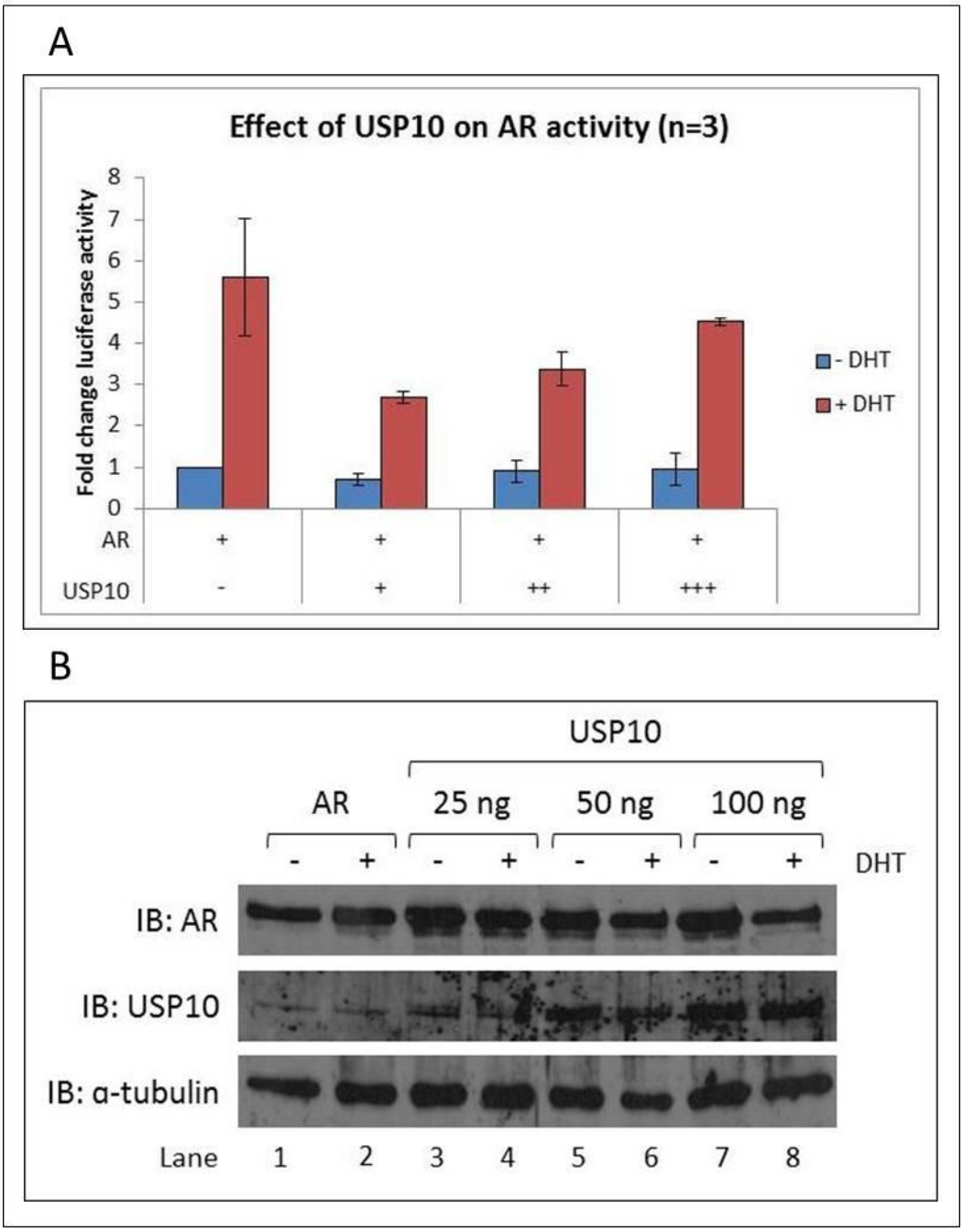

Figure 5.15. Effect of USP10 on AR transcriptional activity in U2OS cells.

U2OS cells were transfected with 100 ng of both ARE3 luciferase and β-gal reporters together with 50 ng androgen receptor (AR) and increasing amounts of USP10; 25 ng (+), 50ng (++) and 100ng (+++). 24 hours after transfection, cells were grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. A) Luciferase reporter activity was measured and normalised against β-gal activity. Data is represented as fold change compared to the AR only - DHT control. Data is the mean of three independent repeats and error bars represent the standard error of the mean of the three replicates. Data was analysed for statistical significance using the Student's t-test, however no data points were significant. B) Luciferase assay samples were analysed by Western blotting with anti-AR and -USP10 antibodies to ensure over-expression of ectopically expressed proteins. α-tubulin was used as a loading control. Western blots are representative of three independent experimental repeats.

5.3.5.2 Effect of MDM2 and USP10 combination on AR transcriptional activity

MDM2 is regulated by auto-ubiquitination and degradation. It was therefore hypothesised that potential deubiquitination of MDM2 by USP10 would protect it from degradation and therefore enable enhanced activity towards AR. Therefore, once the effects of MDM2 and USP10 on AR function had been established in U2OS cells, it was pertinent to assess whether a combination of the two enzymes would further enhance repression of AR activity suggesting a possible role for USP10 as an MDM2 DUB.

U2OS cells were transfected with 100ng of both ARE3 and β-gal reporters together with 50 ng AR, 100 ng MDM2 and 50ng USP10 mammalian expression vectors for 24 hours in serum-containing media prior to growth in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for an additional 48 hours. The amounts of MDM2 (100 ng) and USP10 (50 ng) were chosen based on their ability to repress AR activity separately (Figure 5.14 and Figure 5.15). Data is represented as fold change compared to AR only without DHT treatment. The data presented is from only one experimental repeat and would therefore require repeating in order to substantiate the results of this experiment.

In accordance with the previous data, MDM2 did indeed repress DHT-stimulated AR activity; reducing it from 8.3-fold in the AR only control to 3.4-fold (Figure 5.16, compare lanes 2 and 4). Unfortunately, and inconsistent with data in Figure 5.15, USP10 failed to markedly effect AR activity (Figure 5.16, compare lanes 2 and 6), which meant that the entire experiment designed to test potential co-operativity between the two AR co-regulators had failed. Although disappointing, this experiment has the potential to demonstrate interplay between MDM2 and USP10 within the AR signalling cascade and warrants further exploration.

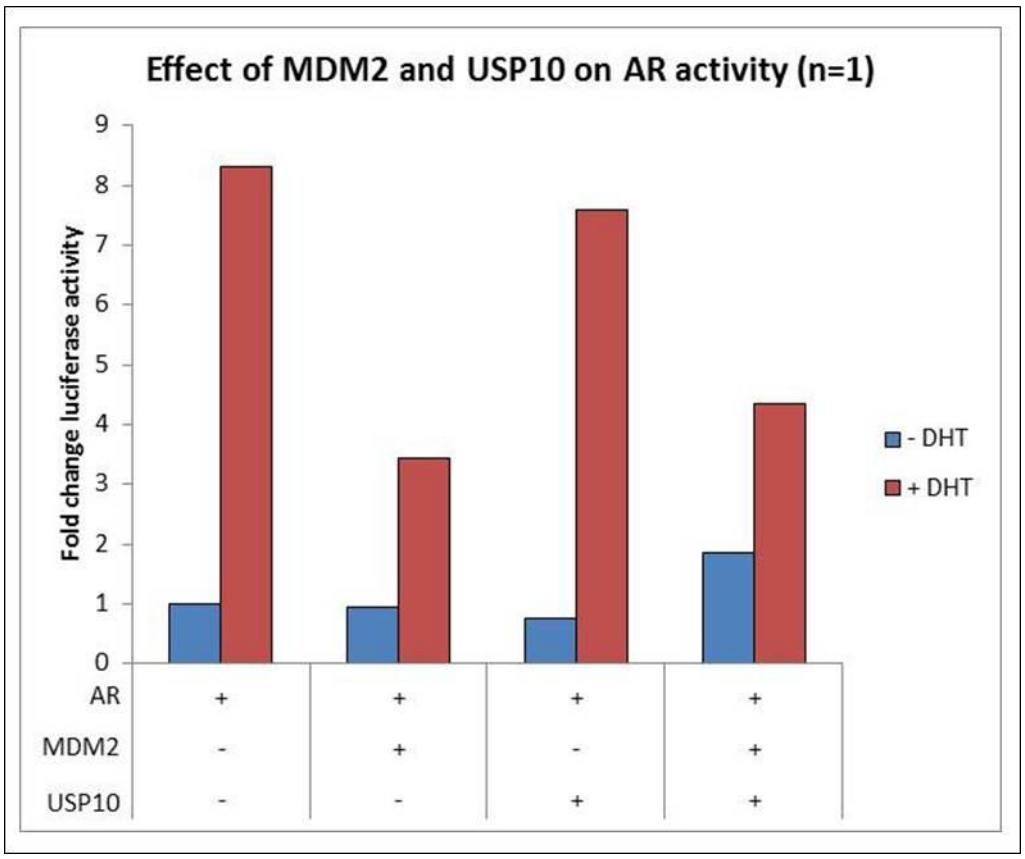

Figure 5.16. Effect of MDM2 and USP10 combination on AR transcriptional activity in U2OS cells.

U2OS cells were transfected with 50 ng androgen receptor (AR), 100 ng of both ARE3 luciferase and β-gal reporters, as well as 100 ng MDM2 or 50 ng USP10 or a combination of both. 24 hours after transfection, cells were grown in steroid-depleted media supplemented with (+ DHT) or without (- DHT) 100 nM DHT for a further 48 hours. Luciferase reporter activity was measured and normalised against β-gal activity. Data is from one experiment and is represented as fold change compared to the AR only - DHT control.

5.4 Discussion

USP10 has been reported to be a direct AR interacting protein that causes an increase in AR transcriptional activity in addition to direct deubiquitination of ubiquitinated histone H2A.Z, influencing AR-regulated gene transcription (Faus *et al.*, 2005; Draker *et al.*, 2011). Validation of targets isolated from the siRNA library screen of DUB enzymes in LNCaP cells conducted in the Solid Tumour Target Discovery Group, identified USP10 as a potential regulator of AR transcriptional activity. However, in contrast to the previous studies knockdown of USP10 increased mRNA expression and protein level of PSA as well as other AR-target genes, including *KLK2*, *TMPRSS2* and *NDRG1*.

Over-expression of USP10 in COS-7 cells revealed that it has a subtle repressive effect on AR transcriptional activity upon the ARE3 luciferase reporter, although this was not statistically significant. However, subsequent extrapolation from these experiments into the U2OS cell line model demonstrated a robust attenuation of AR-mediated transcription using the ARE3 luciferase reporter.

The disparity between these findings, both from the USP10 depletion experiments in LNCaP cells and the reporter studies in U2OS cells, and those of the Faus *et al*., and Draker *et al*., reports, that indicate the DUB as an AR co-activator is intriguing and suggests a potential cell-type and transcriptional assay dependency on the function of USP10 in the AR signalling cascade. The first paper identifying USP10 as a regulator of AR activity utilised a PC-3 cell variant, termed PC-3/AR that stably expressed AR. Although it is known that PC-3 cells become responsive to androgens upon over-expression of AR (Kokontis *et al.*, 1991), they may have lost other factors involved in the AR signalling cascade and therefore may not function identically to an AR-positive cell line. Faus *et al.,* demonstrated that USP10 over-expression in PC-3/AR cells increased AR activity upon the murine *Pem* gene ARE-1 and ARE-2 reporters and that the DUB interacted directly with the receptor both in mass spectrometry analysis of PC-3/AR nuclear extracts and in cell-free experiments. In contrast, the data presented in Figure 5.11 failed to show an endogenous interaction between USP10 and AR in LNCaP cells, suggesting either that the interaction in this cell line is transient and thus difficult to detect by immunoprecipitation, or that the observation by Faus *et al*., is a potential artefact of protein over-expression in a non-androgenic cell line; further studies of the endogenous interaction within other cell lines would be required to

elucidate this. In addition, the luciferase assays performed in PC-3 cells using a triplicate consensus ARE reporter construct (Figure 5.10) failed to demonstrate an effect of USP10 over-expression on AR-mediated transcription, again questioning the role of USP10 as a co-activator of the receptor.

PC-3 cells were originally isolated from a bone metastasis of a grade IV prostatic adenocarcinoma (Kaighn *et al.*, 1979). Analysis of gene expression between LNCaP cells and PC-3 cells has identified nearly 2200 differentially expressed genes; PC-3 cells down-regulate prostate specific genes and up-regulate genes required for angiogenesis and invasion (Dozmorov *et al.*, 2009). Furthermore, many studies have shown that over-expression of AR in PC-3 results in repression of androgen-induced proliferation and invasion, contrary to data from AR positive cell lines where AR is a driver of proliferation and metastasis (Niu *et al.*, 2008; Yuan *et al.*, 2010). Although over-expression of AR in PC-3 cells allows for transcription of known AR regulated genes, these cells do not behave identically to their AR-positive counterparts (Dozmorov *et al.*, 2009), and therefore USP10 may not behave as it would in AR-positive LNCaP cells. Additional AR co-factors may be missing from this cell line, and interestingly, p53, a known target of USP10, is not expressed in PC-3 (Skjoth and Issinger, 2006). The AR and p53 positive LNCaP cell line was utilised throughout the study, including all of the initial validation experiments which is a more physiologically relevant and robust model system to interrogate the function of component proteins of the AR signalling cascade.

USP10 was most recently shown to directly deubiquitinate the histone variant H2A.Z, resulting in reduction of ubiquitinated histone H2A.Z at AREs of AR regulated gene promoters, including *PSA*, and therefore causing increased transcription of AR-regulated genes (Draker *et al.*, 2011). These data was consistent with a previous study reporting that mono-ubiquitination of H2A.Z was linked to silencing of gene transcription (Sarcinella *et al.*, 2007) and suggested that deubiquitination could antagonise the repressive effect of H2A.Z modification. Firstly, Draker *et al* showed that USP10 over-expression decreased the levels of ubiquitinated H2A.Z in HEK293 cell lysates and confirmed ubiquitinated histone H2A.Z as a substrate of USP10 by use of an *in vitro* deubiquitination assay. Furthermore, immuno-fluorescence and cytoplasmic-nuclear extraction in LNCaP cells were used to demonstrate that USP10

localised to both the cytoplasm and the nucleus. Histone H2A.Z was present at the promoters of the *PSA* and *KLK2* genes in LNCaP cells and the levels of ubiquitinated histone were increased upon stable USP10 depletion suggesting retention of this histone modification after USP10 knockdown. PC-3/AR cells over-expressing an ARE3 luciferase reporter were utilised to prove that over-expression of USP10 was linked to transcriptional activation by AR and that knockdown of USP10 led to a reduction in reporter activity. This result was confirmed by decreased mRNA expression of *PSA* and *KLK2* in LNCaP cells in response to stable USP10 knockdown (Draker *et al.*, 2011). The level of USP10 knockdown achieved by stable shRNA depletion of USP10 in LNCaP cells, assessed by detection of USP10 Western blotting, was approximately 80% (Draker *et al.*, 2011); this was in contrast to the USP10 protein levels shown in Figure 5.6c, which were undetectable by Western blotting following siRNA knockdown. Due to the differences in knockdown methodologies and use of the stably transfected PC-3/AR cell lines highlighted in the previous paragraph, it is intriguing to speculate that if these experiments were repeated in LNCaP cells using an shRNA knockdown approach, that the effect of USP10 on AR activity and H2A.Z ubiquitination status may not be the same. It is worth noting, however, that USP10 could be acting at an epigenetic level in addition to directly within the signalling cascade to affect AR transcriptional activity.

From the literature, it is known that USP10 counteracts MDM2-mediated ubiquitination of p53 and hence facilitates p53 function in response to DNA damage (Yuan *et al.*, 2010). Due to the link between MDM2 and the AR, it was hypothesised that USP10 and MDM2 function co-operatively within the AR signalling system to regulate receptor activity. It has been shown previously that MDM2-mediated ubiquitination of the AR causes receptor degradation and transcriptional repression (Lin *et al.*, 2002b; Gaughan *et al.*, 2005). However, this was not observed in the first luciferase experiments in COS-7 cells. This discrepancy may have been due to the use of the ARE3 reporter in these experiments compared to the pPSA luc reporter construct employed in the Gaughan *et al*., study. As such, in contrast to this earlier study, it was found that MDM2 in COS-7 cells failed to affect AR transcriptional activation upon the synthetic ARE3 luciferase reporter (Figure 5.12), while attempts with the pPSA-luc reporter were unsuccessful due to very high background reporter activity resulting in no androgen-induced activation of the AR. It was therefore

important to repeat the luciferase experiments in the osteosarcoma cell line U2OS as it is a human cell line that has high transfection efficiency and thus lends itself to this kind of study. In this cell line, MDM2 was found to repress AR transcriptional activity upon the ARE3 reporter by upwards of 50% (Figure 5.14) indicating a cell line dependency for the function of MDM2 upon the AR using the ARE3 reporter. The differences between the responses of the two cell lines could be due to: (1) species specific differences as COS-7 are a monkey cell line and U2OS are a human cell line; (2) differences in expression of other proteins involved in the ubiquitination or turnover process of the receptor; or (3) the fact that the reporter is a synthetic ARE rather than an endogenous one, hence repeating these experiments with the pPSA luc reporter in U2OS cells would be an important extrapolation to the study.

The next step in the investigation was to assess potential interplay between transcriptional repression of AR by MDM2 and USP10. It was hypothesised that a combination of the two enzymes, both shown to repress AR activity (Gaughan *et al.*, 2005), would enhance the repressive effect on the AR signalling cascade. Unfortunately, this experiment failed, in part, due to the failure of USP10 to down-regulate AR activity and hence the combination effect of USP10 and MDM2 could not be addressed (Figure 5.16). However, this experiment is from only one experiment and should be repeated to further address the hypothesis. One important question from this experiment is why did USP10 not repress AR activity given that it was shown in Figure 5.15 to repress receptor function by 50%? Although difficult to predict, and given that it was only a single experiment, it could be due to the increased levels of plasmid DNA being over-expressed in this system that resulted in transcriptional squelching. However, it is worth noting that MDM2 functioned as expected so this may negate this argument. Obviously, repeats of this experiment are necessary.

In conclusion, USP10 has been identified as a transcriptional co-repressor of AR in contrast to previously published work in this area (Faus *et al.*, 2005; Draker *et al.*, 2011). USP10 knockdown increases the expression of the majority of AR regulated genes investigated. Over-expression of USP10 in three cell lines suggested that it has a repressive effect on AR signalling. However, further studies interrogating the potential interplay between USP10 and MDM2 would be a fascinating extension to the investigation. In addition, assessment of both USP10 and MDM2 catalytic mutants

individually and together on AR activity would be interesting to explore. The link between MDM2 and Akt warrants investigating in this system as it may promote additional effects of MDM2 and potentially USP10. Further investigation of USP10 recruitment to AR regulated promoter and reinvestigation of its role in histone deubiquitination in AR positive cell lines may be vital in solving the conflicts between the data presented above and previous reports (Faus *et al.*, 2005; Draker *et al.*, 2011).

Chapter 6: Generation of recombinant GST-tagged AR C-terminal domain proteins and USP10 as tools for *in vitro* assays

6.1 Introduction

6.1.1 Post-translational modification of the Androgen receptor

Post-translational modifications of nuclear hormone receptors by phosphorylation, acetylation, methylation, SUMOylation and ubiquitination can dramatically alter their functionality and stability (Anbalagan *et al.*, 2012). The AR contains 23 reported residues that are the target of modification (Gioeli and Paschal, 2012), these are summarised in Figure 6.1.

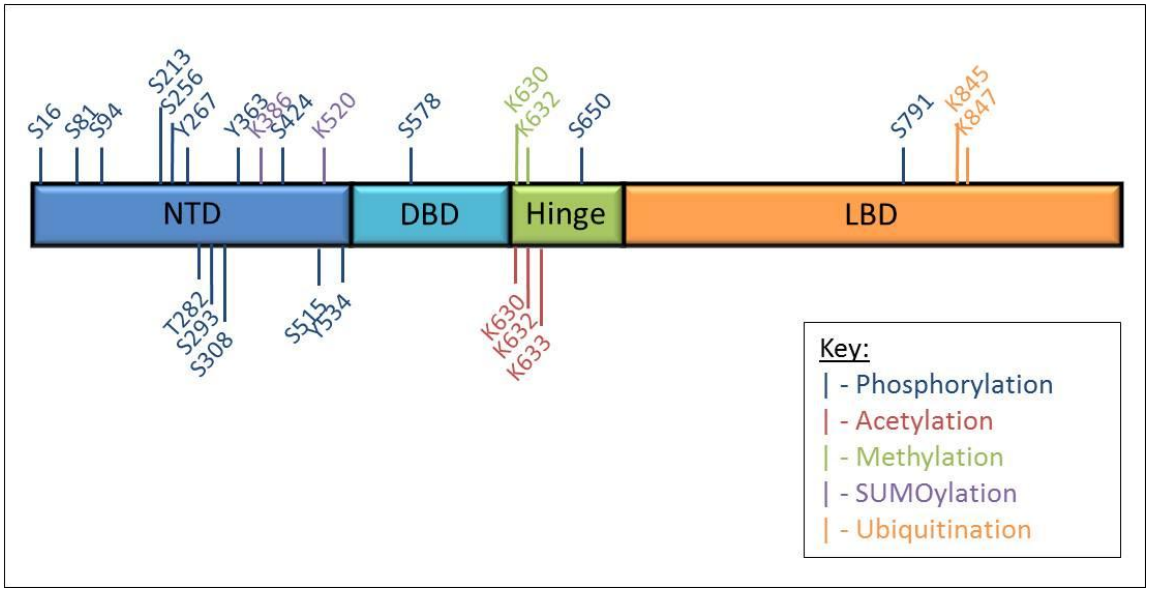

Figure 6.1. The sites of AR post-translational modification.

Post-translational modification of the AR occurs within each of the domains; the N-terminal domain (NTD), DBD-binding domain (DBD), the hinge and the ligand-binding domain (LBD). The AR is modified by phosphorylation, acetylation, methylation, SUMOylation and ubiquitination. Adapted from (Gioeli and Paschal, 2012).

Phosphorylation of the AR at the serine, threonine and tyrosine residues can affect multiple aspects of AR activity including transcriptional activity, cellular localisation and stability (Gioeli and Paschal, 2012). The majority of phosphorylation sites are clustered in the N-terminal transactivation domain (NTD) of the AR, although at least one phosphorylation site is found in each of the other receptor domains, including the DBA-binding domain (DBD) and ligand-binding domain (LBD) (Gioeli and Paschal, 2012). Phosphorylation of many sites is found to increase in the presence of androgen and several sites are phosphorylated in response to epidermal growth factor (EGF) treatment (Yang *et al.*, 2007). Akt, MAPK and members of the stress kinase family are found to influence AR phosphorylation (Lin *et al.*, 2001; Lin *et al.*, 2003; Gioeli *et al.*, 2006; Ponguta *et al.*, 2008). Phosphorylation of S213 and S791 is induced by EGF- and insulin-like growth factor (IGF)-mediated activation of Akt (Lin *et al.*, 2001; Lin *et al.*, 2003). Phosphorylation of these sites was found to have a differential effect on AR transcriptional activity depending on the passage number of the LNCaP cells investigated; low passage number LNCaP cells showed repression of AR activity upon phosphorylation by Akt whereas high passage number LNCaP cells showed increased transcriptional activity (Lin *et al.*, 2003).

The sites of acetylation are found clustered within the conserved KLKK/RXKK motif of the hinge region of the AR (Faus and Haendler, 2006). Mutation of K630, K632 or K633 sites is found to impair recruitment of co-activator proteins and promote recruitment of the N-CoR co-repressor therefore inhibiting AR activation (Faus and Haendler, 2006). Acetylation of AR is mediated by p/CAF, p300 and Tip60 (Fu *et al.*, 2000; Fu *et al.*, 2002; Gaughan *et al.*, 2002). Over-expression of AR K630Q, a mutant in which the lysine at position 630 is mutated to a glutamate and mimics constitutive acetylation, in CaP cells is found to increase cell growth and reduce apoptosis (Gioeli and Paschal, 2012). SET9, a methyltransferase, has also been reported to methylate the AR at K630 and K632 of the KLKK motif (Gaughan *et al.*, 2011). Methylation by SET9 up-regulates AR activity by enhancing the interaction of the N- and C-terminal domains of the AR (Gaughan *et al.*, 2011).

SUMOylation is the conjugation of a small ubiquitin-like protein, SUMO, to the lysines of a target protein in a process similar to ubiquitination (Hay, 2006). Two SUMOylation sites have been reported within the NTD, K386 and K520 (Poukka *et al.*, 2000).

SUMOylation is androgen-induced and mediated by the SUMO E3 ligase Ubc9 (Poukka *et al.*, 1999). SUMOylation is generally repressive for AR activity; SUMOylation is dependent for recruitment of the Co-repressor Daxx (Lin *et al.*, 2004).

Until recently the ubiquitination sites of the AR were unknown despite several E3 ligases being implicated in the regulation of AR activity and stability. Xu *et al*., reported ubiquitination of the AR by the novel E3 ligase RNF6 (Xu *et al.*, 2009). Importantly, this study was the first to report the exact location of ubiquitination sites within the AR. *In vitro* ubiquitination assays and proteomic analysis revealed that RNF6-mediated ubiquitination occurred at K845 and K847 of the AR LBD (Xu *et al.*, 2009). These poly-ubiquitination events promoted AR-mediated transcription of target genes through promotion of ARA54, an AR co-activator, recruitment (Xu *et al.*, 2009). Interestingly, K845 ubiquitination was found to act as a prerequisite for the efficient ubiquitination of K847; mutation of K845 to arginine abolished RNF6-mediated ubiquitination and activation of AR activity whilst mutation of K847 only partially reduced ubiquitination and did not affect AR activity (Xu *et al.*, 2009). Furthermore, examination of a panel of ubiquitin mutants able to form only one type of poly-ubiquitin linkage revealed that RNF6 preferentially formed K6- and K27-linked chains (Xu *et al.*, 2009).

Alternative ubiquitination sites have currently not been identified; however it is possible that additional target sites exist not just within the LBD but also within other AR domains. With this mind, *in vitro* ubiquitination assays provide a possible means to preliminarily identify novel ubiquitination sites with the AR by profiling each of the AR domains with all of the currently known AR-associated E3 ligases. Additionally, these experiments in combination with ubiquitin mutants would allow identification of the specific poly-ubiquitin chain types formed by the specific E3 ligases on the AR.

6.1.2 Deubiquitinase enzymes and the Androgen receptor

Ubiquitination of the AR can be counteracted by DUB enzymes. USP10 and USP26 directly influence the ubiquitination status of the AR (Faus *et al.*, 2005; Dirac and Bernards, 2010). Data from the Faus *et al*., and Draker *et al*., studies shows that USP10 regulates the ubiquitination both the AR and histone H2A.Z via a direct interaction with both proteins leading to stimulation of transcription of AR target genes (Faus *et al.*, 2005; Draker *et al.*, 2011). USP26 is found to directly interact with the AR and is co-localised to the nucleus in an androgen-dependent manner (Dirac and Bernards, 2010).

Unlike USP10 however, USP26 was found to both inhibit and activate AR activity depending on the cell background investigated (Dirac and Bernards, 2010). Despite both enzymes being implicated directly in the deubiquitination of AR neither has been evaluated for their ubiquitin chain specificity or ubiquitination site specificity.

6.2 Aims

In order to build a complete picture of the function of ubiquitination in the context of the AR we need to examine the relationships between AR-associated E3 ligases and their DUB counterparts including analysis of the specific ubiquitin chains they target. *In vitro* assays provide a tool to test hypothesised ubiquitination events in a cell-free environment before more robust characterisation studies are undertaken. To develop this methodology further recombinant AR substrate proteins and enzymes were required.

The specific aims of this Chapter are:

- To generate a series of AR C-terminal domain expression vectors designed to produce GST-tagged recombinant protein in bacterial cells and utilise GST affinity chromatography as a mean to purify these proteins
- To study the specific sites of androgen receptor ubiquitination via *in vitro* ubiquitination assays
- To employ GST affinity chromatography to purify enzymatically active recombinant GST-tagged USP10
- To investigate the enzymatic activity of recombinant GST-tagged USP10 against specific lysine-linked poly-ubiquitin chains *in vitro*

6.3 Materials and methods

6.3.1 Generation of pGEX-6P-1-AR-DBD, pGEX-6P-1-AR-DBD-Hinge and pGEX-6P-1-AR-DBD-Hinge-LBD

6.3.1.1 Agarose gel electrophoresis

All products of PCR, digestion, gel extraction and plasmid preparations were analysed by agarose gel electrophoresis on 1% agarose gels (1% agarose in Tris acetate EDTA (TAE) buffer). Samples were mixed with DNA sample buffer (30% sucrose, 100 mM EDTA pH8, 0.05% bromophenol blue) before loading alongside Hyperladder I, II or IV DNA marker (Bioline). Samples were run at 100 V for approximately 30 minutes and gels were stained with ethidium bromide and visualised using the GelDoc™ DNA analysis equipment (Bio-Rad).

6.3.1.2 PCR

To amplify the different domains of the AR, 100 ng of pYFP-AR was used as a template in a PCR reaction containing 1 nM of each primer (Table 6.1), 1 x reaction buffer (containing $MgCl_2$), 0.2 mM dNTPs and 1.5 units of Taq polymerase (Bioline) in a total volume of 10 µl. PCR reactions were performed on the PCR express thermal cycler HBX110 (Thermo Hybaid).

	Primer sequence
AR Forward 1 (F1)	ATG ATG GGA TCC ACC TGC CTG ATC TGT GGA
AR Reverse 2 (R2)	ATG ATG GAA TTC CCC TGC TTC ATA ACA TTT
AR Reverse 4 (R4)	ATG ATG GAA TTC ATT CAG AAA GAT GGG CTG
AR Reverse 6 (R6)	ATG ATG CTC GAG TCA CTG GGT GTG GAA ATA

Table 6.1. Sequence of PCR primers used in the amplification of the AR C-terminal domains.

6.3.1.3 Diagnostic digestion and vector ligation

Resultant AR amplicons were cut from an agarose gel, DNA extracted using the QIAquick spin gel extraction kit (as described by the manufacturer, Qiagen) and ligated into the pCR2.1 vector using the TA cloning kit according to the manufacturer's recommendations (Invitrogen). Briefly, ligation reactions were set up containing 25 ng of vector and approximately 10 ng of the desired AR amplicon with 4 units of T4 DNA ligase on a total volume of 10 µl. Ligation reactions were incubated at 14 ^{o}C overnight and were transformed into chemically competent E.*coli* as described in Section 2.2.4. Transformants were selected by blue/white selection on ampicillin and bromo-chloro-indolyl-galactopyranoside (X-gal)-containing LB agar plates.

Small-scale plasmid preparations (minipreps) of colonies containing potentially recombinant pCR2.1-AR C-terminal domain vectors were performed using the GenElute plasmid miniprep kit (as described by the manufacturer, Sigma-Aldrich) and resultant plasmid DNA digested with the appropriate restriction enzymes (Fermentas) i.e. *Bam* HI and *Eco* RI for the AR-DBD and DBD-hinge and *Bam* HI and *Xho* I for DBD-hinge-LBD for 1 hour at 37 ^{o}C. Released inserts were size-verified by agarose gel electrophoresis prior to sub-cloning into the pGEX-6P-1 vector (GE healthcare) following the same ligation protocol. Recombinant pGEX-6P-1-AR C-terminal domain vectors were identified by diagnostic restriction enzyme digestion as described above and subsequently transformed into XA90 or BL21 strains of competent *E.coli* using the protocol described in section 2.2.4. Once again, recombinant vectors were identified by diagnostic digestion and subsequently subject to large-scale plasmid preparation (maxiprep) using an endotoxin-free plasmid maxiprep kit (Qiagen). Resultant plasmids were sequenced by Cogenics (UK).

6.3.2 Protein purification

6.3.2.1 Small scale protein induction

A positive XA90 or BL21 colony from specific pGEX-6P-1-AR recombinants were inoculated into 10 ml of LB media containing 50 µg/ml ampicillin and grown overnight. This colony was also streaked onto a fresh ampicillin-containing LB agar plate and incubated over-night at 37 ^{o}C.

500 µl of over-night culture was added to fresh LB media with 50 µg/ml ampicillin and grown for a further 3 hours at 37 ^{o}C before 9 µl of 1 M isopropyl B-D-1-thiogalactopyranoside (IPTG) was added to induce protein production. 1 ml samples were collected at the point of induction and each hour after induction for a total of 4 hours as shown in Figure 6.2. These samples were pelleted by centrifugation and the pellets resuspended in 150 µl of water before sonication for 3 x 5 min using the Bioruptor system (Diagenode). 150 µl of SDS sample buffer was added and the samples separated by gel electrophoresis on 15% poly-acrylamide gels as described in section 2.8.1 and stained with Coomassie as described below.

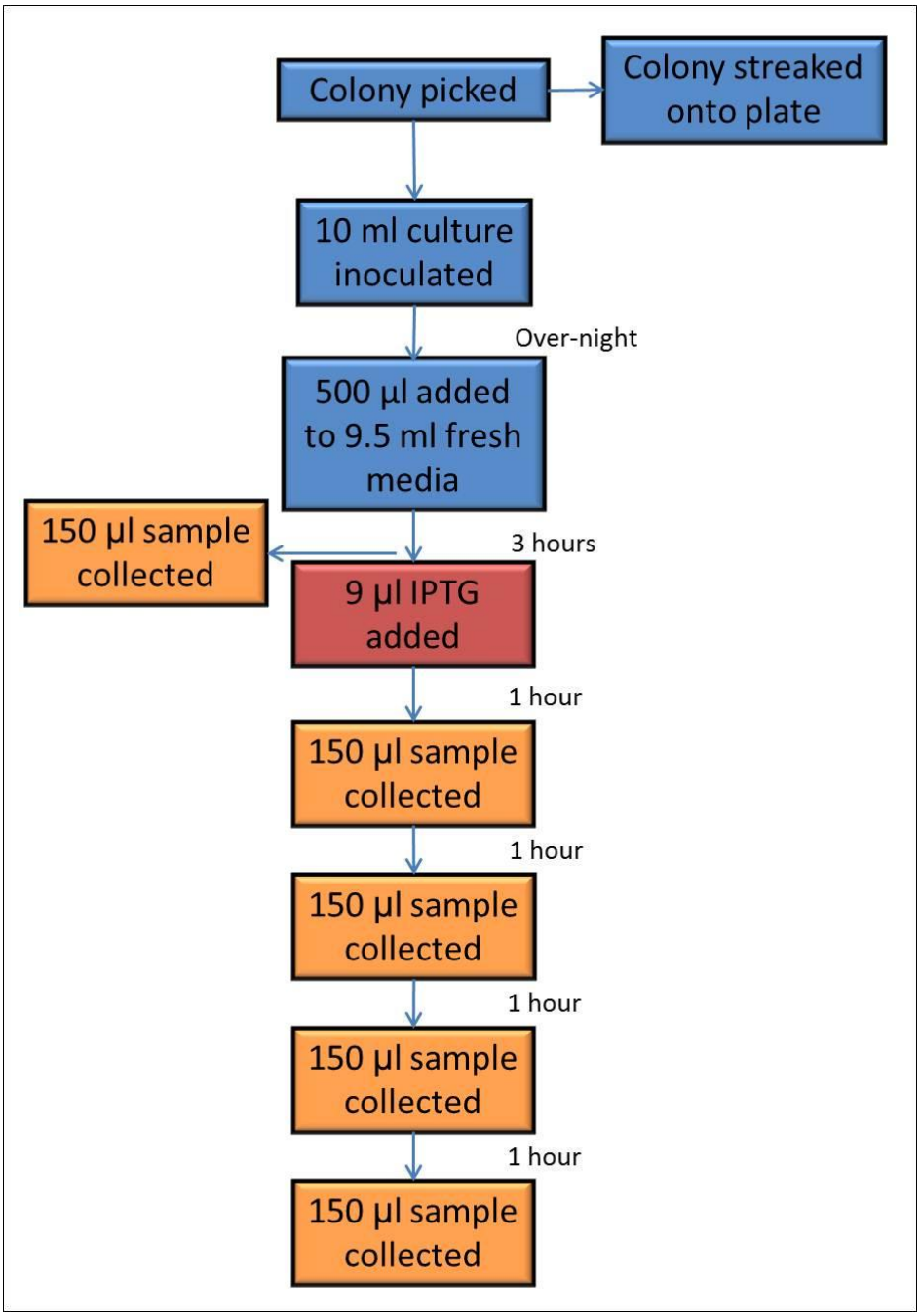

Figure 6.2. Schematic of small-scale protein induction.

XA90 or BL21 E. coli cells transformed with pGEX-6P-1 vectors and cultured overnight at 37 ^{o}C. 500 µl of culture was inoculated into 9.5 ml fresh media and grown for 3 hours before stimulation of protein production by addition of IPTG. Samples were collected at the points shown for analysis by SDS-PAGE and Coomassie staining.

6.3.2.2 Large scale protein induction

A positive colony was picked from XA90 or BL21 plates and inoculated into 10 ml of LB media containing 50 µg/ml ampicillin and grown overnight at 37 ^{o}C. 5 ml of over-night culture was added to 500 ml fresh LB media with 50 µg/ml ampicillin. 1 ml samples were collected each hour and the optical density at 600 nm measured using the SmartSpec Plus spectrophotometer (Bio-Rad) until 0.5 was achieved.

500 µl of 1 M IPTG was added to induce protein production and the culture incubated for a further 4 hrs. 1 ml samples were collected at the point of induction and after 4 hour incubation as shown in Figure 6.3. These samples were pelleted by centrifugation and resuspended in 150 µl of water and 150 µl of SDS sample buffer added.

After 4 hours of protein induction, bacterial cells were pelleted using the Avanti J-26 XP centrifuge (Beckman Coulter) and the pellets resuspended in 40 ml ice cold PBS before being stored at -20 ^{o}C.

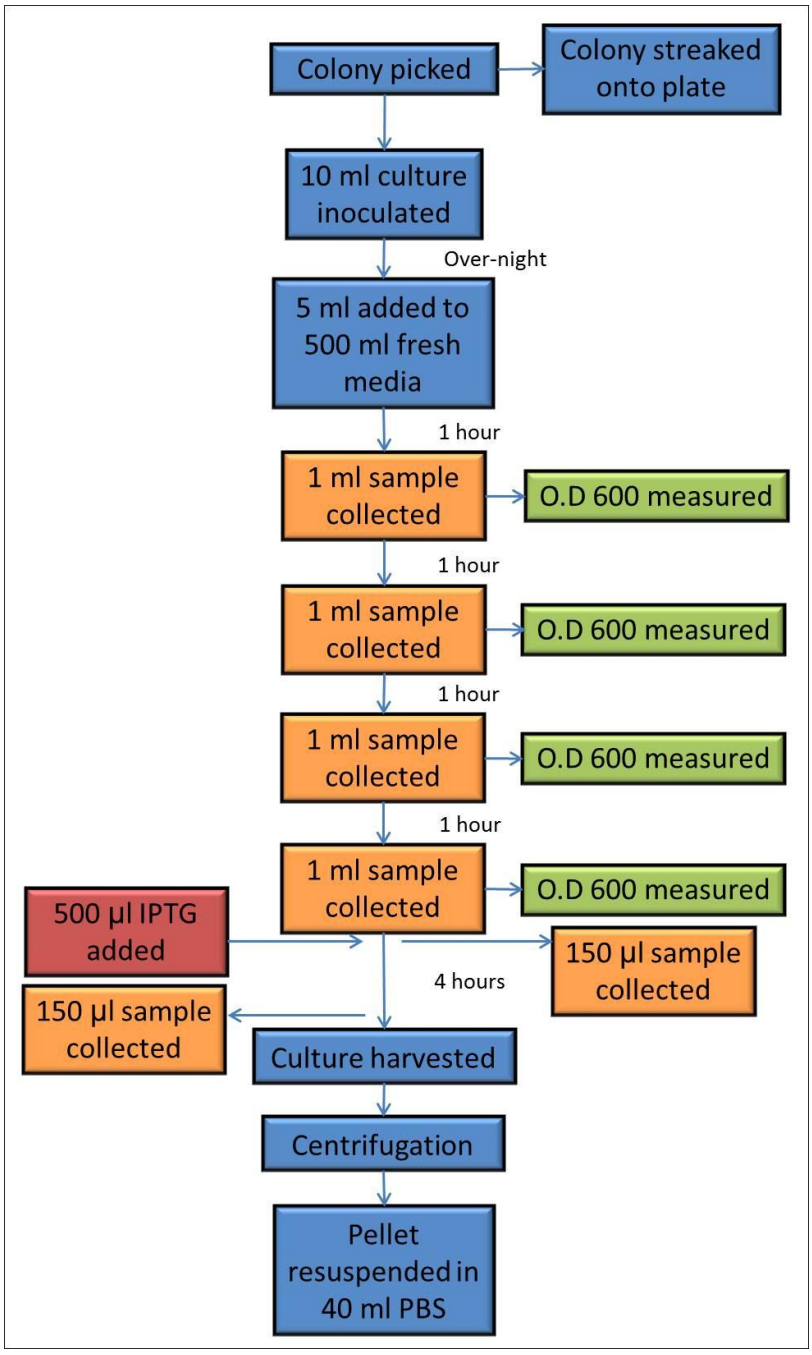

Figure 6.3. Schematic of large-scale protein induction.

XA90 or BL21 E. coli cells transformed with pGEX-6P-1 vectors were cultured overnight at 37 ^{o}C. 5 ml of culture was inoculated into 500 ml fresh media and grown until an optical density (O.D) 600 of 0.5 was achieved. Protein production was stimulated by addition of IPTG. Cultures were grown for a further 4 hours before cells were pelleted and resuspended in 40 ml PBS. Samples were collected at the points shown for analysis by SDS-PAGE and Coomassie staining.

6.3.2.3 GST affinity chromatography

Bacterial cell resuspensions produced from large-scale protein inductions (40 ml) were thawed on day of purification and the following added; 1 ml PBS containing 4 protease inhibitor tablets and 200 µl 1 M Phenylmethylsulfonyl fluoride (PMSF). The cultures were sonicated for 6 x 15 sec with 1 min intervals using the Soniprep 150 plus sonicator (MSE), centrifuged and the supernatant passed through Mira cloth (Calbiochem) to remove any cellular debris. The filtered sample was then loaded onto a column containing 2.5 ml glutathione sepharose (GE healthcare) and incubated for 5 minutes.

The initial flow through from the column was collected. Subsequently, the column washed twice with 20 ml ice cold PBS and the washes were also collected. The GST-tagged protein was then eluted using 15 ml of elution buffer (10 mM reduced glutathione, Tris pH 8.0) and collected in approximately 1 ml fractions. Fractions were then pooled together and subject to buffer exchange by dialysis overnight at 4 ^{o}C. Dialysis buffer A (50 mM NaCl and 50 mM HEPES pH8.0) was used for all non-enzymatically active substrate proteins whilst dialysis buffer B (50 mM NaCl, 50 mM HEPES pH8.0, 5mM DTT and 15% glycerol) was used for all recombinant enzymes. Fractions were stored at -80 ^{o}C. The process is summarised in Figure 6.4.

150 µl samples were collected at each step of the purification process and 150 µl SDS sample buffer added. These samples and those collected from the large scale induction were separated by SDS-PAGE.

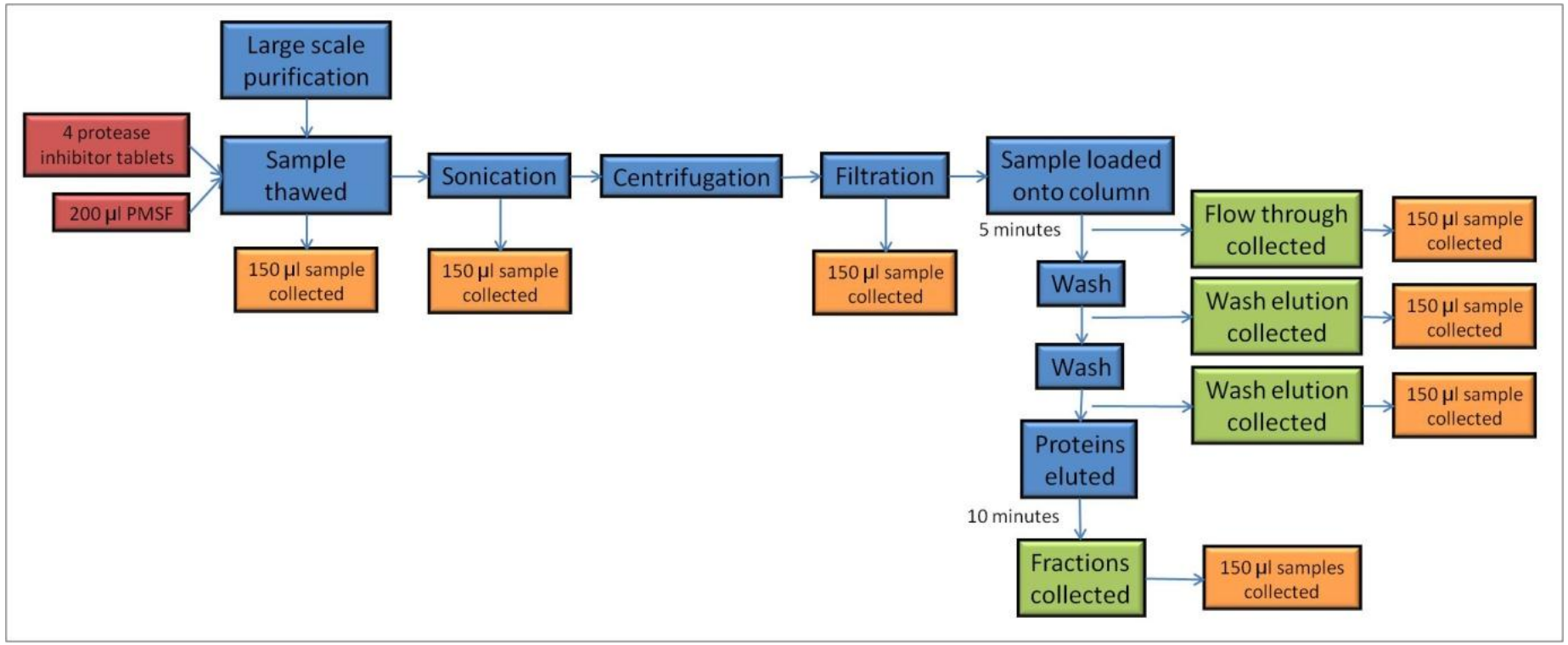

Figure 6.4. Schematic of the GST affinity chromatography protocol.

Protease inhibitors and PMSF were added to bacterial resuspensions from the large-scale protein induction process before samples were sonicated and filtered. Samples were loaded onto freshly prepared glutathione sepharose columns and incubated for 5 minutes. The sample flow through was collected and the column was washed twice with PBD. Proteins were eluted into approximately 1 ml fractions by addition of elution buffer to the column. Samples were collected at the points shown for analysis by SDS-PAGE and Coomassie staining.

6.3.3 In vitro ubiquitination assay

In vitro ubiquitination assay reagents were purchased from Boston Biochem (Boston, MA) with the exception of GST-tagged AR C-terminal domain proteins that were generated in-house (see Section 6.4.1.2). To investigate ubiquitination of the AR C-terminal domains by MDM2, *in vitro* ubiquitination assays containing 2 µl reaction buffer, 100 nM UBE1 (E1 enzyme), 5 µM UbcH5a (E2 enzyme), 1 µM MDM2 (E3 ligase), 200 µM K7R ubiquitin, 3.5 µl AR substrate and 10 mM ATP in a total volume of 20 µl were set up. Reactions were incubated at 37 ^{o}C for 4 hours before termination by addition of 1 µl E1 stop buffer. SDS sample buffer with 1% β-mercaptoethanol was added to all samples before analysis by SDS-PAGE, Coomassie staining and Western blot.

6.3.3.1 In vitro deubiquitination assay

To investigate the enzymatic activity of recombinant GST-tagged USP10 *in vitro* deubiquitination assays were set-up containing 2 µl reaction buffer, 50 ng/µl penta-ubiquitin (either K48- or K63 linked) and 0-5 µl of GST-tagged USP10 in a total volume of 20 µl. Reactions were incubated at 37 ^{o}C for up to 4 hours and terminated by addition of SDS sample buffer containing 1% β-mercaptoethanol. Samples were analysed by SDS-PAGE and Western blotting.

6.4 Results

6.4.1 Generation and purification of recombinant GST-tagged AR C-terminal domain proteins

6.4.1.1 Generation of pGEX-6P-1-AR C-terminal domain bacterial expression vectors

To increase our understanding of AR ubiquitination an *in vitro* ubiquitination assay approach would be utilised. This cell-free methodology allows investigation of different E3 ligases in their ability to ubiquitinate the AR as well as enabling the exact sites of ubiquitination to be identified by subsequent mass spectrometry analysis.

In order to generate a series of recombinant AR substrate proteins for in vitro ubiquitination assays a series of AR expression vectors were generated. These vectors encompassed the domains of the AR C-terminal namely the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD). Specific forward and reverse primers were designed to amplify the AR-DBD, AR-DBD-Hinge and AR-DBD-Hinge-LBD from the pYFP-AR vector by PCR. These primers contained specific sites for restriction enzyme cleavage; *Bam* HI and *Eco* RI sites for the AR-DBD and DBD-Hinge and *Bam* HI and *Xho* I for the DBD-Hinge-LBD. Figure 6.5 shows a schematic representation of the AR domains amplified.

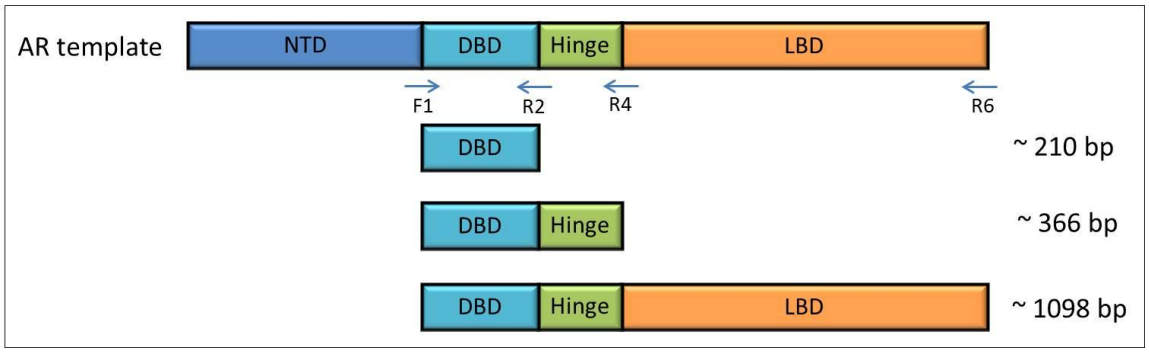

Figure 6.5. Schematic of the AR C-terminal domains amplified by PCR.

AR-DBD, DBD-Hinge and DBD-Hinge-LBD cDNA was successfully amplified by PCR (Figure 6.6). Amplification of the AR-DBD and DBD-Hinge was performed by Miss Lynsey Rogerson. Figure 6.6a shows the products of these PCR reactions after gel extraction. The DBD is approximately 300 bp whilst the DBD-Hinge is approximately 500 bp. Figure 6.6b shows the result of PCR of the AR-DBD-Hinge-LBD. An amplicon of approximately 1100 bp is seen in the PCR reaction containing template whilst no amplification was see in the no template control.

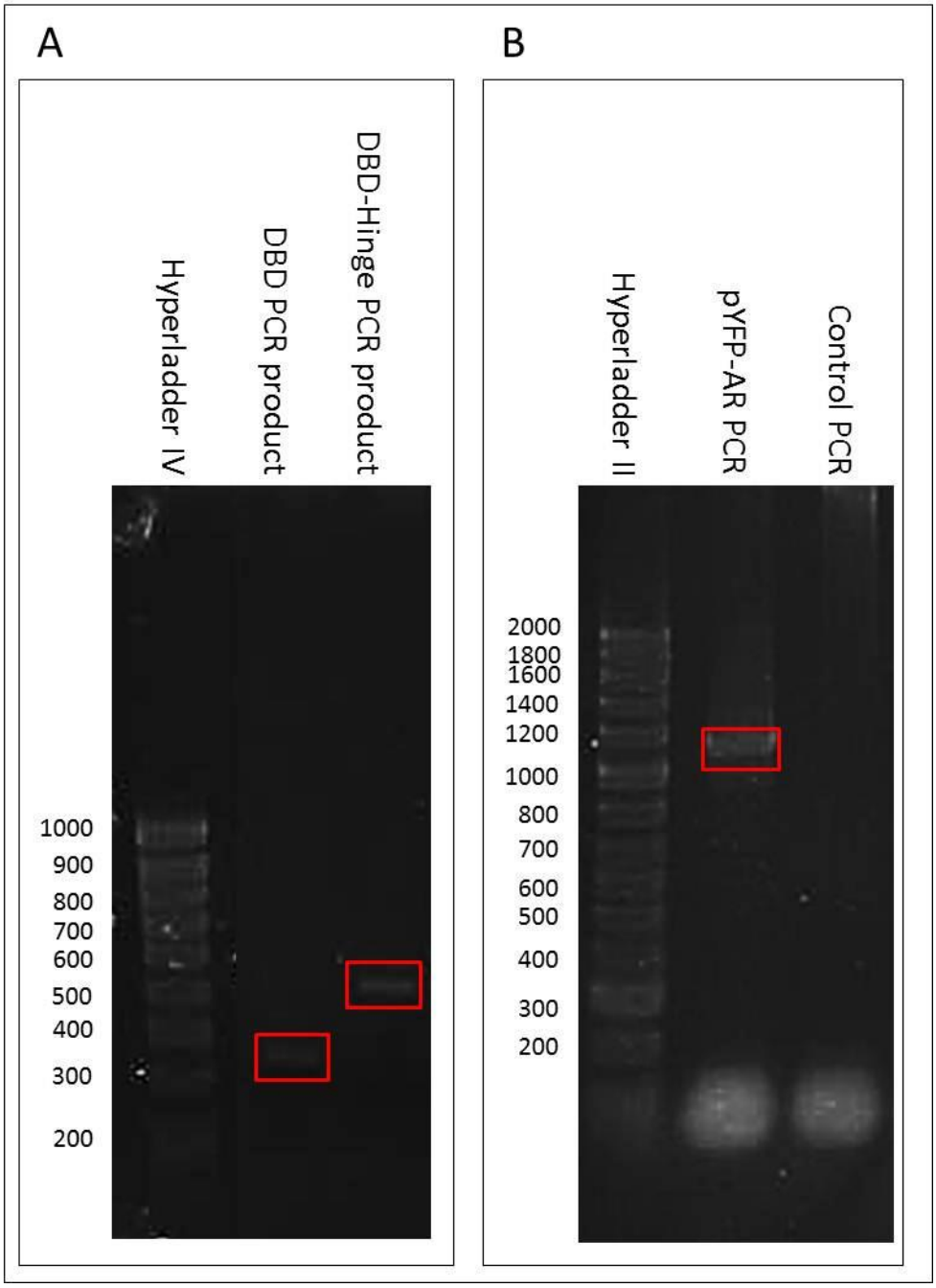

Figure 6.6. AR C-terminal domain amplicons were amplified from pYFP-AR.

A) Androgen receptor (AR)-DBD and AR-DBD-Hinge were amplified by PCR from the pYFP-AR vector before gel extraction. These products were of the correct predicted size of approximately 300 bp and 500 bp (indicated by the red boxes). This work was performed by Miss Lynsey Rogerson. B) AR-DBD-Hinge-LBD was amplified by PCR from the pYFP-AR vector. This product was of the correct predicted size of approximately 1100 bp (indicated by the red box).

Each AR C-terminal domain amplicon was successfully ligated into the pCR2.1 vector (Invitrogen) and subsequently excised using the appropriate restriction enzymes (Figure 6.7a). The amplicons were then sub-cloned into the pGEX-6P-1 vector. Diagnostic digestion of the recombinant pGEX-6P1-1-AR-DBD, pGEX-6P-1-AR-DBD-Hinge and pGEX-6P-1-AR-DBD-Hinge-LBD vectors was achieved using the appropriate restriction enzymes (Figure 6.7b and c). The recombinant vectors were sequenced to ensure no point mutations had been introduced during the cloning process. Restriction maps and sequencing details of the vectors generated can be seen in the appendix.

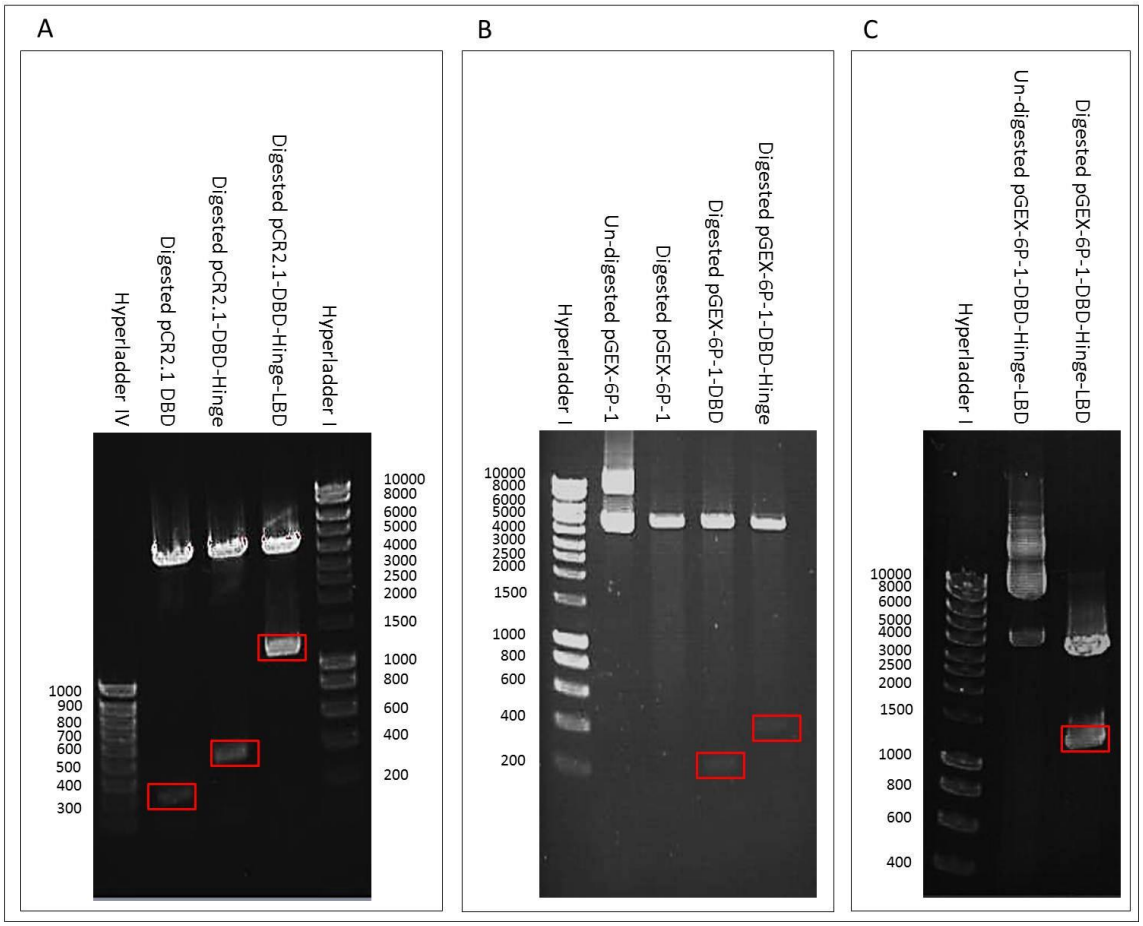

Figure 6.7. AR C-terminal domain fragments were successfully sub-cloned

A) The androgen receptor (AR) C-terminal domain PCR products were ligated into the pCR2.1 vector. Positive transformants were digested with Bam HI and either Eco RI or Xho I to release the amplicon sequences (indicated by the red boxes). B) The restriction enzyme digested AR-DBD and DBD-Hinge fragments were ligated into the pGEX-6P-1 vector via Bam HI and Eco RI restriction sites. Recombinant vectors were assessed by digestion with Bam HI and Eco RI (indicated by the red boxes). C) The restriction enzyme digested AR-DBD-Hinge-LBD was ligated into the pGEX-6P-1 vector via Bam HI and Xho I restriction sites. Recombinant vectors were assessed by digestion with Bam HI and Xho I (indicated by the red box).

6.4.1.2 Purification of recombinant GST-tagged AR C-terminal domain proteins

The next stage, once the pGEX-6P-1 vectors were generated and verified, was to produce large quantities of our GST-tagged AR C-terminal domain proteins in bacterial cells. These proteins could then be purified using affinity chromatography. As a comparison for protein induction and quality of purification, the pGEX-6P-1 empty vector which would generate the recombinant GST-tag alone was included in the purification series alongside the AR C-terminal domain vectors. Recombinant GST-tag would also be useful for subsequent *in vitro* experiments as a control. Figure 6.8 shows a schematic of the GST-tagged proteins to be generated and the approximate molecular weight of each of these proteins.

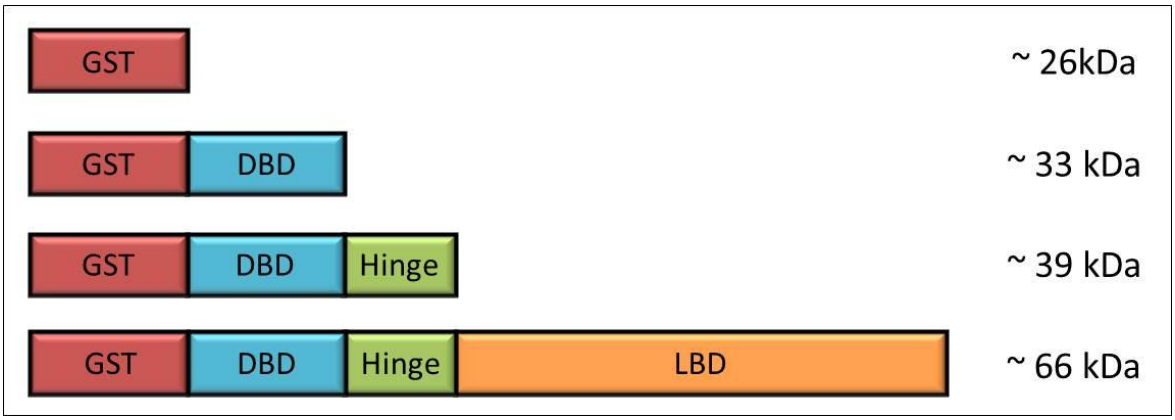

Figure 6.8. Schematic of the GST-tagged recombinant proteins generated by bacterial expression of the pGEX-6P-1-AR C-terminal vectors.

Firstly, the pGEX-6P-1 empty vector and pGEX-6P-1-AR-DBD were transformed into the XA90 strain of *E. coli*, a strain commonly used for production of large quantities of recombinant proteins. Then, to test the protein production capabilities of this bacterial strain, a small-scale protein induction was performed. A 10 ml culture of each vector was grown overnight at 37 ^{o}C before 500 µl was added to fresh culture medium and grown for a further 3 hours. Protein production was induced by addition of 1M IPTG, a non-hydrolysable lactose analogue that can be used by the *lac* operon to drive transcription, and propagated for a further 4 hours. Samples from the culture were collected before induction and after each hour of IPTG treatment for analysis by SDS-PAGE and Coomassie staining.

As seen in Figure 6.9a, low levels of corresponding weight GST protein was present before treatment with IPTG, but was induced after one hour of treatment and was greatest after 4 hours. This was also the case for the recombinant GST-tagged AR-DBD; although the overall levels of protein were much lower (Figure 6.9b).

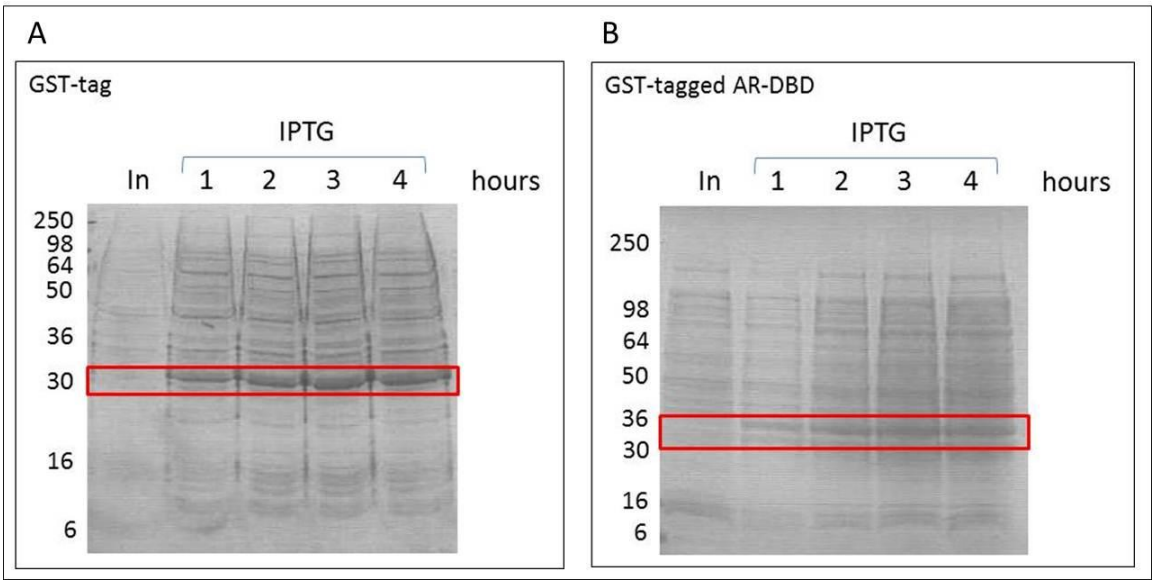

Figure 6.9. Small-scale induction of recombinant GST-tag and GST-tagged AR-DBD protein in XA90 E. coli.

XA90 bacterial cultures transformed with pGEX-6P-1 empty vector or pGEX-6P-1-AR-DBD were induced to produce recombinant GST-tagged proteins by treatment with 1 M IPTG for 4 hours. The input (In) sample represents the un-stimulated culture and was collected before treatment. Samples were collected after 1, 2, 3 and 4 hours of IPTG stimulation. Samples were analysed by SDS-PAGE and Coomassie staining. A) Small-scale protein induction of pGEX-6P-1 empty vector. Recombinant GST-tag was induced with addition of IPTG (indicated by the red box). B) Small-scale protein induction of pGEX-6P-1-AR-DBD. Recombinant GST-tagged AR-DBD was induced with addition of IPTG (indicted by the red box).

Once satisfied that the addition of IPTG drove production of recombinant proteins, large-scale inductions were set up. Similar to the small-scale cultures, a 10 ml culture for each vector was grown overnight at 37 ^{o}C. 5 ml of this culture was added to 500 ml of fresh culture medium and grown until an optical density 600 (OD600) of 0.5 was achieved. Protein production was induced by addition of 1M IPTG and the culture grown for a further 4 hours. Bacterial cells were then pelleted by centrifugation and resuspended in PBS. At this stage protease inhibitors and PMSF were added to block proteolytic degradation of the proteins. Soluble and non-soluble proteins were released from the cells by a series of sonication steps and the sample was loaded onto a freshly prepared glutathione sepharose column. The sample was incubated on the column for 5 minutes before it was allowed to flow through. After washing, the bound proteins were eluted from the column using reduced glutathione. At each stage, a sample was taken for analysis by SDS-PAGE and Coomassie staining. Protein elution fractions were pooled together and subject to buffer exchange by dialysis overnight into dialysis buffer A.

Figure 6.10a and b show the purification profiles of recombinant GST and GST-tagged AR-DBD respectively. Little protein of interest is lost in the column flow through (FT) or washes indicating that these proteins have successfully bound to the column. The eluted fractions of the GST-tag purification show large amounts of recombinant protein present (expected size approximately 26 kDa)(Figure 6.10a), however the amount present in the fractions of the GST-tagged AR-DBD is much smaller (expected size approximately 33 kDa)(Figure 6.10b). It is also important to note that a large amount of background proteins were also eluted from the columns; in the case of the GST-tagged AR-DBD these background proteins are of similar size and amount to the protein of interest making it difficult to determine whether this purification had been successful.

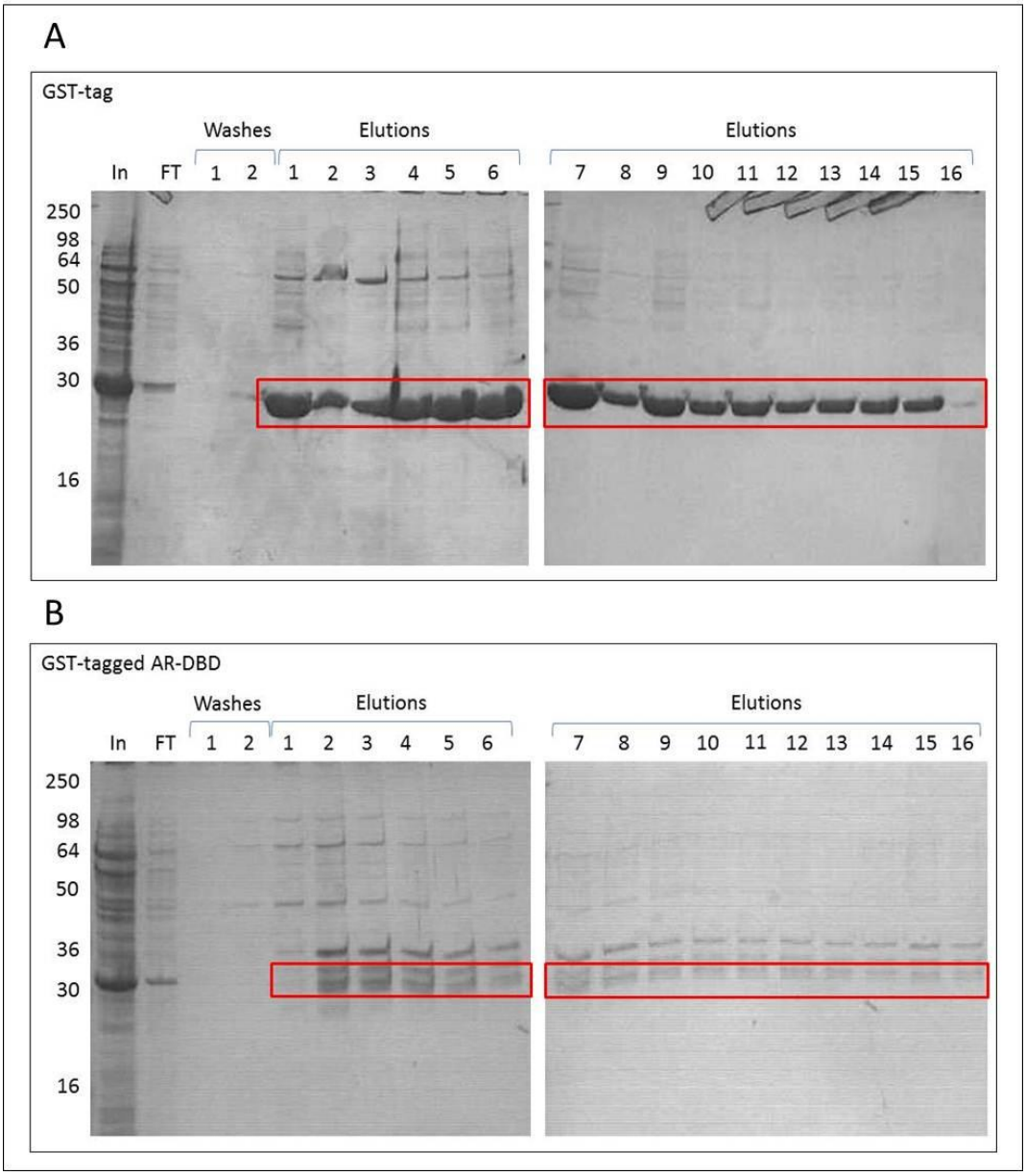

Figure 6.10. Purification of recombinant GST-tag and GST-tagged AR-DBD from XA90 E.coli cultures by GST affinity chromatography.

XA90 bacterial cultures transformed with pGEX-6P-1 empty vector or pGEX-6P-1-AR-DBD were subject to large-scale culturing and protein induction by treatment with 1 M IPTG for 4 hours. Bacterial cells were pelleted and resuspended in PBS before addition of protease inhibitors and PMSF. Suspensions were loaded onto a glutathione sepharose column and allowed to flow through. Columns were washed with PBS before bound proteins were eluted. Eluted recombinant proteins are indicated by the red boxes. The input (In) sample represents the un-stimulated culture and was collected before IPTG treatment. Samples were collected from the flow through (FT), washes and eluted fractions. Samples were analysed by SDS-PAGE and Coomassie staining. A) Purification profile of recombinant GST-tag. B) Purification profile of recombinant GST-tagged AR-DBD.

In order to address the problems highlighted above, the expression vectors were transformed into another *E. coli* strain, BL21, and subject to the same process described above. The BL21 strain of *E. coli* is useful for protein expression, as similar to the XA90 strain, it produces T7 polymerase upon IPTG treatment. However, this strain lacks several proteases thus potentially reducing the amount of background breakdown products that may be seen during the purification process.

Initial small-scale induction tests were successful for the GST-tag, GST-tagged AR-DBD and GST-tagged AR-DBD-Hinge with protein induction observed after 1 hour of IPTG treatment (Figure 6.11a, b and c). Unlike the XA90 inductions, however, increased time of treatment did not appear to increase the amount of protein induction observed. GST-tagged AR-DBD-Hinge-LBD induction was not detectable on Coomassie stained gels (Figure 6.12a), however induction was clearly visible via Western blot (Figure 6.12b). As Western blot is the end-point assay for the *in vitro* ubiquitination assays, large scale culturing for this protein was continued.

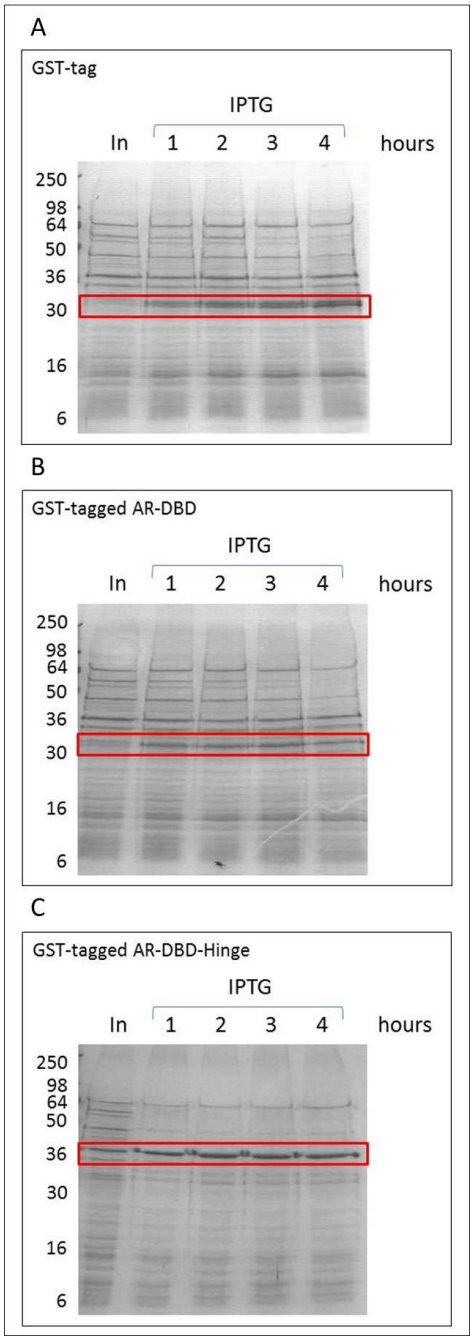

Figure 6.11. Small-scale induction of recombinant GST-tag, GST-tagged AR-DBD and GST-tagged AR-DBD-Hinge proteins in BL21 E. coli.

BL21 bacterial cultures transformed with pGEX-6P-1 empty vector, pGEX-6P-1-AR-DBD or pGEX-6P-1-AR-DBD-Hinge were induced to produce recombinant GST-tagged proteins by treatment with 1 M IPTG for 4 hours. The input (In) sample represents the un-stimulated culture and was collected before treatment. Samples were collected after 1, 2, 3 and 4 hours of IPTG stimulation. Samples were analysed by SDS-PAGE and Coomassie staining. A) Small-scale protein induction of pGEX-6P-1 empty vector. Recombinant GST-tag was induced with addition of IPTG (indicated by the red box). B) Small-scale protein induction of pGEX-6P-1-AR-DBD. Recombinant GST-tagged AR-DBD was induced with addition of IPTG (indicted by the red box).C) Small-scale protein induction of pGEX-6P-1-AR-DBD-Hinge. Recombinant GST-tagged AR-DBD-Hinge was inducted by addition of IPTG (indicated by the red box).

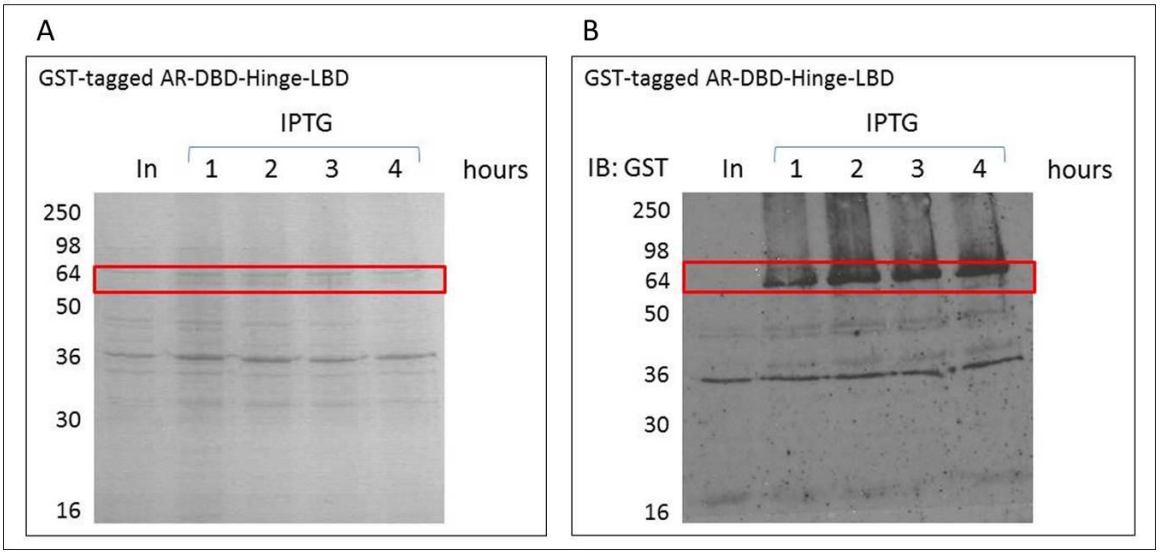

Figure 6.12. Small-scale induction of recombinant GST-tagged AR-DBD-Hinge-LBD in BL21 E. coli.

BL21 bacterial cultures transformed with pGEX-6P-1-AR-DBD-Hinge-LBD were induced to produce recombinant GST-tagged AR-DBD-Hinge-LBD by treatment with 1 M IPTG for 4 hours. The input (In) sample represents the un-stimulated culture and was collected before treatment. Samples were collected after 1, 2, 3 and 4 hours of IPTG stimulation. Samples were analysed by SDS-PAGE, Coomassie staining and Western blot. A) Small-scale protein induction of pGEX-6P-1-AR-DBD-Hinge-LBD was not detected by Coomassie staining (indicated by the red box). B) Western blot analysis of small-scale protein induction of pGEX-6P-1-AR-DBD-Hinge-LBD samples with anti-GST antibodies (indicted by the red box).

Samples for SDS-PAGE were collected as described previously, in addition to samples were collected after the sonication and filtering steps. Figure 6.13 shows the purification profiles of the recombinant GST-tag, GST-tagged AR-DBD and GST-tagged AR-DBD-Hinge proteins (Figure 6.13a, b and c respectively). In each case little protein was lost into the flow through (FT) or during the washing stages, again indicating good binding of the GST-tagged proteins to the affinity column.

Compared to the previous purifications performed in the XA90 *E. coli* strain, there was a reduction in the amount of recombinant GST-tag eluted from the column (Figure 6.13a). Also, there appears to be a greater amount of GST-tagged AR-DBD produced although this could in part be accredited to the reduction in the total number of background proteins also being produced and eluted from the column (Figure 6.13b). The purification for GST-tagged AR-DBD-Hinge generated similar amounts of protein as the GST-tagged AR-DBD (Figure 6.13c), whilst GST-tagged AR-DBD-Hinge-LBD was barely visible on Coomassie stained gels particularly in the later elution fractions (Figure 6.14a). Western blot analysis of these samples with anti-GST antibodies revealed that protein was present in small amounts in all fractions (Figure 6.14b). The elution fractions 2-5 and 6-16 for each protein were pooled together and subject to buffer exchange by dialysis into dialysis buffer A. The protein elutions were then aliquoted into stocks for use in *in vitro* ubiquitin assays and stored at -80 ^{o}C.

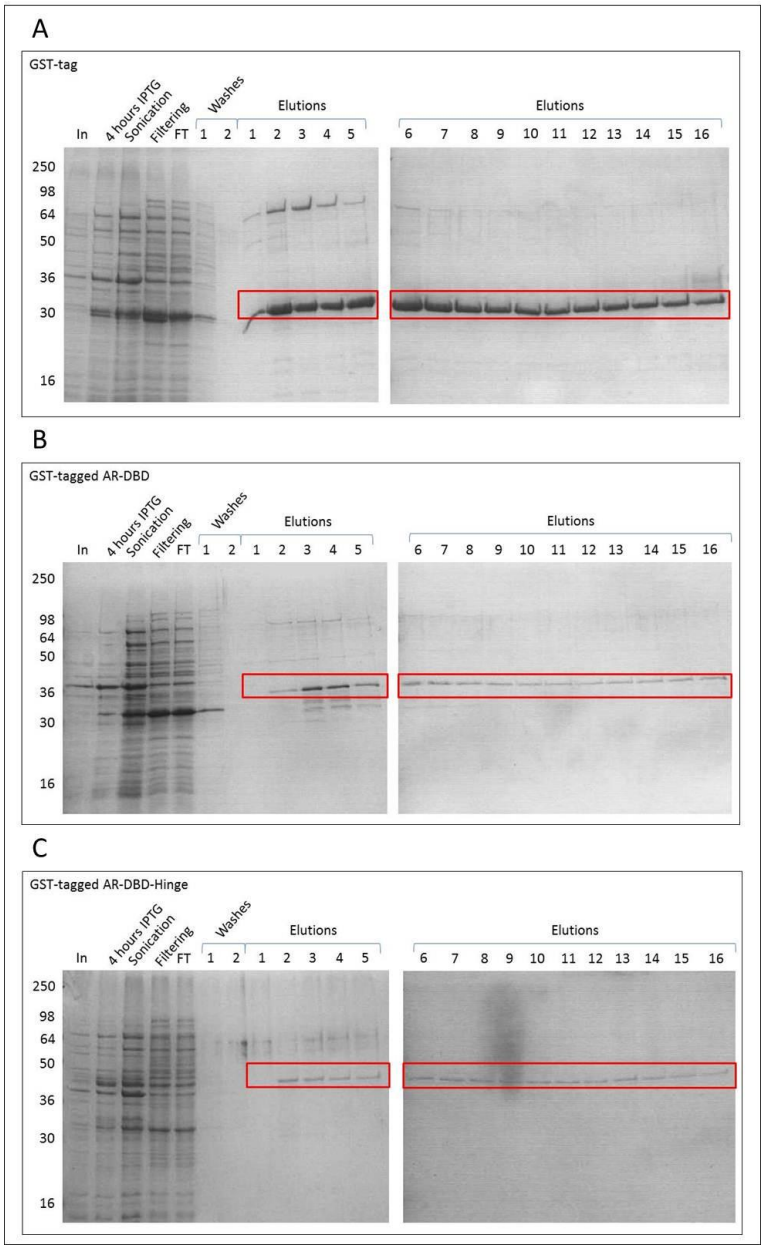

Figure 6.13. Purification of recombinant GST-tag, GST-tagged AR-DBD and GST-tagged AR-DBD-Hinge from BL21 E.coli cultures by GST affinity chromatography.

BL21 bacterial cultures transformed with pGEX-6P-1 empty vector, pGEX-6P-1-AR-DBD or pGEX-6P-1-AR-DBD-Hinge were subject to large-scale culturing and protein induction by treatment with 1 M IPTG for 4 hours. Bacterial cells were pelleted and resuspended in PBS before addition of protease inhibitors and PMSF. Suspensions were loaded onto a glutathione sepharose column and allowed to flow through. Columns were washed with PBS before bound proteins were eluted. Eluted recombinant proteins are indicated by the red boxes. The input (In) sample represents the un-stimulated culture and was collected before IPTG treatment. Samples were collected from the flow through (FT), washes and eluted fractions. Samples were analysed by SDS-PAGE and Coomassie staining. A) Purification profile of recombinant GST-tag. B) Purification profile of recombinant GST-tagged AR-DBD. C) Purification profile of recombinant GST-tagged AR-DBD-Hinge.

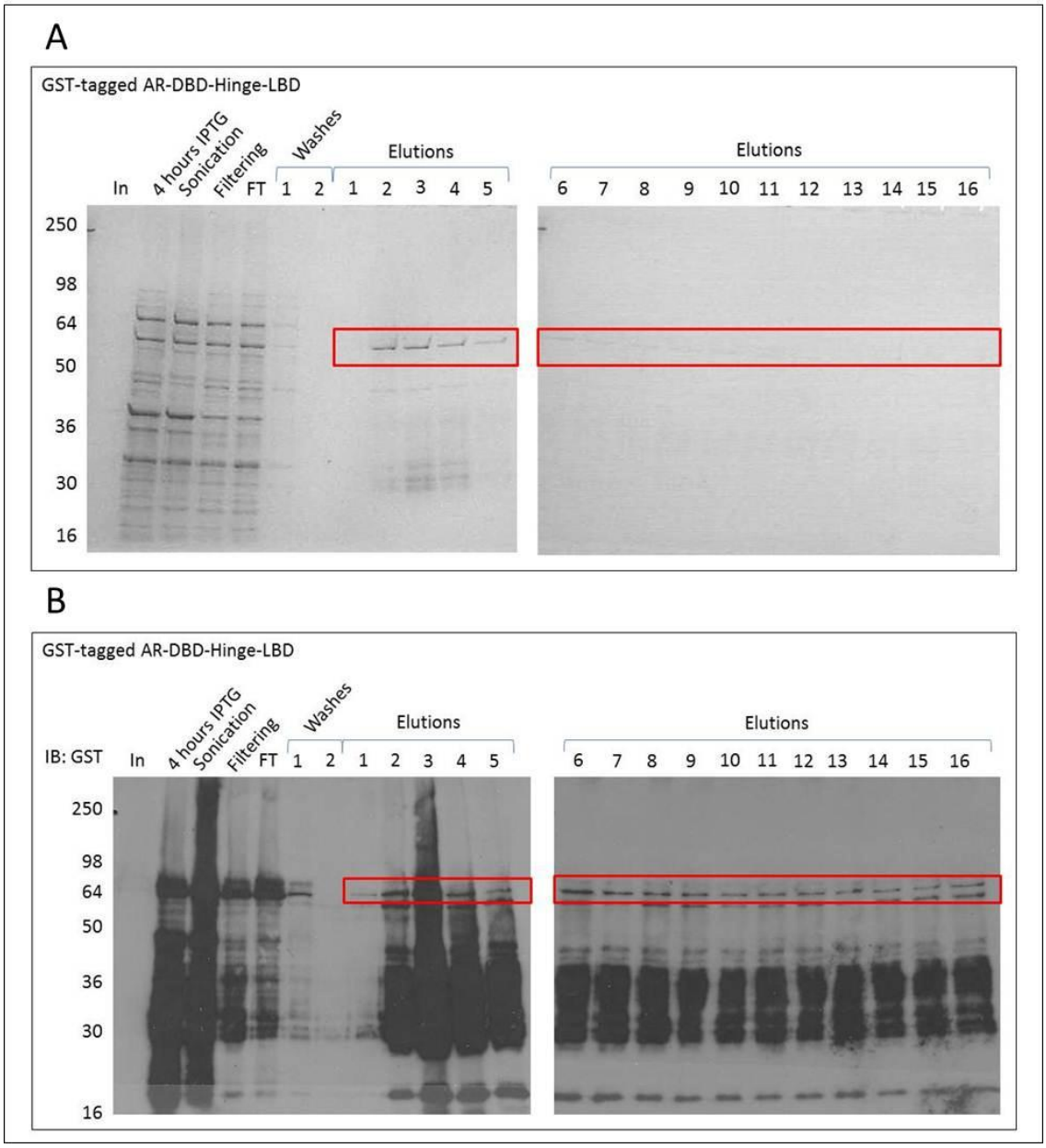

Figure 6.14. Purification of recombinant GST-tagged AR-DBD-Hinge-LBD from BL21 E.coli cultures by GST affinity chromatography.

BL21 bacterial cultures transformed with pGEX-6P-1-AR-DBD-Hinge-LBD were subject to large-scale culturing and protein induction by treatment with 1 M IPTG for 4 hours. Bacterial cells were pelleted and resuspended in PBS before addition of protease inhibitors and PMSF. Suspensions were loaded onto a glutathione sepharose column and allowed to flow through. Columns were washed with PBS before bound proteins were eluted. Eluted recombinant GST-tagged AR-DBD-Hinge-LBD is indicated by the red boxes. The input (In) sample represents the un-stimulated culture and was collected before IPTG treatment. Samples were collected from the flow through (FT), washes and eluted fractions. Samples were analysed by SDS-PAGE, Coomassie staining and Western blot. A) Purification profile of recombinant GST-tagged AR-DBD-Hinge-LBD. B) Western blot analysis of the purification profile of recombinant GST-tagged AR-DBD-Hinge-LBD with an anti-GST antibody.

6.4.1.3 MDM2 ubiquitinates the AR C-terminal in vitro

As a preliminary assay to test whether the recombinant AR C-terminal domain proteins were suitable for *in vitro* ubiquitination assays, GST-tagged AR-DBD-Hinge-LBD was incubated with the E1 enzyme UBE1, the E2 enzyme UbcH5a and the E3 ligase enzyme MDM2 for 4 hours at 37 ^{o}C in the presence and absence of the reaction activator 10 mM ATP. This reaction also contained mutant ubiquitin which is incapable of forming poly-ubiquitin chains due to mutation of all seven ubiquitin acceptor lysine residues to arginine, termed K7R ubiquitin.

As expected, Coomassie staining of the resultant reaction detected both AR and MDM2 (Figure 6.15a). Although ubiquitination of the GST-tagged AR-DBD-Hinge-LBD, as evidenced by higher molecular weight species, was not clearly detected by Coomassie staining, it is interesting that the band corresponding to the AR C-terminal protein became more pronounced after the addition of ATP to the reaction (Figure 6.15a). Additionally, auto-ubiquitination of MDM2 was observed upon activation of the reaction, this is an internal positive control and indicates that the reaction was indeed activated (Figure 6.15a). Analysis of the reaction by Western blot with an anti-C-terminal AR antibody revealed the presence of a 10 kDa larger molecular weight band above the GST-tagged AR-DBD-Hinge-LBD indicating a possible mono-ubiquitination event by addition of a single K7R ubiquitin molecule (Figure 6.15b). Further work is required to elucidate whether the AR C-terminus is indeed a site of ubiquitination by MDM2 and also the other C-terminal domain proteins can be utilised to pin-point the exact domains involved. Importantly, this experiment did not include the recombinant GST-tag protein as a negative control and so it cannot be determined whether the ubiquitination event seen is directly on an AR domain or on part of the GST-tag.

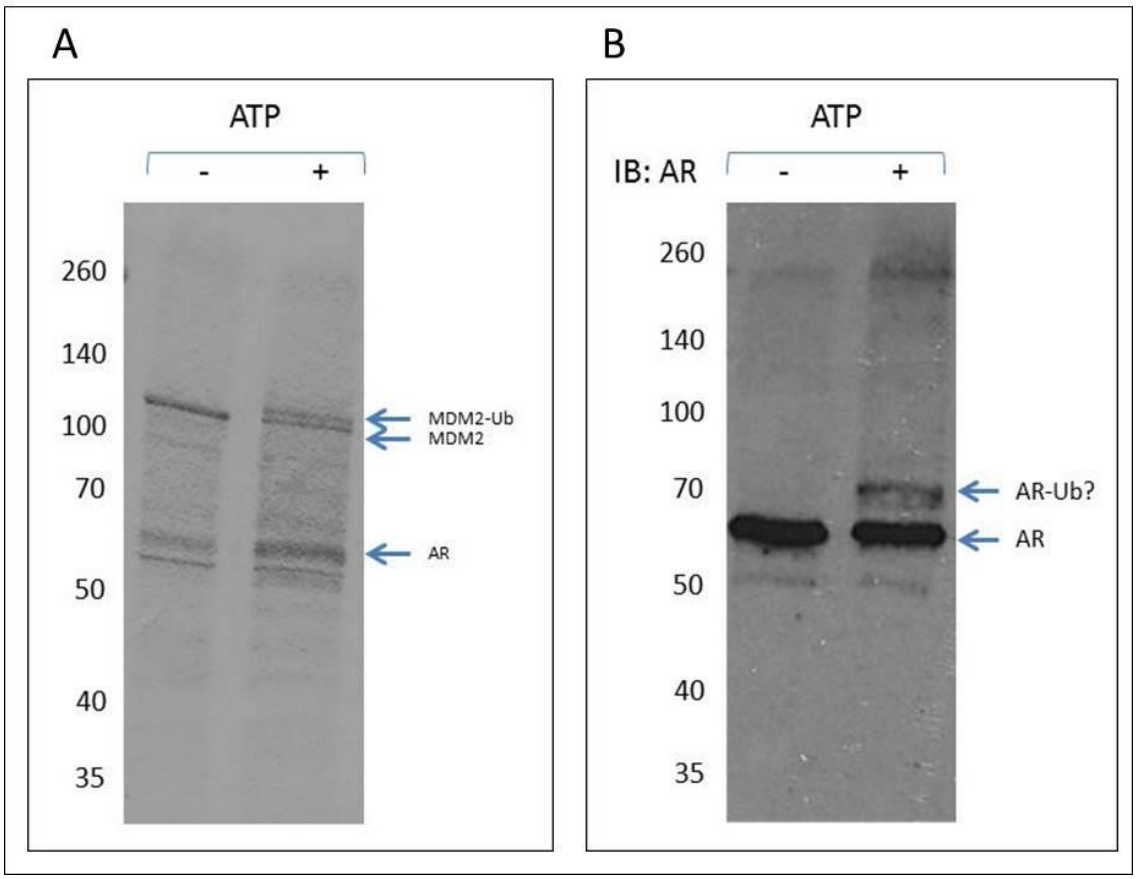

Figure 6.15. MDM2 ubiquitinates GST-tagged AR-DBD-Hinge-LBD in vitro.

In vitro ubiquitination assays were set up containing 100 nM UBE1, 5 µM UbcH5a, 1 µM MDM2, GST-tagged AR-DBD-Hinge-LBD and 200 µM K7R ubiquitin. Reactions were activated by addition of 10 mM ATP (+). Control reactions contained all components but were not stimulated with ATP (-). Reactions were incubated for 4 hours at 37 ^{o}C before being terminated by addition of stop buffer. Samples were analysed by SDS-PAGE, Coomassie staining and Western blot. A) Coomassie staining of in vitro ubiquitination assay samples. AR-DBD-Hinge-LBD and MDM2 are indicated by blue arrows. Ubiquitinated MDM2 (MDM2-Ub) was observed in the + ATP reaction (indicated by a blue arrow). B) Western blot analysis of the in vitro ubiquitination assay samples with anti-AR antibodies. AR is indicated by a blue arrow. A potentially mono-ubiquitinated form of AR (AR-Ub?) was observed in the + ATP reaction (indicated with a blue arrow).

6.4.2 Generation of recombinant USP10

Previous studies of USP10 have revealed that it is a direct interacting partner of the AR acting to stimulate transcriptional activity through regulation of Histone H2A.Z ubiquitination (Draker *et al.*, 2011). Data presented within this thesis is contradictory to these previous reports; showing USP10 does not interact with the AR and has some negative effect on transcriptional activity.

The specificity of USP10 towards different types of poly-ubiquitin chain is unknown and could provide an insight into which type of linkages are preferentially recognised by USP10. Furthermore, once established, *in vitro* deubiquitination assays utilising USP10 or other DUBs could be used to address whether the AR is a direct target of these DUB enzymes. Enzymatically active recombinant GST-tagged USP10 was cultured and purified using a modification of the protocol described above. This work was performed by Miss Susan Hill under my supervision.

pGEX-6P-1-USP10 was firstly sequenced to verify that it did not contain any point mutations or other alterations that would affect the enzymatic activity of recombinant USP10 protein (Faus *et al.*, 2005). Secondly, this vector was transformed into BL21 bacterial cells and a small-scale protein induction performed to ensure that protein was induced with IPTG treatment similar to those above. Figure 6.16 shows that USP10 protein was induced after 1 hour of IPTG treatment and was greatest after 4 hours of treatment.

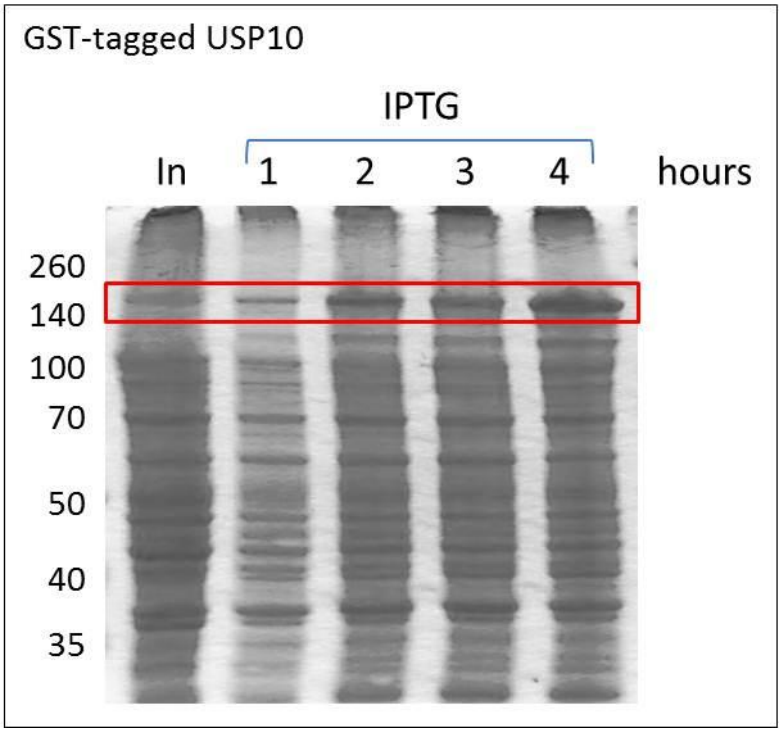

Figure 6.16. Small-scale induction of recombinant GST-tagged USP10 in BL21 E. coli.

BL21 bacterial cultures transformed with pGEX-6P-1-USP10 were induced to produce recombinant GST-tagged USP10 by treatment with 1 M IPTG for 4 hours. The input (In) sample represents the un-stimulated sample and was collected before treatment. Samples were collected after 1, 2, 3 and 4 hours of IPTG stimulation. Samples were analysed by SDS-PAGE and Coomassie staining. Recombinant GST-tagged USP10 was induced with addition of IPTG (indicated by the red box). This work was performed by Miss Susan Hill.

Unlike the AR C-terminal domain proteins which were intended to be used as substrates in the *in vitro* ubiquitination assays, the *in vitro* deubiquitination assays required functional enzymatic activity of purified USP10 hence large-scale induction and purification of GST-tagged USP10 was performed consecutively to avoid unnecessary freezing and thawing of samples and the dialysis buffer was altered to contain glycerol to help preserved the protein structure and function. This work was performed by Miss Susan Hill under my supervision.

Figure 6.17 shows the purification profile of recombinant GST-tagged USP10. Once again, production of USP10 was induced with addition of IPTG and, like the previous purifications, very little protein was lost in the flow through and washes (Figure 6.17a). GST-tagged USP10 was successfully eluted from the column in the early fractions (elutions 2-5), although it was hardly visible in the later fractions (Figure 6.17a). In agreement with the Coomassie gel, analysis of the samples by Western blot showed large amounts of GST-tagged USP10 were present in elutions 2-5, while elutions 6-16 contained markedly less USP10 (Figure 6.17b). The elution fractions 2-5 and 6-16 were pooled together and subject to buffer exchange by dialysis overnight in dialysis buffer B.

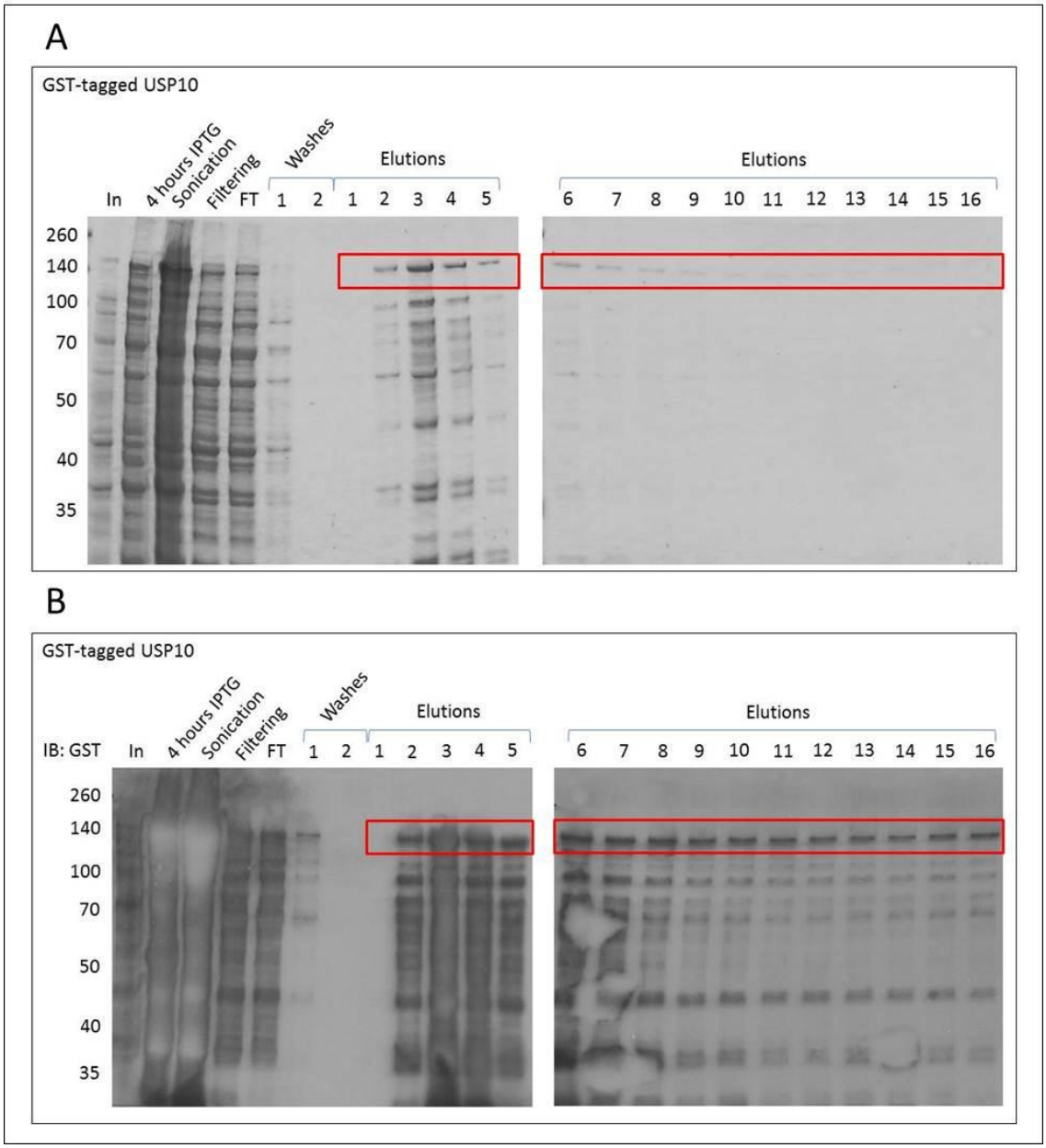

Figure 6.17. Purification of recombinant GST-tagged USP10 from BL21 E.coli cultures by GST affinity chromatography.

BL21 bacterial cultures transformed with pGEX-6P-1-USP10 were subject to large-scale culturing and protein induction by treatment with 1 M IPTG for 4 hours. Bacterial cells were pelleted and resuspended in PBS before addition of protease inhibitors and PMSF. Suspensions were loaded onto a glutathione sepharose column and allowed to flow through. Columns were washed with PBS before bound proteins were eluted. Eluted recombinant GST-tagged USP10 is indicated by the red boxes. The input (In) sample represents the un-stimulated culture and was collected before IPTG treatment. Samples were collected from the flow through (FT), washes and eluted fractions. Samples were analysed by SDS-PAGE, Coomassie staining and Western blot. This work was performed by Miss Susan Hill. A) Purification profile of recombinant GST-tagged USP10. B) Western blot analysis of the purification profile of recombinant GST-tagged USP10 with an anti-GST antibody.

6.4.2.1 USP10 deubiquitinates penta-ubiquitin chains in vitro

To test the enzymatic activity of the purified recombinant GST-tagged USP10, an *in vitro* deubiquitination assay was utilised. Varying amounts of GST-tagged USP10 (2.5–5 µl) were incubated with 50 ng/µl of either K48-linked or K63-linked penta-ubiquitin chains for up to 4 hours at 37 ^{o}C. Control reactions containing all of the necessary components but no recombinant USP10 were also set up. Reactions were terminated by addition of SDS sample buffer and analysed by Western blotting using the anti-K48 ubiquitin or K63 ubiquitin specific antibodies (Figure 6.18). This work was performed by Miss Susan Hill under my supervision.

Figure 6.18a shows the results of the *in vitro* deubiquitination assay for the K48-linked penta-ubiquitin chains. It is important to note that tetra-ubiquitin chains were observed under all conditions suggesting that this cleavage of one ubiquitin moiety was not due to addition of USP10 (Figure 6.18a). Moreover, addition of recombinant GST-tagged USP10 to these reactions had no effect of cleavage of these ubiquitin chains.

Interestingly, addition of recombinant USP10 to K63-linked penta-ubiquitin chain reactions did have an effect on chain cleavage with the appearance of mono- and di-ubiquitin chains (Figure 6.18b). Similar to K48 penta-ubiquitin, some cleavage products were present before the addition of enzyme, tetra- and tri-ubiquitin chains. Deubiquitination was also increased with time as more prominent mono- and di-ubiquitin bands were observed after 4 hours reaction time (Figure 6.18b).

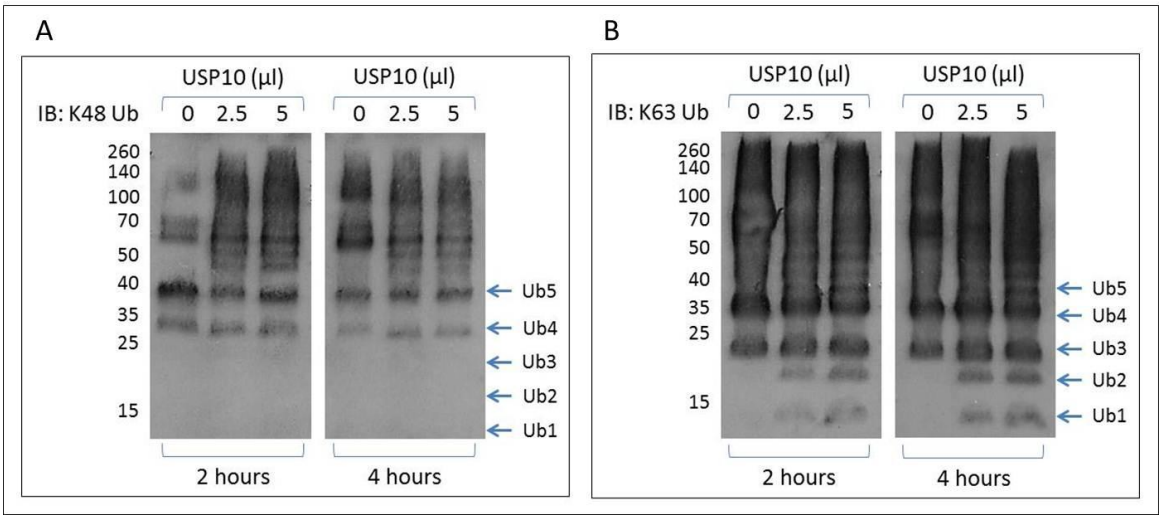

Figure 6.18. GST-tagged USP10 deubiquitinates K63-linked penta-ubiquitin chains in vitro.

In vitro deubiquitination assays were set up containing GST-tagged USP10 and 50 ng/µl K48- or K63-linked penta-ubiquitin chains. Increasing volumes of GST-tagged USP10 were added to the reactions and reactions without GST-tagged USP10 served as controls. Reactions were incubated for up to 4 hours at 37 ^{o}C before being terminated by addition of SDS sample buffer. Samples were analysed by SDS-PAGE and Western blot with anti-K48- and K63-specific ubiquitin antibodies. Penta-, tetra-, tri-, di- and mono-ubiquitin chains are indicated by the blue arrows (Ub5, Ub4, Ub3, Ub2 and Ub1 respectively). This work was performed by Miss Susan Hill. A) Western blot analysis of in vitro deubiquitination assays containing K48-linked penta-ubiquitin chains with anti-K48 ubiquitin specific antibodies. B) Western blot analysis of in vitro deubiquitination assays containing K63-linked penta-ubiquitin chains with anti-K63 ubiquitin specific antibodies.

6.5 Discussion

Three GST-tagged AR C-terminal fragments were cloned and purified alongside the GST-tag control. These proteins were intended to serve as substrates for *in vitro* ubiquitination assays to help elucidate sites of the AR targeted for ubiquitination. An in vitro mono-ubiquitination assay was established as a valid starting point for identification of novel sites of AR ubiquitination by candidate E3 ubiquitin ligase enzymes. Use of the AR fragments generated in this Chapter would potentially identify domains of the receptor that are modified and thus provide protein for subsequent unequivocal site identification by mass spectrometry.

Although time constraints did not allow for investigation of the AR NTD to be performed, this is another key area for exploration. Xu *et al*., reported two ubiquitination sites within the LBD but their study did not include the NTD. Interestingly, the NTD is the site of the majority of phosphorylation events on the AR and confers more transcriptional activity than the C-terminal AF-2 domain (Gelmann, 2002). Two of these modifications, phosphorylation at S515 and Y534 have been linked to AR stability (Ponguta *et al.*, 2008; Chymkowitch *et al.*, 2011). Mutation of the S515 phosphorylation site increases AR stability due to reduced recruitment of MDM2 (Chymkowitch *et al.*, 2011). Conversely, phosphorylation of Y534 by Src reduces AR ubiquitination by reducing the interaction with CHIP (DaSilva *et al.*, 2009). To enable study of the ubiquitination sites of this domain purified recombinant GST-tagged AR NTD protein has been obtained from Iain McEwan (University of Aberdeen).

Unfortunately, the AR substrates which were purified in this Chapter would not be suitable for proteomic analysis. Although the *in vitro* assay performed with GST-tagged AR-DBD-Hinge-LBD revealed a potential ubiquitination event by Western blot analysis (Figure 6.15b) this band was not visible on the respective Coomassie stained gel (Figure 6.15a). To identify the exact residues that are the targets for modification, bands must be excised precisely from the gels and subject to analysis by proteomic techniques. To achieve this more concentrated AR substrates are required. One possibility is to immunoprecipitate the recombinant AR C-terminal proteins from the elution fractions using an AR C-terminal specific antibody thereby allowing concentration of the proteins whilst removing any residual non-specific proteins.

Although, the identification of the sites of AR ubiquitination is a key area of research it has not been the main focus of the current study. *In vitro* ubiquitination assays, in this case, would serve as a tool to examine the validated DUBs in their ability to remove ubiquitin from AR. Using this we can learn about the complex interactions between different E3 ligases and DUBs. For example, MDM2-induced ubiquitination may be removed exclusively by theoretical DUB X whereas CHIP-induced ubiquitination may be removed by both DUBs X and Y. Steinkamp *et al*., identified androgen ablation therapy-inducted mutations in the AR NTD and LBD of patients with CRCaP (Steinkamp *et al.*, 2009). Importantly, one mutation in the CHIP interaction motif within the NTD, E255K, led to stabilisation of AR and increased ligand-independent nuclear translocation (Steinkamp *et al.*, 2009). The consequences of mis-regulated DUB association or expression could provide a parallel mechanism of aberrant AR activation and potentially targets for CaP therapy.

Recombinant GST-tagged USP10 was successfully purified using an adapted purification protocol. Although the purification process did not yield high concentrations of protein (Figure 6.17) it was found to be enzymatically active (Figure 6.18). The results of the current study and the previously published work on USP10 and the AR have been contradictory (Faus *et al.*, 2005; Draker *et al.*, 2011). *In vitro* deubiquitination assays were set up incorporating USP10 and penta-ubiquitin chains of the most studied lysine linkages, K48 and K63. Intriguingly, USP10 failed to elicit DUB activity towards the K48-linked penta-ubiquitin chains whereas the K63-linked chains were disassembled to di- and mono-ubiquitin moieties. K63-linked ubiquitin chains are associated with increased transcriptional activation(Hochstrasser, 1992); therefore the consequence of disassembly of these chains would be a decrease in activity. This data suggests that USP10 may have a role in removing transcriptionally activating ubiquitination events from the AR although much more thorough investigation is required to support this conclusion.

USP12 was also identified by the siRNA screen as a DUB which effected AR transcriptional activity. Unlike USP10, USP12 was not a candidate for purification and *in vitro* deubiquitination assays in this study as it requires UAF1 and WDR20 to increase its enzymatic activity that would have required substantial work (Cohn *et al.*, 2009; Kee *et al.*, 2010). Instead, to investigate the specificity of USP12, an in-cell ubiquitination

assay could be a more efficient method for studying enzymatic activity. Ectopic expression of USP12, in the presence or absence of UAF1, WDR20 and ubiquitin followed by immuno-precipitation of AR could provide an insight into whether USP12 directly affects the ubiquitination status of AR. This type of experiment is also important to test the activity of USP10 as *in vitro* assays do not recapitulate the physiological complexity of the ubiquitination-deubiquitination systems. Indeed, the in vitro system utilised here is stripped of other factors that may affect the process of ubiquitin removal, such as USP10 may not be contained within the same cellular compartment as AR or it may be sequestered by interacting-proteins. Nonetheless, *in vitro* assays can be used as a relatively high-throughput methodology providing preliminary results for further characterisation experiments in cell-line based studies.

In conclusion, purification of the GST-tagged AR-DBD, DBD-Hinge, DBD-Hinge-LBD and USP10 was successful. The AR C-terminal domains are suitable for exploratory experiments looking at the general region of ubiquitination but are not concentrated enough for proteomic analysis of the exact sites of ubiquitination. GST-tagged USP10 was found to be enzymatically active towards K63-linked penta-ubiquitin chains. To continue this study, the potential of direct AR deubiquitination by USP10 would be assessed and if this process was inherently linked with controlling transcriptional activity of the receptor.

Chapter 7: General Discussion

7.1 USP12 as a co-activator of AR transcriptional activity

USP12 was identified as a regulator of AR activity as a result of a siRNA library screen investigating at the effect of DUB enzyme knockdown on AR-regulated gene transcription in LNCaP cells. Depletion of USP12 resulted in decreased *PSA* mRNA and protein expression whilst also decreasing the expression of the other androgen-responsive genes *KLK2*, *TMPRSS2*, *NKX3.1* and *NDRG1*. *USP12* expression was found not to be regulated by androgen and AR mRNA and protein levels remained unaffected by *USP12* knockdown.

Luciferase reporter assays in the LNCaP-7B7 pPSA cell line following USP12 depletion confirmed that USP12 regulated AR transcriptional activity. Furthermore, over-expression of ectopic USP12 increased AR transcriptional activity upon the ARE3 luciferase reporter in COS-7 cells, and this transcriptional activation was dependent on the deubiquitinase activity of USP12.

In order to investigate the potential mechanism by which USP12 regulated AR activity, characterisation of the effect of USP12 on AR movement and promoter binding was assessed. Cytoplasmic-nuclear fractionation of USP12-depleted LNCaP cells following 8 hours DHT stimulation demonstrated an increased level of AR in the nucleus of untreated LNCaP cells as well as lower AR in the nucleus after 8 hours DHT stimulation. These results suggested that USP12 may have a role in retaining or stabilising the AR in the nucleus. This result is in conjunction with recent evidence implicating USP12 in histone deubiquitination and thus it was hypothesised that USP12 may affect AR recruitment at androgen-regulated gene promoters in response to androgen treatment. Knockdown of *USP12* resulted in reduced AR association at the ARE I of the proximal promoter and ARE III of the enhancer of the *PSA* gene providing evidence of a direct role for USP12 in regulation of AR-mediated gene transcription. Moreover, a direct interaction between USP12 and AR was observed in COS-7 cells with ectopic over-expression suggesting USP12 may potentiate AR recruitment to target promoters by physically tethering the receptor to *cis*-regulatory elements. Use of RNAi rescue to incorporate wild-type and deubiquitinase-dead USP12 in similar chromatin immunoprecipitation experiments would address whether the deubiquitinase activity of USP12 is required for this effect on AR recruitment to target gene promoters.

Post-translational modification of histones provides an important mechanism for controlling gene transcription (Zhang and Reinberg, 2001; Weake and Workman, 2008). Furthermore, epigenetic events have been shown to influence the transcription of AR-responsive genes. JHDM2A, a JmjC-containing histone H3K9 demethylase, is recruited to AR-target gene promoters in response to androgen treatment resulting in removal of mono- and di-methylation marks on histone H3K9 and activation of transcription (Yamane *et al.*, 2006). USP12 directly deubiquitinates histones H2A and H2B, directly linking USP12 transcriptional regulation at the epigenetic level in *Xenopus* (Joo *et al.*, 2011) although this has not been studied in human cells. Investigation of histone ubiquitination status at AR-responsive gene promoters, such as *PSA* and *TMPRSS2*, by chromatin Immunoprecipitation utilising anti-ubiquitinated histone H2A and H2B antibodies in cells subject to USP12 depletion would be important in elucidating if USP12 influences AR-mediated transcriptional events through deubiquitination of histone H2A and H2B.

Several difficulties were encountered in the characterisation USP12 including a lack of available reagents. A purchased HA and FLAG-tagged USP12 mammalian expression vector (Addgene) (Sowa *et al.*, 2009) did not over-express an HA or FLAG-tagged protein corresponding to the predicted size of USP12. Generation of a FLAG-tagged USP12 vector was therefore undertaken to facilitate the project. Unfortunately, no commercially available USP12 antibodies were able to detect endogenous or ectopically expressed USP12, and hence the confirmation of *USP12* knockdown at the protein level could not be performed as well as, restricting the types of characterisation experiments that could be performed utilising endogenous USP12. Although, immunoprecipitation of ectopically expressed FLAG-tagged USP12 with an anti-FLAG antibody identified an interaction between USP12 and AR, repeating this in LNCaP cells would be critical in confirming that this interaction is genuine under physiological conditions. Moreover, immuno-fluorescence studies of the cellular localisation of USP12 have not been performed but this could further confirm a role forUSP12 in regulation of AR nuclear function.

The effect of USP12 on the ubiquitination status of the AR was not studied due to time constraints, however this in an important area that requires addressing. Performing immunoprecipitation of AR in the presence and absence of USP12 under denaturing

conditions would give an insight into whether the enzyme directly affects AR ubiquitination. Furthermore, purification of USP12 from cells by IP and incorporation into *in vitro* deubiquitination assays could elucidate which type of ubiquitin chain USP12 recognises and catalyses. The above data would suggest that USP12 reverses ubiquitination events that lead to AR repression however as USP12 does not affect the protein levels of AR it is unlikely that it reverses lysine 48-linked degrading chains.

Examination of PSA protein levels in response to USP12 depletion in LNCaP cells showed a reduction in α-tubulin, suggesting USP12 may have an effect on LNCaP cell proliferation or viability. SRB staining of LNCaP cells, measuring total protein, confirmed this finding. Moreover, flow cytometry analysis of LNCaP cells following USP12 depletion caused G1 arrest and increased subG1 suggestive of apoptosis. Caspase-3 specific flow cytometry, as well as cleaved PARP-1 Western blotting, showed an increase in LNCaP apoptosis after *USP12* knockdown. These data, taken together, suggests that USP12 may have a pro-proliferative and anti-apoptotic role which may be attached to its role in AR regulation, as AR is pro-proliferative. These results imply that USP12 may be a prospective therapeutic target in prostate cancer. Repeating the phenotypic experiments in an AR-negative cell line would elucidate whether the effects of USP12 observed in LNCaP cells are directly linked to the function of USP12 in the AR signalling cascade or an indirect effect. One simple experiment to address this question would be to combine *USP12* and *AR* knockdown in LNCaP cells to examine if the effect of USP12 depletion is further potentiated by reduction to AR levels.

Another important area for expansion in this study is the interplay between USP12 and PHLPP, an Akt phosphatase. Sowa *et al*., identified PHLPP as an interacting partner of USP12 in mass spectrometry analysis of 75 DUBs but did not validate or characterise this interaction (Sowa *et al.*, 2009). Immunoprecipitation studies would be important for validating that this interaction is genuine. Preliminary data showed that USP12 depletion in LNCaP cells resulted in a decrease in Akt phosphorylation at serine 473, which would theoretically result in a down-regulation of Akt pro-proliferative activity. This reduction in phospho-Akt could result from a potential increase in the activity of PHLPP. If USP12 counteracts PHLPP-mediated de-phosphorylation of Akt, the knockdown of PHLPP in combination with USP12 may result in a rescue of the phosphorylation levels of Akt and the phenotypic effects observed with USP12

depletion alone. As Akt-mediated phosphorylation of both AR and MDM2 have been demonstrated to cooperate in AR down-regulation and degradation (Lin *et al.*, 2002b), USP12 may have an additional role in this pathway. Figure 7.1 illustrates the known dynamics between the above mentioned proteins and the AR and highlights where USP12 may fit into this model (Figure 7.1, red lines).

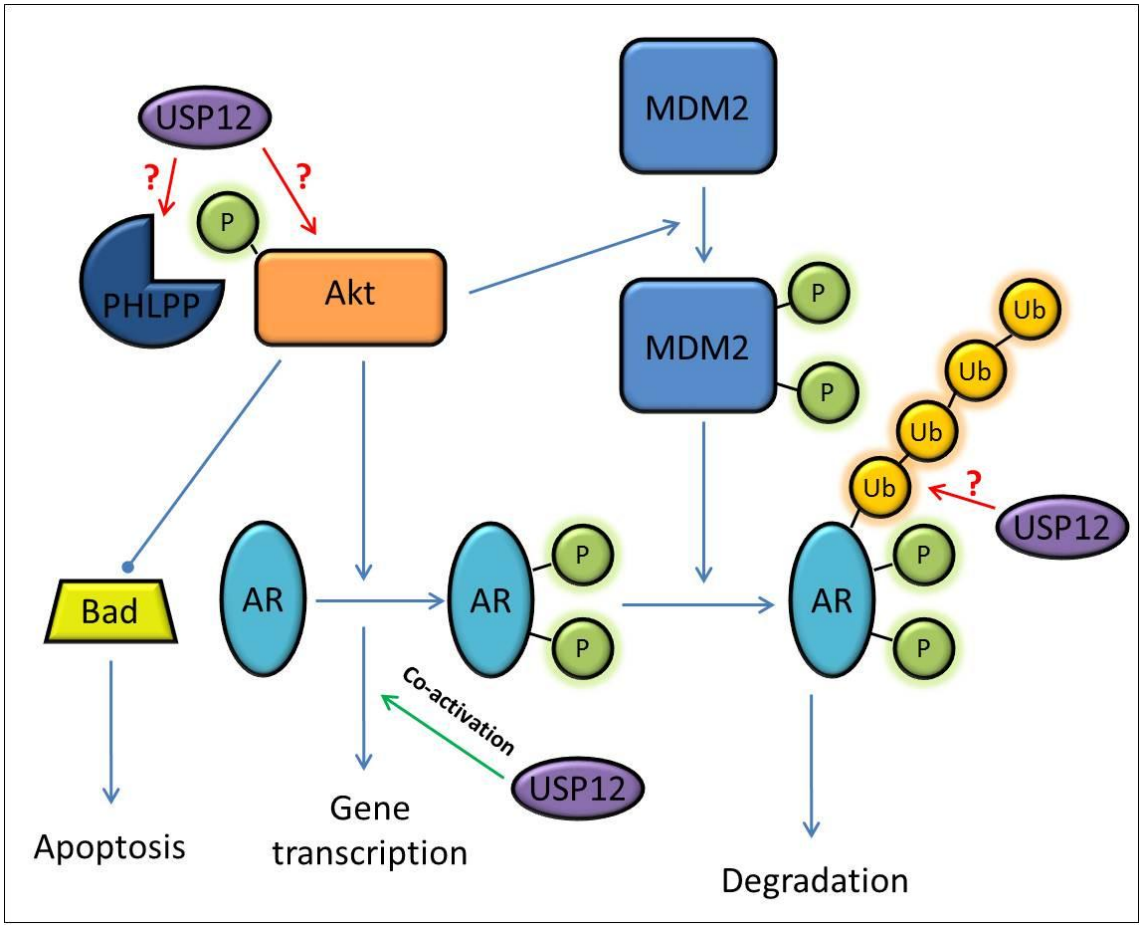

Figure 7.1. The potential role of USP12 in the AR signalling cascade.

USP12 co-activates AR transcriptional activity but may also have a role in direct deubiquitination of AR and regulating the activity of PHLPP towards Akt.

7.2 USP10 as an AR co-repressor

USP10 was identified as a regulator of AR activity in the same siRNA library screen described above. Unlike, *USP12* however, USP10 depletion increased the expression and protein levels of PSA in LNCaP cells. Knockdown of *USP10* had a similar effect on the expression of *KLK2*, *TMPRSS2* and *NDRG1* but had little effect on *NKX3.1*. *USP10* knockdown had no effect on AR protein levels as demonstrated by Western blotting and did not appear to be androgen regulated.

USP10 over-expression in ARE3 luciferase reporter assays in three cell lines, COS-7, PC-3 and U2OS, had a repressive effect on AR activity, suggesting that USP10 is a co-repressor of AR transcriptional activity. These data are contrary to the published literature implicating USP10 in AR signalling, where USP10 is reported to increase AR activity (Faus *et al.*, 2005; Draker *et al.*, 2011). However, use of stably transfected AR-null cell lines, different reporter constructs and insufficient knockdown may account for these discrepancies.

PC-3 cells are AR-null and although they have been shown to respond to androgens upon reintroduction of AR, they are known not to behave identically to AR-positive prostate cancer cell lines. Therefore, stable over-expression of AR in PC-3 cells is not a valid model for the role of USP10 in AR signalling. Further to this, USP10 and AR have been shown to be interacting partners in PC-3/AR cells (Faus *et al.*, 2005), which could not be confirmed in the endogenously expressing LNCaP cell line in this study, suggesting the interaction may be either cell type-dependent or an artefact of over-expression.

USP10 was recently observed to counteract MDM2-mediated ubiquitination of p53 in unstressed cells and in response to DNA damage (Yuan *et al.*, 2010). Given that MDM2 is an E3 ligase that down-regulates AR activity and promotes degradation it was hypothesised that in the context of AR signalling MDM2 and USP10 may cooperate in repressing AR activity. Verification of the repressive nature of MDM2 towards AR in luciferase-based reporter assays was initially problematic; increasing levels of MDM2 had no effect on AR activity in COS-7 cells with the ARE3 luciferase reporter and the pPSA-luc reporter used in the Gaughan *et al*., study showed no androgenic stimulation of AR activity. Once the effect of MDM2 was confirmed in U2OS cells with the ARE3 reporter, an experiment to test the above hypothesis was performed. Unfortunately,

due to time constraints only one experimental repeat was completed and in this experiment USP10 did not have the previously observed repression of AR activity. However, with further optimisation and repeats, this experiment could provide the preliminary evidence to support or disprove the hypothesis and warrant further investigation.

In vitro ubiquitination and deubiquitination experiments will allow for further investigation of whether USP10 and MDM2 cooperate to lead to the K48-linked poly-ubiquitination of AR. Initial *in vitro* ubiquitination assays testing the activity of MDM2 towards the AR-DBD-Hinge-LBD indicated that mono-ubiquitination of the C-terminal of AR can be catalysed by MDM2 (Figure 6.15b). Studies conducted with specific mutant ubiquitin molecules will identify which, if any, lysine-linked poly-ubiquitin chains are catalysed by MDM2 on the AR. Furthermore, *in vitro* deubiquitination assays testing the enzymatic activity of USP10 showed a preferential disassembly of K63-linked penta-ubiquitin chains (Figure 6.18). This data may suggest that USP10 removes transcriptionally activating ubiquitination events from AR. One hypothesis may be that MDM2-mediated ubiquitination and degradation of AR may require the removal of K63-linked poly-ubiquitin chains from the AR by USP10. Figure 7.2 illustrates the hypothesis that USP10 may counteract MDM2-mediated auto-ubiquitination leading to its stabilisation and therefore increased ability to act upon AR and repress its activity.

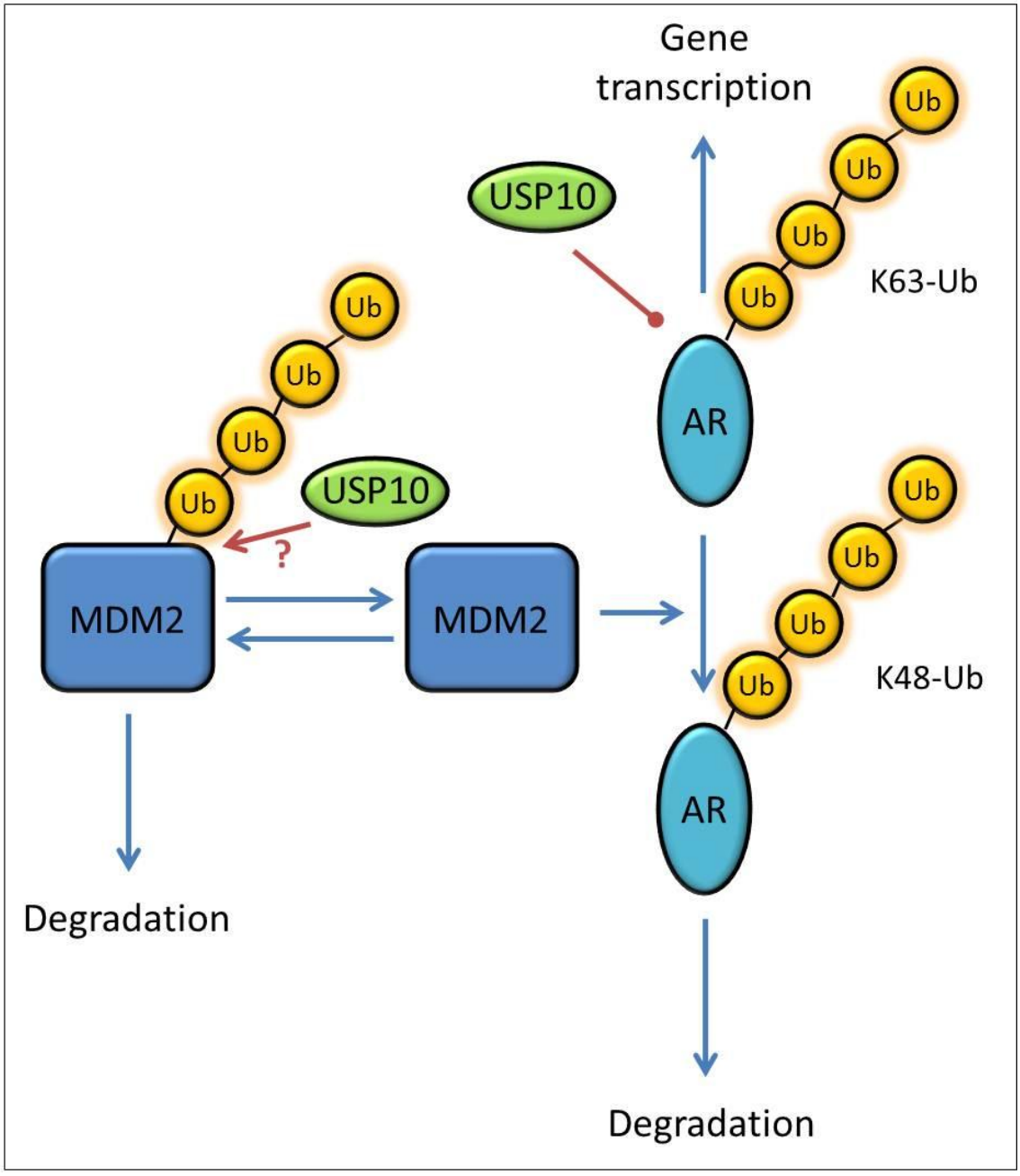

Figure 7.2. The potential role of USP10 in the AR signalling cascade.

USP10 has a repressive effect on AR transcriptional activity and may counteract MDM2 auto-ubiquitination and degradation to exert this effect.

Given that the investigation into the interplay between USP10 and MDM2 and AR signalling are incomplete and that no direct interaction between USP10 and AR in LNCaP cells was observed, a large knowledge gap still remains and interpretation of the results is made more difficult by the discrepancies between the current study and the two previous studies of USP10 and AR (Faus *et al.*, 2005; Draker *et al.*, 2011). To this end, investigation of both AR and MDM2 ubiquitination status in the presence and absence of USP10 may provide an insight into whether USP10 is stabilising MDM2, allowing potentiation of its activity towards AR, or whether USP10 is an AR-specific DUB removing activating ubiquitin chains mediated by another E3 ubiquitin ligase. Furthermore, USP10, like USP7, may have altered affinities towards its substrates under different cellular conditions. USP7 preferentially deubiquitinates MDM2, allowing increased degradation of p53, in unstressed cells whereas under stress p53 is the preferred substrate for USP7 (Vucic *et al.*, 2011).

7.3 Conclusion

In conclusion, USP12 and USP10 were identified by the Solid Tumour Target Discovery Group, Newcastle University, as DUB enzymes that could modulate AR-mediated transcription. USP12 acts as a transcriptional activator of AR which also appears to protect AR-positive prostate cancer cells from apoptosis driving proliferation. Further studies into whether this effect is directly linked with AR activity may provide therapeutic potential of USP12 inhibitors in prostate cancer. On the other hand, USP10 was found to be a co-repressor of AR activity for the majority of androgen-regulated genes studied with the exception of *NKX3.1*. This effect is potentially through mediation of MDM2 activity, although this DUB requires much more extensive investigation in both AR ubiquitination and MDM2 regulation.

Chapter 8: Appendix

8.1 Expression vectors maps

8.1.1 Generation of p3xFLAG-CMV-10™-USP12 and p3xFLAG-CMV-10™-USP12$_{C48A}$ mammalian expression vectors

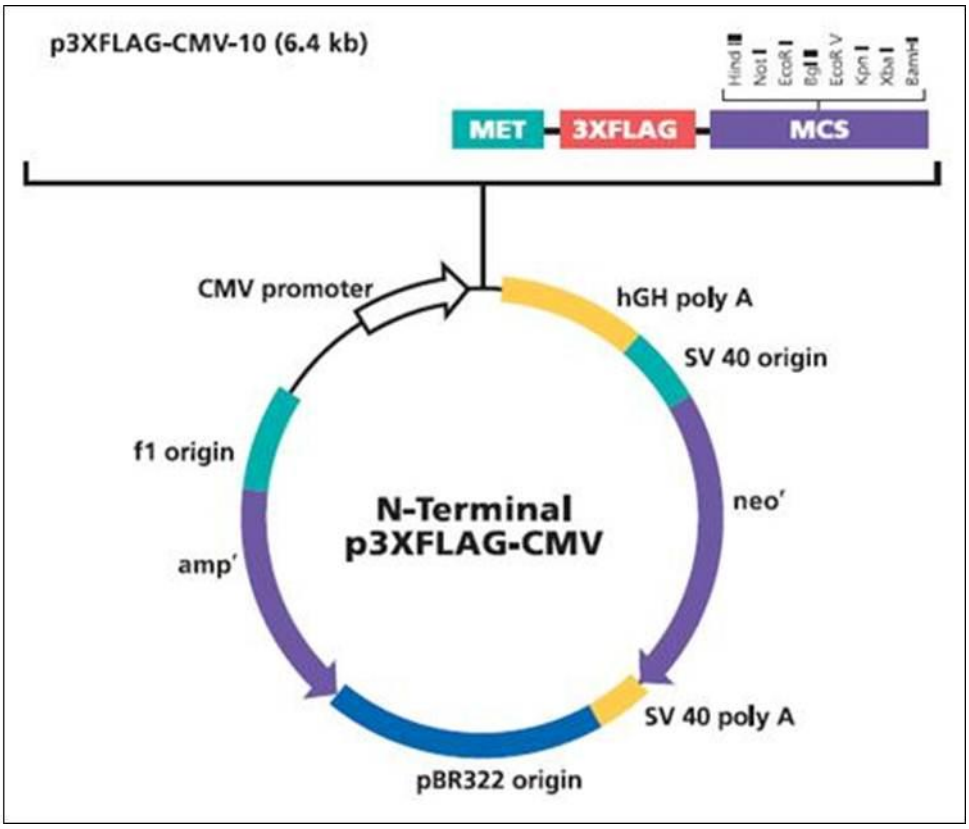

Figure 8.1. Restriction map of the p3xFLAG-CMV-10™ vector.

A schematic representation of the features of the p3xFLAG-CMV-10™ vector including the position of the 3xFLAG tag. Taken from (Sigma-Aldrich., 2012).

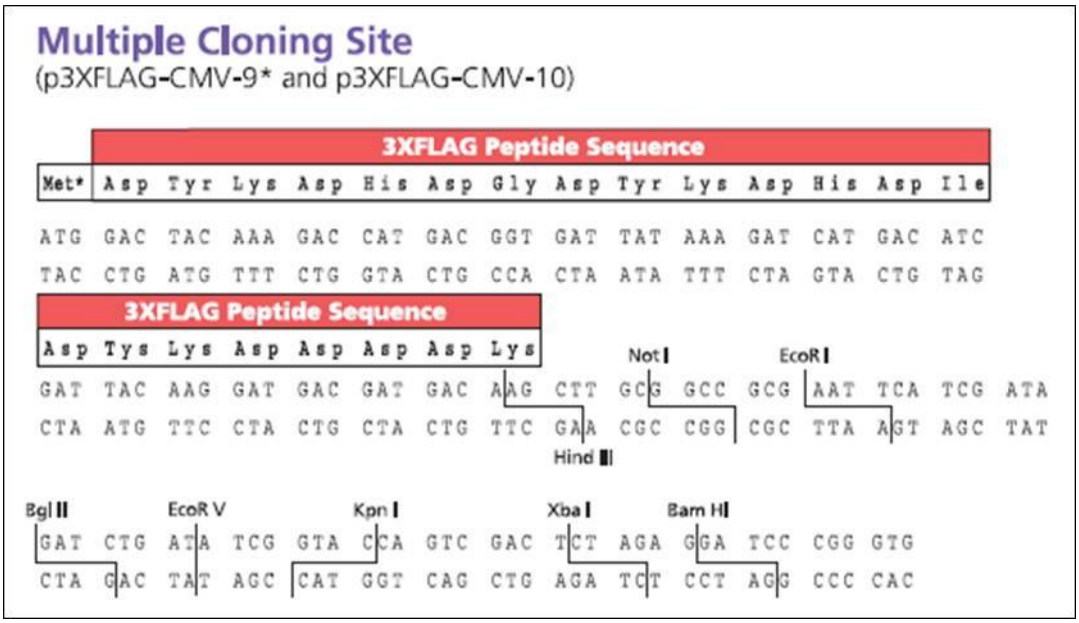

Figure 8.2. Sequence of the p3xFLAG-CMV-10™ vector multiple cloning site.

Sequence of the 3xFLAG tag and multiple cloning site of the p3xFLAG-CMV-10™ vector. Restriction enzyme recognition sites are indicated. Taken from(Sigma-Aldrich., 2012).

Figure 8.3. Sequencing of p3xFLAG-CMV-10™-USP12 expression vector.

USP12 was amplified by PCR from the pDEST-FLAG-HA-USP12 vector and ligated into the pCR2.1 vector. Positive transformants were digested with Xba I and Bam HI to release the USP12 sequence which was subsequently ligated into the p3xFLAG-CMV-10™ vector via Xba I and Bam HI restriction sites. Sequencing of p3xFLAG-CMV-10™-USP12 was performed by Cogenics (UK) using the CMV forward sequencing primer. The 3xFLAG tag, vector sequence and USP12 insert are indicated by green, black and blue text respectively. The underlined region represents the initial PCR primer site used to introduce the Xba I restriction enzyme site. Restriction enzyme recognition sites are indicated.

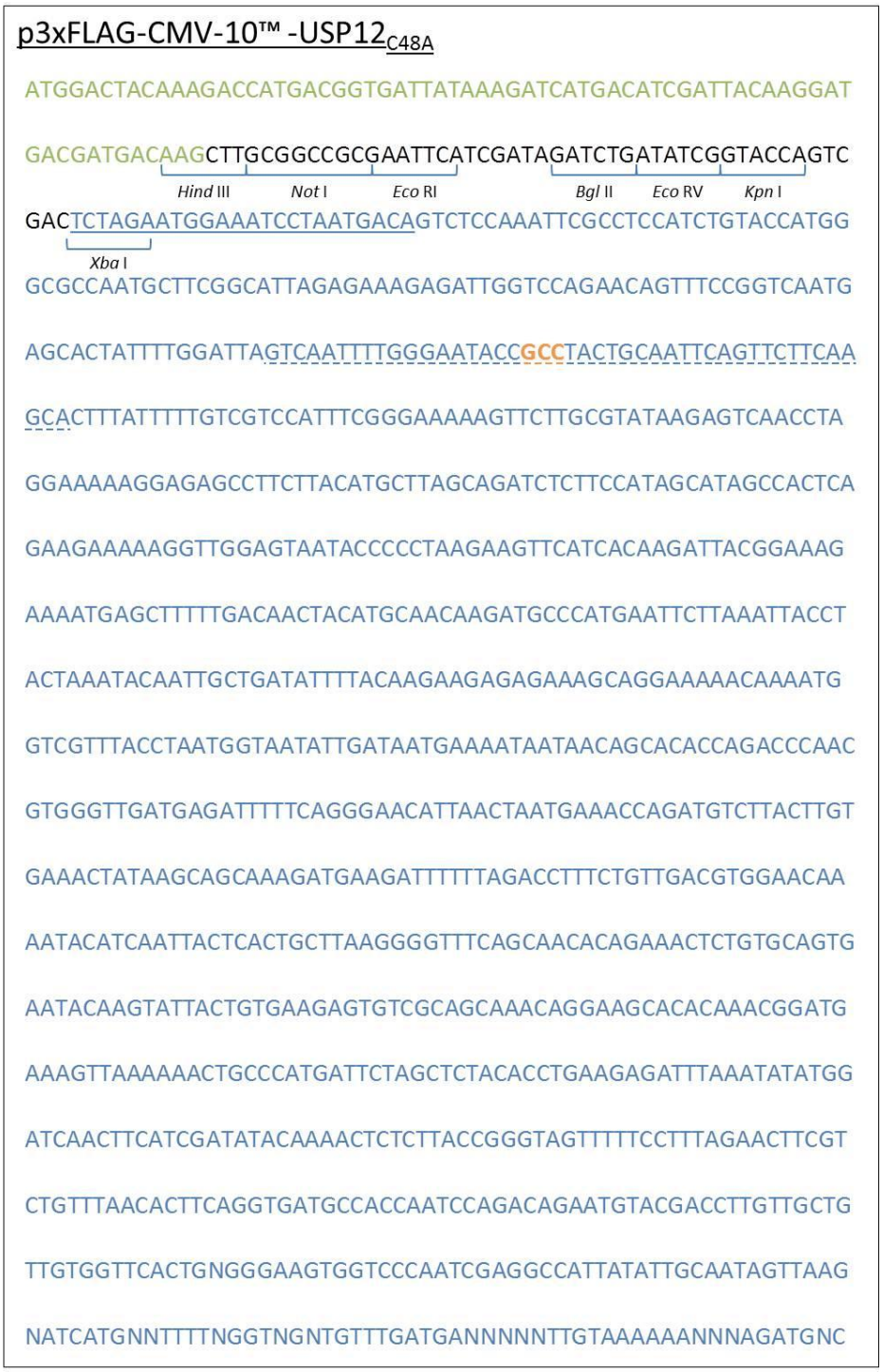

Figure 8.4. Sequencing of the p3xFLAG-CMV-10™-USP12$_{C48A}$ expression vector.

USP12C48A was generated by site-directed mutagenesis of p3xFLAG-CMV-10™-USP12. Sequencing of p3xFLAG-CMV-10™-USP12C48A was performed by Cogenics (UK) using the CMV forward sequencing primer. The 3xFLAG tag, vector sequence and USP12 insert are indicated by green, black and blue text respectively. The region underlined with a dashed line is the area of annealing of the site-directed mutagenesis primers. The mutated region is indicated by orange text; cysteine (TGC) at position 48 has been mutated to alanine (GCC). Restriction enzyme recognition sites are indicated.

8.1.2 Generation of the pGEX-6P-1-AR C-terminal domain bacterial expression vectors

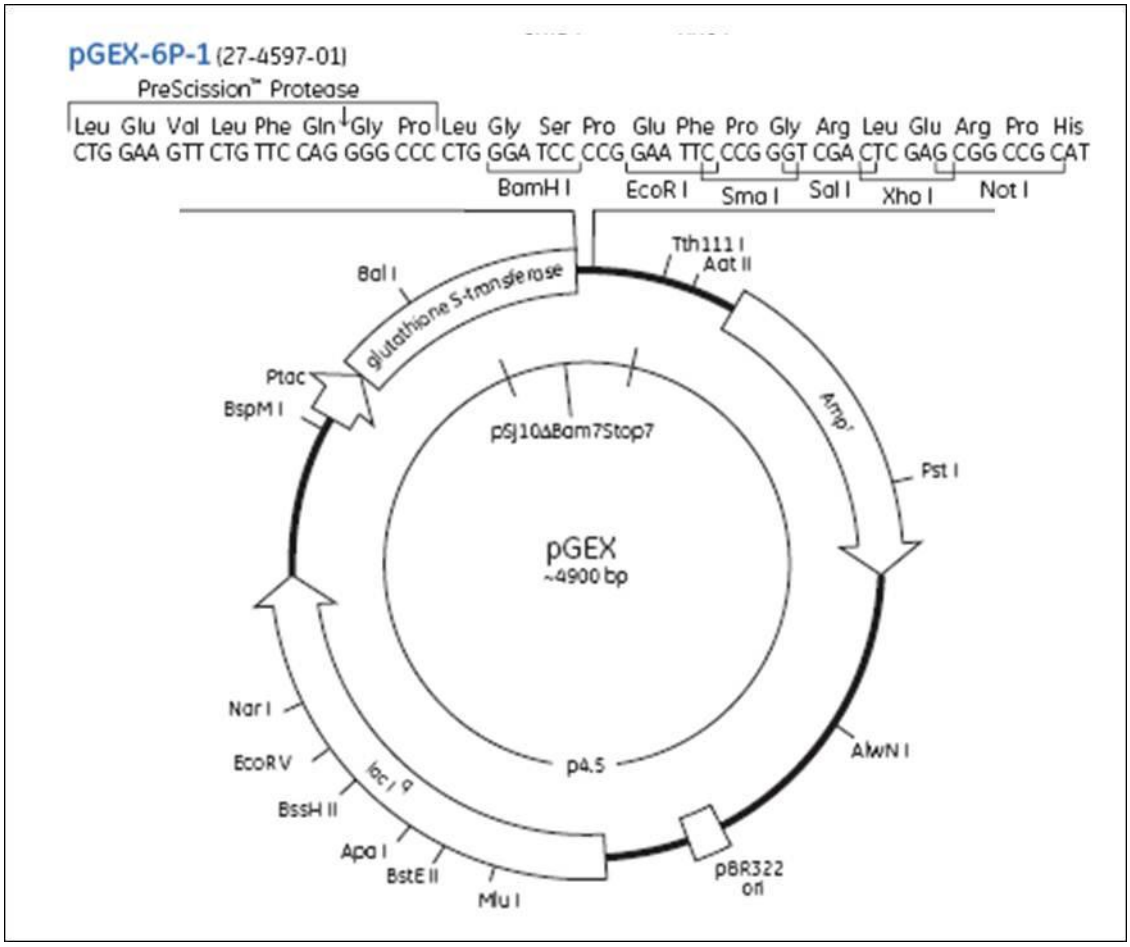

Figure 8.5. Restriction map of the pGEX-6P-1 expression vector

A schematic representation of the features of the pGEX-6P-1 vector including the position of the glutathione-S-transferase (GST) tag and sequence of the multiple cloning site. Restriction enzyme recognition sites are indicated. Taken from(GE healthcare., 2012).

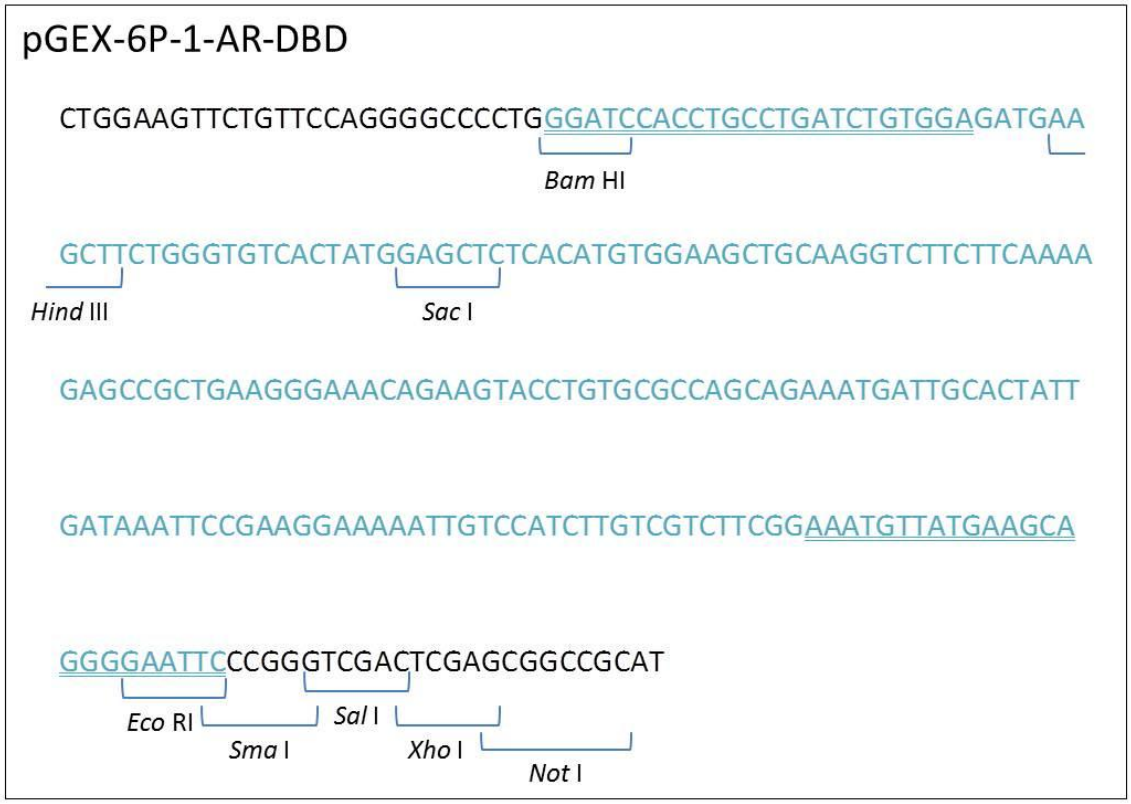

Figure 8.6. Sequence of the pGEX-6P-1-AR DBD expression vector

AR DNA-binding domain (DBD) was amplified by PCR from the pYFP-AR vector and ligated into the pCR2.1 vector. Positive transformants were digested with Bam HI and Eco RI to release the AR-DBD sequence which was subsequently ligated into the pGEX-6P-1 vector via Bam HI and Eco RI restriction sites. Sequencing of pGEX-6P-1-AR-DBD was performed by Cogenics (UK) using the pGEX forward sequencing primer. The vector sequence is indicated by black text. AR-DBD (indicated by blue text) was found to have no alterations compared to with the known AR sequence. Underlined regions represent the initial PCR primer sites used to introduce the Bam HI and Eco R1 restriction enzyme sites. Restriction enzyme recognition sites are indicated.

Figure 8.7. Sequence of the pGEX-6P-1-AR DBD-Hinge expression vector.

AR DNA-binding domain (DBD) and Hinge were amplified by PCR from the pYFP-AR vector and ligated into the pCR2.1 vector. Positive transformants were digested with Bam HI and Eco RI to release the AR-DBD-Hinge sequence which was subsequently ligated into the pGEX-6P-1 vector via Bam HI and Eco RI restriction sites. Sequencing of pGEX-6P-1-AR-DBD-Hinge was performed by Cogenics (UK) using the pGEX forward sequencing primer. The vector sequence is indicated by black text. AR-DBD and Hinge (indicated by blue and green text respectively) were found to have no alterations compared to with the known AR sequence. Underlined regions represent the initial PCR primer sites used to introduce the Bam HI and Eco R1 restriction enzyme sites. Restriction enzyme recognition sites are indicated

Figure 8.8. Sequence of the pGEX-6P-1-AR DBD-Hinge-LBD expression vector.

AR DNA-binding domain (DBD), Hinge and ligand-binding domain (LBD) were amplified by PCR from the pYFP-AR vector and ligated into the pCR2.1 vector. Positive transformants were digested with Bam HI and Xho I to release the AR-DBD-Hinge-LBD sequence which was subsequently ligated into the pGEX-6P-1 vector via Bam HI and Eco RI restriction sites. Sequencing of pGEX-6P-1-AR-DBD-Hinge-LBD was performed by Cogenics (UK) using the pGEX forward sequencing primer. The vector sequence is indicated by black text. AR-DBD, Hinge and LBD regions (indicated by blue, green and orange text respectively) were found to have no alterations compared to with the known AR sequence. Underlined regions represent the initial PCR primer sites used to introduce the Bam HI and Eco R1 restriction enzyme sites. Restriction enzyme recognition sites are indicated.

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