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Functional Characterisation of Phosphodiesterase 4D7 in Prostate Cancer

A Thesis Presented by
Ashleigh Maria Byrne

To the



In accordance with the requirements for the degree of

Doctor of Philosophy

In

Integrated Biology

Institute of Cardiovascular and Medical Sciences
College of Medical, Veterinary and Life Sciences

2014

CERTAVI ET VICI

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Abstract

3',5'-cyclic adenosine monophosphate (cAMP) is the best studied intracellular second messenger. Adenylyl cyclase (AC) catalyses the synthesis of cAMP from ATP following the stimulation of a G protein coupled receptor (GPCR), and its degradation is catalysed by cAMP phosphodiesterases (PDEs) to allow cessation of signal. cAMP can act to bring about a multitude of varying and often opposing cellular responses, which depend on the stimulus received by the GPCR, the cell type, the cell cycle stage, and the complement of downstream effector molecules within that cell. The cAMP PDE subfamilies express multiple splice variants, which possess unique N-termini and non-redundant functional roles. By virtue of this, they are targeted to specific and discrete subcellular locations, where they may form highly specific interactions with scaffold proteins and other enzymes. Here, in these discrete locales, PDEs act to hydrolyse local cAMP, thereby underpinning the spatial and temporal compartmentalisation of cAMP gradients. This fine-tuned balance of synthesis and degradation is paramount for the dynamic cellular responses to extracellular stimuli, allowing differing signal transduction cascades to occur simultaneously in the crowded macromolecular environment of the cell. The compartmentalisation of cAMP signalling is, thus, essential for maintaining cellular homeostasis, and is subject to perturbation in various diseases, including prostate cancer (PC).

Despite the wealth of literature implicating cAMP signalling in the progression of PC, little work has been done on the expression or function of PDE splice variant in this disease. Our group, in collaboration with Philips Research and the Prostate Cancer and Molecular Medicine (PCMM) group in the Netherlands, set out to investigate the changes in cAMP signalling during PC progression by studying the expression of cAMP PDE isoforms, with the aim of identifying a novel PC biomarker, as the current standard biomarker (PSA) is not disease-specific and leads to much over-diagnosis and over-treatment of otherwise non-life threatening prostate tumours. Interestingly, we found PDE4D7 to be dramatically downregulated as PC progresses from an androgen sensitive (AS) to an androgen insensitive (AI) state, and, indeed, this enzyme is showing promise as a novel, disease-specific PC biomarker.

In this thesis, I report my efforts to characterise a function of PDE4D7 within prostate cancer. Firstly, I report the raising of a novel highly specific PDE4D7 antibody and describe the differential expression of this isoform, at the protein level, between AS and AI PC cell models. I present evidence to suggest that PDE4D7 mediates PC cell growth and migration, and that its loss may play a role in PC progression. I propose that an altered epigenome plays a role in the downregulation of PDE4D7 expression. I then report on the raising of a novel phospho-specific antibody and present evidence to show that PDE4D7 is regulated by PKA phosphorylation within its unique N-terminal region, and that this event confers negative regulation on enzyme activity. Finally, I describe my endeavours to elucidate a PDE4D7 protein-protein interaction that may help transduce PDE4D7-specific signals and maintain the enzymes cellular location.

Author's Declaration

I hereby declare that the work presented in this thesis has been carried out by me unless otherwise cited or acknowledged. The work is entirely of my own composition and has not been submitted, in whole or in part, for any other degree at the University of Glasgow or any other institution.

Ashleigh Maria Byrne

January 2014

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Abbreviations

AC	Adenylyl cyclase
ADT	Androgen deprivation therapy
AKAP	A-kinase anchoring protein
AI	Androgen Insensitive/Independent
AMP	Adenosine monophosphate
ARE	Androgen Response Element
AR	Androgen receptor
AS	Androgen Sensitive
ATP	Adenosine Triphosphate
BAR	β -adrenergic receptor
BPH	Benign prostatic hyperplasia
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
cGMP	Cyclic Guanosine monophosphate
cis-NAT	cis-Natural Antisense Transcript
CNG	Cyclic Nucleotide gated
CRE	cAMP response element
CREB	cAMP response element binding (protein)
CRPC	castrate resistant prostate cancer
dn	dominant negative
dNTP	deoxy nucleotide tri phosphates
DISC1	Disrupted in Schizophrenia 1
DHT	Dihydrotestosterone / 17 β -diol-glucuronide
DMEM	Dulbecco's modified eagle's medium
DMSO	Di methyl Sulfoxide
DNA-PK	DNA damage activated protein kinase
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Diamino ethane tetra acetic acid
EGTA	Ethyelene Glycol tetra acetic acid
EPAC	Exchange protein for activated cAMP
ERK	Extracellular-signal Regulated Kinase

FAK Focal Adhesion Kinase
FBS foetal bovine serum
FRET Fluorescence Resonance Energy Transfer
GAF GTPase activating factor
GC Guanylyl cyclase
GDP Guanosine di phosphate
GEF GTP exchange factor
GOI Gene of Interest
GPCR G-protein coupled receptor
GST Glutathione-s-transferase
HARBS High affinity rolipram binding site
HEK human embryonic kidney
LARBS Low affinity rolipram binding site
LB Luria-Bertini
LH Leutinising Hormone
LPS Lipopolysaccharide
LUTS Lower urinary tract symptoms
IP Immunoprecipitation
MAPK Mitogen activated protein kinase
MK2 MAPKAPK2
mRNA messenger ribonucleic acid
ncRNA non-coding ribonucleic acid
NO Nitric Oxide
PAGE Polyacrylamide gel electrophoresis
PC Prostate cancer
PDE Phosphodiesterase
PKA Protein Kinase-A
PKC Protein Kinase C
PLA Proximity ligation assay
PSA Prostate specific antigen (gene: KLK3)
RACK1 receptor of activated C-kinase
SDS Sodium-dodecyl-sulfate
siRNA small interfering ribonucleic acid
SH3 Src-homology 3
SUMO Small Ubiquitin like modifier

TAPAS1 domain tryptophan anchoring phosphatidic acid selective domain

TBE Tris Buffered EDTA

TBST Tris Bufferes Saline-Tween

TCR T-Cell receptor

TE Tris-EDTA

TNF Tumor necrosis factor

tRNA Total ribonucleic acid

UCR Upstream conserved region

VSMC Vascular Smooth Muscle Cell

WT wild-type

Publications

Ashleigh M. Bryne*, David J P Henderson*, Kalyan Dulla, Guido Jenster, Ralf Hoffmann, George S Baillie, Miles D Houslay. The cAMP phosphodiesterase 4D7 is down-regulated in androgen-independent prostate cancer and mediates proliferation by compartmentalizing cAMP at the plasma membrane of VCaP prostate cancer cell. 2014 Br J Cancer 4;110(5):1278-87.

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Apostolos Zarros, Ashleigh-Maria Byrne, Stephanie D. Boomkamp, Stylianos Tsakiris, George S. Baillie. Lanthanum-induced neurotoxicity: solving the riddle of its involvement in cognitive impairment? 2013 Archives of Toxicology, 87 (11) 2031-2035.

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Ashleigh M. Byrne and George S. Baillie. PDE4D7 Activity is Regulated by Phosphorylation Within its Unique N-Terminal Region.

Abstracts and Posters

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Prostate Cancer UK conference 2011.

1 cAMP Signalling, Phosphodiesterases and Prostate Cancer

Signal transduction is essential for the survival of multi-cellular organisms as it enables them to adapt to their environment, and cells to adapt to microenvironment changes. Signal transduction may involve simple diffusion of molecules across the plasma membrane, such as class 1 steroid hormones, which bind intracellular receptors to bring about transcriptional regulation. However, the majority of signalling molecules cannot diffuse across the plasma membrane, and instead must bind cell surface receptors to have their effect. As a result, such molecules are called “first messengers”. Once activated, these cell surface receptors elicit a downstream response to bring about the desired physiological effect on the cell. Eliciting specific cellular responses is an amazing feat given the abundance of first messengers that may bombard the cell at once, and the abundance of receptors expressed on a cell at any one time. This specificity relies on the ability of receptors to employ distinct mechanisms of ligand recognition and compartmentalised second messenger activation.

Cyclic adenosine monophosphate (cAMP) was the first second messenger to be identified, and has remained the best studied. It was discovered by Earl Wilbur Sutherland and colleagues while working on glycogen metabolism in rat liver homogenates (Robison, Butcher et al. 1968). Since then, cAMP has been found to mediate a vast array of cellular activities, which depend on the cell type studied, the receptor activated, adenylyl cyclases expressed and cAMP effector proteins available in the specific cell type. cAMP regulates many cellular events such as cell differentiation, proliferation, apoptosis and learning and memory (Shaulsky, Fuller et al. 1998; Richards 2001; Hochbaum, Hong et al. 2008; Rutten, Wallace et al. 2011; Insel, Zhang et al. 2012). The other related second messenger cyclic guanine monophosphate (cGMP), was later discovered and was initially believed to antagonise the cAMP pathway, but it is now known there is much cross talk between both networks and their relationship is complex (Weissmann, Goldstein et al. 1975; Zaccolo and Movsesian 2007; Levy 2013). The levels of intracellular cAMP and cGMP are tightly regulated by their synthesis by

adenylyl and guanylyl cyclases respectively, and their degradation by phosphodiesterases (PDEs). The balance of synthesis and degradation of the second messengers is stringently controlled. cAMP PDEs are targeted to discrete subcellular localisations by their unique N-termini, where they interact with various proteins such as cAMP effector molecules. Within their location, PDEs mould cAMP gradients and allow differential activation of subsets of cAMP effectors. This compartmentalisation of cAMP signalling is essential for eliciting pleiotropic cellular effects, and is paramount to the homeostasis of the tissue (Houslay 2010; Francis, Blount et al. 2011). Compartmentalisation of cyclic nucleotide gradients allows multiple signalling transduction events to occur simultaneously, and crucially underpins the specificity of receptor action (as many receptors use cAMP to trigger intracellular responses). Another point worth noting is that cell signalling is not linear, but transduction pathways often cross talk with each other in various directions to bring about the desired cellular effect (Bhalla and Iyengar 1999). A Schematic showing an example of cAMP signalling is shown in figure 1.1.

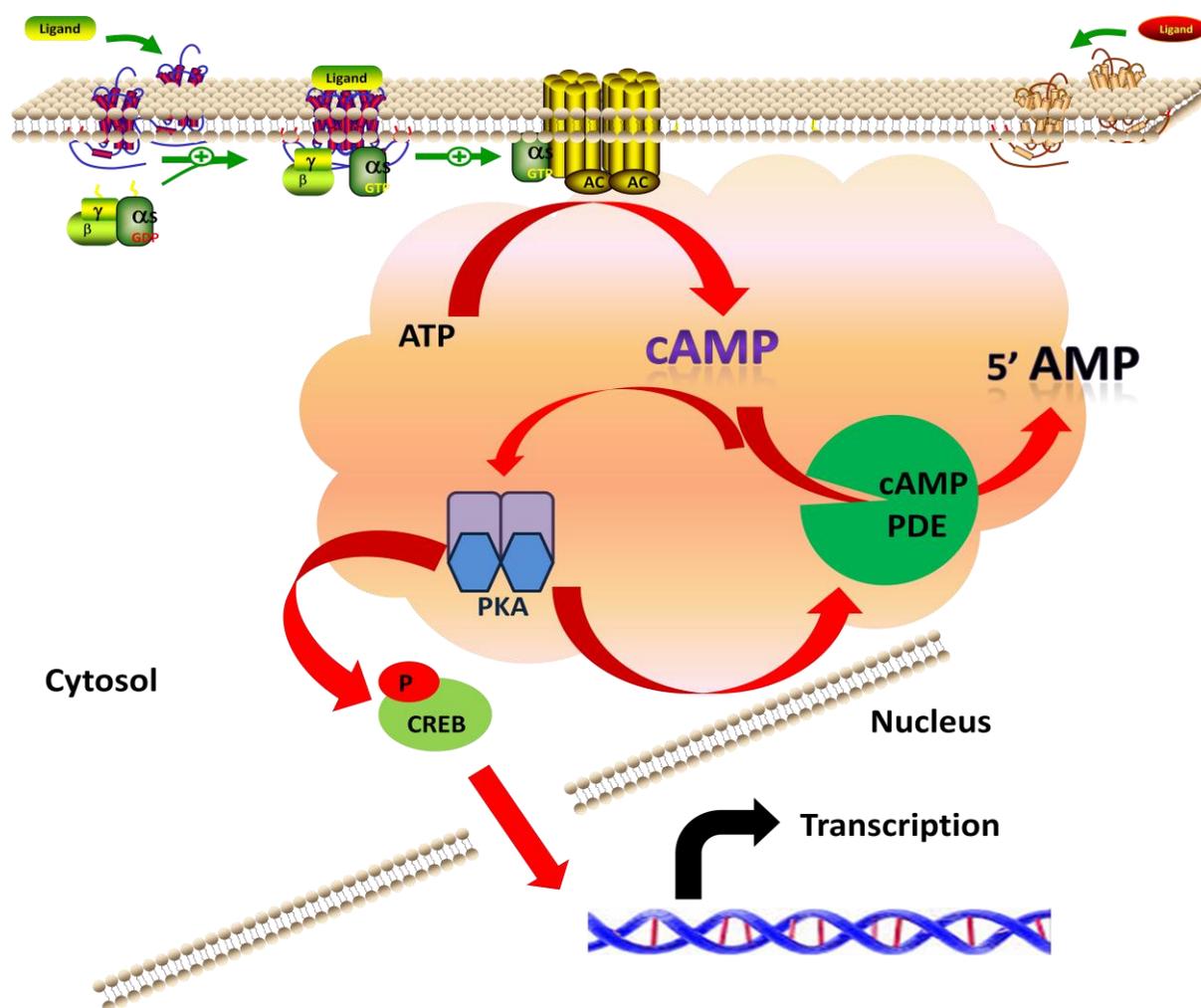


Figure 1.1. An example cAMP signalling pathway.

Upregulation of target gene transcription by phospho-CREB is an example of classical Gs-coupled GPCR activation of cAMP signalling, which is described in more detail below. Briefly; ligand binding to a GPCR brings about synthesis of cAMP from ATP and adenylyl cyclases (AC). cAMP then activates local downstream effector proteins, such as PKA, which phosphorylates target substrates such as CREB, and long form cAMP PDEs. Activated cAMP PDEs then hydrolyse cAMP to 5'AMP to attenuate the signal, thereby sculpting and compartmentalising the cAMP pool.

Extracellular signals modulate intracellular cAMP via at least three components; a heptahelical G protein coupled receptor (GPCR), a heterotrimeric G protein, and adenylyl cyclase.

1.1 G protein Coupled Receptors and Heterotrimeric G proteins.

The G protein Coupled Receptor (GPCR) superfamily and heterotrimeric guanine nucleotide exchange proteins (G proteins) play pivotal roles in signal transduction. They are activated by various external stimuli such as hormones, peptides, nucleotides, Ca^{2+} , ions, lipids, amines, photons and organic odourants (Bockaert and Pin 1999; Fredriksson and Schiöth 2005). GPCR activation allows the receptor to couple to heterotrimeric G proteins which then act on effectors in a cyclical activation-inactivation mechanism. Via ligand binding or constitutive activation, GPCRs can undergo a conformational change that alters their affinity for the G protein, which in turn results in signal transduction to downstream effectors such as adenylyl cyclases (ACs) (Kleuss, Raw et al. 1994). G proteins are composed of 3 subunits, α , β and γ . At the unbound GPCR, $\text{G}\alpha$ is GDP bound and associated with the β/γ subunits. Once the GPCR is activated, it more efficiently binds the G protein, and, acting as a guanine exchange factor (GEF), causes $\text{G}\alpha$ to swap GDP for GTP, resulting in its activation and dissociation from the β/γ subunits. The free $\text{G}\alpha$ -GTP and/or the β/γ complex can then interact with effector molecules. The β and γ subunits function only as a tightly bound complex in which the β subunit binds the effector protein, thereby directly modulating the downstream effect (Downes and Gautam 1999; Milligan and Kostenis 2006; Oldham and Hamm 2008). However, one study shows that the γ subunit may also be involved in effector binding (Bell, Xing et al. 1999). Following effector stimulation, $\text{G}\alpha$ acts as an intrinsic GTPase and initiates its own inactivation through nucleophilic attack on GTP, reverting to the GDP bound G protein, a reaction which in some cases is accelerated by GTPase activating proteins such as the regulators of G protein signalling (RGS) proteins. The GDP bound $\text{G}\alpha$ then re-associates with the β/γ dimer bringing

about cessation of signal from the GPCR (Kleuss, Raw et al. 1994; Milligan and Kostenis 2006). Agonist stimulation of the GPCR is diminished also by receptor desensitisation due to phosphorylation by kinases such as PKA (Ferguson, Zhang et al. 1998; Kohout and Lefkowitz 2003). The mechanism of $G\alpha$ dissociating from the β/γ complex is well described, but doubt has been cast as to whether it occurs *in vivo* as FRET studies have suggested that the subunits actually undergo a rearrangement and remain associated (Bunemann, Frank et al. 2003). The crystal structure of activated $G\alpha$ was used to elucidate the arginine that is essential for mechanism of action, mutation of which leads to a constitutively active protein (Noel, Hamm et al. 1993). Although rare, such a mutation has been documented to be involved in pituitary adenomas, where constitutive cAMP synthesis is pro-proliferative (Landis, Masters et al. 1989).

Humans express 21 $G\alpha$, 16 $G\beta$ and 12 $G\gamma$ subunits from 16, 5, and 12 genes respectively (Downes and Gautam 1999). If each $G\beta$ subunit could dimerise with each $G\gamma$ subunit, there would be over 60 different possible dimers (Cabrera-Vera, Vanhauwe et al. 2003). However, it is more likely that only certain β/γ dimers function within tissues, for example $G\beta_1\gamma_1$ is the predominant dimer found in the retina (Fawzi, Fay et al. 1991). The $G\alpha$ subunit confers the basic properties of a G protein, and can be one of four families based on sequence similarity; $G\alpha_s$, $G\alpha_i$ / $G\alpha_o$, $G\alpha_q$ / $G\alpha_{11}$ and $G\alpha_{12}$ / $G\alpha_{13}$. Each family contains a number of isoforms and may be ubiquitously or specifically expressed. G proteins containing $G\alpha_s$ couple many GPCRs to ACs to bring about increases in intracellular cAMP (Wettschureck and Offermanns 2005).

G proteins are subject to post-translational modifications that affect their association with various GPCRs and effectors, and the G protein subunits themselves (Chen and Manning 2001). GPCRs are the most common membrane bound protein receptor families, accounting for >1% of the vertebrate genome, and represent one the largest and most diverse protein superfamilies in mammals. They are encoded by ~800 different genes in humans (Bockaert and Pin 1999). Human GPCRs can be grouped into 5 different families based on phylogenetic analysis, called; glutamate, rhodopsin, adhesion, frizzled and secretin (Fredriksson, Lagerstrom et al. 2003). The rhodopsin family is the

largest, and much structural information on this GPCR family has been gained from the 2D crystal structures of frog and bovine rhodopsin (Unger, Hargrave et al. 1997). Common to all GPCRs are 7-transmembrane spanning α -helices (TMI-VII), connected by 3 intracellular and 3 extracellular loops that anchor the receptor within the plasma membrane. The end of the extracellular loop forms the N-termini, which contain functional and/or ligand binding domains, whereas the intracellular loops end with the C-termini. The N- and C-termini differ in sequence and length between different GPCRs (Fredriksson and Schiöth 2005). There has been much interest in GPCRs due to their implication in disease, e.g. germline loss of function mutations in rhodopsin cause retinitis pigmentosa, and most notably, cholera (Spiegel 1996). Endotoxins produced by *Vibrio cholerae* and other bacteria that target the $G\alpha$ subunit have been widely used in the characterisation of the function of G proteins (Milligan and Kostenis 2006).

GPCRs are some of the most pharmaceutically targeted proteins. From the ~ 800 genes encoding GPCRs, ~400 are considered to be potential drug targets. The cognate ligands are known for over 200 GPCRs, with many other receptors deemed 'orphan'; with no known function. Currently, ~30 GPCRs are drug targets, being targets of ~30% of all current marketed drugs. Targeted GPCRs include opioid receptors for pain, β 2-adrenoreceptors for asthma and angiotensin receptors for hypertension (Hopkins and Groom 2002; Wise, Gearing et al. 2002). Alfred Gilman and Martin Rodbell were awarded the 1994 Nobel Prize for Physiology or Medicine for their work on the structure and function of heterotrimeric G proteins (Gilman 1995; Rodbell 1995). In 2012, the Nobel Prize for Chemistry was awarded jointly to Robert J. Lefkowitz and Brian K. Kobilka for their work on the structure and function of GPCRs over the past few decades.

1.2 Adenylyl Cyclases

Adenylyl cyclases (ACs) catalyse the synthesis of cAMP from ATP following hormonal activation. They were first described in the 1970s (Neer 1974; Ishikawa and Homcy 1997), with the first AC isoform cloned from brain (Krupinski, Coussen et al. 1989; Tang, Krupinski et al. 1991). Originally all ACs were thought to be transmembrane bound (tmAC), and activated only following GPCR stimulation (Geng, Wang et al. 2005). To date, nine such mammalian tmAC isoforms have been described (ACI-ACIX), which share a membrane bound topological structure, but differ in expression pattern and mode of regulation by G protein subunits, forskolin, Ca^{2+} influx and phosphorylation by protein kinases (Iwami, Kawabe et al. 1995; Zimmermann and Taussig 1996; Dessauer, Scully et al. 1997; Tesmer, Sunahara et al. 1997; Willoughby and Cooper 2007). A class of soluble AC (sAC) enzymes were subsequently identified in rat testes (Buck, Sinclair et al. 1999), which is regulated by bicarbonate ions and pH (Chen, Cann et al. 2000).

ACs I, III and VIII are activated by Ca^{2+} influx via calmodulin binding, inhibited by $\text{G}\beta\gamma$, and insensitive to PKC regulation (Tang and Gilman 1991; Levin and Reed 1995; Willoughby and Cooper 2007). They are expressed in brain (I and III) and olfactory cells (VIII) (Ishikawa and Homcy 1997).

ACs II, IV and VII are $\text{G}\alpha_s$, $\text{G}\beta\gamma$ and PKC activated, $\text{G}\alpha_i$ inhibited, and Ca^{2+} insensitive. They are expressed in lung (II) and ubiquitously (IV and VII) (Levin and Reed 1995; Ishikawa and Homcy 1997; Cooper 2003).

ACs V and VI are $\text{G}\alpha_s$ activated and inhibited by Ca^{2+} , PKC and PKA. They are expressed mostly in heart and brain tissues (Ishikawa and Homcy 1997; Cooper 2003).

ACIX is the most inactive of the known isoforms. It is inactivated by Ca^{2+} signalling via the phosphatase calcineurin. It is notably expressed in the pituitary gland (Cooper 2003).

ACX, or sAC, is activated by bicarbonate ions under strict pH conditions. It was first identified in the testes and found to be involved in sperm activation following ejaculation, and was considered a target for male contraceptives. It was later found to be expressed in many other tissues including the brain and kidneys (Buck, Sinclair et al. 1999; Chen, Cann et al. 2000; Geng, Wang et al. 2005; Wang, Lin et al. 2009).

All mammalian ACs except IX are activated by the naturally occurring labdane diterpene, forskolin, produced by the plant *Coleus forskohlii*. Forskolin is widely used to study cAMP signalling. It directly activates ACs, without the need for GPCR stimulation, thereby increasing intracellular cAMP and thus PKA activity (Seamon, Vaillancourt et al. 1984; Hurley 1999; Onda, Hashimoto et al. 2001). Forskolin has been widely used in studying the structure and function of ACs (Tang and Hurley 1998). It has been suggested that there are endogenous forskolin-like small molecules that regulate ACs.

Regulation of the tmACs adds further layers to the complexity of cAMP signalling. Not only is their activity modulated by GPCR and calcium signalling, but they may be subject to post-translational modifications by protein kinases and phosphatases in signalling feedback loops. Their isoform-specific regulation, tissue expression and/or specific subcellular localisation also lend to cAMP signalling control. Given the various G protein families, the 9 tmACs with the regulation of each G protein and AC, the potential plethora of G protein/AC interactions further makes for convoluted multifaceted cAMP signalling. sACs suggest cAMP can be synthesised closer to intracellular downstream effectors, and not necessarily at the plasma membrane, thereby adding a spatial component to the regulation of cAMP signalling. It may be that tmACs and sACs cooperate in the same signalling pathway. ACs are targeted to and act in microdomains of the cell, in association with other signalling proteins such as PKA, AKAPs and PDEs. Specific AC interactions within microdomains facilitate compartmentalisation of cAMP signalling and underpin receptor specificity (Willoughby and Cooper 2007; Wang, Lin et al. 2009).

1.3 Guanylyl Cyclases

Guanylyl cyclases (GCs) catalyse the synthesis of cGMP from GTP in response to nitric oxide (NO), peptide hormones, free radicals, bacterial toxins and Ca^{2+} among other stimuli. The GC family also contains membrane bound and soluble isoforms that are widely expressed (Lucas, Pitari et al. 2000).

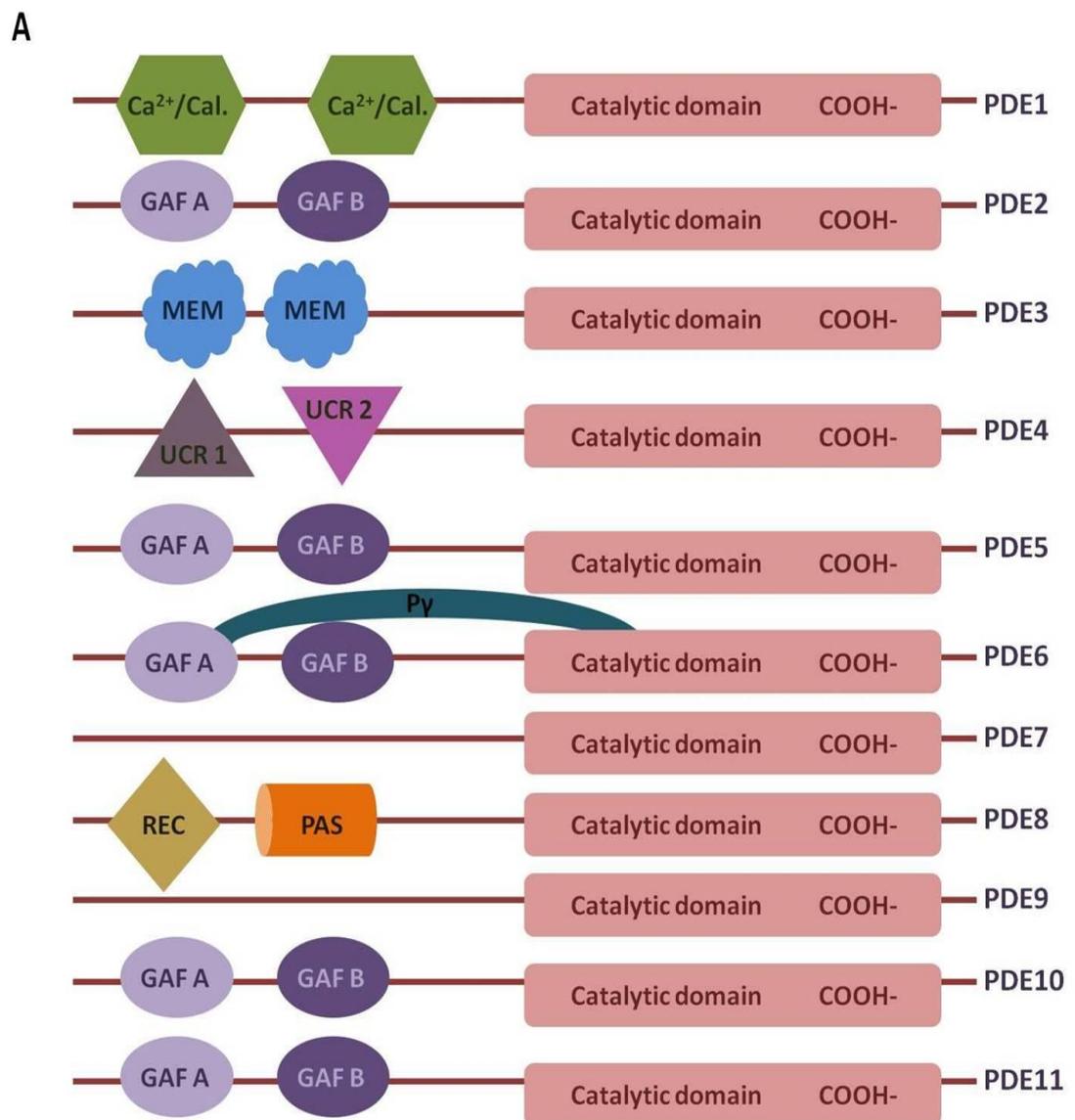
1.4 Phosphodiesterases

Phosphodiesterases (PDEs) are a large divergent superfamily of enzymes that act as the sole route for hydrolysis of the cyclic nucleotides, cAMP and cGMP, both of which function as major intracellular second messengers. They cleave the phosphodiester bond of the cyclic nucleotide to generate inactive 5'-nucleotide metabolites. Along with ACs, PDEs determine the intracellular concentrations of cyclic nucleotides and thus are pivotal in maintaining cAMP and cGMP homeostasis. PDEs are central to the preservation of compartmentalised cyclic nucleotide signalling. It is suggested that they may also alter protein-protein interactions by conformational change induced through allosteric binding to cyclic nucleotides (Bender and Beavo 2006). PDEs are themselves subject to post-translational modifications such as phosphorylation, SUMOylation and ubiquitination. PDEs were first described in early studies by Sutherland and colleagues who noted the formation of 5'AMP in tissue fractions, in a manner that was potentiated in the presence of divalent cations and inhibited by methylxanthines, such as caffeine. This activity was subsequently identified as that of a PDE (Rall and Sutherland 1958; Francis, Turko et al. 2001). PDEs have been studied for more than 50 years but still a great deal is unknown due to the complexity of this superfamily. However, in recent years a lot of knowledge has been gained on the structure, physiological functions and regulation of mammalian PDEs.

1.4.1 Overview of PDE Families

PDEs have long been divided into two main classes based on their primary structure; PDEI and PDEII. Identification of the low affinity cAMP PDEI and the high-affinity cAMP PDEII in *S. cerevisiae* showed how PDEs had diverged throughout evolution (Sass, Field et al. 1986; Nikawa, Sass et al. 1987). Subsequently, isolation of the dual specificity *Candida albicans* PDEI (Hoyer, Cieslinski et al. 1994) was found to share homology to the *S. cerevisiae* PDEI (Nikawa, Sass et al. 1987). Originally, *Escherichia coli* (*E. coli*) cAMP was believed to be under the control of only its synthesis, but in the 1960s a PDE was described from lysed bacterial cells (Brana and Chytil 1966). Subsequently this *E. coli* PDE was characterised, cpdA, but showed no homology to class I or II PDEs and so represented a third class of PDE (Imamura, Yamanaka et al. 1996; Richter 2002). It seems, so far, that class I PDEs include those of higher eukaryotes (vertebrates, *Drosophila melanogaster*, *Caenorhabditis elegans*, and a few fungal PDEs), class II is comprised of mostly fungal enzymes and *Vibrio fischeri*, and class III is made up of PDEs from prokaryote enzymes (Richter 2002).

Most human PDEs belong to class I PDEs. 21 PDEI genes have to date been identified in humans, rat and mouse, which give rise to eleven families of PDEs (PDE1-11). PDEI enzymes are grouped based on 270 conserved amino acids in the C-terminal catalytic domain, and around 35-50% sequence identity is found between the 11 different families (Richter 2002; Omori and Kotera 2007). The PDE1 families are grouped according to sequence homology, presence of certain regulatory domains, sensitivity to specific inhibitors and whether they hydrolyse cAMP, cGMP or both (figure 1.2). Most families are composed of 2-4 subfamily genes, each of which may give rise to splice variants. The subfamilies show >70% sequence identity and have identical regulatory domains that lie to the N-terminal side of the catalytic domain (Omori and Kotera 2007). Distinct genes, alternative splicing and use of alternative promoters give rise to the various isozymes, of which there may be 100 (Francis, Houslay et al. 2011). The PDE splice variants are unique in terms of tissue expression, subcellular localisation, regulation by phosphorylation or Ca²⁺/calmodulin and their interactome.



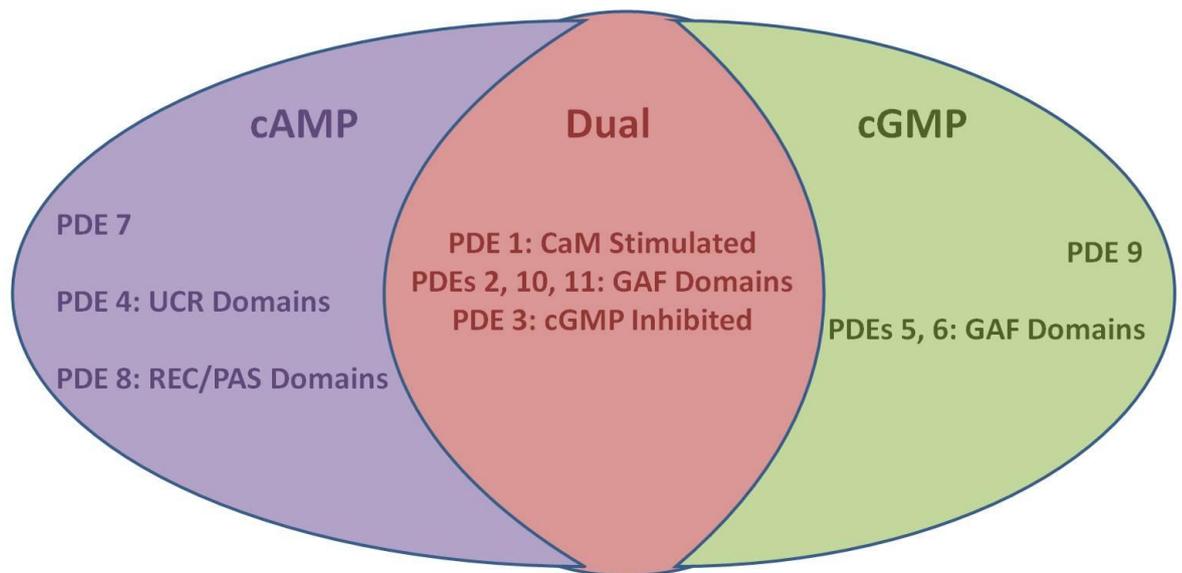
B

Figure 1.2. Phosphodiesterase regulatory domains and substrate specificity.

A; Unique N-terminal regulatory domains that affect PDE activity are expressed in a family specific fashion. Calcium/calmodulin binding domains (Ca²⁺/Cal.) in PDE1, GAF domains in PDEs 2,5,6,10,11, membrane associated domains (MEM) in PDE3, upstream conserved regions (UCR) in PDE4, Rec and Pas domains in PDE8, and no known regulatory domains in PDE9. **B;** the substrate specificities of the PDE families.

1.4.2 Nomenclature

A nomenclature classification system devised by Beavo and colleagues in 1994 is widely used. The acronym PDE is preceded by letters representing the species (e.g. Hs for *Homo sapiens*), it is then followed by the Arabic number designating the family (1-11), which is followed by a capital letter indicating the subfamily gene (A-D), this is further followed by a number to assign the splice variant of that subfamily; example HsPDE4D7 (Beavo, Conti et al. 1994). The splice variant number is simply its order of appearance in GenBank. Inconsistencies exist due to different researchers working on the same variant in different species and entering their findings. Based on the mouse genome a new, slightly modified system has been logged; <http://www.informatics.jax.org/mgihome/nomen/gene.shtml>. Both classifications are very similar and acceptable; indeed both are used depending on the journal.

Further classification can be based on the presence or absence of GAF domains which are involved in cyclic nucleotide allosteric regulation and dimerisation of the PDEs. There are five GAF-PDE families; PDE2, PDE5, PDE6, PDE10 and PDE11, each of which contains two GAF domains, and the others are non-GAF-PDE families (Martinez, Wu et al. 2002; Omori and Kotera 2007). The acronym GAF comes from the first three protein families found to contain this domain; GMP binding PDE, *Anabaena* adenylyl cyclase and *E. coli* FhlA domain, (Aravind and Ponting 1997). GAF domains are actually very rare in human proteins but are widespread in other species and well conserved (Heikaus, Pandit et al. 2009). They were first recognised to play a regulatory role in PDE5, following the discovery that one of the GAF domains allosterically binds cGMP along with the catalytic site (Turko, Francis et al. 1998). This binding brings about conformational change and potentiates activity of the enzyme and also regulates its activity via phosphorylation by cGMP dependant kinase (PKG) (Francis, Bessay et al. 2002; Martinez, Wu et al. 2002; Rybalkin, Rybalkina et al. 2003; Heikaus, Pandit et al. 2009). GAF domains present quite a kinetic puzzle; as cGMP acts as both a substrate and an allosteric modulator of GAF-PDEs.

Based on the amino acid sequences of the mammalian PDEs 1-7, the *S. cerevisiae* PDEII, the two nematode PDEs 1 and 4, and the four *Ephydatia fluviatilis* (freshwater sponge) PDEs 1-4, a phylogenetic tree was constructed. This arrangement clearly shows how vertebrate PDEs diverged from a common ancestral gene by gene duplication and domain shuffling before the parazoan-eumetazoan split (Koyanagi, Suga et al. 1998).

The PDE superfamily is a major pharmacological target for a variety of human illnesses (Xu, Hassell et al. 2000; Francis, Turko et al. 2001). Immediately after Sutherland and colleagues discovered PDE activity, it was found that they could be inhibited by caffeine. Indeed, a caffeine analogue, theophylline, has been used as a non-selective PDE inhibitor in a therapeutic setting for many years. Most early PDE inhibitors acted on every PDE in every tissue due to lack of specificity and thus had a low therapeutic index (Bender and Beavo 2006). The presence of multiple PDEs, expression of various splice variants, specific tissue distribution and intracellular localisation and different conformations of PDE enzymes have been challenging to the development of anti-PDE drugs, but these attributes are also promising in the development of more specific compounds. The crystal structures of the catalytic domain and active site have been solved, and this has facilitated the rational design of more specific and efficacious PDE inhibitors.

1.4.3 PDE Structure

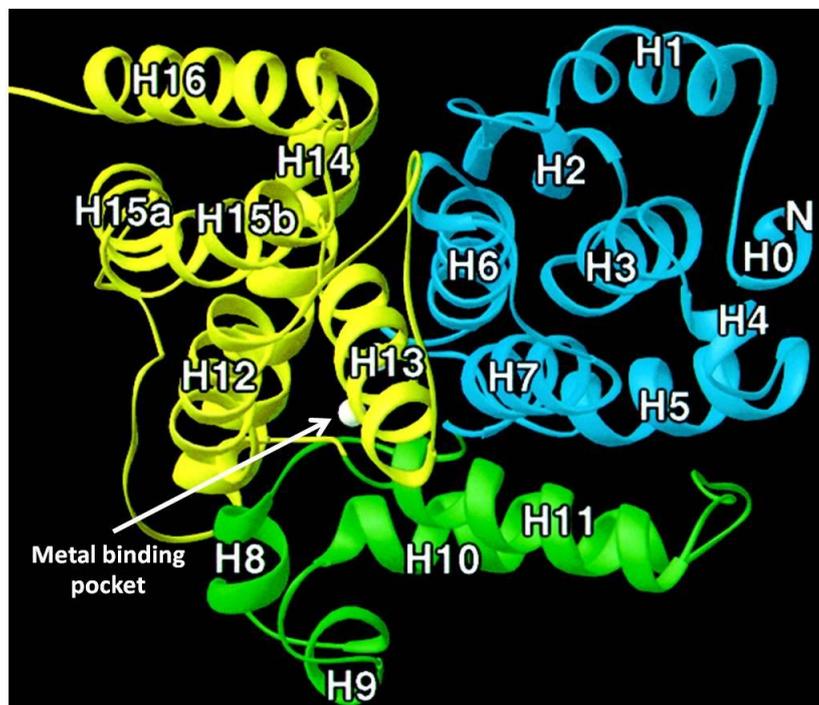
1.4.3.1 The Catalytic Site

Within recent years, the crystal structures for the catalytic domains of a number of PDE families have been solved (Bender and Beavo 2006). This is also true for the structure of the GAF domains of PDE2A (Martinez, Wu et al. 2002). Much information was gained from the first crystal structure of the PDE4B catalytic domain (Xu, Hassell et al. 2000) and the later structure of the PDE4D2 catalytic domain in complex with AMP (Huai, Colicelli et al. 2003). The models of cyclic nucleotide-bound PDEs are based on the co-crystal structures of the isolated

catalytic domains of the PDE bound to its 5' metabolite product or a substrate analog inhibitor. Co-crystals of PDEs bound to substrates are not available, nor are crystal structures of a PDE holoenzyme.

Around 300 amino acids in the region of the active site are conserved across all 11 PDE families and these give rise to similar 3D catalytic structures (Richter 2002; Ke 2004; Omori and Kotera 2007). The catalytic site is composed of 15-17 α -helices and an extended β -hairpin loop, which folds into a compact structure of 3 subdomains. For PDE4 (based on PDE4B2), there are of 17 α -helices (figure 1.3) (Xu, Hassell et al. 2000). The junction of the helices forms a hydrophobic pocket large enough to accommodate cyclic nucleotide or 5' nucleotide metabolite. This represents the cyclic nucleotide-binding pocket. Several amino acids found within the catalytic domain are conserved across all PDEs. Two metal ions are bound to the PDE catalytic domain; tightly bound Zn^{2+} and more loosely bound Mn^{2+} or Mg^{2+} , which are essential for stabilisation of the active site structure and catalysis of cyclic nucleotide (Xu, Hassell et al. 2000; Huai, Wang et al. 2003; Zhang, Card et al. 2004). This can be termed the metal binding pocket, and is highly conserved and histidine rich. The metal cations also bind a solvent water molecule that likely acts as a nucleophile in the hydrolysis reaction.

Zn^{2+} has been identified in most PDE catalytic domains; however, preference for the second metal cation varies and may add another layer of PDE regulation. Phosphorylation of long form PDE4 isozymes leads to an increased affinity for Mg^{2+} (Sette and Conti 1996), whereas PDE9 activity has a preference for Mn^{2+} (Wang, Wu et al. 2003). The concentrations of these cations differ between tissues and this would influence the metal cation complement and therefore PDE activity. Studies with PDE inhibitors show the metal cation pair influence the PDE-inhibitor interaction, for example, PDE4D in complex with the PDE4 inhibitor rolipram (Huai, Wang et al. 2003).



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Figure 1.3. Ribbon diagram of the PDE4B2 catalytic site secondary structure.

The 17 α helices H0-H16 form 3 subdomains; the N-terminal domain (cyan), the middle domain (green) and the C terminal domain (yellow). The metal binding pocket is found in the catalytic domain behind helix 13.

1.4.3.2 cAMP or cGMP or Both?

Selectivity of PDEs for the two different cyclic nucleotides (cAMP/cGMP) is complex and the mechanism is not fully understood.

It has been suggested that a glutamine residue conserved in all PDE families underscores the mechanism of cyclic nucleotide specificity (Xu, Hassell et al. 2000; Xu, Rocque et al. 2004). This mechanism was later coined the 'Glutamine Switch' (Zhang, Card et al. 2004). From high resolution co-crystal structures of

the cAMP PDEs 4B and 4D with AMP, the cGMP PDE5A with GMP, and the apo-structure of dual specificity PDE1B, Zhang and colleagues described how the invariant glutamine determines the specificity of each PDE, by recognising the purine moiety in the cyclic nucleotide. The hydrogen bond arrangement surrounding the conserved glutamine 'lock' it in a specific orientation so as only cAMP or cGMP is recognised. In dual specificity enzymes, a neighbouring histidine residue enables flexibility of the hydrogen-bond network and allows free 'rotation' of the glutamine and so either cyclic nucleotide may be bound. A hydrophobic 'clamp' also holds the purine base tightly within the active site. The crystal structure analysis uncovered four key interactions that affect cyclic nucleotide selectivity and catalysis; 1) metal binding; 2) coordination to the phosphate group; 3) a hydrophobic 'clamp'; and 4) a hydrogen-bonding arrangement that determines cyclic nucleotide specificity. These elements may also determine inhibitor selectivity (Huai, Wang et al. 2003). However, there are many critics of this proposal, stating it is too simple a mechanism as evidenced by studies on PDE 9 and PDE10 (Ke, Wang et al. 2011). PDE9A2 has specificity for cGMP, but the 'glutamine switch' is flexible, and its catalytic domain more closely resembles that of cAMP specific PDE4D2 and not PDE5A1, another cGMP PDE (Huai, Wang et al. 2004). In this case, the authors stated that PDE9A2 only weakly binds the non selective inhibitor 3-isobuty-1-methylxanthine (IBMX) due to this flexibility. Another argument against this mechanism involves the locked glutamine switches in PDE10A2 (Wang, Liu et al. 2007) and PDE2A3 (Iflland, Kohls et al. 2005), both dual specificity PDEs. It is proposed that substrate specificity is more complex and is due not to a single glutamine residue, but the complement of many residues within the binding pockets of a specific PDE (Wang, Liu et al. 2005; Wang, Liu et al. 2007).

1.4.4 PDE Oligomerisation

Observations from the crystal structure studies on the PDE catalytic sites suggest that oligomerisation is not necessary for PDE function in cell free systems. There is, however, much evidence to suggest that PDEs can oligomerise under physiological conditions. The GAF domains of PDEs 2, 5 and 10 mediate their

homo-dimerisation via intermolecular interactions, and this dimerisation regulates enzyme activity (Martinez, Wu et al. 2002; Zoraghi, Bessay et al. 2005; Handa, Mizohata et al. 2008). PDE1 and PDE6 exist as heterotetramers with their cognate regulatory proteins, two calmodulin molecules and two γ subunits respectively (Sonnenburg, Seger et al. 1995; Barren, Gakhar et al. 2009).

1.4.4.1 PDE4 Dimerisation

Long form PDE4 isozymes act as dimers via intermolecular interactions of their UCR1 and UCR2 regions, whereas as short form PDE4s that lack UCR1 exist only as monomers (Richter and Conti 2002). It has also been demonstrated that long form PDE4D dimerisation is a structural requirement for activation of the enzyme by PKA phosphorylation or PA binding, and that it acts to stabilise the enzymes in a high affinity rolipram binding site (HARBS) conformation. It is also suggested that both oligomers must be phosphorylated by PKA for enzyme activation (Richter and Conti 2004).

1.4.5 PDEI family

1.4.5.1 Regulation Overview

PDEs are firstly regulated at the level of their transcription and differential expression patterns in cells/tissue. Another level of regulation is afforded by protein domains specific to each family, such as GAF domains, described above. These domains; GAF, PAS, Ca^{2+} /Cam, UCR are responsible for receiving stimulatory or inhibitory signals from the signalling pathway within which a specific PDE acts. Fine tuning of PDE activity and function is achieved via an array of biochemical alterations of the PDE, such as phosphorylation/dephosphorylation, binding of Ca^{2+} /calmodulin and protein-protein interactions.

1.4.5.2 Overview of Families 1-3, and 5-11

1.4.5.2.1 PDE1

PDE1 enzymes are part of the Ca^{2+} / calmodulin-dependant group of PDEs (CaM-PDEs), which have been well studied and characterised. Three subfamily genes, PDE1A-C, give rise to a number of splice variants with dual specificity, that contain Ca^{2+} /calmodulin regulatory domains towards their N-terminus. The isozymes share similar kinetics, displaying the same V_{max} values for both cGMP and cAMP, but sensitivity to CaM varies between them (Yan, Zhao et al. 1996). All CaM PDEs are activated by calmodulin binding in the presence of calcium, with a 10-fold increase in V_{max} and are negatively regulated by phosphorylation, which acts to decrease their affinity for Ca^{2+} / calmodulin (Huang, Chau et al. 1981; Sharma and Wang 1985; Hashimoto, Sharma et al. 1989; Beavo 1995; Kakkar, Raju et al. 1999). Due to its regulation by Ca^{2+} /calmodulin, PDE1, along with the Ca^{2+} /calmodulin regulated ACs I III and VIII, provide a link between the cAMP and Ca^{2+} second messenger signalling systems (Beavo 1995; Kakkar, Raju et al. 1999).

1.4.5.2.2 PDE2

One gene, PDE2A, is expressed as three splice variants, PDE2A1-3, all of which show dual specificity for cGMP and cAMP. Isozymes are notably expressed in cardiac myocytes with different subcellular locations due to their N-termini, which may impact on cyclic nucleotide preference (Stephenson, Coskran et al. 2009; Lee and Kass 2012). GAF domains regulate PDE2s; GAF A and B, and they exist as homodimers. The C-terminal end of GAF A is linked to the N-terminal end of GAF B by a linker helix (LH1), with GAF A-GAF A and GAF B-GAF B associations between the dimers. Binding of cyclic nucleotide to GAF B initiates enzyme activation (Martinez, Wu et al. 2002; Pandit, Forman et al. 2009). It has been suggested that activation occurs with different conformational changes in the catalytic domain depending on which cyclic nucleotide binds (Wu, Tang et al. 2004). Evidence also suggests that cyclic nucleotide hydrolysis depends on co-activation by the other cyclic nucleotide, placing PDE2 as a regulator of cyclic nucleotide crosstalk (Lee and Kass 2012).

1.4.5.2.3 PDE3

Two PDE3 subfamily genes are known currently, PDE3A and PDE3B. Both hydrolyse cGMP and cAMP. PDE3A is more highly expressed in oocytes and cells of the cardiovascular system, whereas PDE3B is more highly expressed in cells involved in energy homeostasis such as pancreatic β -cells, adipocytes and hepatocytes (Beavo 1995; Ahmad, Degerman et al. 2012). Although PDE3s show dual selectivity, the V_{max} for cGMP is quite low, conferring higher specificity for cAMP. However cGMP binds with high affinity and low hydrolysis, and it is suggested that cGMP acts to inhibit cAMP hydrolysis by these PDE isozymes. PDE3 is activated by insulin via Akt phosphorylation (Loten and Sneyd 1970; Kitamura, Kitamura et al. 1999) and by leptin via PI3K (Zhao, Shinohara et al. 2000). Many studies show that PDE3 is pivotal in maintaining insulin homeostasis and modulation of insulin in erythrocytes. It also may be involved in vascular disease in prediabetes patients (Hanson, Stephenson et al. 2010). PDE3, particularly PDE3A, is of major importance in the heart. It has long been a target of inhibition by inotropics, such as milrinone, for chronic heart failure. This drug provides short term reprieve, but is associated with long term increases patient mortality (Packer, Carver et al. 1991). PDE3 has been shown to be downregulated in heart failure, which may be a causative factor, and the reason for treatment failure (Ding, Abe et al. 2005).

1.4.5.2.4 PDE5

PDE5A encodes three isoforms; PDE5A1-3, from two alternative promoter regions (Lin, Chow et al. 2002), and was the first cGMP PDE identified. PDE5 was first described in lung (Francis, Lincoln et al. 1980) and heart (Lugnier, Schoeffter et al. 1986) tissue. PDE5 is a GAF-PDE. It contains a GAF tandem domain towards its N-terminus that modulates its activity. PDE5 can be phosphorylated and activated by PKA following cGMP binding, another link between cAMP and cGMP signalling networks (Thomas, Francis et al. 1990). However, PKG seems to be the preferred kinase, which upon cGMP directly binding the catalytic site (and allosterically to a GAF domain), phosphorylates and activates PDE5 (Corbin, Turko et al. 2000). It has also been shown that high-affinity binding of cGMP to GAF A can directly activate PDE5 without phosphorylation and that GAF B may

confer regulatory properties on this interaction and thus enzyme activation (Rybalkin, Rybalkina et al. 2003; Zoraghi, Bessay et al. 2005).

PDE5 expression is normally low and is increased in disease states leading to vasoconstriction. It is involved in NO/cGMP signalling pathways. PDE5 inhibitors were originally developed to treat cardiac disease and hypertension via NO mediated vasodilation, but had little efficacy in trials. However due to their famous side effects, such inhibitors are now used to treat erectile dysfunction (Schwartz, Levine et al. 2012). Recently, the inhibitor sildenafil, was approved for treating pulmonary hypertension, and research on the inhibition of PDE5 in heart disease is ongoing (Nagendran, Archer et al. 2007; Kanwar, Agarwal et al. 2013). PDE inhibition is also showing promise as a therapeutic strategy to reduce the symptoms of Huntington's disease, in which NO-cGMP signalling is downregulated (Saavedra, Giralt et al. 2013). PDE5 inhibitors are also showing promise in treating the lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (Giuliano, Ückert et al. 2013).

1.4.5.2.5 PDE6

PDE6 is a cGMP specific, GAF-PDE. PDE6 is highly expressed in the rods (PDE6R) and cones (PDE6C) of photoreceptors. PDE6 exists as a heterotetramer composed of α , β and γ subunits. The PDE6R arrangement is $\alpha\beta\gamma\gamma$, whereas PDE6C is $\alpha\alpha\gamma\gamma$ (Deterre, Bigay et al. 1988; Cote 2004). The $\alpha\beta$ or $\alpha\alpha$ dimers constitute the catalytic site and the $\gamma\gamma$ ($P\gamma$) dimer acts as an inhibitory element. Under dark conditions, cGMP is bound to the GAF A domain, which increases the affinity of $P\gamma$ for the catalytic dimer, thereby inhibiting PDE6 (D'Amours and Cote 1999). Once a photon of light is received by rhodopsin, it activates the retinal G protein transducin, which brings about dissociation of $P\gamma$ from the catalytic site and PDE6 is activated. cGMP then dissociates from GAF A and is hydrolysed by PDE6, leading to reassociation of the $\alpha\beta/ \alpha\alpha$ and $P\gamma$, closure of a cyclic nucleotide gated channel, and generation of a photoresponse (Artemyev, Arshavsky et al. 1998; Guo, Grant et al. 2005). Mutations in PDE6R cause simplex and familial retinitis pigmentosa (RP) (Daiger, Bowne et al. 2007). Currently there is no cure, but gene therapy is a promising treatment for RP (Wert, Davis et al. 2013).

1.4.5.2.6 PDE7

The PDE7 family is cAMP specific and is encoded by two genes; PDE7A and PDE7B. The family is notably involved in inflammation (Safavi, Maliheh, et al. 2013). PDE7A is highly expressed in cells of the immune system and PDE7B is highly expressed in pancreas (Hetman, Soderling et al. 2000; Dong, Zitt et al. 2010). Early studies recognised rolipram insensitive PDE activity in T cells, later identified as PDE7, which was subsequently shown to be required for T cell activation due to inhibition of PKA. Inhibition of PDE7 decreased T cell proliferation and showed promise as a therapeutic target for T cell-mediated immune diseases (Ichimura and Kase 1993; Bloom and Beavo 1996; Li, Yee et al. 1999). However, subsequent investigations demonstrated that PDE7 knockout mice maintained fully functioning T cells (Yang, McIntyre et al. 2003), and selective PDE7 inhibition did not affect naive T cell function (Nueda, Garcia-Roger et al. 2006). PDE7 over expression is implicated in leukaemia and has been shown to be a promising therapeutic target, especially with co-inhibition of PDE3 and PDE4 (Zhang, Murray et al. 2008; Dong, Zitt et al. 2010). More recent investigations place PDE7, along with PDE4, as an important mediator of the inflammatory response following spinal cord injury (SCI). Two PDE7 inhibitors were successful in reducing the degree of neuroinflammation and tissue damage in SCI mice (Paterniti, Mazzon et al. 2011). PDE4 inhibition by rolipram was previously shown to promote axon regeneration following SPI (Nikulina, Tidwell et al. 2004) and dual inhibition of PDE7 and PDE4 may be a promising therapeutic intervention in the treatment of SPI. A recent study also shows PDE7 inhibition is efficacious in reducing the Alzheimer's disease phenotype (Perez-Gonzalez, Pascual et al. 2013)

1.4.5.2.7 PDE8

PDE8 encodes PDE8A and PDE8B subfamilies, which are cAMP-specific and sensitive to the PDE inhibitor dipyrindimole. PDE8A gives rise to five isoforms through alternative splicing (Wang, Wu et al. 2001), and PDE8B to two; PDE8B1 and PDE8B2 (Hayashi, Shimada et al. 2002). PDE8A is expressed in many tissues with highest expression in testes, ovary, small intestine and colon. PDE8B is mostly expressed in the thyroid gland (Fisher, Smith et al. 1998; Hayashi,

Matsushima et al. 1998). Not much is known on the function of PDE8 enzymes due to the lack of specific inhibitors, however PDE8A knockout mice have shown that PDE8 mediates androgenic steroidogenesis in the testes and adrenal gland (Tsai and Beavo 2011). PDE8A plays a crucial role in luteinising hormone (LH)-mediated testosterone production in the testis, which is mediated by an increase in cAMP. PDE8A KO mice displayed increases in testosterone levels (Vasta, Shimizu-Albergine et al. 2006). A testosterone deficit is responsible for many conditions, such as idiopathic male infertility. Current treatments for such ailments are unfavourable and inhibiting PDE8 may be a novel approach. Similarly, PDE8 activating agents could be considered as male contraceptives. As a result, PDE8B has been identified as a mediator of glucocorticoid steroidogenesis in the adrenal gland fasciculata cells using knockout mice, which exhibited an increase in urinary corticosterone levels (Tsai, Shimizu-Albergine et al. 2011). These findings place PDE8 as a mediator of the endocrinal regulation of the prostate and thus the dysregulation involved in prostate cancer (PC). PDE8A is also suggested to mediate Raf-1 and ERK signalling (Brown, Day et al. 2013). Some of the PDE8 isozymes possess N-terminal regulatory domains called PAS, and acronym of the proteins in which they were first identified; Per, ARNT, and Sim. These domains have been identified in several proteins in archaea, eubacteria and lower eukaryotes and seem to be involved in binding of small molecules and protein-protein interactions (Soderling, Bayuga et al. 1998; Wang, Wu et al. 2001).

Some PDE8 isozymes contain a REC (receiver) domain in addition to the PAS domain. REC domains play a crucial role in bacterial signalling systems. They may also be involved in protein-protein interactions (Galperin 2010). Phosphorylation of the REC domain may be involved in enzyme activation (Thomason, Traynor et al. 1999). Indeed our group found PDE8 to be activated by PKA (Brown, Lee et al. 2012). However, little is known about the function of these domains in mammals, and PDE8 is the only PDE family that contains such motifs.

1.4.5.2.8 PDE9

The PDE9 family hydrolyses cGMP specifically, with the highest affinity of all the cGMP PDEs (Soderling, Bayuga et al. 1998). The PDE9A gene gives rise to over 21 isoforms through alternative splicing. It was characterised as part of a study looking at Down syndrome and other genes on chromosome 21 and was suggested to be involved in the Downs phenotype (Guipponi, Scott et al. 1998). It is sensitive to the PDE5 inhibitor zaprinast and like PDE5 is implicated in NO/cGMP signalling. But unlike cGMP PDEs 2, 5 and 6, PDE9 does not possess GAF, or any N-terminal protein domains (Fisher, Smith et al. 1998). As a result, its regulation is ill understood. PDE9 seems to be expressed in all tissues except blood. It shows wide distribution in the brain, having the same expression pattern of as soluble guanylyl cyclase (sGC) and nitric oxide synthase (NOS) in rat brain. High relative PDE9 expression was identified in areas of the brain in which the nitric oxide (NO)-sGC-cGMP signalling pathway operates, suggesting this PDE is involved in processes such as learning and behavioural state regulation (Andreeva, Dikkes et al. 2001). Indeed, alteration of NO-cGMP signalling via PDE9 inhibition improves cognition and LTP. This is a promising therapeutic strategy for AD and other diseases involving cognitive impairment, indeed the selective PDE9 inhibitor; PF-04447943, has just completed phase II clinical trials as an AD drug (Wunder, Tersteegen et al. 2005; Zhihui 2013). PDE9 has also been shown to be regulated by testosterone via NO-cGMP signalling in the Leydig cells of rats (Andric, Janjic et al. 2010) and recent evidence suggests that PDE9 inhibition could also be efficacious in the treatment of erectile dysfunction (da Silva, Pereira et al. 2013), and that it is expressed in LUTS (Nagasaki, Nakano et al. 2012). Other recent studies implicate PDE9, along with PDE5, in breast cancer cell proliferation, and inhibition of PDE9 has been shown to bring about apoptosis via caspase 3 induction (Saravani, Karami-Tehrani et al. 2012).

1.4.5.2.9 PDE10

PDE10(A) is a dual specificity PDE. In this case, cAMP inhibits cGMP hydrolysis and cGMP inhibits cAMP hydrolysis, so one cyclic nucleotide may somehow act to regulate the other in PDE10 localised regions. The cyclic nucleotides bind the active site with different orientations and interactions (Wang, Liu et al. 2007).

Two human variants are known; PDE10A1 and PDE10A2, which sensitive to the inhibitor dipyridimole. PDE10 does not contain PKA or PKG phosphorylation sites, but instead is regulated by PKC (Fujishige, Kotera et al. 1999). PDE10 shows an interesting brain expression pattern, being in the same locations as dopamine receptors, where it may serve to regulate cAMP and cGMP during neuronal signal transduction (Fujishige, Kotera et al. 1999; Fujishige, Kotera et al. 1999). Many studies suggest its involvement in neurological disorders such as Huntington's disease (HD) and recent findings show PDE10 inhibition improves motor skills and cognition in HD mice (Giampa, Laurenti et al. 2010; Giralt, Saavedra et al. 2013). It is also expressed in rat testes and may mediate spermatogenesis along with PDE4 (Fujishige, Kotera et al. 1999). PDE10 is a GAF-PDE, but unlike all the others (PDEs 2, 5 and 6), the GAF domain is allosterically bound by cAMP rather than cGMP (Gross-Langenhoff, Hofbauer et al. 2006). The effect on PDE activity of cAMP binding GAF B of PDE10 is unclear. Some studies show no effect (Matthiesen and Nielsen 2009), while others report that the interaction activates PDE10, like for PDE2 and PDE5 (Jager, Russwurm et al. 2012). It may be that interactions at the GAF tandem bring about a conformational change and affect enzyme activity indirectly. The crystal structure of PDE10 GAF domain implies it is involved in dimerisation like in PDE2, but in an asymmetric manner (Handa, Mizohata et al. 2008).

1.4.5.2.10 PDE11

PDE11 is the latest addition to the PDE superfamily (Fawcett, Baxendale et al. 2000). It is a dual specificity, GAF-PDE and shows sensitivity to IBMX and dipyridimole. PDE11A is expressed as four transcripts, PDE11A1-4, with differential tissue expression in skeletal muscle, prostate, testis, kidney, liver, pituitary, brain and salivary glands. PDE11A1 and PDE11A2 contain only a single GAF domain, PDE11A3 contains one full GAF and a truncated GAF, and PDE11A4 contains the GAF tandem seen in all other GAF-PDEs (Hetman, Robas et al. 2000; Yuasa, Kotera et al. 2000). The differences in the N-termini suggest diverse regulation between the isozymes. It would seem that cGMP regulates PDE11A via these domains, as it does in other GAF-PDEs, but studies are conflicting (Gross-Langenhoff, Hofbauer et al. 2006; Matthiesen and Nielsen 2009; Jager, Russwurm

et al. 2012). A recent study found PDE11A functions in the CNS, and polymorphisms in this gene are implicated in major depressive disorder (MDD) (Wong, Whelan et al. 2006). This is unsurprising, as many studies implicate cGMP hydrolysing PDEs in neurological disorders. A PDE11A deficit has also been connected to spermatogenesis and fertility (Wayman, Phillips et al. 2005).

1.4.6 The Phosphodiesterase 4 Family

The PDE4 family is cAMP specific, and comprises 4 subgenes, PDE4A-D. PDE4 has been the subject of much research due to the fact that its inhibition leads to potent anti-inflammatory effects. Rolipram is the archetypal PDE4 inhibitor, and rolipram-sensitive PDE activity had been documented for some time, finally to be classified as the PDEIV family (Reeves, Leigh et al. 1987). A mutant *dunce* gene which caused *Drosophila* to exhibit learning defects (Dudai, Jan et al. 1976), was also found to cause abnormal cAMP metabolism, and so, the *dunce* gene was characterised as a cAMP PDE (Byers, Davis et al. 1981; Davis and Kiger 1981). *Dunce* cDNA was then used to search for homologs in mammals, leading to the first molecular cloning of a PDE4, indeed of any PDE, from rat (Davis, Takayasu et al. 1989). This PDE shared >70% sequence identity with *dunce* in the conserved region, underscoring the conservation of PDEs throughout evolution and highlighting their importance. Subsequent cloning experiments isolated four groups of rat cDNA clones that showed homology to *dunce* and corresponded to four different genes (Swinnen, Joseph et al. 1989). These were later found to have human homologs (Bolger, Michaeli et al. 1993) encoding four subfamilies; PDE4A, PDE4B, PDE4C and PDE4D, which gave rise to splice variants. The PDE4 family was found to express distinct N-terminal motifs sharing no homology to each other, but with homologous regions on rat PDEs. These class specific motifs were termed upstream conserved regions 1 and 2 (UCR1 and UCR2) (Bolger, Michaeli et al. 1993).

Human PDE4A and C genes map to chromosome 19 (19p13.2 and 19p13.1 respectively), PDE4B to chromosome 1 (1p31.3) and PDE4D is located on

chromosome 5 (5q12.1) (Milatovich, Bolger et al. 1994; Horton, Sullivan et al. 1995; Szpirer, Szpirer et al. 1995; Sullivan, Olsen et al. 1999)

The PDE4 subfamily genes encode over 25 splice variants. It is likely that no two isozymes will exhibit redundancy due to the unique extreme N-terminal domains. The unique N-termini of most isozymes are encoded by a single exon; however multiple exons are employed for PDEs 4B1, 4C1 and 4D7. One exception to this is the identical N-terminal regions expressed on the super short isoforms PDE4D6 and PDE4B5 (Cheung, Kan et al. 2007).

1.4.6.1 PDE4A

There are 11 recorded PDE4A splice variants, but some are rodent specific and the human PDE4As are PDE4A1, PDE4A4, PDE4A7, PDE4A10 and PDE4A11.

PDE4A1 is a super-short, exclusively membrane-associated isozyme (Shakur, Wilson et al. 1995). It is regulated by Ca^{2+} and binds phosphatidic acid (PA) via a TAPAS domain in its unique N-terminal region. An increase in intracellular Ca^{2+} brings about binding of PDE4A1 to PA and insertion into lipid bilayers in an irreversible manner (Baillie, Huston et al. 2002). Deletion of the PDE4A1 N-terminus prevented its membrane insertion and created a soluble enzyme, giving evidence for the hypothesis that PDEs are targeted to distinct subcellular locations via their unique N-termini (Shakur, Pryde et al. 1993).

PDE4A4 is a long form isozyme, homologous to rat PDE4A5, and is the most studied PDE4A isozyme. It is associated with neural signalling and the isozymes have been shown to be differentially expressed across the brain and CNS (D'Sa, Eisch et al. 2005). Expression in the hippocampus suggests PDE4A4 is involved in learning/memory processes and cAMP dependant neurogenesis. Anti-depressive treatment with various PDE4 inhibitors led to region-specific upregulation of some PDE4A isoforms (Ye, Jackson et al. 2000; D'Sa, Eisch et al. 2005). This compensatory mechanism likely affects the efficacy of antidepressants and isoform-selective inhibitors would serve as more efficacious antidepressant drugs. PDE4A has also been shown to be involved in impaired LTP and memory

due to sleep deprivation. Mice showed an upregulation of PDE4A5 (human PDE4A4) when cAMP/PKA dependant LTP was impaired, and PDE4 rolipram inhibition rescued this phenotype (Vecsey, Baillie et al. 2009).

It has been shown that PDE4A4 undergoes a conformational change following rolipram challenge, and this induces reversible association with p62, a protein involved in protein aggregation disorders. It is proposed that this event would sequester PDE4A4 in a p62 aggregate, away from its functional sites within the cell, and thereby be an additive affect to therapeutic inhibition. It may also be a means of sequestering PDE4A4 and therefore adapting cAMP signalling during time of cellular stress (Christian, Anthony et al. 2010). It was later found that this rolipram-induced conformational change is mediated by activation of the p38 MAPK pathway and consequent phosphorylation of PDE4A5 by MAPKAPK2 (MK2) (MacKenzie, Wallace et al. 2011). This study also found that such MK2 phosphorylation of long form PDE4s somewhat inhibits their phosphorylation by PKA, thereby lessening the desensitisation response to cAMP activation mediated by long form PDE4s.

PDE4A5 was found to undergo apoptosis-induced caspase 3 cleavage of its N-terminus, and is the only know PDE isoform which encodes a caspase 3 cleavage site (Huston, Beard et al. 2000). The location of this isozyme changed following cleavage, again showing how the N-termini of PDEs are involved in their subcellular targeting. PDE4A5 can interact with the SH3 domains of SRC family kinases (O'Connell, McCallum et al. 1996; Beard, Huston et al. 2002), an action which is inhibited following cleavage by caspase 3. Binding of PDE4A5 to SH3 domains may be to alter the local cAMP environment as required by these kinases.

A study on groups of smokers (with and without COPD) tested for changes in PDE4 isoform expression and found that PDE4A4 was uniquely upregulated in the macrophages of COPD patients (Barber, Baillie et al. 2004). This underscored the importance of the isozyme in inflammatory related diseases. PDE4A5 was found to bind the immunophilin XAP2/AIP/ARA9 via its N-terminus and probably also its UCR2 domain (Bolger, Peden et al. 2003). This interaction causes reversible

inhibition of PDE4A5, potentiates inhibition caused by rolipram, and it inhibits phosphorylation of PDE4A5 by PKA; effects which are likely mediated by conformational changes in this isozyme. The consequence of the XAP2/PDE4A4/5 interaction is unknown but it may be to sequester the PDE to a certain locale/complex. XAP2 also binds and stabilises the aryl hydrocarbon receptor (AHR) which is the receptor for carcinogens found in cigarette smoke (Meyer and Perdevew 1999; de Oliveira and Smolenski 2009). This is another link between PDE4A4/5 and inflammation, as cigarette smoke is main cause of COPD. A more recent study showed a rolipram-sensitive upregulation of PDE4A5 in SCI, again placing PDE4A4/5 in an inflammatory setting (Schaal, Garg et al. 2012).

An unusual splice variant; the short form PDE4A7, has a unique C-terminus as well as a unique N-terminus, with a conserved core region. Atypically, PDE4A7 is also catalytically inactive (Horton, Sullivan et al. 1995; Sullivan, Rena et al. 1998). It is widely expressed and its function is unclear. It may oligomerise with other PDE isoforms to modulate their activity or intracellular targeting (Johnston, Erdogan et al. 2004), or may indeed be a scaffold protein.

The long forms PDE4A10 and PDE4A11 are the most recently identified PDE4As. They show different localisation and sensitivity to PDE4 inhibitors when compared with PDE4A4/5, again, suggesting different targeting and catalytic site conformations due to their unique N-termini (Rena, Begg et al. 2001; Wallace, Johnston et al. 2005). PDE4A10 is widely expressed in rat tissues, but is concentrated in the heart, with high basal expression. Unusually, this isoform is downregulated following an increase in cAMP levels, a process that is observed in neonatal rat hearts. The significance of this is unknown, but it may be to remodel the signalling in a distinct, as yet unknown, cAMP pool (McCahill, Campbell et al. 2008). The converse of this can be seen during monocyte to macrophage differentiation which involves a complex remodelling of the PDE4 profile, including upregulation of the normally absent PDE410 (Shepherd, Baillie et al. 2004). These isozymes are only beginning to be studied so other facets of their function should be discovered soon. Recent evidence shows PDE10A is upregulated during the inflammatory response following traumatic brain injury (TBI) (Oliva, Kang et al. 2012)

1.4.6.2 PDE4B

PDE4B encodes 5 isozymes; the long PDE4B1, PDE4B3 and PDE4B5; the short PDE4B2 and the supershort PDE4B5.

Many studies have linked the long arm of chromosome 1 and mutated disrupted in schizophrenia 1 (*DISC1*) gene to schizophrenia and related mental disorders (Blackwood, Fordyce et al. 2001; Waterworth, Bassett et al. 2002; Hennah, Varilo et al. 2003; Ozeki, Tomoda et al. 2003; Kamiya, Kubo et al. 2005; Millar, Pickard et al. 2005). A study of a Scottish family found the schizophrenic phenotype to be caused by a balanced (1;11)(q42;q14.3) chromosomal translocation resulting in disruption of the *DISC1* gene by the breakpoint on chromosome 1 (Blackwood, Fordyce et al. 2001). A subsequent study found another balanced t(1;16)(p31.2;q21) translocation associated with schizophrenia (Millar, Pickard et al. 2005). The translocation breakpoint on chromosome 16 lies within an intron of cadherin 8, and the 1p31.2 breakpoint lies within a PDE4B1 intron, placing PDE4B as a susceptibility gene for schizophrenia and related mental maladies. The t(1;16) translocation event caused a 50% reduction in PDE4B1 protein expression. It has also been found that wild type *DISC1* binds a number of PDE4 isoforms via sites in the UCR2 and catalytic domain, indeed isolated UCR2 region is sufficient to bind *DISC1*, (Millar, Pickard et al. 2005; Murdoch, Mackie et al. 2007). It has been suggested that *DISC1* binds to the low activity form of PDE4B and activation of the enzyme by PKA leads to dissociation of its interaction with *DISC1* and an increase in PDE4B activity (Millar, Pickard et al. 2005). However, a study using a full length *DISC1* isoform suggests that an increase in cAMP does not cause such dissociation from PDE4B1 (Murdoch, Mackie et al. 2007). The differences may be due to different isoforms of PDE4B and *DISC1* being used in each study. In fact, the latter report found that *DISC1* interactions with various PDE4 isoforms are differentially regulated by cAMP/PKA, suggesting these interactions are very complex. Two point mutations in mouse *DISC1* located within the PDE4B binding region inhibited the *DISC1*/PDE4B interaction and conferred psychiatric phenotypes (Clapcote, Lipina et al. 2007; Murdoch, Mackie et al. 2007). Single nucleotide polymorphisms (SNPs) within the PDE4B gene, particularly within PDE4B2 introns, have been shown to positively correlate with

the incidence of schizophrenia (Fatemi, King et al. 2008). PDE4B1, PDE4B2, PDE4B3 and PDE4B4 protein levels were found to be decreased in post-mortem samples from schizophrenic patients, and PDE4B3 was decreased in bi-polar samples. These findings suggest that PDE4B plays a role in the etiology of schizophrenia and related psychoses. Truncated DISC1 and/or PDE4B1 due to the translocation events, mutations within DISC1 and PDE4B genes, or SNPs, may affect the DISC1/PDE4B interaction thereby affecting cAMP signalling in specific brain regions and may lead to the schizophrenic phenotype.

As previously discussed, PDE4 is involved in memory and cognition. Using hippocampal slices from rat, it was found that PDE4B3 expressed in neurons is regulated by NMDA-receptors and LTP, suggesting that cAMP modulation is important for synaptic plasticity and LTP (Ahmed and Frey 2003), and a number of studies suggest a role for glutamatergic signalling in schizophrenia. Interestingly, altered PDE4A and PDE4B profiles were observed in the brains of autism patients' post-mortem (Braun, Reutiman et al. 2007).

A number of studies have used PDE4B knockout mice to study its role in cognition and psychiatric illnesses. These mice displayed; the antipsychotic ability of rolipram PDE4 inhibition (Siuciak, Chapin et al. 2007); abnormal information processing and attention deficits (PPI) due to altered dopaminergic signalling (Siuciak, McCarthy et al. 2008); an increase in anxiety associated behaviour and corticosterone production (Zhang, Huang et al. 2008); and augmented long term depression (LTD) and reversal learning phenotype (Rutten, Wallace et al. 2011). Also, nicotine can exert antidepressant effects, and rats which underwent chronic nicotine treatment displayed a downregulation in PDE4B mRNA in selective brain regions (Polesskaya, Smith et al. 2007).

Lipopolysaccharide (LPS) is a potent inflammatory agent, which can cause tissue damage and even death via tumour necrosis factor (TNF) α , which is produced by monocytes and macrophages, cells that play an important role in alcohol-induced liver disease and hepatitis due to dysregulated cytokine signalling. PDE4B has been associated with this disease, where LPS induces its upregulation in monocytes and macrophages following chronic ethanol exposure. The

resultant decrease in cAMP causes an LPS-induced increase in TNF. (Jin and Conti 2002; Gobejishvili, Barve et al. 2008). Further work showed that the anti-inflammatory S-adenosylmethionine (SAM), a biological methyl donor, inhibits the LPS-induced upregulation of PDE4B2 due to hypermethylation of the PDE4B2 promoter region. This leads to an increase in cAMP and PKA, inhibition of TNF induction, and anti-inflammatory effects (Gobejishvili, Avila et al. 2011). A recent study shows that PDE4B inhibits transcription of the bacterial induced anti-inflammatory mediator, cylindromatosis (CYLD). Inhibition of PDE4B with rolipram led to upregulation of CYLD and an anti-inflammatory response (Komatsu, Lee et al. 2013). These studies, once again, display the importance of PDE4 in inflammation and support the notion that PDE4 inhibition has huge therapeutic potential. They suggest a role for PDE4B2 in bacterial infection mediated inflammation. Using SAM to inhibit LPS/PDE4B2/TNF-mediated inflammation is a promising treatment for liver disease, especially due to the safe tolerability profile of SAM.

The supershort PDE4B5 is the most recently identified isoform (Cheung, Kan et al. 2007). It is highly conserved across species and is brain specific. It is highly unusual, with a 'unique' N-terminus identical to that of supershort PDE4D6, which is also brain specific. However, PDE4B5 and PDE4D6 do not share any upstream transcription factor binding sites, suggesting they are indeed independent PDE4 isoforms. Interestingly PDE4B5 also binds DISC1, suggesting the binding site for DISC1 resides in the part of UCR2 left in this supershort isozyme.

1.4.6.3 PDE4C

The PDE4C subfamily comprises the long forms PDE4C1, PDE4C2 and PDE4C3. It is the least studied and characterised PDE4 subfamily due to its limited expression. Initial clones of PDE4C isoforms turned out to be mRNA artefacts (Engels, Sullivan et al. 1995; Obernolte, Ratzliff et al. 1997; Sullivan, Olsen et al. 1999). A number of screens carried out to look at differential PDE4 expression within various tissues have shed some light on PDE4C expression.

In a screen of rat retina samples, various PDE4 proteins were detected except PDE4C. However, a small amount of mRNA was detected, suggesting a possible low level of PDE4C protein may exist in this tissue. It is worth noting that, abundant PDE4C protein was seen in a lung sample, which was used as a positive control. (Whitaker and Cooper 2009). In a study looking at the PDE4 profile of inflammatory cells (monocytes, macrophages, T-cells and neutrophils) from COPD patients, PDE4C was found to be expressed in the macrophages, monocytes and neutrophils of COPD, non-COPD smokers and non-smokers. It was poorly expressed in blood monocytes but was upregulated following their differentiation to macrophages in the lung, in all samples (Barber, Baillie et al. 2004). This study suggests PDE4C is involved in inflammatory cell signalling, but not perhaps in the disease phenotype of COPD. However, in contrast to these findings, PDE4C expression was detected in monocyte to macrophage differentiation in U937 monocytic cells (Shepherd, Baillie et al. 2004) and it showed very low expression, with no activity in lung fibroblasts compared to PDE4B and PDE4D isoforms (Selige, Hatzelmann et al. 2011). PDE4C is suggested to be upregulated in the autoimmune disease Wegener's, which is associated with neutrophil activation. Healthy donor granulocytes that were TNF α -primed were stimulated with the antibody responsible for this disease, resulting in upregulation of a number of genes, including PDE4C (Surmiak, Kaczor et al. 2012). Neutrophils play an important role in the inflammatory response to bacterial infection and so this study again places PDE4 as an inflammatory mediator, and particularly PDE4C, as a mediator of bacterial toxin mediated inflammation, underscoring the importance of cAMP signalling in these responses.

PDE4C also showed no expression in the heart (Richter, Day et al. 2008), but was found to be the major PDE4 isozyme in pancreatic islet cells (Heimann, Jones et al. 2010). This may suggest a role in homeostasis, like PDE3, and a recent study identified PDE4C mRNA in rat penile smooth muscle cells (RPSMCs) (Chung, Jung et al. 2012). PDE4C has also recently been identified as a novel p53 target gene (Garritano, Inga et al. 2013).

1.4.6.4 PDE4D

The PDE4D subfamily is the largest to date, comprising 9 human isoforms; PDE4D1-9. PDE4D10 and PDE4D11 have been identified in rodents (Chandrasekaran, Toh et al. 2008; Lynex, Li et al. 2008), with possible PDE4D11 sequence in the human genome, but no evidence for any protein expression. PDE4D3, PDE4D4, PDE4D5, PDE4D7, PDE4D8 and PDE4D9 are long form variants, PDE4D1 and PDE4D6 are short and PDE4D2 is a super-short form. The PDE4D subfamily represents the highest PDE4 expression in many tissues including the prostate and adenocarcinoma of the prostate (Henderson D., thesis 2011).

1.4.6.4.1 PDE4D1 and PDE4D2

cAMP and follicle stimulating hormone (FSH) induce transcription of PDE4D1 and PDE4D2 from an intronic PDE4D promoter, within Sertoli cells (Vicini and Conti 1997). The promoter contains a cAMP response element binding protein (CREB) and cAMP response elements (CRE). mRNA of both enzymes was increased, but only PDE4D2 was translated. cAMP-inducible transcription of PDE4D1/2 constitutes a feedback loop involved in desensitisation of chronic cAMP signalling and is paramount to homeostasis in these cells. These isoforms are also upregulated in response to cAMP elevation in Jurkat T cells, with higher transcription of PDE4D1 (Erdogan and Houslay 1997). Healthy (quiescent) and diseased (activated) vascular smooth muscle cells (VSMCs) were found to have different PDE4D profiles, with an increase in the expression of short form variants following cAMP elevation in only diseased VSMCs (Tilley and Maurice 2005). This represents phenotype-dependent differential PDE4 expression, similar to that during monocyte-macrophage differentiation (Shepherd, Baillie et al. 2004). These findings suggest short form-specific PDE4D inhibitors would be of therapeutic value to augment cAMP signalling in activated VSMCs, without affecting normal VSMCs in the same artery.

1.4.6.4.2 PDE4D3

A number of signalling pathways and binding partners have been elucidated for PDE4D3.

In overexpressing cells, PDE4D3 was shown to directly bind phosphatidic acid (PA) (Grange, Sette et al. 2000) which caused an increase in PDE activity. No such increase for PDE4D1 was observed; suggesting PA binding regulates long form PDE4D isoforms. PDE4D3 also binds relaxin family peptide receptor 1 (RXFP1) via B arrestin2 and AKAP79, and acts to negatively regulate signalling by the hormone relaxin. The signalosome involved here also includes PKA and a constitutively active GPCR and the complex acts to mediate low level basal relaxin signalling (Halls and Cooper 2010).

cAMP enhances skin pigmentation following signals from melanocyte-stimulating hormone (MSH), which, in turn, stimulates the transcription factor MITF, a signalling pathway that plays a crucial role in the proliferation of skin pigment cells. PDE4D3 was specifically found to be upregulated in melanocytes following forskolin treatment. This increase was ablated when MITF was knocked down by siRNA, suggesting MITF regulates PDE4D3 expression in melanocytes. This is another example of a feedback loop, whereby MSH activates a cAMP “pool” that stimulates MITF, which in turn induces PDE4D3 expression which dampens the cAMP signal. Targeting this MITF-PDE4D3 loop may be of therapeutic value for skin cancer (Khaled, Levy et al. 2010; Ohanna, Bertolotto et al. 2011).

Various PDE4 isoforms have been found to interact with the neuronal scaffolding protein Ndel1, but it uniquely interacts with PDE4D3 in a dynamic manner. PKA phosphorylation on Ser13 within the N-terminus of PDE4D3 causes dissociation from Ndel1 (Collins, Murdoch et al. 2008). This event mirrors the differential PDE4 isoform release from N-terminally truncated or full length DISC1 (Millar, Pickard et al. 2005; Murdoch, Mackie et al. 2007)

Interestingly, Ser13 falls within the region on which mAKAP binds PDE4D3 and it has been suggested that PKA phosphorylation acts to increase the affinity of this PDE for mAKAP (Carlisle Michel, Dodge et al. 2004), suggesting a ‘switching of

partners' mechanism. Later, it was shown that within this mAKAP/PKA/PDE4D3 complex is also EPAC1, members of the ERK5 MAPK pathway and protein phosphatase PP2A (Dodge-Kafka, Soughayer et al. 2005; Dodge-Kafka and Kapiloff 2006; Dodge-Kafka, Bauman et al. 2010). This complex serves to tightly regulate localised cAMP signalling in cardiac cells in an extremely elegant and efficient manner (figure 1.5). Interestingly, Collins et al. had independently found EPAC to interact with Ndel1. This multifaceted signalosome displays interweaving negative and positive cAMP regulatory loops to fine tune the cAMP signalling response, further exemplifying the complexity of cAMP signalling, and how tightly regulated complexes are paramount to modulating the compartmentalisation of this signalling to allow pleiotropic responses to occur.

PDE4D3 is also part of the cardiac ryanodine receptor (RyR2)/ calcium-release-channel complex (Lehnart, Wehrens et al. 2005). This macromolecular complex is crucial to excitation-contraction (EC) coupling in the heart. Lehnart and colleagues observed a reduction in PDE4D3 levels within the RyR2 complex, which resulted in PKA hyperphosphorylation and 'leakiness' of the complex in failing hearts. This suggests PDE4D3 deficiency may play a role in the pathogenesis of heart failure (HF). This work also underscores how important it may be to find isoform-specific inhibitors when treating heart disease.

PDE4D3 has been found to also bind LIS1 (Murdoch, Vadrevu et al. 2011), a protein essential for neuronal migration where loss of function mutations are the causative factor of the neurodevelopmental disorder lissencephaly (Cardoso, Leventer et al. 2000). LIS1 is a microtubule-associated protein, maintaining the cell's microtubule network integrity. It interacts with dynein, and perturbation of this interaction likely plays a role in lissencephaly. Murdoch et al. found that PDE4D3 and other long form PDE4 isozymes bind LIS1 following PKA phosphorylation, and in fact, compete with dynein for binding. This interaction would act to sequester LIS1, inhibit its association with dynein and thus downregulate dynein activity. This study suggests that PDEs can modulate signalling by acting as scaffold proteins, and not just by cAMP regulation. This may explain the expression of the catalytically inactive PDE4A7 isoform (Horton, Sullivan et al. 1995; Sullivan, Rena et al. 1998).

To date, PDE4D3 is the only documented case of PKA phosphorylation within the unique N-terminus of any long form PDE4 (Sette and Conti 1996). However, results presented in this thesis suggest that the long form PDE4D7 is also PKA phosphorylated within its N-terminus, and that this affects its activity.

1.4.6.4.3 PDE4D4

PDE4D4 is a long form isozyme with the ability to bind the SH3 domain of src, lyn and fyn kinases (Beard, O'Connell et al. 1999). A perturbed PDE4D4 epigenome has been implicated in PC. Gestational low-dose exposure to environmental oestrogens, such as bisphenol A, causes susceptibility to later hormonal hits and the development of PC in adult life. Rat neonatal prostate glands represent those of human foetal prostates. Following various oestrogenic exposures of rat neonates, PDE4D4 was found to be hypomethylated in the prostate. Normally PDE4D4 expression is decreased in the aging prostate due to gradual hypermethylation. However brief neonatal exposure to oestrogens led to continued hypomethylation of PDE4D4 throughout the life of the rats. These changes in methylation status and gene expression were identified prior to any histopathologic changes, suggesting such events are a driving force of prostate carcinogenesis (Ho, Tang et al. 2006; Prins, Tang et al. 2008).

PDE4D4 interacts with spectrin to maintain integrity of the lung microvascular endothelial cell barrier (Creighton, Zhu et al. 2008). It has also been implicated in depression, where knockdown of this isoform along with PDE4D5 produced antidepressant effects (Wang, Zhang et al. 2013)

1.4.6.4.4 PDE4D5

Historically, PDE4D5 was the first example of a PDE4 isoform having a specific function within the cell. PDE4D5 acts in complex with PKA, AKAP79 and the scaffold protein β -arrestin to regulate the switch from β 2-adrenoreceptor (β 2-AR) coupling to G_i mediated ERK activation, thereby helping to compartmentalise cAMP signalling at the β 2-AR in cardiac cells (Perry, Baillie et al. 2002; Lynch, Baillie et al. 2005). PDE4D5 also binds the scaffold protein receptor for activated c-kinase (RACK1) (Yarwood, Steele et al. 1999). In fact, β -arrestin and RACK1

interact with overlapping sites within the PDE4D5 N-terminus and therefore compete for binding to regulate PDE4D5 signalling (Bolger, Baillie et al. 2006). RACK1 sequestration of PDE4D5 ultimately limits the amount of PDE4D5 available for recruitment to the β 2-AR. This is an example of yet another layer of regulation within the cAMP/PDE signalling network. Recent work has also demonstrated that, following β 2AR activation, PDE4D5 competes with EPAC1 for binding to β -arrestin2. β -arrestin2 recruits EPAC1 to β 1AR, resulting in H-ras activation (Berthouze-Duquesnes, Lucas et al. 2013). Therefore PDE4D5 may also exert its function by acting as a scaffold, to sequester EPAC1 thereby modulating and offering specificity to BAR signalling. This is another example of how a PDE4D may regulate signalling aside from cAMP hydrolysis. It also exemplifies the intricate and complex nature of compartmentalised cyclic nucleotide signalling. This signalling axis is involved in cardiac cell remodelling, and perturbation of this BAR regulation may be involved in HF.

PDE4D5 has also been placed in a signalling system with RACK1 and focal adhesion kinase (FAK) whereby the PDE4D5/RACK1/FAK complex regulates cell polarity (Serrels, Sandilands et al. 2010). Knockdown of PDE4D5 pinpointed its role in cell spreading and signal-induced polarisation, and the PDE4D5/RACK1/FAK complex localises to nascent adhesion structures and the leading edge of polarising cells. A follow on study found this signalling pathway to employ EPAC and its downstream effector Rap1 (Serrels, Sandilands et al. 2011). Perturbation of this 'direction sensing' signalling complex to cause constitutive EPAC/Rap1 activation resulted in lack of nascent adhesion stabilisation and impaired cell spreading and responses to polarisation signals. Indeed FAK and PDE4D5 are upregulated in mouse squamous cell carcinomas (SCCs), suggesting that perturbation of this direction sensing complex is involved in cancer cell migration and invasiveness. One study employed the *Sleeping Beauty* (SB) transposon system to identify novel oncogenes involved in PC (Rahrmann, Collier et al. 2009). Transposon based mutagenesis caused altered prostate histology and an increase in proliferation of the epithelium, features associated with PC precursor lesions. Within these lesions, candidate cancer genes were identified, among them PDE4D. This study also found PDE4D to be upregulated in prostate adenocarcinoma patient samples compared to those

with BPH. Additionally, shRNA PDE4D knockdown decreased cell proliferation. The report went on to suggest that PDE4D5 is the main player in cAMP dysregulation in PC. PDE4D overexpression may occur in the aging prostate and lead to increased risk for PC. This is in contrast to a number of PC expression profiling studies that are listed in the Oncomine Database. Within these, no change of PDE4D mRNA expression, or a decrease in expression with increasing tumour grade, were observed. It may be that an mRNA decrease does not represent post transcriptional regulation of PDE4D. Indeed, poor correlation between the mRNA and protein levels of many PDE4D isoforms has been noted in rats due to post transcriptional modifications (Rhodes, Yu et al. 2004; Richter, Jin et al. 2005; Rahrman, Collier et al. 2009). However, the protein level of PDE4D7 matches that of its mRNA, as shown in this thesis.

1.4.6.4.5 **PDE4D6**

PDE4D6 is a supershort isozyme, sharing its unique N-terminal region with the super short PDE4B5. Both are exclusively expressed in brain, and no specific function has been assigned to either (Wang, Deng et al. 2003; Cheung, Kan et al. 2007).

1.4.6.4.6 **PDE4D7**

This long form isozyme was first described alongside PDE4D6 (Wang, Deng et al. 2003). PDE4D7 has been implicated in carotid (common) stroke. The PDE4D gene was linked to ischemic stroke in Northern European populations via its role in atherosclerosis, which involves proliferation and migration of VSMCs. A genomewide search for stroke susceptibility genes within an Icelandic cohort identified the susceptibility locus to be located at 5q12 (Gretarsdottir, Sveinbjornsdottir et al. 2002) which was in fact pinpointed to the PDE4D locus (Gretarsdottir, Thorleifsson et al. 2003). They further pinpointed the stroke-associated haplotype/SNPs to extend over the 5' exon and promoter regions of PDE4D7, and found a significant correlation between the stroke-associated haplotype and PDE4D7 mRNA levels. Since the disease haplotype is located at the 5' end of PDE4D7, it may affect transcription of this isoform. Surprisingly, no

further studies on PDE4D7 have been carried out to look at the behaviour of its promoter region or the function of the PDE4D7 protein.

1.4.6.4.7 PDE4D8

PDE4D8 is a long form PDE4. In mice it shows restricted expression that is highest in lung, heart and liver (Richter, Jin et al. 2005). Studies using cardiomyocytes have found β 1-adrenoreceptor (β 1AR) to associate preferentially with PDE4D8, which dissociates from the receptor following agonist activation (De Arcangelis, Liu et al. 2009; De Arcangelis, Liu et al. 2010). PDE4D8 also associates with β 2AR by β arrestin recruitment, much like PDE4D5. However, while PDE4D5 mediates β 2AR signalling following receptor activation, it seems PDE4D8 (along with PDE4D9) acts to regulate basal cAMP signalling around the β 2AR (De Arcangelis, Liu et al. 2009). So, following receptor stimulation PDE4D8 is released from β 1AR, but recruited to β 2AR. Perhaps simultaneous activation of both β ARs causes PDE4D8 to move from β 1AR to β 2AR via arrestins, which would allow a local increase in cAMP at the β 1ARs, so as to elicit downstream signalling and bring about contractility of cardiac cells. This is yet another example of the complexity and elegance that underpins tightly regulated, compartmentalised cAMP signalling.

1.4.6.4.8 PDE4D9

Very little is known about this long form PDE. It is widely expressed in rat tissue (Richter, Jin et al. 2005). Akin to PDE4D8, it is involved in β adrenergic signalling in cardiomyocytes. It has been shown to bind β 2AR, but in contrast to PDE4D8, they are associated in the resting state and receptor stimulation causes dissociation of PDE4D9 (De Arcangelis, Liu et al. 2009). Recent work places PDE4D9 within the dopaminergic signalling network. ATF4 (CREB2) and DISC1 associate to act as a transcriptional repressor complex on the PDE4D locus, and suppress PDE4D9 promoter activity (Soda, Frank et al. 2013). It seems PDE4D9 transcription is regulated via dopamine receptor signalling. PKA phosphorylation of DISC1 inhibits its nuclear localisation and association with ATF4, thereby leading to an upregulation of PDE4D9. PDE4D9 can also interact with DISC1, but this interaction decreases with dopamine treatment. These results add to the

growing amount of evidence that PDE4 isoform functionality is regulated by DISC1. Again, this represents an example of feedback signalling where activated dopamine receptor leads to an increase in cAMP, activating PKA which phosphorylates DISC1 thereby relieving the transcriptional constraint on the PDE4D9 promoter, leading to an increase in PDE4D9 expression and attenuation of the cAMP signal. Mutations in DISC1, such as those described by Clapcote et al., would then inhibit formation of the repressor complex resulting in PDE4D9 overexpression (Clapcote, Lipina et al. 2007). Indeed, perturbation of this signalling axis within the dopaminergic system may play a role in psychiatric pathophysiology.

1.4.7 PDE4 Regulation

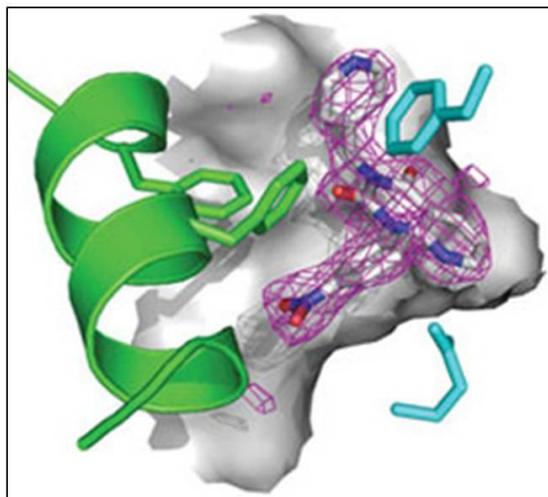
1.4.7.1 UCRs and PKA Phosphorylation

The UCR1 and UCR2 domains confer important regulation on PDE4 isozymes. UCR2 underpins an auto-inhibitory mechanism. Deletion of short form PDE4D1 sequences encompassing the UCR2 region, led to increases in V_{max} of 2-3 fold (Jin, Swinnen et al. 1992), and 6 fold (Kovalala, Sanwal et al. 1997), with little change in K_m . Deletion of PDE4A5 N-terminal region up to the and including UCR2, also caused a 3-fold increase in activity (Beard, Huston et al. 2002). All long form PDE4 isoforms are activated by PKA phosphorylation within the conserved RRESF motif in the UCR1 region (Sette, Iona et al. 1994; Sette and Conti 1996; MacKenzie, Baillie et al. 2002). Long form PDE4D3 is activated upon cleavage of its UCR2 domain, and also by antibody binding to the UCR2, which acted to ablate its inhibitory constraint (Lim, Pahlke et al. 1999). This activation had the same characteristics as that produced following PDE4D3 phosphorylation, giving credence to the idea that enzyme activation involves release of an auto-inhibitory mechanism and a conformational change around the catalytic site. This idea was confirmed with the elucidation of the crystal structure of the PDE4 UCR2 domain (Burgin, Magnusson et al. 2010). PDE4D UCR2 and catalytic domains were co-crystallised, with and without the presence of inhibitor molecules. The UCR2 domain displayed shape complementarity with

the catalytic site. The Inhibitors acted to close the UCR2 around the active site, precluding cAMP from the binding pocket (figure 1.4 A). This investigation clearly supported the UCR1-UCR2 model for activation of long form PDE4s. In the absence of UCR1 phosphorylation by PKA, UCR2 straddles the active site, keeping the enzyme in a 'closed' conformation. PKA phosphorylation of UCR1 then causes release of the UCR2 constraint, and the active site is available for cAMP to bind (figure 1.4 B). Since PDE4 isoforms dimerise, inhibition by UCR2 may be intra- or intermolecular. Intermolecular regulation is likely to occur, highlighting the functional importance of PDE4 dimerisation (Gurney, Burgin et al. 2011).

Another regulatory mechanism stems from the fact that UCR1 and UCR2 are capable of forming independent protein folds and interacting with each other and with other regions of the PDE4s to regulate enzyme activity (Beard, Olsen et al. 2000). The carboxyl end of UCR1 interacts with the amino end of UCR2 via electrostatic interactions. PKA phosphorylation of UCR1 inhibits this interaction, leading to conformational change and enzyme activation (figure 1.4 B).

A

Burgin, Magnusson et al. *Nature Biotechnology* 2010; 28, 63–70

B

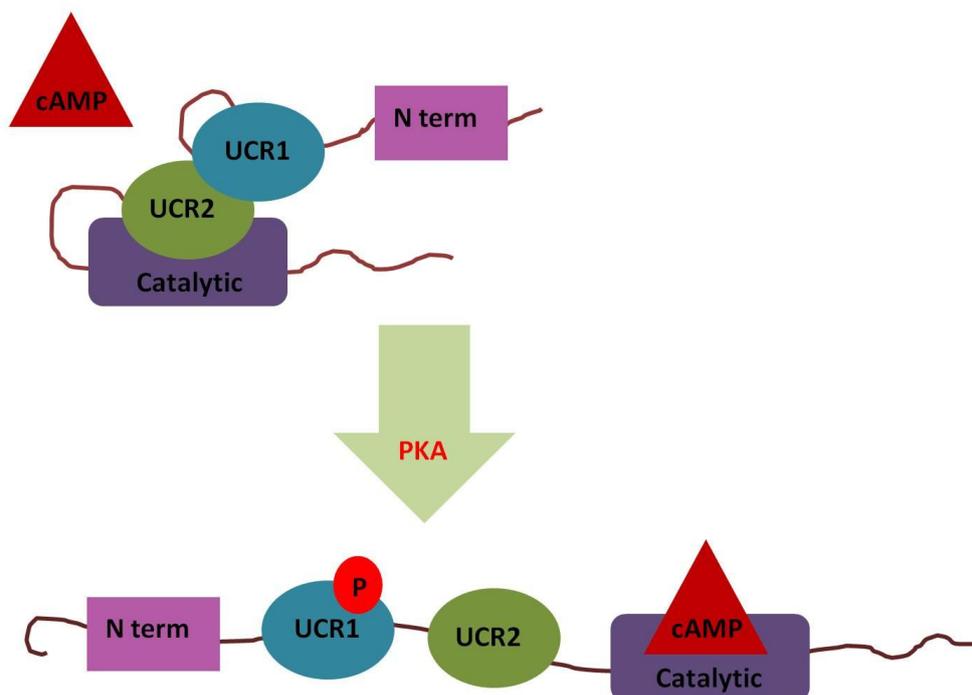


Figure 1.4. Long form PDE4 regulation by the UCR2 domain.

A; a surface representation of the PDE4D catalytic domain (grey) bound by inhibitor interacting with UCR2 (green). **B;** UCR2 and UCR1 interact via electrostatic interactions to form an inhibitory module. UCR2 straddles the active site, precluding cAMP from entering the catalytic pocket. PKA phosphorylation of UCR1 relieves this constraint, leading to enzyme activation.

1.4.7.2 PDE4 Conformers

Although the catalytic site is highly conserved across all PDEs, PDE4 is suggested to exist in one of two different conformers, adding another layer of enzyme regulation. These are the high affinity rolipram-binding state (HARBS) and the low affinity rolipram-binding state (LARBS) (Souness and Rao 1997). The significance of these different conformations is unclear. PDE4 isoforms may exist in one or the other, depending on tissue/cell expression, as different affinities for rolipram have been seen in different tissues for the same isoform. UCR2 is involved in HARBS, as it binds both the active site and inhibitor via a Phe residue (Burgin, Magnusson et al. 2010). The two conformers are a consideration when designing PDE4 inhibitors.

1.4.7.3 ERK Phosphorylation

ERK is a member of the MAPK superfamily signalling pathway. This mitogen-activated protein kinase pathway consists of sequential phosphorylation events from MAPKKK to MAPKK to MAPK, each activating the next kinase. ERK is a MAPK. The pathway is conserved from yeast to mammals (Widmann, Gibson et al. 1999). It is both activated by, and acts on a diverse range of signalling molecules, including androgens (Nguyen, Yao et al. 2005).

ERK phosphorylates long form PDE4B/C and D isozymes on a PQSP consensus site, C terminal to the catalytic domain. EGF-stimulated phosphorylation of PDE4D3 and PDE4D5 by ERK2 was shown to markedly decrease their activity, with a 75% reduction in V_{max} for PDE4D3 and no change in K_m (Hoffmann, Baillie et al. 1999). This inhibition was ablated by a MEK inhibitor. Here we see another example of a signalling feedback loop where ERK inactivation of the PDEs permits a local increase in cAMP, followed by activation of PKA which phosphorylates and activates the PDE to override ERK phosphorylation-mediated inhibition.

The catalytic region of all PDE4 isoforms contains two ERK docking sites necessary for ERK association with PDE4s. This is a prerequisite for phosphorylation. The docking sites are made up of an FQF site, C-terminal to the substrate serine, and a KIM domain situated N-terminal to the serine (MacKenzie, Baillie et al. 2000). ERK phosphorylation also inhibits super short isoforms, albeit weakly, but intriguingly activates short PDE4 isoforms, as demonstrated with PDE4D2 (MacKenzie, Baillie et al. 2000), and PDE4D1 and PDE4B2 (Baillie, MacKenzie et al. 2000; MacKenzie, Baillie et al. 2000). Interestingly, ERK cannot phosphorylate PDE4A isoforms due to the presence of arginine instead of a proline in the ERK consensus site; RQSP instead of PQSP. Thus the PDE4 profile of a tissue/cell will dictate the outcomes from ERK signalling. ERK therefore provides cross-talk between cAMP and MAPK signalling.

1.4.7.4 Phospholipids and Phosphatidic Acid

Long PDE4 isoforms may be activated by phosphatidic acid (PA). For example, PDE4D3 shows a PA-induced 2-3 fold increase in V_{max} and no change in K_m , similar to PKA activation. However, little effect is seen on short form PDE4s, such as PDE4D1 (DiSanto, Glaser et al. 1995; Némoz, Sette et al. 1997). This suggests UCR1 is involved in phospholipid binding. Phosphatidic Acid (PA) also altered the sensitivity of PDE4D3 to Mg^{2+} in a manner akin to PKA phosphorylation. PKA and PA do not activate long form PDE4s simultaneously, as they do not have an additive effect (Némoz, Sette et al. 1997). This suggests a distinct mode of regulation by PA. PA binding sites are not well characterised. However, the N-terminus of super short PDE4A1 was found to contain a novel tryptophan anchoring phosphatidic acid selective binding domain 1 (TAPAS1) PA binding site which mediates its membrane insertion (Baillie, Huston et al. 2002). An insertion helix of LWWF lies opposite to the Ca^{2+} binding helix DKR. Submicromolar Ca^{2+} is required to gate the insertion process, likely through conformational change of the second helix. TAPAS1 is not expressed on long form PDE4s, and PA binding to the UCR1 is via an unknown mechanism.

1.4.7.5 PDE4 Intracellular Targeting

The unique N-termini of PDE4 isoforms are paramount to their intracellular targeting. Evidence also suggests that the UCR2 domain contributes to targeting, except in cases of super-short isoforms such as PDE4A1, which is targeted to the membrane solely by its N-terminus (Houslay and Adams 2003). Protein-protein interactions involving the N-termini (and UCR2) determine the subcellular localisation of most PDE4s. Targeting of PDE4 isoforms to specific intracellular locales and their functional coupling to specific receptors and scaffold proteins underpins the spatial dynamics of cAMP gradients. The importance of PDE locations was demonstrated by Mongillo et al. in neonatal rat cardiomyocytes. Using FRET-based cAMP reporter constructs to measure cAMP concentrations, they found that PDE4 and PDE3 showed distinct responses to BAR agonists due to their location (Mongillo, McSorley et al. 2004).

1.4.7.6 AKAPs

A kinase anchoring proteins (AKAPs) also play an important role in signal compartmentalisation. They act to sequester and target PKA to discrete cAMP pools, and keep PKA close to substrate proteins such as PDE4s. AKAPs act as scaffold proteins that ensure strict organisation of cAMP signalling proteins. They allow the broad spectrum kinase that is PKA to exert distinct cellular responses. AKAPs are well conserved across species and more than 50 have been identified based on their ability to co-purify with PKA. They are structurally diverse but functionally similar as all possess a PKA anchoring domain, unique targeting regions and all have the ability to form multiprotein complexes (Carnegie and Scott 2003; Wong and Scott 2004; McConnachie, Langeberg et al. 2006). Microtubule associated protein 2 (MAP2) was the first identified AKAP, having co-purified with PKA RII subunit from brain extract (Lohmann, DeCamilli et al. 1984). Most AKAPs bind the RII subunit of PKA, but RI-binding AKAPs have been identified (Angelo and Rubin 1998). AKAPs with dual specificity have also been described (Huang, Durick et al. 1997; Huang, Durick et al. 1997; Ruppelt, Mosenden et al. 2007).

PDE4D3 interacts and co-localises with muscle specific mAKAP at the nuclear membrane in cardiomyocytes (Dodge, Khouangsathiene et al. 2001). mAKAP directly binds to the N-terminus of PDE4D3 lending specificity to this interaction, as PDE4D5 was not seen to associate with mAKAP. mAKAP associated PDE4D3 maintains basal cAMP levels, and upon stimulation of GPCRs, increased cAMP production overcomes this inhibition and brings about release of PKA C subunits from the complex, followed by PDE activation and cessation of the signal. Later, it was shown that this mAKAP/PKA/PDE4D3 complex also contained EPAC1, members of the ERK5 MAPK pathway, ryanodine receptor (RyR) and protein phosphatase 2A (PP2A) (Kapiloff, Jackson et al. 2001; Dodge-Kafka, Soughayer et al. 2005; Dodge-Kafka and Kapiloff 2006; Dodge-Kafka, Bauman et al. 2010). It also seems that phosphorylation of PDE4D3 by PKA increases its affinity for mAKAP (Carlisle Michel, Dodge et al. 2004). This macromolecular complex orchestrates concomitant cAMP regulatory loops. For example, cAMP increases result in activated PKA, which, by being bound to mAKAP, phosphorylates and activates PDE4D3, which in turn hydrolyses cAMP. Another loop involves mAKAP binding and activating PP2A, which in turn dephosphorylates PDE4D3, inactivating the PDE, thereby regulating PKA phosphorylation of the RyR Ca²⁺ sensitive channel. Such an intricate signalosome highlights the complexity of compartmentalised signalling, and provides cross-talk between the cAMP and Ca²⁺ networks (figure 1.5).

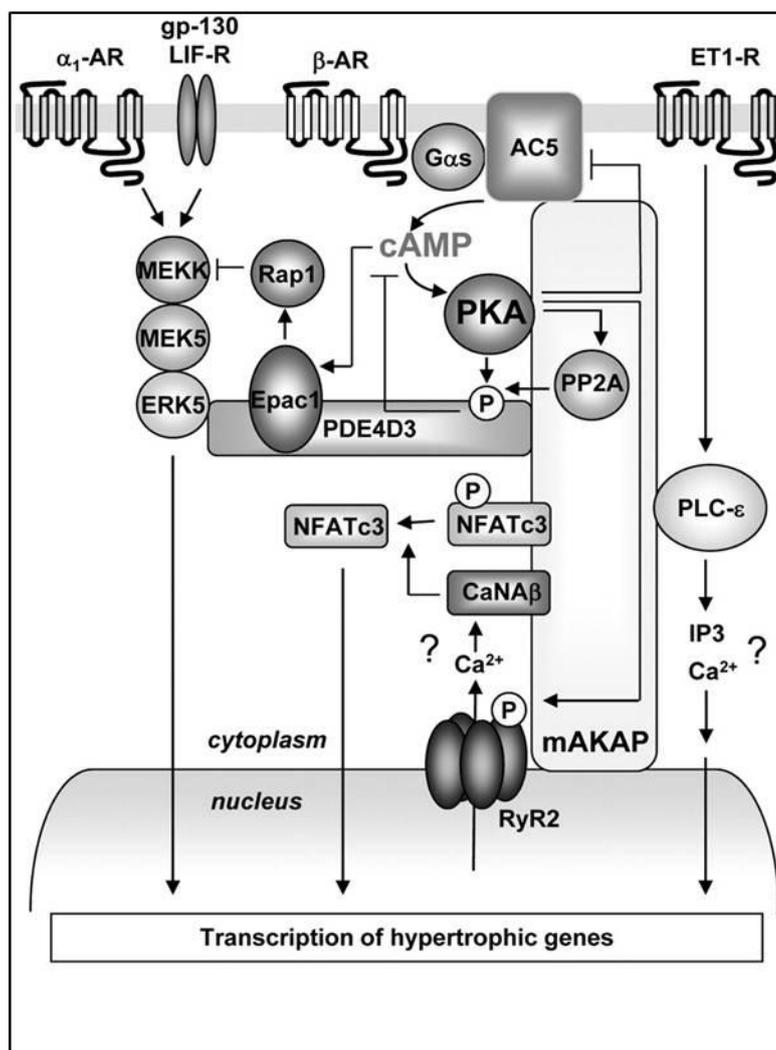
A study on the effect of naturally occurring mAKAP coding polymorphisms revealed perturbations in the mAKAP/PKA/PDE4D3 complex (Rababa'h, Craft Jr et al. 2013). The mutations either affected the AKAP binding to PKA or PDE4D3 with consequent alterations in PKA activity and Ca²⁺ signalling. There was also an increase in calcineurin expression and CREB phosphorylation, suggesting such mutations may induce pro-hypertrophic gene expression. Indeed, such mAKAP polymorphisms may be involved in hypertrophy, an element of HF.

The mAKAP/PKA/PDE4D3 complex has been extensively studied in the perinuclear region of cardiomyocytes but PKA/AKAP/PDE4 complexes are involved in many processes within various tissues and cell types. PDE4 has been shown to associate with the AKAP gravin, where it is in complex with PKA at the

sub-plasma membrane region (Willoughby, Wong et al. 2006). PDE4D3 also associates with AKAP450, PKA and other proteins at the centrosomes, implying a role in cell division (Taskén, Collas et al. 2001). PDE4A1 can associate with three different AKAPs in T cells; AKAP149, AKAP95 and MTG (Asirvatham, Galligan et al. 2004). This demonstrates that a certain PDE4 can act in different regions within the cell with distinct functional outcomes by being anchored to different AKAPs complexes.

AKAPs are a key component of T cell signalling. PKA plays an unusual role in T cell activation as it acts in lipid rafts to negatively regulate T cell receptor (TCR) immune responses. PKA phosphorylates C-terminal Src kinase (Csk), which subsequently phosphorylates and inhibits lymphocyte specific protein tyrosine kinase (Lck). Lck is required for T cell signalling by phosphorylating the TCR-CD3 complex, thus, PKA brings about inhibition of TCR responses (Halle, Haus-Seuffert et al. 1997; Ramstad, Sundvold et al. 2000; Ruppelt, Mosenden et al. 2007). PDE4 recruitment to lipid rafts via β arrestin plays an important role in this signalling axis (Abrahamsen, Baillie et al. 2004) and the dual specificity AKAP EZRIN has been found to target PKA to the lipid rafts where it will be close to its substrate, Csk (Ruppelt, Mosenden et al. 2007). It is therefore likely that PKA, EZRIN and PDE4 act in a signalling complex to mediate T cell activation. Indeed PDE4A has been shown to bind three different AKAPs in distinct subcellular regions within T cells (Asirvatham, Galligan et al. 2004).

Some AKAP genes express splice variants that locate to different subcellular locations (McSorley, Stefan et al. 2006), and AKAPs can also bind ACs (Kritzer, Li et al. 2012), presumably different AC isoforms may bind different AKAPs, maybe different AKAP isoforms. This multifariousness adds yet another level of complexity to cAMP compartmentalised signalling.



Diviani D et al. Am J Physiol Heart Circ Physiol
2011;301:H1742-H1753

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Figure 1.5. The mAKAP/PDE4D3/PPP2A/PKA/EPAC/MAPK/RyR multienzyme signalosome in the heart.

B-adrenergic stimulation brings about synthesis of cAMP which activates anchored PKA. This in turn activates PDE4D3 in a negative feedback loop, and increases the affinity of PDE4D3 for mAKAP. PKA also activates RyR2 and PP2A, the latter dephosphorylates and thereby inactivates PDE4D3. EPAC1 activates Rap1 which inhibits the hypertrophic ERK5 MAPK pathway. The latter dephosphorylates and thereby inactivates PDE4D3. EPAC1 activates Rap1 which inhibits the hypertrophic ERK5 MAPK pathway.

1.4.7.7 β -arrestins

β -arrestins are scaffold proteins that modulate the activity of Gs at GPCRs, as already discussed. There are two types of β -arrestins, the visual arrestins found in the retina involved in photoreceptor signalling, and the non-visual β -arrestins 1, 2 and 3 which are ubiquitous and regulate the function of a variety of GPCRs and the internalisation of GPCRs in clathrin-coated pits (Attramadal, Arriza et al. 1992; Gurevich and Benovic 1992; Krupnick, Goodman et al. 1997; Laporte, Oakley et al. 1999).

β -arrestin recruitment of PDEs to GPCRs is necessary to bring about receptor desensitisation and regulate cAMP signalling that emanates from activated Gs-coupled GPCRs. β -arrestins can potentially bind to all PDE4 isoforms via their conserved catalytic domains (Perry, Baillie et al. 2002). However not all PDE4s seem to be recruited by β -arrestins. Upon isoproterenol challenge of cardiomyocytes and other cell types, PDE4D5 is preferentially recruited to β 2AR compared to PDE4D3, the other major isoform in the cells studied (Bolger, McCahill et al. 2003). The C-terminus of β -arrestin binds the N-terminus of PDE4D5, and the N-terminus of β -arrestin interacts with the conserved motifs FXFELXL and FQFELTLEED in the PDE4D5 catalytic region (Baillie, Adams et al. 2007). The affinity of this interaction is increased by PDE4D5 ubiquitination (Li, Baillie et al. 2009). Thus, it may be that only certain PDE4 isoforms have the ability to bind these scaffold proteins via their N-termini, and this is influenced by the post-translational modification status of the isozyme.

Signalling via β 2AR, cAMP and PKA has long been implicated in environmental chronic stress responses (Thaker, Lutgendorf et al. 2007). β -arrestin2 has been implicated in PC. It was found to negatively regulate AR dependant gene expression. It binds the AR in complex with the E3 ligase MDM2, thereby bringing about AR ubiquitination and degradation (Lakshmiathan, Zou et al. 2009). The authors proposed that upregulation of β -arrestin2 would lead to downregulation of AR. This may play a role in the switch to 'non-classical' AR signalling, an event observed in advanced PC. Indeed, activation of β -arrestin2 has been shown to increase the proliferation and migration rate of PC cells (Zhang, He et

al. 2011). β -arrestin2 interacts with c-Src to bring about an increase in ERK activation and cAMP concentration. This would suggest that a long form PDE4 is involved, and is hyperphosphorylated by ERK, leading to its inhibition and an increase in cAMP.

1.4.7.8 RACK1

RACK1 was first identified as a PKC binding protein (Mochly-Rosen, Khaner et al. 1991). It is a ubiquitously expressed WD repeat scaffold protein, with homology to the G protein β subunit (McCahill, Warwicker et al. 2002). RACK1 acts as a scaffold for many proteins involved in a variety of signalling pathways, many of which are involved in G protein signalling (Dell, Connor et al. 2002; Chen, Spiegelberg et al. 2004). It is also involved in cell cycle progression via sequestration of Src (Mamidipudi, Zhang et al. 2004), and is implicated in cancer cell migration via its role in cell polarity (Serrels, Sandilands et al. 2010). RACK1 is an important scaffold in the brain, and has been linked to neurological disorders (Sklan, Podoly et al. 2006). RACK1 also binds the androgen receptor (AR). In a PKC dependent manner it allows the AR to shuttle to the nucleus in absence of ligand, thereby repressing androgen activation of the AR and thus expression of androgen responsive genes, such as PSA (Rigas, Ozanne et al. 2003). As discussed, RACK1 interacts with PDE4D5. Interaction is via the WD repeat propeller structure in a highly specific manner. PDE4D5 does not bind other WD repeat proteins, nor does RACK1 bind any other PDE isoforms (Yarwood, Steele et al. 1999). The interaction is via the PDE4D5 N-terminus, but also via a conserved motif in the catalytic region, with RACK1 essentially straddling PDE4D5. This binding mode provides steric hindrance, which aids the sequestration of PDE4D5, and may prevent it binding other proteins at that time (Bolger, Baillie et al. 2006). The N-terminal binding region actually overlaps with the β -arrestin binding site ensuring that binding of these scaffolds to PDE4D5 is mutually exclusive.

RACK1 is pivotal to many signalling pathways, helping to keep them linear and coordinated, and may allow different networks to cross talk. Aberrations in any

of these pathways due to RACK1 are likely involved in various pathologies and RACK1 is a potential therapeutic target (Ron, Adams et al. 2013).

1.4.7.9 SH3 Domains

Protein-protein interaction via SH3 domains is a well conserved mechanism throughout eukaryotes. Src, Lyn and Fyn are tyrosine kinases that are involved in a variety of cellular events, and bind target proteins via their SH3 domains (Houslay and Adams 2003). These SH3 domain proteins play important roles in intracellular targeting of other proteins. PDE4D4 and PDE4A5 can bind SH3 domains via a proline rich region in their N-termini. PDE4D4 can bind the SH3 domains of many proteins, whereas PDE4A5 selectively interacts with the tyrosyl kinases Src, Lyn and Fyn. (Beard, O'Connell et al. 1999). This is due to the difference in their N-terminal proline rich SH3 binding motifs where PDE4D4 contains a polyproline motif, whereas that of PDE4A5 is PxxPxxP. These differences may affect the targeting of the PDEs, and their functional outcome. Indeed, the binding of PDE4A5 to SH3 brings about its membrane and perinuclear localisation, which is ablated when the proline rich region in its N-terminus is cleaved. Along with the N-terminal SH3 binding motif, PDE4A isoforms also contain such a motif in their LR2 domain (McPhee, Yarwood et al. 1999). Binding at this site to the SH3 domain of Lyn increases PDE4A5 sensitivity to rolipram putting it into the HARBs state by causing a conformational change (McPhee, Yarwood et al. 1999). PDE4A5 actually possess three proline rich SH3 consensus motifs within its N-terminal region, only one of which binds Lyn. PDE4A5 actually localises to two subcellular regions; membrane ruffles at the cell margin and the perinuclear region (Huston, Beard et al. 2000). It seems the N-terminal SH3 binding motif determines targeting to the membrane ruffles, and SH3 binding at UCR2 targets PDE4A5 to the perinuclear region (Beard, Huston et al. 2002). PDE4A5 localisation is clearly a complex issue, and may vary between tissues and cell cycle stage. It may depend on synergism between SH3 binding of the N-terminal region and the UCR2 domain.

1.5 cAMP Effector Proteins

Second messengers require effector proteins in order to elicit their desired downstream effects. Any proteins that directly bind a second messenger are termed second effector proteins. cAMP and cGMP each have specific effectors.

1.5.1 PKA

Protein kinase A (PKA) is one of the highly conserved eukaryotic protein kinases (EPKs) which function as molecular switches to activate downstream signalling cascades (Taylor, Zhang et al. 2013). The notion that proteins could be regulated by phosphorylation was first observed by Krebs and co-workers, who saw that glycogen phosphorylase could be reversibly activated by addition of a phosphate moiety (Krebs and Fischer 1956). This paved the way for the discovery of EPKs, which comprise 2% of the human genome, translating to more than 500 proteins (Manning, Whyte et al. 2002). The EPKs all share a common kinase core and together are known as the kinome (Taylor, Keshwani et al. 2012).

PKA is assembled as an inactive tetrameric holo-enzyme composed of two catalytic (C) and two regulatory (R) subunits, C_2R_2 ; where upon cAMP binding, active C_2 dissociates from R_2 (figure 1.6) (Døskeland, Maronde et al. 1993; Kim, Xuong et al. 2005; Kim, Cheng et al. 2007). Different genes give rise to four different R subunits (RI α , RII α , RIB and RII β) and 3 different C subunits (C α , C β and C γ), resulting in different PKA holo-enzymes, which lend specificity to the signalling system. The holoenzyme is named according to the R subunits present. The two major forms of PKA; PKAI and PKAII, are made of RI and RII, respectively, interacting with identical subunits (Merkle and Hoffmann 2011). PKAI and PKAII show significant differential tissue expression. The R subunits share a common domain organisation but differ in biochemical characteristics (Døskeland, Maronde et al. 1993; Heller, Vigil et al. 2004; Vigil, Blumenthal et al. 2004). The R subunits possess dimerisation/docking (D/D) domains in their N-terminus via which they bind the relevant AKAP, allowing PKA to be in the

vicinity of its substrate. This D/D domain is connected to two tandem C-terminal cAMP binding domains A and B (CBD A/B) by a flexible linker region. The linker region contains an autoinhibitory pseudosubstrate motif that sits on and inhibits the active sites of the C subunits in the absence of cAMP. Four molecules of cAMP bind the R homodimers to cause release of the auto-inhibition of the C subunits (Heller, Vigil et al. 2004; Wong and Scott 2004; Taylor, Kim et al. 2005; Taylor, Zhang et al. 2013). The activated C subunits are then free to phosphorylate target proteins by phosphoryl transfer of the ATP γ -phosphate. The optimal PKA consensus site is RRxS/T(h), where h is any hydrophobic residue (Songyang, Blechner et al. 1994). Differences in the length and amino acid composition of the linker regions seem to be the determinant for conferring different quaternary structure between the R isoforms (Vigil, Blumenthal et al. 2004). Such differences may grant steric specificity for associating with signalling complexes.

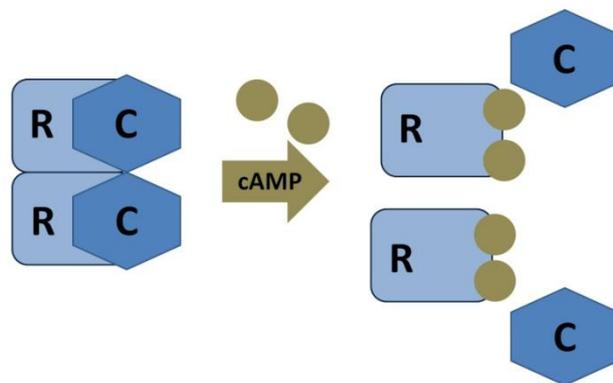


Figure 1.6. PKA.

In the inactive state PKA exists as a holoenzyme composed of two regulatory (R) and two catalytic (C) subunits. Following GPCR activation, two molecules of cAMP bind each R subunit, liberating the C subunits which are then free to phosphorylate downstream targets.

PKA possess the conserved hydrophobic, bi-lobal EPK core. It comprises a small N-lobe and large C-lobe, between which, is a deep cleft that houses the active site of the C domain (Bossemeyer 1995). This cleft accommodates ATP and substrate. The N-terminal (N-) lobe comprises a 5 stranded antiparallel β sheet and 2 helices. The C-terminal (C-) lobe comprises 7 helices and 4 short β strands. Several residues in the C lobe interact with the ATP triphosphate group. The β strands form the base of the cleft, the catalytic loop and a metal binding loop. The C lobe also possesses an activation loop, phosphorylation of which is essential for catalysis and binding to the R α subunit (Adams, McGlone et al. 1995; Taylor, Zhang et al. 2013). This phosphorylation brings about the assembly of a hydrophobic spine that connects the lobes; it comprises 2 residues from each of the lobes. This spine is termed the regulatory (R-) spine as it seems to 'break' while acting as a switch during PKA activation (Knighton, Zheng et al. 1991; Kornev and Taylor 2010; Taylor, Keshwani et al. 2012). There is also a catalytic (C-) spine that runs parallel to the R-spine, formation of which is induced upon cAMP binding the CBDs. This brings about conformational change and priming of PKA for catalysis.

The core itself is not sufficient for catalysis, and requires a short N-tail and C-tail that extend from the C subunit and wrap around the core. These tails may be post-translationally modified themselves, and act as *cis*-regulatory elements, providing both stability to the holoenzyme and allosteric regulation (Taylor, Kim et al. 2008; Taylor, Keshwani et al. 2012). They are also essential for protein-protein interactions (Taylor, Kim et al. 2005).

Many studies on the crystal structure of PKA have been carried out. Catalysis involves dynamic conformational changes, not just at the active site, but throughout the whole enzyme, notably within the linker region of the R subunits (Heller, Vigil et al. 2004). cAMP binding to the CBD induces a small local conformational change, followed by a large conformational change in R and release of C (Vigil, Blumenthal et al. 2004). This larger change occurs more slowly, allowing attenuation of upstream cAMP prior to reformation of the holoenzyme. To date, the data suggest an opening-closing mechanism for PKA

catalysis. ATP and cAMP bind PKA leading to an orchestrated movement of residues in the PKA backbone, resulting in a closed conformation which is poised for catalysis. (Masterson, Cheng et al. 2010; Masterson, Shi et al. 2011). Thus, the process of PKA activation and subunit disassociation is highly dynamic.

The C subunit also acts as a scaffold to mediate protein-protein interactions, particularly the large C-lobe (Taylor, Kim et al. 2008). NMR studies showed that the D/D domain of RII α provides a hydrophobic groove for AKAP binding (Newlon, Roy et al. 2001). Although the D/D domain is conserved, this groove can discriminate between the different R dimers, contributing to specificity of PKA signalling. RI α can accept bulky side chains into its groove, RII α cannot but has a more flexible groove (Kinderman, Kim et al. 2006).

Mg²⁺ ions have been shown to affect PKA activity, and due to their differential concentration throughout the cell cycle and in different subcellular locations these ions may represent another level of regulation of PKA signalling specificity (Masterson, Shi et al. 2011).

Binding of cAMP to the CBDs occurs in a sequential manner, whereby cAMP firstly binds CBD-B, priming CBD-A to accept another cAMP molecule, with consequent release of the active C subunit (Kim, Cheng et al. 2007; McNicholl, Das et al. 2010).

The differences in R subunit function have been demonstrated in knockout (KO) mice. RI α KO is embryologically lethal, whereas RII β KO led to a compensatory mechanism by RI α and a deficit in neural plasticity (Hensch, Gordon et al. 1998). Protein kinases are involved in all signalling pathways, and they are desirable drug targets due to their implied roles in disease. Such compounds could inhibit activity, activation or localisation of PKA (Taylor, Kim et al. 2008). Dysregulation of PKA holoenzyme construction may play a role in PC progression. C and R splice variant switching has been observed with a downregulation of CB2; upregulation of CB1 CB3 and CB4; and upregulation of RII β during neuroendocrine differentiation (NED) (Kvissel, Ramberg et al. 2007). Differential expression of the C isoforms was also observed between non-tumour and tumour

samples and between AS and AI PC cell lines. Interestingly, CB2 is upregulated in tumour compared to normal tissue, and so is involved in aberrant proliferation. Changes in the PKAI and PKAII ratio is an important part of cellular differentiation but is also implicated in cancer progression (Merkle and Hoffmann 2011). PKAI is normally transiently expressed in response to stimuli but is often constitutively expressed in malignancies. PKI upregulation in PC brings about the excretion of a cAMP independent extracellular PKA (ECPKA), which may act as a growth factor to promote tumourgenesis. Upon overexpression of exogenous PKAII, PKAI and ECPKA are downregulated and the cancer phenotype is reversed (Cho, Lee et al. 2000).

1.5.2 Exchange Proteins Directly Activated by cAMP (EPAC)

EPAC1 and EPAC2 are guanine nucleotide exchange factors (GEFs) for the ubiquitous GTPases Rap1 and Rap2 (de Rooij, Zwartkruis et al. 1998; de Rooij, Rehmann et al. 2000). The cAMP activation of Rap1 in a PKA-independent manner led to notion that not all effects of cAMP are mediated by PKA or cyclic nucleotide gated ion channels (de Rooij, Zwartkruis et al. 1998).

EPACs possess N-terminal regulatory, and C-terminal catalytic domains. The regulatory domain contains a cAMP binding site, which shares sequence homology to those found in PKA and acts as an autoinhibitory element on the GEF domain. Such autoinhibition is alleviated upon cAMP binding. Also found here is a Dishevelled, Egl-10, Pleckstrin (DEP) domain. This is not involved in cAMP binding, but is required for localisation to membrane structures (EPACs are most often localised to membranes). The catalytic domain comprises the GEF domain and a Ras exchange motif (REM), which lends stability to the GEF domain. It also possesses a CDC25-homology domain (CDC25HD) and in EPAC2, a Ras-association domain (RA) is present (de Rooij, Zwartkruis et al. 1998; de Rooij, Rehmann et al. 2000; Rehmann, Das et al. 2006; Niimura, Miki et al. 2009). EPAC2 has an additional, lower affinity, cAMP binding domain that lies N-terminal to the DEP motif (de Rooij, Rehmann et al. 2000). The functional consequence of cAMP binding here is unknown.

Similar to the case with PKA, the binding of cAMP to EPAC involves small local conformational changes followed by huge conformational change throughout the rest of the enzyme in order to lift the stable autoinhibitory element and allow Rap1/2 access to the catalytic GEF site (Harper, Wienk et al. 2008; Rehmann, Arias-Palomo et al. 2008).

EPAC2 is also involved in oncogenic Ras signalling (Li, Asuri et al. 2006). Growth factor activation of Ras can recruit EPAC2 to the plasma membrane in a bid to activate local Rap1; however, EPAC2 must be cAMP bound and activated in order for transport to the plasma membrane. Thus, EPAC2 provides cross talk between growth factor signalling and cAMP signalling.

PKA- and cAMP-gated ion channels are understood to underpin cAMP and Ca^{2+} signalling in the heart (Fink, Zakhary et al. 2001). However, EPAC1 associates with the mAKAP/PKA/PDE4D3/RyR macromolecular complex in cardiomyocytes (Dodge-Kafka, Soughayer et al. 2005). Different studies have shown EPAC to be both pro- and anti- hypertrophic, but evidence leans towards the former (Morel, Marcantoni et al. 2005; Schmidt, Sand et al. 2007). EPAC can regulate Ca^{2+} oscillations in cardiomyocytes and ventricular myocytes as part of a signalling pathway involving PKC, CaMKII and RyR (Pereira, Metrich et al. 2007; Oestreich, Malik et al. 2009). EPAC/Rap1 mediates the formation of gap junctions in neonatal rat cardiac myocytes by enhancing accumulation of connexin 43 at cell-cell boundaries, thus coordinating with PKA signalling to regulate gap junction function and contractility (Somekawa, Fukuhara et al. 2005).

EPACs locate to various subcellular regions and so have been suggested to play roles in several biological functions, notably in integrin-mediated cell adhesion and cell-junction formation (Bos, de Rooij et al. 2001; Bos 2006). Activation of EPAC with cAMP analogs can potentiate the therapeutic efficacy of human endothelial progenitor cells (EPCs), which are administered to treat ischemic tissue, via increased integrin-dependent migration to sites of ischemia (Carmona, Chavakis et al. 2008). EPAC localisation changes throughout the cell cycle in a manner which suggests an important role in the coordination of microtubule transport and organelle partition during division into daughter cells

during mitosis (Qiao, Mei et al. 2002). A small nuclear pool of EPAC appears to be involved in DNA repair (Huston, Lynch et al. 2008). EPAC/Rap2 initiates the nuclear exit of DNA-dependent protein kinase (DNA-PK) in a PDE4B-regulated manner. Interestingly, PKA mediates the re-entry of this enzyme, giving us an example of an antagonistic interlacing cAMP signalling network.

EPAC also plays an important role in the immune system. EPAC1/Rap1 potentiates cell adhesion, polarisation and chemotaxis of, and regulates apoptosis of leukocytes (Shimonaka, Katagiri et al. 2003; Lorenowicz, van Gils et al. 2006; Gerard, Mertens et al. 2007; Grandoch, Bujok et al. 2009). Along with PKA, EPAC modulates the activity of macrophages by inhibiting phagocytosis (Aronoff, Canetti et al. 2005). In monocytes cAMP signals exclusively through PKA, but upon differentiation to macrophages, EPAC1 is upregulated and works with PKA to mediate signalling in these cells (Bryn, Mahic et al. 2006). However, EPAC1 inhibition of macrophage phagocytosis may be Rap1 independent (Brock, Serezani et al. 2008).

EPAC has also been implicated in PC. It has been found that EPAC1/Rap1 action increases PC cell proliferation and survival by activation of B-Raf/ERK, PI3K/AKT and mTOR signalling, all of which cross talk to bring about an upregulation in pro-proliferative genes (Misra and Pizzo 2009). However, another study suggests EPAC inhibits PC proliferation and migration (Grandoch, Rose et al. 2009). This involves an inhibition of MAPK and RhoA signalling and collapse of the F-actin cytoskeleton. The former study employed early stage, androgen sensitive (AS) cells, whereas the latter employed later stage, androgen insensitive (AI) PC cells. This suggests a possible differential role of EPAC depending on PC stage. As PKA signalling is perturbed during PC progression, compensatory cAMP signalling may come into play, via EPAC. A recent follow on study by Misra et al. found that activation of EPAC also brings about an upregulation of pro-inflammatory markers in both AS and AI PC cells, and acts via MAPK-COX2-PGE₂-mTOR signalling to bring about proliferation (Misra and Pizzo 2013).

1.5.3 Cyclic Nucleotide gated Ion Channels

Cyclic Nucleotide-gated (CNG) Ion Channels were first identified in retinal rod and cone cells and olfactory sensory neurons (OSNs) (Fesenko, Kolesnikov et al. 1985; Haynes and Yau 1985; Nakamura and Gold 1987). CNG channels have since been described in many sensory organs, neuronal function and in spermatozoa, although their function is not fully understood (Kaupp and Seifert 2002). Rod and cone CNG channels show cyclic nucleotide selectivity, but OSN CNG channels are activated by both cAMP and cGMP. CNG ion channels belong to an ion channel superfamily, based on a shared transmembrane topology, pore structure and C-terminal cyclic nucleotide binding domain (Kaupp and Seifert 2002). cAMP or cGMP binding to the intracellular cyclic nucleotide binding domain results in opening of the channel and the passage of both alkali and divalent ions, particularly Ca^{2+} , and consequent electrical and chemical response (Kaupp and Seifert 2002). Six genes encode human CNG ion channels, which fall into two gene subfamilies. The genes show differential tissue expression and some give rise to splice variants. One subfamily comprises of CNG α 1- α 4, whereas the second is made up of CNGB1 and CNGB3. These proteins can associate to form hetero- or homo-trimeric channels. There is no desensitisation of signal at the channels, but they are regulated by CaM binding and Ca^{2+} and post-translational modifications. Pseudechetoxin from snake venom is a potent CNG channel antagonist. Mutations in CNG channel are linked to sight defects and KO mice suggest they are involved in LTP in the hippocampus (Parent, Schrader et al. 1998; Kaupp and Seifert 2002).

1.6 The Human Male Prostate Gland-a Brief Synopsis of Biology and Structure

1.6.1 Function of the Male Human Prostate

The male prostate gland is about the size and shape of a walnut, it surrounds the urethra at the neck of the bladder, weighing in at about 20g in the young adult (Kumar and Majumder 1995). The prostate is a secretory endocrine gland that contributes 0.5ml to the total 3ml of ejaculated seminal fluid (Steers 2011). These secretions are antibacterial and alkaline in order to protect the reproductive glands from infection, and protect sperm in the acidic environment of the vagina. Prostatic secretions are high in electrolytes, especially citrate, zinc, calcium, magnesium and potassium; which are important for metabolism and DNA stability of spermatozoa (Kavanagh 1985; Canale, Bartelloni et al. 1986). The electrolyte composition of prostatic fluid changes during early in PC, with a marked decrease in the levels of citrate and zinc (Costello and Franklin 2009). The serine protease prostate specific antigen (PSA) is also a constituent of prostatic secretions; it presumably acts to liquefy semen prior to ejaculation (Malm, Hellman et al. 2000).

1.6.2 Structure of the Male Human Prostate

The anatomy of the prostate gland is divided into four main zones fused together within a capsule (McNeal 1988; Cornud and Papanicolaou 2011) (figure 1.6). Three zones consist of glandular tissue, and each zone constitutes a certain proportion of the gland. These are; the peripheral (70%), central (25%), transitional (5%) and the fibromuscular anterior zone, named according to the anatomical relationship of the zone to the urethra. 60-65% of adenocarcinomas arise in the peripheral zone, <5% in the central zone, 25-30% in the transitional zone, and no pathology is found in the fibromuscular anterior zone as it is non glandular. Benign prostatic hyperplasia (BPH) arises in the transitional zone. The fibromuscular anterior zone forms a connective tissue and smooth muscle layer

that makes up the entire anterior surface of the gland (Cornud and Papanicolaou 2011). Whether prognosis can be inferred from the zone in which the carcinoma arises is unclear, but certainly, cancers arising in the transitional zone have a more favourable outcome than those from the peripheral zone, and express a number of different markers (Sakai, Harada et al. 2005).

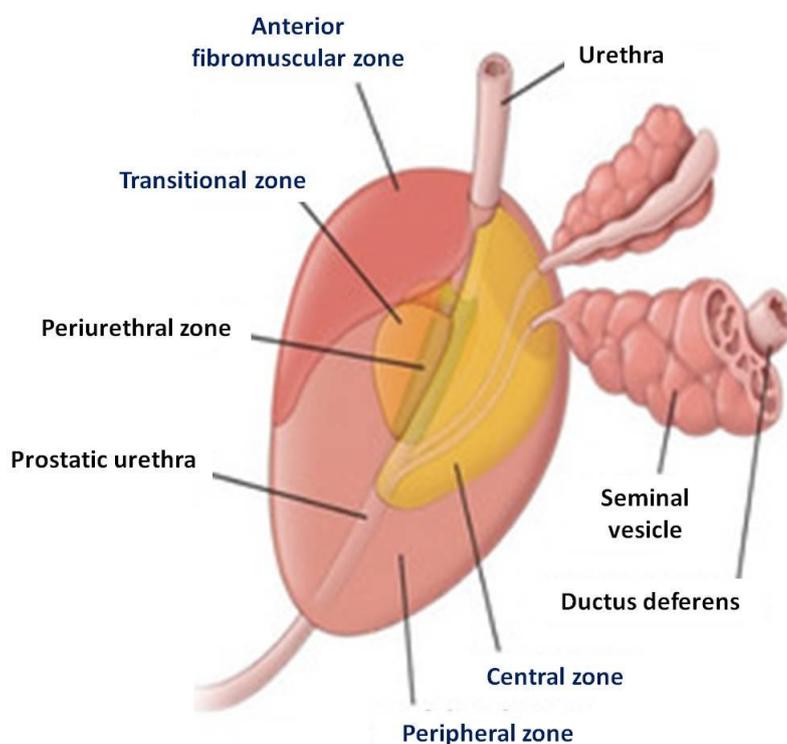


Figure 1.7. The male human prostate gland.

The gland is anatomically divided into four zones; the central, peripheral, transitional and anterior fibromuscular zones.

The prostate gland possesses two main cell types. Firstly, epithelium; which forms glands composed of secretory luminal cells, basal stem cells and neuroendocrine (NE) cells, and secondly stroma; which consists of smooth muscle and fibroblast cells and surrounds the glands (Liu and True 2002). The androgen receptor (AR) and PSA are expressed in the luminal secretory cells of all zones. The epithelium is separated from the basement membrane and stroma by the basal cells. Other cells include nerves, blood vessels and infiltrating immune cells. These cells are highly organised within the normal prostate, and this organisation becomes perturbed during normal aging and in PC (McNeal 1988; Liu and True 2002). Throughout the life of a man, the cellular make up of the prostate changes, for example, during development there is an abundance of stem cells (Schalken and van Leenders 2003). The balance of cells and histology of the prostate is paramount to the health of the gland, and is perturbed in adenocarcinoma.

1.7 Signalling in the Prostate

1.7.1 Androgens and the AR

1.7.1.1 Androgens

Testosterone has been of scientific interest for over 160 years, but was called testosterone only 77 years ago. It is mainly synthesised in the Leydig cells of the testes, but also the adrenal gland (Freeman, Bloom et al. 2001). Along with oestrogen, testosterone and its derivatives are paramount to the growth, differentiation and homeostasis of the prostate gland, and of male secondary sexual characteristics, and its importance has been demonstrated in AR knockout (ARKO) mice (Hess, Bunick et al. 1997; Kurita, Wang et al. 2001; Matsumoto, Shiina et al. 2008; Walters, Simanainen et al. 2010). Testosterone and its androgen metabolite 5 α -dihydrotestosterone (DHT) are members of the steroid hormone superfamily, and are both ligands for the AR, which acts to bring about transcription of target genes (Matsumoto, Shiina et al. 2008).

Synthesis of testosterone and DHT occurs in testes following Luteinising hormone stimulation of its cognate GPCR (Payne and Hales 2004). Figure 1.8 outlines the synthesis pathway, and how testosterone ties into the ‘female’ oestrogen signalling network. DHT possess far more potency for the AR than testosterone, with >2-5 fold increased affinity for, and >2-10 fold slower dissociation from the receptor (Toth and Zakar 1982; Kemppainen, Lane et al. 1992; Bruchovsky and Wilson 1999; Jarow, Wright et al. 2005). Testosterone and DHT activation of the AR occurs in different tissues and serves different functions (Deslypere, Young et al. 1992; George 1997; Hsiao, Thin et al. 2000). Testosterone is more important for Wolffian duct development during embryogenesis, whereas DHT mediates the development of the prostate gland and external genitalia, and virilising events during puberty (Deslypere, Young et al. 1992). Thus, two hormones activate the same receptor with quite different outcomes.

Testosterone is found in the blood stream in one of three states; bound to albumin, bound to sex hormone-binding globulin (SHBG), or unbound. To enter target cells and be metabolised to DHT or other derivatives, or access the AR, it must dissociate from any protein-binding partner; thus there is cycling between free and bound testosterone (Rosnera, Hryb et al. 1999; Vermeulen, Verdonck et al. 1999). Studies done on rats demonstrated that within cells, 90% of testosterone is rapidly metabolised to it derivatives, mostly DHT (Bruchovsky and Wilson 1968; Foley and Kirby 2003).

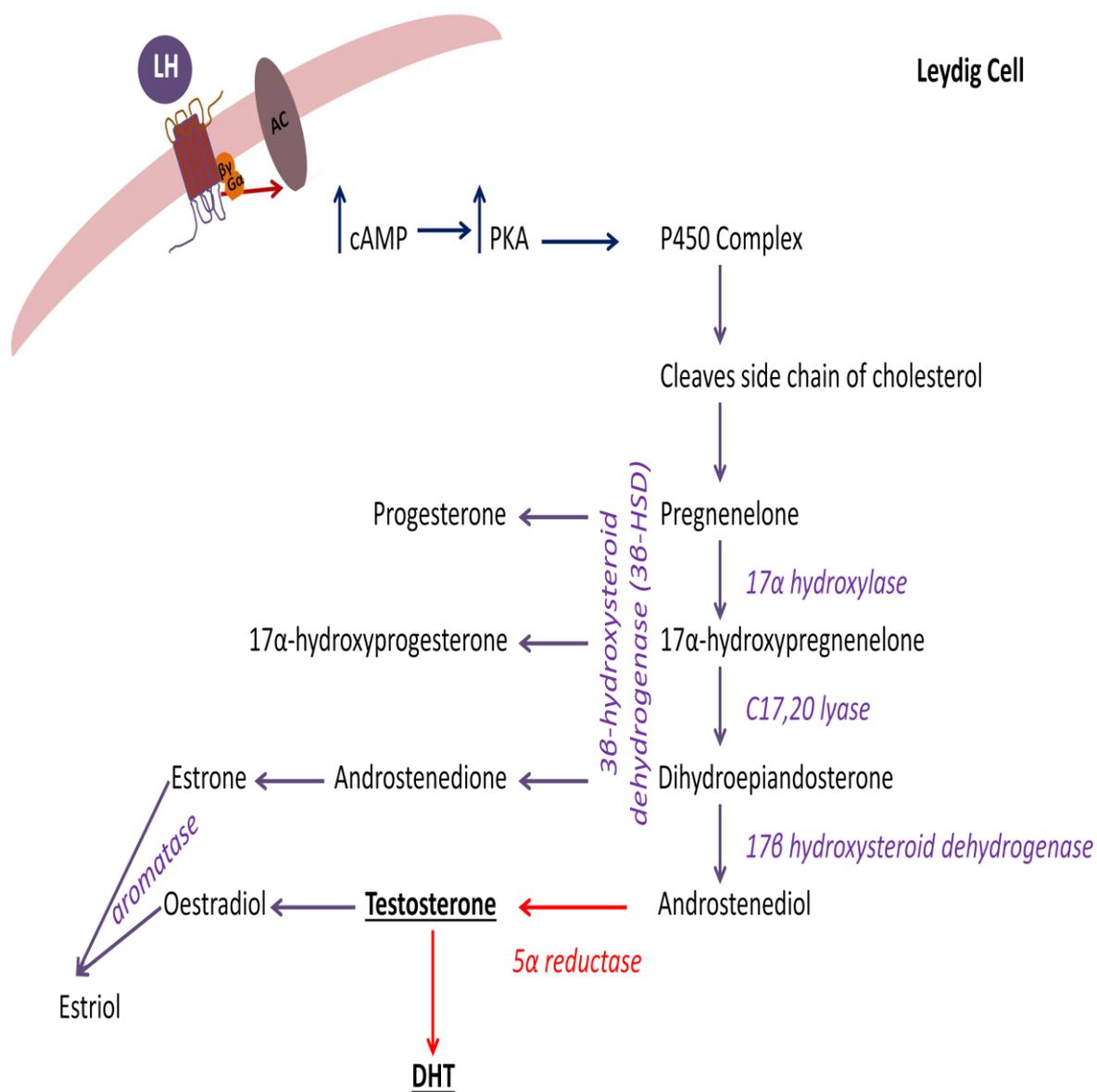


Figure 1.8. Synthesis of androgens.

The androgens; testosterone and DHT, are synthesised in the Leydig cells of the testes from cholesterol, following luteinising hormone stimulation of a cognate GPCR. DHT is synthesised from testosterone by 5 α reductase. Androgens and oestrogens are essential for homeostasis of the prostate gland.

1.7.1.2 The AR

The androgen receptor is a 110kDa protein that belongs to the steroid receptor (SR) superfamily. SRs are bound in the cytosol by heat shock proteins (HSPs). Upon binding of cognate steroid hormone, the HSPs are released, exposing the nuclear localisation signal on the SR, which then translocates to the nucleus. Here, the AR binds to androgen response elements (AREs) within target genes to bring about their transcription (Bain, Heneghan et al. 2007; Dehm and Tindall 2007). Genomic signalling following DHT or testosterone binding is the major function of the AR, but it has been shown to participate in other pathways such as that of ERK (Dehm and Tindall 2007). The AR gene is found on the long arm of the X chromosome (Xq11-X12) (Brown, Goss et al. 1989). Two isoforms of the AR, AR-A and AR-B, have been reported, with differing functions in mesenchymal tissue (Liegibel, Sommer et al. 2003). The existence of AR-B has been disputed, and is possibly an artefact of caspase-3 cleavage (Gregory, He et al. 2001), but a lot of evidence does point towards its existence (Hirawat, Budman et al. 2003). The AR structure includes a C-terminal ligand (androgen)-binding domain (LBD), followed by a hinge region which houses the nuclear translocation signal (NTS), a DNA-binding domain (DBD) and an N-terminal domain (NTD). Within the NTD and LBD are activation function 1 and 2 (AF1/AF2), respectively. AF1 and AF2 don't share sequence homology, and recruit exclusive co-activators to facilitate specific gene transcription when the AR is bound to AREs. Mutations within AR domains are linked to disease, such as PC (Glass and Rosenfeld 2000; Rosenfeld and Glass 2001; Hirawat, Budman et al. 2003; Pemberton and Paschal 2005; Wilson 2009).

When unbound, the AR exists as a monomer in the cytoplasm bound by immunophilins and many HSPs, such as HSP90. HSP90 stabilises the unbound AR, and facilitates activation following ligand binding (Georget, Térouanne et al. 2002). Androgen binding initiates release of these binding partners, major conformational change in the LBD, and the formation of homodimers via inter-molecular interactions between the LBDs and NTDs of two AR monomers (Chang and McDonnell 2005; Wilson 2009).

All classical SRs, such as the AR, can bind identical DNA response elements (DREs). However, the AR has a selective preference for AREs. The AR DBD contains two zinc finger motifs in which Zn^{2+} is coordinated by 4 cysteine residues, and these contribute to ARE selectivity (Shaffer, Jivan et al. 2004; Helsen, Kerkhofs et al. 2012). The presence of a serine in the AR DBD, head-to-head binding to the DNA rather than the usual head-to-tail, hydrogen bonding, and Van der Waals forces also contribute to AR-ARE specificity (Shaffer, Jivan et al. 2004).

Post-translational modifications of the AR are also paramount to its activation. It is subject to phosphorylation, methylation, acetylation, SUMOylation, and ubiquitination. This suggests that the SR provides a point of cross talk between androgen signalling and other signalling cascades (Gioeli and Paschal 2012). There are a number of phosphosites on the AR, mostly within the NTD. They are differentially phosphorylated depending on whether the AR is in the cytosol or the nucleus. Phosphorylation is potentiated upon androgen binding of the AR, and dephosphorylation, which can be stimulated by PKA signalling, may impair androgen binding (Blok, de Ruiter et al. 1998). Perturbation of PKA signalling crosstalk with the AR is implicated in PC, and is the subject of many investigations.

1.7.1.2.1 AR Nuclear Transport and Transcription Regulation

Ligand binding is necessary for nuclear translocation of the AR. The LBD modulates the unbound receptor; and LBD deletion mutants are constitutively nuclear with decreased transcriptional activity (Simental, Sar et al. 1991). Androgen binding causes rapid nuclear translocation. The NTS within the hinge region of the AR contains two clusters of basic residues that are essential for translocation, which is common amongst steroid hormone receptors (Jenster, Trapman et al. 1993). AR nuclear entry employs the classical β -karyopherin pathway of other SRs where the NTS binds the importin- α/β heterodimer and the AR is facilitated through nuclear pore complexes (NPCs) (Stewart 2007; Cutress, Whitaker et al. 2008). Mutations in this region are implicated in diseases, such as PC. Following transcriptional modulation, the AR exits the nucleus to re-enter

the cytosol, completing the cycle. The double strand break repair enzyme DNA dependant protein kinase (DNA-PK) directly binds the AR and acts both as a transcriptional coactivator, and to recycle the transcription machinery, thus bringing about disassembly of the AR /transcription factor complex (Mayeur, Kung et al. 2005). This stimulates AR nuclear export and may involve phosphorylation of the AR by DNA-PK, and the exportin pathway (Shank, Kelley et al. 2008). cAMP has been shown to mediate DNA-PK nuclear translocation as PKA signalling promotes its entry and retention, while EPAC/Rap2 signalling brings about its nuclear export (Huston, Lynch et al. 2008). This suggests a possible role for cAMP signalling in the nuclear/cytosol shuttling of SRs.

Once imported into the nucleus, the AR binds to AREs that contain the consensus nucleotide sequence 5'-GGA/TACNNNTGTTCT-3', where N is any base (Roche, Hoare et al. 1992). This initiates the recruitment of various transcription coactivators and basal transcription factors which form a stable transcription complex (Hirawat, Budman et al. 2003). The coactivators p160 and CBP/p300 are preferentially recruited to the PSA enhancer region, along with RNA polymerase II (RNA Pol II) (Louie, Yang et al. 2003). These coactivators possess histone acetylation activity and can recruit histone acetyl transferase (HAT), thus 'loosening' the chromatin which is necessary for transcription (Fu, Rao et al. 2004). Acetylation of the AR itself within the hinge region also modulates its activity at ARE loci (Fu, Rao et al. 2004; Lavery and Bevan 2011). Aberrant AR acetylation is implicated in PC.

1.7.1.2.2 AR Signalling Cross-Talk with Other Pathways

Phosphorylation of the AR is essential for its transcriptional activity (Wang, Liu et al. 1999). It can occur with or without the presence of steroid hormone, suggesting that AR activity is modulated by non-steroidal signalling as well as androgens (Gioeli and Paschal 2012). Such pathways may directly activate the AR or increase its affinity for low levels of androgens. MAPK, PKA, PKC, Akt and CDK pathways have been shown mediate phosphorylation of the AR (Nazareth and Weigel 1996; Guo, Dai et al. 2006; Gioeli and Paschal 2012). A number of phosphorylation sites have been identified, including serines 16, 81, 256, 308,

424, 650 and 94 (Zhu, Becklin et al. 2001; Gioeli, Ficarro et al. 2002). Steroidal and non-steroidal pathways differentially modulate the AR phospho-status. Perturbation of AR phosphorylation is associated with PC. Non-steroidal phosphorylation provides an AR activation mechanism in the absence of androgen, which occurs in androgen ablation resistance and late stage androgen insensitive prostate cancer (AI PC) (Nazareth and Weigel 1996; Gioeli, Ficarro et al. 2002; Guo, Dai et al. 2006) (figure 1.9).

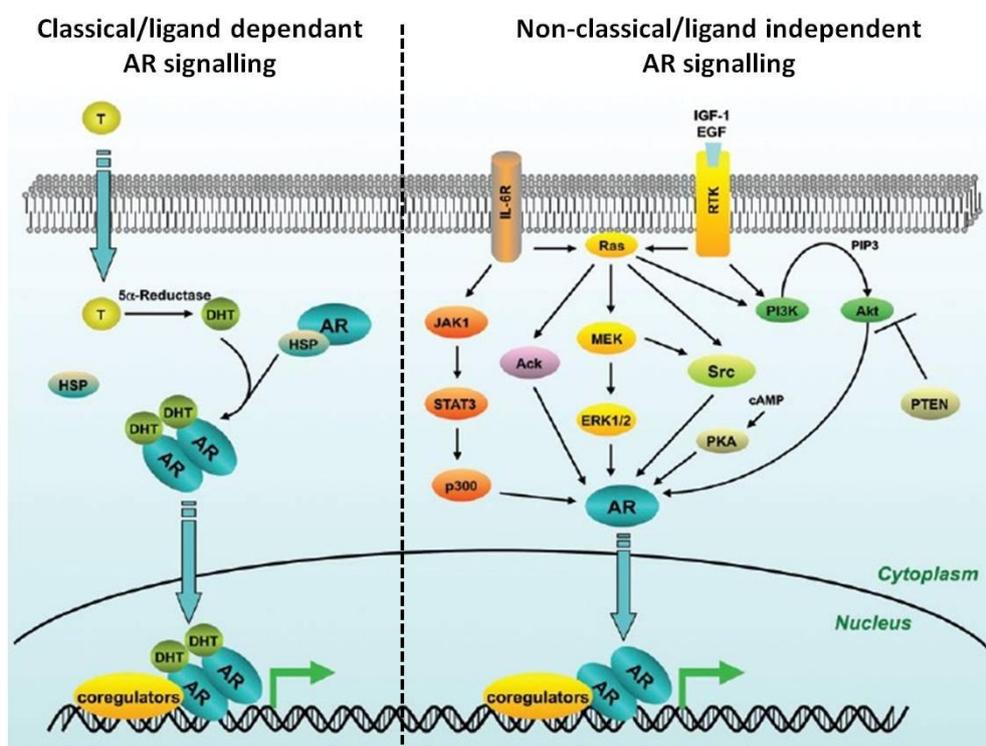


Figure 1.9 AR signalling.

The AR can be classically activated by ligand (testosterone/DHT) or non-classically by crosstalk with other signalling pathways, such as cAMP/PKA. Ligand-independent activation via crosstalk with these pathways is proposed to play a major role in the development and progression of AI PC. (adapted from (Lonergan and Tindall 2011)).

1.8 Prostate Pathogenesis- Benign Disease and Prostate Cancer

1.8.1 Benign Prostatic Hyperplasia (BPH)

Of all mammals, only humans and dogs develop BPH and PC (De Marzo, Coffey et al. 1999). A high fat diet plays a huge role in these pathologies, and humans quickly took on a diet high in animal fat following our descent from the trees. Population studies show that similar endocrine and dietary factors are associated with BPH and PC (Guess 2001). Risk factors also include other lifestyle choices such as smoking, race, ethnicity, familial history and especially age (Ziada, Rosenblum et al. 1999). BPH presents with LUTS and is the commonest benign tumour in men over 60 (Ziada, Rosenblum et al. 1999). While PC is uncontrolled proliferation of genetically-abnormal cells, BPH involves hyperplasia of 'normal' epithelial and stromal cells within the transitional zone of the prostate gland, leading to enlargement of the prostate (De Marzo, Coffey et al. 1999). Whether or not BPH leads to PC has been a topic of controversy, but most evidence suggests it rarely, if ever, is a causative factor. Indeed, there is significantly different gene expression between the two pathologies (Luo, Duggan et al. 2001), but it must be remembered that BPH and PC occur in different regions of the prostate gland that would normally display differential gene expression (Lee and Peehl 2004).

Infection and Inflammation also play a role in BPH (Penna, Fibbi et al. 2009). Prostatitis is the original name for prostate complaints, and is also an inflammation of the gland. BPH and prostatic inflammation (prostatitis) both cause an increase in PSA, which leads to problems when trying to detect cancer (Nadler, Humphrey et al. 1995). The molecular mechanisms underlying BPH are ill understood due to the heterogeneity of the disorder, and it is not always distinguishable from prostatitis (Nickel 2008). Many molecular events seem to underpin the development of BPH, but due to the variety of factors that this umbrella term describes, much work has to be done to better understand BPH (Lee and Peehl 2004).

1.8.2 Prostate Stem Cells and Senescence

The prostatic epithelium contains the basal and luminal layers, which are composed of basal, secretory and neuroendocrine cells and differ in their gene expression and morphology (Miki and Rhim 2008). The AR and PSA are expressed in the luminal secretory cells (Liu, True et al. 1997). All epithelial tissues undergo continual cell replacement due to damage or cell death. They are able to do this due to adult stem cells that have the ability to self-renew and differentiate into cells of their tissue of origin, thereby maintaining tissue homeostasis (Blanpain, Horsley et al. 2007). The wnt, Sonic and Notch signalling pathways are important in this process and perturbations of these pathways in prostatic stem cells (PSCs) have been linked to prostate tumorigenesis (Lawson, Xin et al. 2005). With age, less regeneration occurs, and epithelial cells become replicatively senescent. This may be a mechanism to decrease transcription of proto-oncogenes, and indeed many cells become cancerous because they bypass senescence. However, an amassing of senescent PSCs may be a causative factor in BPH. These cells display an upregulation of cytokines, which are secreted and act as paracrine factors on non-senescent, neighbouring cells, driving their proliferation and increasing the prostate mass (Castro, Giri et al. 2003; Castro, Xia et al. 2004). Senescent aging cells also show resistance to apoptosis and in this way may directly increase the prostate mass. A number of reasons for this have been suggested, such as constitutive expression of the proto-oncogene Bcl2 (Wang 1995), aberrant cell cycle regulation (Schwarze, Shi et al. 2001), and downregulation of pro-apoptotic Bad (Untergasser, Koch et al. 2002).

1.8.3 The Intra-prostatic Immune System; Prostatitis and Inflammation

There is much evidence to suggest that inflammation is a major causative factor of BPH and PC, and the development of metastases (Drake 2010). Prostatitis is chronic inflammation of the prostate. It presents with LUTS and pelvic pain and often overlaps with BPH, where men may have both. Additionally, men with a

history of BPH are more likely to later develop prostatitis. Unlike BPH, prostatitis can affect men of all ages. Factors such as stress, vasectomy, a previous STD and other bacterial infections are associated with prostatitis. However, it can be difficult to distinguish between BPH and prostatitis due to symptom overlap and lack of gold standard diagnostics (Collins, Meigs et al. 2002; St. Sauver, Jacobson et al. 2008).

The prostate seems to possess an intra-immune system, much like the lungs and gut. Normally it is composed of small numbers of mainly CD4⁺ and CD8⁺ T cells, macrophages and B lymphocytes. With age, the immune cell complement ratio changes, with an increase in T cell infiltrates, and this is particularly seen in BPH. The changing testosterone to oestrogen ratio that occurs with age may also upset the immune infiltrate of the prostate. There is also an increase in interferon and cytokine production, which causes increased proliferation of BPH cells, essentially chronic 'wound healing'. The cause of chronic inflammation and BPH is unclear, and may or may not be due to infection, or indeed an autoimmune mechanism (Kramer, Mitteregger et al. 2007; Fibbi, Penna et al. 2010).

Perturbations in the intraprostatic immune cell infiltrate are also associated with PC, and can be caused by androgen deprivation therapy (ADT). An increase in the level of T cells, particularly CD4⁺, and macrophages is observed within primary tumours following ADT (Mercader, Bodner et al. 2001; Gannon, Poisson et al. 2009). Post-ADT inflammation is common but the cause is unclear. It seems that circulating androgens play an immunosuppressive role in the normal prostate. TLR4 is a main player in innate immune responses. Its expression has been observed to be increased in prostatic epithelial and stromal cells following castration (Quintar, Roth et al. 2006), and TLR4 signalling in the prostate has been shown to be pro-inflammatory (Gatti, Quintar et al. 2009). A SNP in the 3'UTR of the TLR4 gene has also been suggested to be a susceptibility factor for PC (Zheng, Augustsson-Balter et al. 2004). TLR4 and NFκB signalling following viral or bacterial infection of the prostate, results in an increase in epithelial proliferation (Kundu, Lee et al. 2008)

Aberrant cytokine and cognate receptor expression is associated with the pathophysiology of PC. The pro-inflammatory cytokines IL-1 α , IL-6 and its receptors, and TNF α have been shown to be upregulated in BPH and PC, and their expression correlates with that of PSA and tumour progression (Bouraoui, Ricote et al. 2008). These cytokines, along with IL-8, are also involved in PC associated cachexia, an ill understood wasting disease associated with many cancers. The cytokine profile of PC changes as the cancer progresses and between patients (with and without cachexia) in a manner that can distinguish PC stage (Tazaki, Shimizu et al. 2011). IL-8 has also been shown to be constitutively and abundantly expressed in AI PC, correlating with cancer progression, angiogenesis and metastasis. It acts via AKT and NF κ B to bring about downregulation of pro-apoptotic proteins and upregulation of anti-apoptotic proteins, and it also renders AI PC cells resistant to ADT (Ma, Ren et al. 2009; Singh and Lokeshwar 2009).

The intra-prostatic immune system thus plays a huge role in the risk and development of PC, and may be a target for pharmacological intervention (Drake 2010).

1.9 Prostate Cancer

Second to lung cancer, PC is the commonest cancer in men, and the second leading cause of all cancer related deaths in the US. 238,590 new cases and 29,720 deaths due to PC are predicted for 2013 in the US. Throughout a man's life, the chance of getting PC is 1 in 6. The disease is more prevalent in African American men than any other race (Siegel, Naishadham et al. 2013). In the UK, over 10,000 men died due to PC in 2010 (Cancer Research UK). With our aging societies there is a great need to better understand the underlying mechanisms of PC, to identify more specific biomarkers, and to develop tailored anti-cancer therapies.

PC shares many features with BPH and they often go hand in hand, but whether or not BPH is causative is unclear. Despite the biochemical similarities, such as

dependence on the AR (Berry, Maitland et al. 2008), differing histology and anatomy suggest BPH is not linked to PC. BPH arises in the transitional zone stroma, whereas PC most commonly originates in the epithelium of the peripheral and central zones (Schenk, Kristal et al. 2011). There is no obvious staged progression of PC because tumours display very high heterogeneity, within the same patient and between patients (Liu, Roudier et al. 2004; Lamont and Tindall 2011). This of course, makes detection and treatment difficult. However, chronic inflammation does play a role in the progression of both BPH and PC (De Nunzio, Kramer et al. 2011).

1.9.1 Steroid Signalling in PC and BPH

AR signalling is essential for development of the prostate and sex organs, bone and male secondary sexual characteristics (Matsumoto, Shiina et al. 2008). However, during prostatic pathologies, AR signalling becomes perturbed, upsetting the balance between proliferation and apoptosis (Merkle and Hoffmann 2011). AR signalling is necessary for early stage PC (AS PC) and the progression into AI PC, even after ADT (Kaarbo, Klokk et al. 2007). As discussed, the AR crosstalks with a number of other signalling pathways such as PKA and PI3K. During PC progression, these networks become perturbed leading to aberrant gene expression (Kaarbo, Klokk et al. 2007; Desiniotis, Schafer et al. 2010; Merkle and Hoffmann 2011). Studies have shown differential gene expression between AS PC, AI PC, and during ADT, including those involved in androgen metabolism and upregulation of the AR (Holzbeierlein, Lal et al. 2004; Chandran, Ma et al. 2007). As well as upregulation of the AR and an increase in its activity, there is significant change in the expression of AR pathway genes as the disease evolves from AS to AI PC. A number of genes involved in adhesion, differentiation and apoptosis are downregulated from AS to AI PC (Hendriksen, Dits et al. 2006). PKA has been shown to be involved in the AR regulation of gene expression in androgen derived cells, suggesting it's a key player in AR reactivation following ADT (Wang, Jones et al. 2006).

Oestrogenic signalling is also necessary for the development of the prostate (El-Shafei, Mostafa et al. 2011) but can be perturbed in BPH and PC (Ellem and Risbridger 2010). The androgen: oestrogen balance is essential for prostatic homeostasis, but this changes with age, leading to a decreased testosterone to oestrogen ratio (Schatzl, Brössner et al. 2000). There are many studies on the affects of oestrogens in BPH and PC but with varied results.

Estrogen receptor-related receptor α (ERR α) is an orphan receptor whose endogenous ligands are unknown. It is activated by environmental chemicals with oestrogen-like activity. It is expressed in the prostate and is important during embryogenesis, but overexpression of EER α has been observed in PC (Miao, Shi et al. 2010). PGE2 has been shown to cause an upregulation of EER α and subsequent increase in aromatase expression, leading to high oestrogen levels in the prostate (Miao, Shi et al. 2010). Indeed, a synergism between overexpressed androgens and oestrogens may be involved in both BPH and PC progression (Roberts, Jacobson et al. 2004; Ricke, McPherson et al. 2008). Perhaps changes in the levels of one hormone leads to compensatory expression of the other, disturbing the steroid hormone equilibrium and homeostasis. Melatonin has also been shown to regulate the oestrogen-androgen balance, and this is perturbed in BPH (Gilad, Matzkin et al. 1997). Further evidence for oestrogens playing a role in PC is seen by the oestrogen-induced upregulation of PSA in PC cells in the absence of androgens (Nakhla, Romas et al. 1997). Some studies suggest a high level of estradiol is anti-proliferative, while other studies suggest the opposite. This may be due to expression of two oestrogen receptors, ER α and ER β , which mediate different cellular responses (Yao, Till et al. 2011). It seems likely that the balance of signalling via these receptors is perturbed during PC. Yao et al. found no correlation between oestrogen and a risk of PC in normal prostates, but did find an upregulation of oestrogens in men who have taken the BPH drug finasteride, and they suggest this creates a low-moderate risk of PC. The breast cancer drug toremifene which inhibits oestrogen receptors has been shown to prevent the progression of PC (Raghow, Hooshdaran et al. 2002; Price, Stein et al. 2006). Clearly the role of oestrogens in the prostate and prostatic pathologies is complex and highly ambiguous.

Stromal-epithelial cell interaction is also very important in prostatic homeostasis. It involves ER α and ER β , which are exclusively expressed in the stroma and epithelium, respectively (Risbridger, Wang et al. 2001). BPH and PC tissues exhibit an increase in these receptors compared to normal prostate, and there is expression of ER β in the stroma in PC only, again suggesting oestrogens are involved in hyperplasia and cancer progression (Royuela, de Miguel et al. 2001). Many studies suggest ER β mediates anti-proliferative signals, while ER α mediates pro-proliferative signals. Indeed ER β KO mice display prostatic hyperplasia (Krege, Hodgin et al. 1998). ER β has also been shown to be downregulated in PC due to hypermethylation, and its re-expression induces apoptosis (Walton, Li et al. 2008). Differential expression of these receptors between normal and diseased prostate tissue, and perturbation of the ratio of these receptors in prostatic stroma and epithelia, would clearly upset the prostatic oestrogen balance, and likely plays a role in the progression of both BPH and PC. This makes these receptors and indeed oestrogen a promising therapeutic target.

Despite the involvement of oestrogens, the AR and androgens still underpin prostatic pathologies, and the AR is still the main therapeutic target in PC (Massie, Lynch et al. 2011). Within PC cells, AR signalling brings about a coordinated system of transcriptional alterations. The AR is essential for tumourigenesis and progression into AIPC (Taplin and Balk 2004).

Androgen deprivation therapy (ADT) and AR antagonists have long been used in the treatment of BPH and PC to inhibit cell proliferation and LUTS. However, a lot of drugs block testosterone, which produces undesirable side effects such as impotence. The advent of 5 α -reductase inhibitors (5- α RI), such as finasteride, showed promise due to their more specific mode of action (Stoner 1990). Finasteride inhibits testosterone catabolism to DHT, therefore reducing levels of the more potent androgen and thus AR stimulation, while not affecting testosterone levels. 5- α RI have since been very successful in treating LUTS and BPH (Robinson, Garmo et al. 2013).

There are two isoforms of 5 α -reductase, 5 α R1 and 5 α R2. Finasteride inhibits 5 α R2, as it was originally thought to be the main player in testosterone metabolism. However, there is an upregulation of 5 α R1 and a downregulation of 5 α R2 along with decreased activity, in early PC. And both isozymes are upregulated in localised high-grade compared to low-grade PC and BPH (Thomas, Douglas et al. 2008; Thomas, Douglas et al. 2008). This is another example of a balance being perturbed and adds to the complexity of BPH and PC. It also explains why finasteride loses potency, and suggests that a dual inhibitor strategy may be the way forward.

High serum testosterone/estradiol levels and a high testosterone:17 β -diol-glucuronide ratio have been associated with a decreased risk of BPH (Kristal, Schenk et al. 2008). This suggests a reduction in 5 α -reductase correlates to reduced BPH risk. Indeed, population studies done on pre-BPH healthy men, with follow-up, found a correlation between BPH risk and high DHT serum levels (Parsons, Palazzi-Churas et al. 2010; Liao, Li et al. 2012). The DHT/testosterone ratio remains constant or changes with age, suggesting an increase in 5 α -reductase activity as men age.

Genetic abnormalities of 5 α -reductase type 2 have also been linked to BPH and PC. Although rare, polymorphisms in the type 2 gene have been associated with an enlarged prostate gland and a predisposition to both BPH and PC (Roberts, Bergstralh et al. 2005; Salam, Ursin et al. 2005). Hypermethylation of the 5 α -reductase type 2 gene promoter region has been suggested to account for the lack of expression of this enzyme in 25-30% of prostate cancers (Niu, Ge et al. 2011). This may explain the decrease in type 2 in early PC seen by Thomas et al., and a compensatory upregulation of type 1. It would also explain the resistance to finasteride seen in a number of BPH and PC patients. Continuous finasteride treatment for BPH has been linked to neuroendocrine differentiation (NED), which is associated with aggressive PC and poor prognosis (Tarle, Spajic et al. 2009). NED plays a major role in PC progression. These cells release neuropeptides that act in an autocrine fashion via GPCRs to promote growth and aggressiveness. The neuropeptides can activate tyrosine kinases such as Src, Lyn

and FAK which cause activation of the AR, supporting androgen independence (Yang, Ok et al. 2009).

1.9.2 Other Genetic Abnormalities in Prostate Cancer

A number of chromosomal deletions and rearrangements are associated with PC. Deletions at 5q are common and become more frequent as tumours advance (Dong 2001). Rearrangements and deletions in chromosome 5 are present in AI PC cell lines, but not in the AS LNCaP cell line. 5q11 is the most common break point (Ozen, Navone et al. 1998). It has been suggested that tumour suppressor genes reside on 5q. This is interesting given that PDE4D is also located on 5q12.

1.9.2.1 TMPRSS2:ERG

One of the commonest genetic abnormalities in PC is the TMPRSS2 and Ets fusion. TMPRSS2 is an androgen responsive transmembrane serine protease that is highly expressed in normal prostatic epithelium, and Ets genes are a family of oncogenic transcription factors (Lin, Ferguson et al. 1999; Kumar-Sinha, Tomlins et al. 2008). The fusion causes overexpression of the Ets isogene; ERG, ETV1 or ETV4 (Tomlins, Rhodes et al. 2005; Macaluso and Giordano 2007). TMPRSS2-ERG is the commonest fusion event, observed in 70% of clinically localised cancers (Mehra, Tomlins et al. 2007). It has shown promise as a biomarker for PC, detectable in urine and far more specific than PSA (Nguyen, Violette et al. 2011; Tomlins, Aubin et al. 2011). The TMPRSS2-ERG gene contains AREs, and the protein product is upregulated via AR signalling, and this also brings about an upregulation of wild-type (wt) ERG (Mani, Iyer et al. 2011).

TMPRSS2 and ERG are both located on chromosome 21q, and the chimera is most commonly caused by deletion of the region in between them, and less commonly due to reciprocal translocation. There are a possible 14 fusion variants, with T1/E4 being the most common (Tu, Rohan et al. 2007). The fusion is composed of the 5' promoter region of TMPRSS2 and the coding region of ERG, thus a

fusion protein is not translated, but most commonly a truncated ERG (T1/E4) (Fernandez-Serra, Rubio-Briones et al. 2011). The truncated ERG brings about aberrant upregulation of its target genes, such as histone modifying enzymes. Prostate tumours that contain the TMPRSS2-ERG chimera are considered to be in a different group to those without. They display very different altered proteomes, with an upregulation of genes involved in oestrogen receptor signalling in those containing the fusion event (Setlur, Mertz et al. 2008). Studies looking at the correlation between the TMPRSS2-ERG and patient outcome have been discordant, but most suggest that tumours harbouring high copy numbers of the chimera are associated with poor clinical outcome compared to those not expressing the fusion, and its presence may be used as a prognostic tool when combined with PSA value and Gleason score (Attard, Clark et al. 2008; Fine, Gopalan et al. 2010). The TMPRSS2-ERG fusion may occur in some tumour foci, but not in other foci, within the same patient, giving credence to the idea of independent origin of separate tumours, which can have different Gleason scores (Furusato, Gao et al. 2008). The fusion is more prevalent in early stage PC compared to high grade aggressive disease, and has been identified in benign lesions and prostatic intraepithelial neoplasia (PIN-widely believed to be a precursor of PC), thus it is likely involved in the onset and/or progression of PC (Perner, Mosquera et al. 2007; Furusato, Gao et al. 2008).

1.9.2.2 **TMPRSS2:ERG and PTEN**

Despite the above observations by Perner et al. and Furusato et al., TMPRSS2:ERG chimeras have been less commonly found in PIN lesions than in PC (Carver, Tran et al. 2009; Carver, Tran et al. 2009; King, Xu et al. 2009). This suggests the fusion event is not a driving force for PC progression. King et al. interestingly found that TMPRSS2:ERG fusion occurs alongside loss of PTEN, the other commonest event in PC. They propose that cooperation of ERG overexpression and PI3K pathway activation in PIN lesions contribute to progression to adenocarcinoma. TMPRSS2:ERG and PTEN loss have been shown to be additive, possibly due to a coordinated increase in epithelium to mesenchymal transition (EMT), which confers motility, and the occurrence of

both events correlates with a reduced time to biochemical recurrence of PC following treatment (Yoshimoto, Joshua et al. 2008; Carver, Tran et al. 2009).

1.9.2.3 AR Mutations

Although rare in untreated PC, a number of AR genetic and epigenetic abnormalities are found in castration resistant PC (CRPC) (AI PC; following ADT). These events mainly fall into 3 categories; (1) AR gene amplification and AR overexpression, which sensitises cells to low levels of androgen and occurs in a third of CRPC cases; (2) point mutations that render the receptor promiscuous, and (3) AR splice variants and truncations that lead to androgen-independent receptor activation (Waltering, Urbanucci et al. 2012). The best studied point mutation is T877A, originally identified in LNCaP cells (Veldscholte, Ris-Stalpers et al. 1990). It mutates the binding pocket, conferring promiscuity on the AR, where even anti-androgen drugs become agonistic, leading to androgen withdrawal syndrome (AWS) (Hara, Miyazaki et al. 2003; Sun, Shi et al. 2006; Waltering, Urbanucci et al. 2012). AWS makes targeting the AR in CR/AI PC extremely complex and difficult. Downstream signalling molecules which may provide better therapeutic targets are becoming the focus of much research (Waltering, Urbanucci et al. 2012).

1.9.3 Androgen-Independent Prostate Cancer

ADT is successful when initially administered, with a reduction in tumour size due to induced apoptosis of cancer cells. However, following treatment the PC invariably reappears, and this constitutes the aggressive androgen-independent/castration resistant-prostate cancer (AI PC/CRPC). AI PC comes about because the PC cells adapt to the androgen-deprived microenvironment and in fact thrive in such conditions (Dutt and Gao 2009). It is suggested that androgen-independence comes about in two stages, called clonal evolution. Stage one involves a transition of a subset of tumour cells, where they remain androgen-responsive but not dependent on androgens. These cells may originally

require androgen for growth, but not survival. Stage two involves clonal selection of such cells and outgrowth of an androgen-independent tumour (Craft, Chhor et al. 1999). PC tumours are highly heterogeneous, even in the same patient. Metastatic tumours from different sites within the same patient can possess different transcriptomes, suggesting that late stage PC represents a heterogeneous group of diseases. AR negative and AR positive tumours can exist in the different sites. Despite being termed androgen-independent cancer, the belief is that progression into AI disease is still reliant on the AR (Shah, Mehra et al. 2004).

1.9.4 Suggested Mechanisms for Progression into Androgen Insensitive Prostate Cancer

Although the development of AI disease is ill understood, several mechanisms have been suggested.

1.9.4.1 Selection for Androgen-Independence - Stem Cells

As discussed earlier, epithelial stem cells are an important part of the basal layer of the prostate (Lawson, Xin et al. 2005). Unlike neighbouring epithelial cells, they do not express the AR and thus are out-with the normal androgen signalling axis. They are quiescent and can self renew, making them valid escapees of ADT (Nikitin, Nafus et al. 2009). These cells are found in primary and metastatic prostate tumours. They undergo extensive proliferation and have the ability to differentiate into the tumour cells from which they were derived, thereby carrying mutations (Collins, Berry et al. 2005). The idea that these androgen-independent cells are selected during ADT is supported by the overexpression of stem and basal cell genes in AI PC, such as Bcl2 (Lawson, Xin et al. 2005). Indeed upregulation of Bcl2 seems to be a main player in the transition to AI disease. Bcl2 overexpression occurs as a result of ADT and subsequent selection of stem cells, and its overexpression then allows cells to rapidly proliferate in the absence of androgens (Catz and Johnson 2003). Recent

evidence suggests that selection of stem cells may be a very early event following ADT, and these may be cancer initiating cells (CICs) (Finones, Yeargin et al. 2013). The AR is expressed in most AI tumours, and is lost only in very late stage metastatic disease (Balk 2002), therefore prostate tumours are not solely composed of aberrant stem cells, and as stated earlier, are highly heterogeneous.

1.9.4.2 AR Amplification

Amplification of the AR gene, resulting in overexpression of the AR is a common feature of prostate tumours following ADT. It sensitises the cells to low levels of androgens enabling them to maintain AR signalling in an androgen deprived environment (Visakorpi, Hyytinen et al. 1995).

1.9.4.3 Non-steroidal AR Signalling

The early stages of ADT are successful in repressing AR signalling. However, a host of other signalling pathways have been suggested to cross-talk with the AR, and may act to stabilise the receptor and its transcriptional activity, and compensate for AR signalling during ADT (Shafi, Yen et al. 2013). Indeed, some signalling pathways have been found to be upregulated in AI PC, suggesting the cells try to compensate for lack of AR signalling, or they may enable AR activation by residual levels of androgens.

Both PKA and NF κ B/p52 can activate the AR in the absence of androgens by interacting directly with NTD of the AR, enhancing its translocation and recruiting coactivators, thereby bringing about transcription of genes such as PSA (Nazareth and Weigel 1996; Sadar, Hussain et al. 1999; Nadiminty, Lou et al. 2010).

As discussed earlier, inflammation and cytokine signalling are important factors in PC progression. Upregulation of the atypical protein kinase C (aPKC), which is

involved in epithelial signalling, brings about an concomitant upregulation and secretion of IL-6, thereby contributing to AI PC progression via autocrine and paracrine signalling (Ishiguro, Akimoto et al. 2009; Malinowska, Neuwirt et al. 2009). IL-6 overexpression can activate the AR and is also associated with distant metastases, particularly bone, and poor patient outcome (Hobisch, Eder et al. 1998; Shariat, Andrews et al. 2001). Studies on the mechanism of IL-6 action have been confusing as it has been shown to activate the AR NTD in a PKA independent, MAPK-mediated process (Ueda, Bruchovsky et al. 2002), and to mediate NED in a PKA- and MAPK-dependent manner (Deeble, Murphy et al. 2001).

As discussed earlier, IL-8 is also an important cytokine in PC pathogenesis. It has been shown to increase AR expression and activation in an androgen free environment, possibly mediated by NF κ B (Seaton, Scullin et al. 2008). Interferon (IFN) has also been shown to increase protein levels of the AR by stabilising it, with consequent nuclear accumulation and upregulation of AR-responsive genes (Chen and Sawyers 2002; Basrawala, Alimirah et al. 2006). Down-regulation and polymorphisms of the anti-inflammatory IL-10 are positively associated with PC (Faupel-Badger, Kidd et al. 2008).

These are just some examples of how cytokine signalling loops can act on the AR signalling pathway and mediate transcription of androgen-regulated genes. Growth factor (GF) signalling is also important in PC progression. Stromal fibroblasts secrete GFs which bring about autocrine and paracrine proliferative loops, an important process in many cancers (Bhowmick, Neilson et al. 2004). Epidermal, insulin-like, transforming, fibroblast, and vascular endothelial growth factors (EGF, IGF, TGF β , FGF and VEGF, respectively) and their cognate receptors all crosstalk with the AR signalling axis (Zhu and Kyprianou 2008). The AR has been shown to participate in a signalosome involving EGF kinase members, in which there is bidirectional regulation of their transduction pathways (Migliaccio, Castoria et al. 2010). Additionally, the EGF receptor (EGFR) is overexpressed in AI PC (Di Lorenzo, Tortora et al. 2002; Gan, Shi et al. 2010). Upregulation of EGFR can lead to increased secretion of cytokines, such as IL-6, forming positive autocrine and paracrine feedback loops,

thereby promoting PC progression (Sauer, Gitenay et al. 2010). Indeed, treatment of PC cells with either EGF or IL-6 resulted in Src and Ack1 kinase-mediated phosphorylation of the AR, its consequent activation and cell proliferation (Liu, Karaca et al. 2010). EGF/EGFR signalling is suggested to be involved in the transition from AS to AI PC, to be correlated to poor patient outcome, and to be a potential therapeutic target (Di Lorenzo, Tortora et al. 2002; Schlomm, Kirstein et al. 2007). Transactivation of the AR by EGF shows how this steroid receptor acts as a signalling hub, allowing crosstalk between different cascades, which promote PC progression if perturbed.

Nerve growth factor (NGF) signalling via one of its cognate receptors; p75^{NGFR}, is anti-proliferative and important in prostate homeostasis. Expression of NGF and its p75 receptor is lost as PC progresses, and this also contributes to the AI phenotype (Sigala, Bodei et al. 2008).

1.9.5 Prostate Cancer Diagnosis and Treatment

The risk factors for PC are not well understood, but increasing age, ethnicity and heredity are three well known factors (Heidenreich, Bellmunt et al. 2011).

The clinical symptoms of PC often do not show until the disease is advanced and metastatic, therefore, early detection is important and much research is focused on finding specific PC biomarkers for early disease. Prostate specific antigen (PSA) has been used as a biomarker since the 1980s, and has greatly contributed to the early detection of PC. However, although highly tissue specific, it is not disease-specific and the level of circulating PSA can be raised in benign prostatic disease. Therefore, using PSA as a diagnostic tool is controversial, and has led to over diagnosis and over treatment of what would otherwise have been harmless cancers. Despite this, the discovery of PSA as an indicator of disease paved the way for much research into our understanding of PC and treatment regimes. (Stamey, Yang et al. 1987; Lilja, Ulmert et al. 2008).

1.9.5.1 Diagnosis

In the pre-PSA years, PC detection was based on a trans-rectal ultrasound (TRUS) along with an invasive biopsy, at which stage the cancer had most likely spread and was incurable (Jacobsen, Katusic et al. 1995). Following PSA testing, there was a huge increase in the incidence of PC (Potosky, Miller et al. 1995). PSA screening has proved successful in decreasing PC mortality, however only modestly so, and whether or not this outweighs the problem of over detection/treatment is of constant debate (Hugosson, Carlsson et al. 2010). The threshold level of PSA is also being debated. A study using a lower threshold, found lower PC mortality, but higher over diagnosis, and the exact cut off of normal PSA level is unclear (Heidenreich, Bellmunt et al. 2011).

The latest European Association of Urology (EAU) guidelines for PC cancer detection state that PSA screening and digital rectal exam be the first port of call. Following this, TRUS-guided repeated biopsies should be carried out to obtain histological information. PSA level should also be continually monitored (Heidenreich, Bellmunt et al. 2011). If a tumour is confirmed, it is then given a Gleason score (Shah 2009). This score enables the clinician to predict the outcome of the cancer and to determine what treatment, if any, is most suitable. The tumour is assigned a score of 1-5, where 5, represents the most advanced tumour with complex morphology. The tumour is not assigned one grade, but the sum of the most prevalent and the second most prevalent morphological pattern within that tumour. The resulting number (2-10) is the Gleason score for that tumour, and is a strong indicator of survival of men aged 65-75 (Albertsen, Fryback et al. 1995). However, multifocal prostate tumours may have different Gleason scores in different foci, which makes assigning a grade problematic, and consequently, scores may differ between clinicians (Shah 2009).

Over the years, PC care has evolved immensely, with earlier and more effective detection and improved treatments regimes resulting in decreased PC-specific mortality (Heidenreich, Bellmunt et al. 2011)

1.9.5.1.1 Prostate Specific Antigen

PSA is member of the kallikrein family of proteases, it acts to liquefy the semen in order to facilitate ejaculation (Malm, Hellman et al. 2000). PSA is highly tissue specific, with little or no expression elsewhere. It is androgen-regulated and is therefore increased in PC due to increased AR activity (Cleutjens, van Eekelen et al. 1996). This increase, along with a compromised prostate architecture allows extra PSA into the circulation (Barry 2001). As discussed, blood PSA is also raised during benign prostatic diseases, and so this method of detection is very controversial. Also, the threshold level that should prompt a biopsy is unclear, but >4ng/ml blood is the general consensus for a raised PSA level (Barry 2001). However, PC can be present with PSA values below 4ng/ml, and levels above this do not mean a cancer is definitely present, especially in older men.

Although not perfect, PSA testing is widely used as it is simple and non-invasive. Consequently, this has revolutionised patient care. It has allowed for early diagnosis of cancers that otherwise would have presented at an incurable stage, and allows monitoring of indolent cancers. Lead time of a disease is the time gained by screening, the amount of time between the screen and when the disease would otherwise have presented, i.e. with symptoms. The lead time of PC due to PSA screening with a cut off of 4ng/ml has been shown to be between 4-8 years (Finne, Fallah et al. 2010). This provides a vast window of opportunity to implement treatment that otherwise may not be effective and this aspect has resulted in >30% reduction in PC-specific mortality (Roobol, Kerkhof et al. 2009; van Leeuwen, Connolly et al. 2010). The diagnosis of PC, while still organ confined, has also improved biochemical recurrence-free survival, i.e. it has reduced re-emergence of the cancer, again due to improved therapeutic outcome due to the lead time (Han, Partin et al. 2001).

Despite the favourable outcome for many patients, overdiagnosis is a huge problem. It constitutes a large percentage of the lead time through diagnosis of cancers that otherwise would never have presented due to being clinically insignificant and non-life threatening (Draisma, Etzioni et al. 2009). Such overdiagnosis results in men having inappropriate treatment with severe consequences such as impotence, and puts extreme financial pressure on health

care systems. This is why population-based PSA screening is rare, and is not carried out in the UK (van Leeuwen, Connolly et al. 2010).

One study suggests that underdiagnosis more commonly occurs than overdiagnosis (Graif, Loeb et al. 2007). Lowering the PSA threshold for recommending biopsy to 2.5ng/ml decreased underdiagnosis and increased the number of men with 5 year progression-free survival (PFS). However, using this PSA cut off increased the rate of overdiagnosis.

Currently, 15 novel biomarkers are undergoing evaluation as predictors of AI disease (Klotz 2013). Of these PCA3 is becoming widely used. Prostate cancer gene 3 (PCA3) is a non-coding mRNA that is specifically overexpressed in PC and can be detected in urine following prostate massage. Its usefulness as a standalone biomarker has been debated, but it has been successful when tested alongside or following PSA and/or digital rectum exam (DRE) to determine which patients should undergo a second biopsy, following a negative first biopsy (Filella, Foj et al. 2013). It has been suggested that using a low cut-off PCA3 score, following an abnormal PSA test/DRE may prevent unnecessary first biopsies (Crawford, Rove et al. 2012). Currently a PCA3 test is not available on the NHS but can be carried out for £300-£400 (Cancer Research UK).

The search for new biomarkers continues. A highly sensitive disease-specific biomarker that can be detected in urine would be desired. The ideal scenario is to be able to detect patient specific biomarkers based on personalised genome sequencing. This would enable tailored patient care. The field of proteomics research is showing much promise in finding such methods and novel biomarkers (Goo and Goodlett 2010; Pin, Fredolini et al. 2013).

1.9.5.2 Prostate Cancer Treatment

1.9.5.2.1 Watchful Waiting and Active Surveillance

Prostate tumours have often been identified during autopsy on elderly men that died of unrelated causes. Most PCs are in fact indolent, and given the problems associated with treatment, such as impotence and incontinence, treatment is not always the preferred first port of call (Dall'Era and Kane 2008).

Watchful waiting, or deferred treatment, has been in place since pre-PSA times (Adolfsson 2008), where treatment was employed only when the cancer had advanced locally or metastasised (Klotz 2013). It is now used mainly with elderly men, or men who do not expect to live longer than 10 years, and who present with low risk PC. The use of watchful waiting varies worldwide (Adolfsson 2008). If the cancer progresses, palliative hormone therapy is offered (Dall'Era and Kane 2008).

Active surveillance is an evolution of watchful waiting and the technique was developed in response to the problem of overdiagnosis and overtreatment. It is also applied to patients with low risk PC, but usually younger men. The patient is continually monitored and treatment is undertaken if the cancer progresses according to predefined thresholds that define progression (Adolfsson 2008). The criteria for active surveillance are, a low tumour grade (Gleason score <6), a locally confined tumour, a PSA level <10ng/ml, and a history of 3 or less biopsies. Progression is generally defined as a PSA doubling time of <2-4 years, a PSA level of >10ng/ml, and a higher Gleason score on subsequent biopsy (Heidenreich, Bellmunt et al. 2011). In contrast to watchful waiting, treatment following active surveillance is intended to cure the patient (Adolfsson 2008).

The fact that PC with a Gleason score of 6 or lower has a long natural history makes active surveillance a viable option for patients. Although follow up studies are still quite young, (10-15 years), PC-specific mortality is very low in active surveillance cohorts (Klotz 2013). However, the psychological effects of active surveillance need to be better addressed. Often men opt for treatment due to

anxiety caused by the knowledge of having an untreated tumour, despite no evidence of disease progression (Dall'Era and Kane 2008).

1.9.5.2.2 Hormone Therapy

Androgen ablation therapy (ADT) has been the 'gold standard' treatment for advanced, recurrent and high risk PCs since 1941 (Chodak, Sharifi et al. 1995; Gleave, Klotz et al. 2009). It may be the primary treatment or is administered alongside other treatments in patients where locally-advanced cancer has re-emerged following radiotherapy or prostatectomy (Ahmadi and Daneshmand 2013). The European Association of Urology (EAU) recommends ADT for metastatic PC, and as adjuvant treatment for locally advanced PC with radiotherapy, or when the patient is unfit for radical local treatment.

Combined androgen blockade (CAB)/maximal androgen blockade (MAB) is the use of an antiandrogen following surgical or medical castration (McLeod 1997). Antiandrogens compete with steroid androgens for AR binding, leading to apoptosis (Thomas and Neal 2013). The first antiandrogens used were steroidal and produced cardiovascular side effects. Later, non-steroidal agents were developed which were much better tolerated. These include flutamide and bicalutamide (McLeod 1997). Combined therapy is far more effective than castration alone for metastatic disease (Chen, Huang et al. 2010). Chemical castration causes an initial surge/flare in androgens, and so the antiandrogens combat this.

Luteinising hormone-releasing hormone (LHRH) mediates LH production of testosterone. Both LHRH agonists and antagonists are used as chemical castration agents in the treatment of PC. Agonists are the primary ADT agents and cause an initial 'flare' of testosterone, which desensitises the receptor leading to an overall decrease in testosterone production. Antagonists are more recently on the market, and they inhibit the receptor, thereby blocking release of LH and testosterone synthesis. Thus, these agents induce castrate-levels of testosterone (Huirne and Lambalk 2001; Thompson 2001; Thomas and Neal 2013).

ADT prevents progression of PC and associated consequences, but does not cure it nor prolong survival of patients with advanced disease. Despite castrate levels of testosterone, the cancer invariably re-grows and is insensitive to ADT. New drugs that inhibit androgen biosynthesis and AR nuclear translocation are used during this phase (Thomas and Neal 2013). Also, long term ADT has high morbidity with side effects such as impotence, incontinence, hot flushes, gynecomastia, osteoporosis, depression and metabolic complications (Gleave, Klotz et al. 2009; Ahmadi and Daneshmand 2013).

1.9.5.2.3 Radiotherapy

Radiotherapy is used in the management of low-high risk localised PC. Treatment can be either external beam radiotherapy (EBRT) or brachytherapy. The former is external radiation that is targeted to the outside of the body, in the region of the prostate. The latter involves the implantation of radioactive isotopes into the prostate that emit radiation over 60 days (Pieters, de Back et al. 2009; Chen and Zhao 2013). Brachytherapy uses a much lower dose of radiation, is non-invasive, and is commonly used in the treatment of low risk prostate tumours.

External radiotherapy is the preferred option for higher risk PC, although it can be used for low risk cancer also. Different amounts of radiation are used depending on whether the PC is low, intermediate or high-risk (Heidenreich, Bellmunt et al. 2011). Indeed, both radiotherapies may be used together if a clinically occult extraprostatic tumour is suspected, which radiation from the prostatic implants would not reach (Davis, Pisansky et al. 1999).

External beam radiotherapy with long term ADT (3 years) for advanced localised PC has been shown to delay PC-specific mortality longer than radiotherapy alone (Bolla, de Reijke et al. 2009), and is the current standard for radiotherapeutic management of high risk PC (Heidenreich, Bellmunt et al. 2011). Side effects of EBRT were common, due to irradiation of nearby tissue, such as the bladder. However methods such as MRI reconstruction of the prostate and better beam

targeting have greatly reduced damage to peripheral tissue (Dowling, Lambert et al. 2010).

1.9.5.2.4 Prostatectomy

Prostatectomy is the partial or complete (radical-RP) removal of the prostate gland in the treatment of BPH, LUPS and PC. There are a number of different techniques that fall under three main types.

1) Laparoscopic prostatectomy is the least invasive method to remove the whole prostate. It may or may not be robot-assisted and involves only small incisions and little blood loss (Trabulsi and Guillonneau 2005).

2) Open prostatectomy is open surgical removal of the whole gland via a large incisions in the abdomen or perineum (Serretta, Morgia et al. 2002). It is preferred in some countries, such as Italy.

3) Transurethral resection of the prostate (TURP) is carried out to relieve moderate to severe symptoms of LUTS associated with BPH or PC. The surgical instrument is a resectoscope, which also has a camera on the end to guide it to the prostate. Morbidity after TURP used to be high but has improved greatly since the introduction of the modern instrument (Rassweiler, Teber et al. 2006).

1.10 cAMP Signalling in the Prostate and Prostate Cancer

As discussed, cAMP is strongly associated with PC progression via proliferative and proinflammatory signalling. Dysregulation of cAMP signalling in PC was first noted by Shima et al. who identified differential expression of AC in normal and tumour tissue (Shima, Kawashima et al. 1976). Since then, cAMP PDEs and effector proteins have been implicated in prostate carcinogenesis.

Androgen-independent activation of the AR has been reported in response to several signalling pathways such as Rho-GTPase and NF κ B/p52 signalling (Lyons, Rao et al. 2008; Nadiminty, Lou et al. 2010). Such androgen-independent AR transactivation is a matter of debate, but cAMP signalling is implicated in many of the observations. Cross talk between cAMP signalling and the AR was first observed when activated PKA resulted in an androgen-independent rise in PSA expression in PC cells (Sadar 1999). It was later found that PKA can mediate recruitment of the coactivators CREB and CBP/p300, and HAT to the PSA locus (Kim, Jia et al. 2005). Vasoactive intestinal peptide (VIP) which is associated with PC progression has been shown to mediate androgen-independent AR transactivation via a PKA/ERK pathway (Xie, Wolff et al. 2007). PKA-mediated enhancement of AR activity in an androgen-free environment places cAMP as a main player in the progression of AI PC. Also, PKA isoform switching occurs as PC progresses into an AI phenotype (Merkle and Hoffmann 2011).

1.10.1 cAMP PDEs in Prostate Cancer

Given the vast amount of evidence suggesting a role for cAMP in the progression of PC and the transition to AI PC, there is surprisingly little in the literature on the role of cAMP PDEs in this disease.

In order to gain knowledge of the role of PDEs in the prostate, Uckert et al. sought to characterise the expression of PDE isoforms in the various prostatic zones (Uckert, Kuthe et al. 2001). mRNA was detected for PDEs 1, 2, 4, 5, 7, 8, 9

and 10. Later they confirmed the presence of PDE4, PDE5 and PDE11 protein in the transitional zone of the human prostate (Uckert, Oelke et al. 2006). PDE4 was later found to be abundant in the fibromuscular stroma and to regulate prostate smooth muscle tone in complex with PKA and actin (Waldkirch, Uckert et al. 2010). These studies highlight the need for more research into the implications of dysregulated cAMP compartmentalisation in PC.

PDE4B has been shown to be downregulated in AIPC due to oxidative stress, which results in an increase in PKA and phospho-CREB and consequent transactivation of the AR and development of an AI phenotype (Kashiwagi, Shiota et al. 2012).

As discussed earlier, the *Sleeping Beauty transposon* study by Rahrman et al. identified PDE4D as a candidate PC gene (Rahrman, Collier et al. 2009). They claimed that knockdown of PDE4D via shRNA decreased the proliferation of AI DU145 cells. A more recent study showed that inhibition of all PDE4 subfamily isoforms led to an increase in the expression of the AR and upregulation of PSA in AS LNCaP cells (Sarwar, Sandberg et al. 2013).

1.10.2 Protein Kinase A and Prostate Cancer

As discussed, several studies show that the cAMP/PKA pathway crosstalks with the AR in androgen-deprived conditions (Merkle and Hoffmann 2011).

The regulatory subunits of PKA undergo differential expression during PC progression. PKA-II is the main isoform expressed in normal tissue, but during prostate carcinogenesis there is an increase in the expression of R1 α , shifting the main PKA isoform to PKA-I (Cho, Lee et al. 2000). An increase in R1 α expression has also been demonstrated in many other cancers (Kvissel, Ramberg et al. 2007), and it has been shown that overexpression of R1 α negatively correlates with PC disease outcome (Merkle and Hoffmann 2011). It has also been postulated that overexpression of R11 α can reverse the tumour phenotype (Cho, Lee et al. 2000; Neary, Nesterova et al. 2004). In contrast, a recent study found

that R1 α was downregulated in tumour tissue compared to normal prostatic tissue, and in AI cells compared to AS cells (Sarwar, Sandberg et al. 2013). Sarwar et al. also found that a forskolin-induced increase in cAMP led to the upregulation of R1 α in AS LNCaP cells, whereas it abolished expression of R1 α in AI PC3 cells. These findings are confusing given the results of the aforementioned studies. However, the cAMP-induced increase in R1 α expression in AS cells suggests that an increase in cAMP due to downregulation of PDE4, (Kashiwagi, Shiota et al. 2012) and consequent upregulation of R1 α is involved in the AS to AI transition of PC. Indeed, Kvissel et al. found an upregulation of R1 α in PC cells but not in primary tumour samples, and they suggest it may be a late event in disease progression (Kvissel, Ramberg et al. 2007). Another study which also found an upregulation of R1 α to be neoplastic speculated that cancer associated R1 α may function independently of PKA (Bossis and Stratakis 2004).

As discussed, it is the R subunit that confers binding interactions of PKA, and thus switching of this subunit may perturb the signalosome in which PKA is involved, even possible placing PKA in a different complex at a different subcellular location.

Although PKA is an intracellular enzyme for the most part, a catalytically active, extracellular PKA (ECPKA) isoform that is cAMP-independent has been found to be secreted from malignant cells (Cho, Lee et al. 2000; Cvijic, Kita et al. 2000). ECPKA expression is correlated with that of R1 α and it may be involved in adherence and invasion of cancer cells.

The catalytic subunits of PKA are also differentially expressed during PC progression (Kvissel, Ramberg et al. 2007). An increase in CB2 is associated with increased proliferation, and an increase in CB3 and CB4 is associated with NED. CB2 was increased in specimens from localised untreated tumours of Gleason score 6-7.

Thus, these studies suggest that an aberration in the PKA-I:PKA-II ratio is indicative of tumour formation, and may be a driving force for PC progression.

As discussed, the cAMP-responsive transcription factor, CREB, is phosphorylated and activated by PKA and provides a point of crosstalk between PKA and AR signalling cascades. It has been found that oestrogens and androgens can activate IGF-1R via CREB phosphorylation in PC cells, and this may mediate cell survival (Genua, Pandini et al. 2009). This, and the work described above, show that PKA can mediate steroid-independent transactivation of the AR; highlighting the importance of cAMP-mediated autocrine and paracrine signalling loops within the prostate and PC progression.

1.10.3 EPAC and Prostate Cancer

As discussed above, there have been some conflicting results on the role of EPAC in PC. Chronic inflammation leads to activation of the COX₂-PGE₂-cAMP signalling pathway which promotes tumourigenesis and metastasis in various cancers (Misra and Pizzo 2013). Misra et al. have found that EPAC1 is involved in PC progression via upregulation of inflammatory pathways. They found that EPAC1/Rap1 activates the B-Raf/ERK, PI3K/AKT and mTOR signalling networks, all of which cross talk to bring about an upregulation of pro-proliferative genes (Misra and Pizzo 2009). A recent follow up study by the same group found that EPAC activation also brings about an upregulation of pro-inflammatory markers in PC cells, and acts via MAPK-COX₂-PGE₂-mTOR signalling to bring about an autocrine proliferation loop (Misra and Pizzo 2013). However, another study suggested that EPAC inhibits PC proliferation and migration (Grandoch, Rose et al. 2009). This involves an inhibition of MAPK and RhoA signalling and collapse of the F-actin cytoskeleton. Both studies employed AI PC3 and DU145 cells; however, in the study by Grandoch et al., the cells were serum starved prior to EPAC activation. This meant that the cell medium contained no hormones such as androgens. Perhaps, in the presence of androgens EPAC elicits a pro-inflammatory, pro-proliferative effect, but in the absence of androgens the opposite is true. This suggests a mechanism whereby residual androgens, following ADT, potentiate PC progression during the later stages of the disease.

There is little else in the literature on the direct role of EPAC, but studies looking at the activity of Rap proteins in PC give an indication of the role of EPAC.

As discussed earlier, EPAC1/Rap1 signalling plays an important role in cell adhesion and chemotaxis. Constitutive Rap1 activation has been shown to promote angiogenesis via VEGF (Menon, Doebele et al. 2012). The authors show that PKA antagonises this pro-angiogenic effect of Rap1, suggesting interplay between PKA and EPAC signalling loops in PC. They propose that PKA activation may explain the anti-proliferative effects of EPAC1 reported by Grandoch and colleagues.

It has also been demonstrated that an increase in cAMP in AS PC cells leads to Rap1/PKA/EGF-dependent activation of MAPK (Chen, Cho et al. 1999). Again this represents an example of EPAC/PKA synergism in PC. Increased Rap1 activity in AI PC cells promotes migration and invasion via a process that involves integrins (Bailey, Kelly et al. 2009). This likely involves EPAC, which is known to play a key role in integrin-mediated cell adhesion (Bos 2006).

Rap2 has also been shown to mediate androgen-dependent PC cell growth (Bigler, Gioeli et al. 2007) Its expression is decreased in AI PC, suggesting loss of Rap2 may be involved in AI PC progression. When overexpressed in AS cells, Rap2 inhibited MAPK activation and PSA expression. This suggests a possible antagonistic relationship between Rap1 and Rap2, and would explain why activating EPAC can induce either pro- or anti-proliferative cellular effects.

1.10.4 Neuroendocrine Differentiation in Prostate Cancer

Neuroendocrine (NE) cells are found dispersed around the stromal and luminal epithelium cells in the normal prostate gland, the amount of which can vary between individuals (Sun, Niu et al. 2009; Li, Chen et al. 2013). They are non-mitotic and do not express the AR or PSA, are distinct from stem cells, and are most closely related to the secretory cells (Huang, Yao et al. 2006). Little is known of their function, but they are presumed to play paracrine and secretory roles in the development and function of the gland (Cox, Deeble et al. 1999). NE cells make up only 1% of untreated prostate tumours, but become abundant in AI PC, following ADT (Li, Chen et al. 2013). This is likely because they do not express the AR so are resistant to ADT, and there may be selection for NE cells during PC progression, like with stem cells. The higher the density of NE cells, the more poorly differentiated the tumour is (Sun, Niu et al. 2009).

It has long been known that an increase in cAMP can trigger neuroendocrine differentiation (NED) characteristics in AS cells, likely through PKA signalling (Bang, Pirnia et al. 1994; Cox, Deeble et al. 2000). NE cells secrete neuropeptides and cytokines that act via autocrine and paracrine signalling to facilitate NED and increase the proliferation of neighbouring non-NE cells (Gkonos, Krongrad et al. 1995; Juarranz, Bolanos et al. 2001; Li, Chen et al. 2013). An increasing NE cell population and thus level of secreted neuropeptides is believed to be involved in the transition to AI PC (Jongsma, Oomen et al. 2000). It has also been suggested that neuropeptides may be involved in atypical androgen signalling in the transition to AI PC (Gkonos, Krongrad et al. 1995). NE cells also highly express the potent angiogenic peptide VEGF, with higher levels in AI cells compared to AS cells (Harper, Glynne-Jones et al. 1996). This likely facilitates metastasis and neovascularisation, giving NE cells another driver for the progression of PC. The neuropeptide vasocactive intestinal peptide (VIP) has been highly implicated in NED-mediated PC progression, as it potentiates angiogenesis through upregulation of VEGF in AS cells (Collado, Gutierrez-Canas et al. 2004; Collado, Sanchez et al. 2005). This pathway may involve PKA, ERK and PI3K signalling cascades (Gutiérrez-Cañás, Juarranz et al. 2005). VIP has also been shown to increase PSA secretion (Gkonos, Ashby et al. 1996). Thus

increasing levels of VIP during NED and PC progression may be involved in the increase in serum PSA levels.

Interestingly NED is reversible (Cox, Deebie et al. 1999). Exposure to neuropeptides brings about conversion to a NE morphology. When differentiating agents are withdrawn or decreased, the cells can re-enter the cell cycle and proliferate. This mechanism may provide PC with a double-edged sword. Under the right conditions, with increasing cAMP, cells may become NE, secreting growth stimulating agents to neighbouring cells, and eventually these agents may act in an autocrine manner to reverse the NED, returning the cells to a proliferative state. If this occurs *in vivo*, it may occur in a cyclical manner, increasing tumour mass and potentiating androgen-independence.

A recent study has linked inflammation to NED, where differentiated AS LNCaP cells displayed increased secretion of the pro-inflammatory cytokine MIF (Tawadros, Alonso et al. 2013).

A small subset of men develops small cell neuroendocrine carcinoma (SCNC) following ADT (Sun, Niu et al. 2009; Li, Chen et al. 2013). SCNC cells are all NE-like cells, but they are highly proliferative and metastasise rapidly with very poor prognosis. An SCNC can exist alone or alongside a conventional PC tumour which is more common, and may develop following tumour suppressor gene mutation in normal NE cells.

Despite the apparent importance of NED in PC progression and transition into androgen independence, little is known on the cAMP-dependent mechanisms involved.

Aims of Research

In summary, there is much evidence implicating cAMP signalling in the progression of PC. Despite this, the roles of individual cAMP PDE isoforms in PC have not been investigated. Given their highly specific and non-redundant roles, this seems pertinent in order to understand the effects of changes in cAMP signalling during the progression of this disease. Due to the current lack of a disease-specific biomarker, our group undertook such an investigation; in the hope that changes in cAMP signalling would present a novel biomarker. Indeed, we identified a significant change in the expression of PDE4D7 mRNA between AS and AI disease, such that it successfully discriminates between normal and tumour prostate tissue, and as such is a promising novel PC biomarker. This thesis focuses on characterising a function of PDE4D7, as none have yet been assigned to this enzyme.

This study is divided into three parts;

- 1) Investigations into the expression of PDE4D7 protein in AS versus AI PC cell lines, and the effect of PDE4D7-mediated cAMP hydrolysis on PC cell proliferation and migration.
- 2) Identification of a novel mode of PDE4D7 isoform-specific regulation.
- 3) Investigations into the PDE4D7 interactome.

It is expected that the findings presented here may enhance our current knowledge about cAMP and in the progression of PC, and that PDE4D7 may provide a novel node for therapeutic manipulation in the treatment of PC.

2 Materials and Methods

2.1 Molecular Biology

2.1.1 Cloning and PCR

2.1.1.1 Primer Design

Primers of 15-20 nucleotides in length were designed for optimal annealing between 55°C and 65°C using the formula;

$$Tm (oC) = 2 (NA+NT) + 4 (NG+NC)$$

Where N= number of the corresponding bases (A,T,G and C).

They were designed with complementarity to the sense and 3' antisense end of the target DNA, with or without the introduction of mutations. Primers were tested *in silico* using Geneious bioinformatics software (Biomatters). Following this they were made and purified by Thermo-Fischer Scientific.

2.1.1.2 Site Directed Mutagenesis (SDM)

The following primers were used in this study for SDM, to create PDE4D7 Ser42 mutants using VSV-PDE4D7 as template DNA;

PDE4D7 S42A forward:

5' CGG AGA CTT GCA TGT CGC AAT ATT CAG CTT CCC CCT CTC 3'

PDE4D7 S42A reverse:

5' ATT GCG ACA TGC AAG TCT CCG GAC AAG ATA GGG TTC CAT TCC 3'

PDE4D7 S42D forward:

5' CGGAGACTTGACTGTCGCAATATTCAGCTTCCCCCTCTC 3'

PDE4D7 S42D reverse:

5' GGGATAGAACAGGCCTCTGAACTGACAGCGTTATAAGTCG 3'

2.1.2 PCR Product Clean-up

All PCR products were purified using Qiaquick PCR Purification Kit (Qiagen) according to manufacturer's protocol. Briefly, high salt buffers are used to bind DNA to a silica membrane in a spin column. Contaminants are washed away-they pass through the membrane. DNA was then eluted in nuclease free water.

2.1.3 Restriction Endonuclease Digestion of DNA

All restriction endonucleases and corresponding buffers were sourced from Roche. Sub-cloning of all cloned human genes used within this study was performed using two differing restriction sites so as to ensure correct orientation of insertion into vector plasmids.

Digestion of purified PCR products were performed in 100µl volumes with the following composition:

Enzyme 1: 1.5ul
Enzyme2: 1.5ul
10x buffer: 10ul
Eluted PCR product: 87ul

Plasmid vector was digested using the following reaction composition:

Enzyme 1: 1.5ul
Enzyme2: 1.5ul
10x buffer: 2ul
Plasmid vector: 100ng
dH2O: up to 20ul

The digestion reactions were allowed to proceed for 1h at 37°C.

2.1.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse 5ul of PCR reaction mixtures. 1% agarose (w/v) was dissolved in TAE (Tris-acetate-EDTA) buffer (40mM Tris-Cl; pH 8.5, 0.114% (v/v) glacial acetic acid and 2mM EDTA). 0.001% Ethidium Bromide was added to visualise the separated DNA fragments under UV light. The DNA was added to loading buffer containing (final concentration 5% [v/v] glycerol solution, 0.06% [w/v] bromophenol blue, 0.06% [w/v] xylene cyanol). A 1kb DNA ladder (Promega) was used as a marker. The gel was run for ~45 minutes at 100 V. The gel was removed from the tank and analysed using the Gel Doc XR+ System (Bio-Rad) using the ultra-violet light setting. If the cloning was successful; i.e. DNA bands at the correct molecular weight, the remainder of the PCR reaction product was purified as described in section 2.1.2.

2.1.5 Ligation

Ligation of PCR fragments and digested plasmids was conducted using the Rapid DNA Ligation Kit from Roche as per the manufacturer's instruction. All buffers were supplied with the kit. Briefly; vector and insert DNA fragments were combined in a 1:4 molar ratio (of vector/insert) in 10µl of 1x DNA dilution buffer. 10µl of ligation buffer was then added to the mixture. Ligations were incubated at room temperature for 20 minutes before transformation into competent DH5α *E.coli* cells from Invitrogen. Competent cells were then transformed with 2ul of ligation reaction as described in section 2.1.6. Plasmid DNA was extracted, purified and sequenced (GATC Biotech).

2.1.6 Transformation of Competent Cells

Transformations were carried out using One Shot TOP10 or DH5α *E. coli* competent cells (Invitrogen) for protein expression or plasmid amplification respectively. 2ul of a plasmid DNA or ligation mixture was gently added to 50ul of cells and incubated on ice for 30mins. Cells were then subjected to heat-shock at 42°C for 30 seconds, and placed back on ice for 1-2 minutes. 250ul of LB

medium was then added and the cells were placed in a 37°C shaking incubator for 45mins-1 hour to recover. The cell suspension was then plated onto LB-agar plates containing 100ug/ml of the appropriate antibiotic. The plates were left to dry and incubated at 37°C overnight.

2.1.7 Sequencing

The day following transformation of *E. coli*, a number of colonies were picked with a sterile pipette tip and placed in to 50ml Falcon tubes containing LB and 100ug/ml of the appropriate antibiotic and inoculated overnight at 37°C. Following this the plasmid DNA was isolated using a Qiaprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Plasmid samples were then sent to GATC sequencing services, where they sequence 30-100ng/ul of plasmid DNA in 20ul samples. The sequencing results for each colony were analysed *in silico* using Geneious bioinformatics package (Biomatters), to determine the sequence was correct and void of any unwanted mutations.

Sequencing was performed using single GATC universal primers;

For pcDNA3.1: 5'CAAACAACAGATGGCTGGC3'

For pGEX: 5'CTGGCAAGCCACGTTTGG3'

2.1.8 Storage of Plasmid DNA

For plasmid storage, sterile glycerol was added to an overnight culture of cells to make a final concentration of 20% glycerol. The glycerol stock was then snap-frozen on dry ice and stored at -80°C until required.

Glycerol stocks could then be used to inoculate culture media by scraping the frozen stock with a sterile pipette tip and then transferred into 5 ml LB media containing the appropriate antibiotic. Plasmid isolation was then performed as described in Section 2.1.9.

2.1.9 Isolation of plasmid DNA

Plasmid DNA was harvested subsequent to the overnight incubation of glycerol stock sample. For small scale harvesting (5ml), DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. For large scale harvesting, the bacterial cell culture was centrifuged at 6000xg for 15 minutes and plasmid DNA was subsequently purified by QIAGEN Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. Both kits work on the principles of alkaline lysis of the bacterial cells followed by anion exchange on a column and precipitation of DNA under low salt and pH conditions. RNA, proteins and other low molecular weight contaminants are washed away using medium salt buffers and high purity DNA is then eluted in high salt buffer. Isopropanol precipitation is used to desalt and concentrate the DNA.

2.1.10 Bioinformatic Analysis

All bioinformatic analysis conducted within this study was performed using the Geneious® bioinformatics package (v5.3) from Biomatters. All sequence and accession files were sourced from the NCBI database.

2.1.11 RNA Extraction and Purification

RNase Zap (Invitrogen) was used on the bench, pipettes and all other items used when extracting tRNA. Filter tips (Rannin) were used to also prevent contamination and degradation of RNA samples. tRNA isolation was carried out using an RNeasy Kit midi from Qiagen. Prior to harvesting the RNA, cells were grown in the appropriate cell culture dish, all medium was removed, and they were directly harvested using RLT buffer. RLT contains highly denaturing guanidine-thiocyanate and immediately inhibits any RNases. The amount of RLT buffer added was based on cell density, as outlined in the manufacturer's protocol. For example, 200ul was used for one well of a 6 well plate and if 3 wells were used this was pooled to make 600ul final volume. Samples were then added to Eppendorf tubes and homogenised by vortexing for 1 min and being

passed through a 19 gauge needle a number of times (5-7). Following this, all steps listed in the manufacturer's protocol were carried out. Briefly, tRNA was ethanol-precipitated and applied to an RNeasy spin column. High salt buffers bind tRNA longer than 200bps to the silica column membrane, thereby enriching mRNA isolation. On-column DNase digestion was then performed using DNase1 (Qiagen) for 30mins at RT, in order to remove any genomic DNA contamination. Bound tRNA was then washed to remove other contaminants. RNA was then eluted in nuclease-free water.

2.1.12 Nucleic acid Quantification

Isolated DNA and RNA samples were assessed for quantity and purity using a Nanodrop spectrophotometer (Thermo-Scientific) Nucleic Acid concentration was quantified by absorbance at A260nm and purity (protein contamination) was assessed by the A260/A280 nm ratio, with 1.7-1.8 being optimal values for protein/nucleic acid ratio for DNA, and 1.8-2 being optimal for RNA.

2.1.13 cDNA Synthesis

1ug of RNA was reverse transcribed into cDNA using Affinty Script Multiple Temperature cDNA synthesis Kit (Agilent) according to manufacturer's instructions. Briefly, random primer amplification synthesises cDNA from tRNA. RNA is incubated with the random primers at 65°C for 5 minutes followed by cooling to RT at which point the primers anneal to the RNA. When cooled, dNTP mix and the reverse transcriptase are added, along with buffer and an RNase inhibitor. cDNA was then synthesised at 42°C for 60minutes followed by termination of the reaction at by heat inactivation at 85°C for 5 minutes. The reverse transcriptase enzyme provided in this kit is a genetically modified version of MMLV RT, which is highly thermostable. The cDNA was quantified using the Nanodrop.

2.1.14 Quantitative Real Time PCR

2.1.14.1 Primer Design

Primers with melting temperatures of 55-60°C were designed to amplify an 80-120bp segment of target cDNA within the gene of interest (GOI). The primers contained 3' TAMRA and 5' FAM labels. All primers were BLAST-searched against the NCBI mRNA database to ensure specificity. If any displayed affinity for a possible off-target region of the cDNA it was ensured that the cognate oligonucleotides used in the probe-set showed no such affinity or homology to that off-target transcript. These primers were then designed and HPLC purified by Eurofins MWG Operon. Taqman Probe sets were used to increase specificity of the qPCR reaction for the desired cDNA target transcripts and reduce post assay analysis time (table 2.2).

Accession	Gene	Forward	Reverse	Probe
	panPDE4D	ACCATTACCAT GCTGATGTGGC CT	ACACAGCCTCCAAAGC AGGTG	TCCATGCTGCAGATGTTGTCC AGT
AF53696.1	PDE4D7	TGCCTCTGAGG AAACACTAC	GCTGAATATTGCGACA TGAAAG	CCAGTAATGAAGAGGAAGAC CCTTCCGC
AF012073	PDE4D5	AACGAAGACCT TGTGAAATCC	GCTGCTGAGAAGCATT CTG	ATCTCTCCGAGAAATTCCCCCA GGCT

Table 2.1. List of Primers and Probes used in this Study

2.1.14.2 qPCR Protocol

qPCR was carried out using a Prism 7300 qPCR cyler from Applied Biosystems. The qPCR buffer used was PerfeCTa qPCR FastMix Reaction Mix (Quanta Biosciences). Reactions were carried out in MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems).

15ul reaction mixes were added to each well:

REAGENT	FINAL CONCENTRATION/VOLUME IN 15uL MIX
qPCR FastMix buffer	7.5ul
Forward Primer	500nM (0.1ul)
Reverse Primer	500nM (0.1ul)
Dual Labelled Probe	500nM (0.1ul)
Nuclease free water	2.2ul
Diluted cDNA (10ng/ul)	5ul

The qPCR reaction was carried out in 2 segments:

qPCR Protocol			
Segment	Cycles	Temperature	Time
1	1	1	30 sec
		1	2 min
2	40	1	15 sec
		1	1 min

Cycle Threshold (Ct) values were obtained for each GOI and GAPDH and used to calculate the fold expression of a GOI and as a percentage of GAPDH control.

2.1.14.3 qPCR data analysis

Relative changes in gene expression were quantified using the comparative Ct ($\Delta\Delta C_t$) method as described by Livak and Schmittgen (2001). Here the PCR signal of the transcript of interest in a treated sample is compared to that of a control sample, after both are normalised to an endogenous housekeeping gene such as GAPDH. The objective of this method is to compare the PCR signal of a target gene in a treatment group to the untreated control after normalising to an endogenous reference gene. Firstly, the point at which the PCR was detected above a set threshold in exponential phase, termed threshold cycle (Ct), was

obtained from the real-time PCR instrumentation. Ct is determined from a log-linear curve where PCR signal is plotted against the cycle number. The Ct values of each sample were then imported into Microsoft Excel and the average Ct of triplicate samples was calculated. The amount detected at a certain cycle number is directly related to the initial amount of target in the sample. Hence, to determine the quantity of gene-specific transcripts present in cDNA, Ct values for each treatment had to be normalised first to obtain ΔCt . This was accomplished by subtracting the mean Ct value of endogenous reference gene GAPDH of each group from the corresponding mean Ct value of gene of interest (GOI) accordingly ($\Delta\text{Ct} = \text{Ct}_{\text{GOI}} - \Delta\text{Ct}_{\text{GAPDH}}$). The concentration of gene-specific mRNA in treated cells relative to control cells was then normalized through subtraction again to obtain $\Delta\Delta\text{Ct}$ values ($\Delta\Delta\text{Ct} = \Delta\text{Ct}$ of treated cells - ΔCt of control treated cells). Finally, the relative expression which is often termed as RQ value was determined by raising 2 to the power of the negative value of $\Delta\Delta\text{Ct}$ ($2^{-\Delta\Delta\text{Ct}}$) for each sample (Amount of target = $2^{-\Delta\Delta\text{Ct}}$). Alteration in mRNA expression of target genes was defined as fold difference in the expression level in cells after the treatment relative to that of the control treatment. Samples with poor technical replicate values were excluded from analysis.

2.2 Protein Chemistry

2.2.1 Peptide Array Technology

PDE4D7 peptide arrays were produced by automatic SPOT synthesis employing the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments) (figure 2.1 A) and Fmoc (9-fluorenylmethoxycarbonyl) chemistry (Frank 2002). The fundamental principle of this technique is the dispensation of a solvent droplet onto the planar surface of a porous membrane which is absorbed and forms a circular spot; this spot then acts as a reaction vessel. The peptide array can then be overlaid with purified proteins or antibodies to verify PPIs and identify functional binding motifs and antigen epitopes. PDE4D7 peptide arrays were created with overlapping 25mer peptides, each shifted along by 5 amino acids so

each overlaps the next by 20 amino acids, until the full protein was spotted onto the nitrocellulose support (figure 2.1 B).

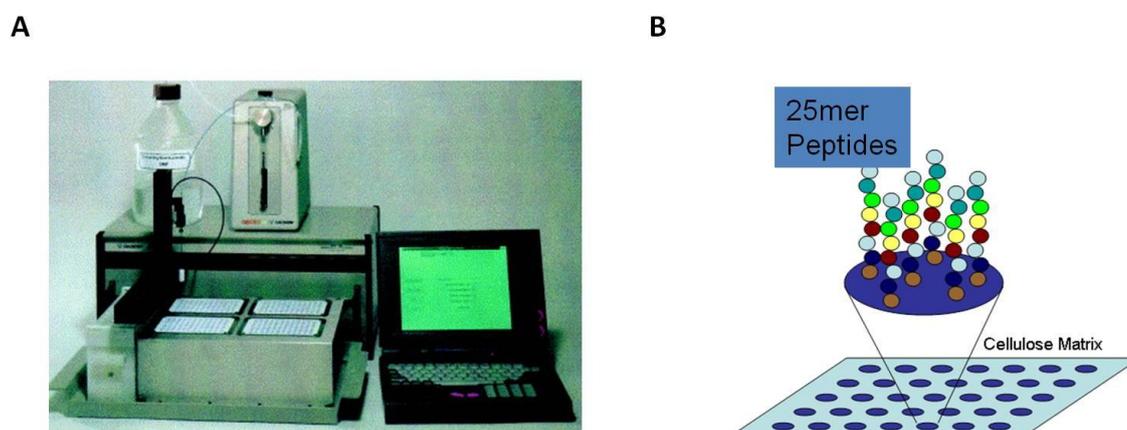


Figure 2.1. SPOT synthesis.

A; the AutoSpot-Robot ASS 222 spotting robot (the peptide sewing machine) (Frank 2002). **B;** 25mer peptides encompassing PDE4D7 were spotted onto a nitrocellulose support, which is on a glass slide.

2.2.1.1 Peptide Array Validation of Novel PDE4D7 Antibody

PDE4D7 arrays were blocked in 5% non-fat milk (Marvel) in TBST for 1h at RT with gentle shaking. Arrays were then incubated with the antibody at 1:1000 in 1% milk overnight with gentle shaking at 4°C. Following 3 TBST washes, arrays were incubated with HRP-conjugated anti-goat at 1:5000 in 1% milk for 1h at RT. The arrays were again washed several times with TBST before subjected to enhanced chemiluminescence (ECL) Western immunoblotting kit for autoradiography as described previously. The dark spots detected were indicative of a positive interaction of the antibody with the peptide array.

2.2.2 Phosphorylation Assays

2.2.2.1 In vitro Phosphorylation of PDE4D7 Peptide Arrays

Peptide arrays were blocked in 5% PhosphoBLOCKER (Cell Biolabs) containing 0.5 mM DTT and 1mM ATP, in TBST for 1h at RT with gentle shaking. They were then incubated in phospho-buffer (20 mM Tris-HCl; pH 7.5, 10 mM MgCl₂, 0.5 mM CaCl₂, 1 mM DTT, 0.2 mg/ml BSA, 1 mM ATP) with or without 100 units of active bovine PKA catalytic subunit (Sigma) for 1h at 30°C with gentle agitation. Following the phosphorylation assay, the arrays were washed thrice in TBST. Phosphorylated peptides were detected using either RXXpS antibody or the custom made PDE4D7-pSer42 antibody.

2.2.2.2 In vitro Phosphorylation of purified PDE4D7

N-terminal only GST-PDE4D7 wt, and GST-PDE4D7 S42A mutant proteins were purified as described. 2ug of each and 2ug of purified GST were incubated with phospho-buffer (20 mM Tris-HCl; pH 7.5, 10 mM MgCl₂, 0.5 mM CaCl₂, 1 mM DTT, 0.2 mg/ml BSA, 1 mM ATP) with or without 25 units of active bovine PKA catalytic subunit (Sigma) for 1h at 30°C with gentle agitation. The proteins were then analysed by SDS-PAGE followed by immunoblotting with either RXXpS antibody or the custom made PDE4D7-pSer42 antibody.

2.2.3 PDE4 Activity Assay

The PDE4 assays carried out were based on that described by Marchmont and Houslay (Marchmont and Houslay 1980). It comprises a two-step radioassay whereby samples are firstly incubated with 8-[³H]-labelled cAMP substrate and the cAMP is hydrolysed to 8-[³H] labelled 5'AMP by PDE4 enzymes in the samples. Secondly, the labelled 5'AMP is dephosphorylated to adenosine by addition of snake venom which contains 5' nucleosidase. Dowex ion exchange resin is then added to separate negatively charged un-hydrolysed cAMP from the uncharged adenosine. The amount of 8-[³H] bound adenosine in the samples is then

determined by scintillation counting which calculates the rate of cAMP hydrolysis.

The following buffers were used;

Buffer A (20mM TRIS-HCL, pH7.4)

Substrate Buffer (20mM TRIS-HCL/10mM MgCl₂, pH7.4)

Dowex (Sigma/Fluka); in a 1:1:1 ratio of Dowex:dH₂O:EtOH

2.2.3.1 Preparation of Dowex Ion-exchange Resin

Dowex was prepared and activated by dissolving 400g of Dowex resin in 4 litres of 1M NaOH. The solution was stirred for 15 minutes at room temperature and the resin was allowed to settle. The supernatant was removed and the Dowex resin was repeatedly washed with dH₂O and allowed to settle after each wash (~30 washes, 4L of dH₂O). After washing with dH₂O the resin was stirred with 4 litres of 1M HCl for 15 minutes at room temperature and allowed to settle. The resin was then washed a further 5 times with dH₂O and stored at 4°C as 1:1 slurry with distilled water. This Dowex slurry was utilised in the PDE assay as a 2:1 solution of slurry to 100% ethanol.

2.2.3.2 Preparation of Cell Lysates

Cells to be assayed were harvested in KHEM buffer (50mM HEPES pH7.4, 50mM KCL, 1.92mM MgCl₂) containing Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche) and PhosStop Phosphatase Inhibitor Cocktail Tablets (Roche). Lysates were diluted to 1ug/ul total protein.

2.2.3.3 PDE Assay Protocol

Lysate samples were prepared in triplicate in 1.5ml Eppendorf tubes on ice. Samples were made to 50ul final volume with Buffer A. Three blank control samples were prepared which contained 50ul of Buffer A only. 50ul of cAMP substrate mix (2uM 8-[³H]-cAMP (Amersham Biosciences) + 2ul 1mM cAMP (sodium salt, Sigma) per ml of Substrate Buffer) was added to each sample, including blanks, giving 2uM unlabelled cAMP and 3uCi/ml 8-[³H] cAMP final. The samples were incubated for exactly 10 minutes at 30°C to start the PDE reaction, and then boiled for 1-2 minutes to terminate the reaction. The samples were placed back on ice and allowed to cool for at least 15 minutes. Following this, 25ul of 1mg/ml snake venom (Sigma, made in Buffer A) was added to all samples (0.2mg/ml final). Again samples were incubated for 10 minutes at 30 °C. 400ul of the Dowex solution was then added and samples were vortexed, followed by cooling on ice for at least 15 minutes. Samples were then centrifuged at 13000rpm for 3 minutes at 4°C. 150ul of supernatant was added to new, labelled eppendorf tubes. To this, 1ml of Opti-flow SAFE 1 scintillant (Fischer Scientific) was added. Standards were made with 1ml of scintillant and 50ul of the cAMP substrate mix. All tubes were vortexed and placed in the scintillation counter (Wallac 1409 Liquid Scintillation Counter).

2.2.3.4 Calculation of PDE Activity

To calculate the specific cAMP hydrolysing activity the following equation was used to give PDE activity in pmoles/min/mg protein:

$$A = \frac{2.61 \times (C - B) \times N \times 10^{12} \times 1000}{S \times p \times t}$$

Where:

A = Specific PDE activity (pmol cAMP/mg/min)

C = Sample value (CPM)

B = Blank value (CPM)

S = Standard value (CPM)

N = Amount of cAMP in substrate (moles)

P = Amount of protein added (μg)

t = Time (min)

2.2.4 Protein-Protein Interactions

2.2.4.1 Pull-Down Assay (IP) and Mass Spectrometry

Hek293 cells were transfected with VSV-tagged PDE4D7 or control vector (pcDNA3.1). Following an appropriate expression time (~30 h), cells were harvested in 3T3 lysis buffer. Cell suspension was gently agitated on an end-over-end wheel at 4°C. The lysate was then centrifuged and the supernatant was assayed for protein concentration by means of a Bradford assay. 500 μg of total protein was used for each IP; untransfected and transfected lysates were immunoprecipitated with 20 μl FLAG (Invitrogen) or VSV (Sigma) tagged agarose beads. The IP samples were incubated at 4°C on an end-over-end wheel overnight. The input samples were boiled in sample buffer and frozen at -20°C. The following morning the beads were pelleted by centrifugation and washed thrice with lysis buffer. 20 μl 2x SDS-PAGE sample buffer was added and the beads were boiled for 5 min. Input and IP samples were then loaded onto a pre-cast polyacrylamide gel which was run for 1h40min at 100V. Following this the gel was Coomassie stained. The gel bands representing proteins pulling down with PDE4D7 were excised, along with the corresponding gel band areas in the control lanes.

These bands were shipped to Mr. Eberhard Krause of the FMP - Leibniz-Institut für Molekulare Pharmakologie Berlin who kindly performed the mass spectroscopic analyses.

2.2.4.2 Yeast Two Hybrid (Y2H) Screen

The Y2H screen was outsourced to Mr. Bangze Jin of Shanghai Genomics Inc. It is described in Chapter 5.

2.2.4.3 ProtoArray

ProtoArray Human Protein Microarray slides V5.0 (Invitrogen) were used for this study. Each array contains over 9,000 human proteins printed in duplicate on nitrocellulose on a 1x3 inch slide. Proteins on the slides are produced from clones in the Invitrogen Ultimate™ ORF Collection. The proteins are expressed as GST fusions using a baculovirus system, after which they are purified under non-denaturing conditions and printed onto the slide. Briefly, a slide is incubated with a purified protein of interest, followed by fluorescent antibody detection. Any proteins on the slide to which the protein of interest has bound are seen as fluorescent dots. Three ProtoArray slides were used in this study. The slides were stored at -80°C. Before beginning the experiment they were removed from the freezer and allowed to equilibrate on ice for at least 15 minutes. All incubations are performed in a 4-chamber incubation tray (Griener).

Following equilibration, the slides were incubated with blocking buffer (50mM HEPES pH7.5, 200mM NaCl, 0.08% Triton X-100, 25% glycerol, 20mM reduced glutathione, 1mM DTT, 1X Synthetic Block) on a rocker at 4°C. The slides were then probed with 1) purified GST tagged PDE4D7, 2) purified GST alone, 3) washing buffer alone (1X PBS, 1X Synthetic Block, 0.1% Tween). 120ul of buffer/probe mix was dropped onto the slide and covered with a LifterSlip (Thermo Scientific) to prevent evaporation. Following a number of washes, slides were incubated with primary antibody (anti-PDE4D7, Millipore). Again slides were washed, followed by probing with fluorescent secondary antibody (Alexa Fluor 647-Goat anti- Rabbit). All antibody incubations were carried out at 4°C for 90 minutes at a dilution of 1 in 1000 in washing buffer.

The slides were allowed to dry at room temperature (RT) overnight before being scanned on a ScanArray Express Microarray Analysis System (PerkinElmer Life

Sciences). Bluefuse software (BlueGnome) was used to organise the data generated by the scanner. It employs object-oriented perl programming to manage microarray files and map data fields in a 'Bluefuse' output. It generates a list of the protein pairs on the microarray along with a confidence score of A-E, representing the confidence that that protein binds the protein of interest, with A being highest confidence score. Protein pairs that scored A or B on the slide probed with PDE4D7, and D or E on both control slides were shortlisted. They were then compared to the image output, and if indeed full fluorescent red dots are present on both of the duplicate proteins, they were considered for further research.

2.2.4.4 Co-Immunoprecipitation (Co-IP)

Co-IP is an *ex vivo* method for the verification of PPIs. For overexpressed PDE4D7, ~500ug of total protein was used for IPs with VSV-tagged agarose beads. For endogenous PDE4D7, 1mg of total protein was used for IPs with 0.65ug of the novel PDE4D7 antibody or 0.65ug of isotype-matched IgG (Jackson ImmunoResearch Laboratories, Inc.) from the same species, with protein G-agarose beads. The cell lysates were precleared with 20ul of the appropriate beads for 30min on an end-over-end wheel at 4°C. The beads were then pelleted, and the supernatant added to fresh Eppendorf tubes. For VSV IPs, 20ul fresh beads were added and the lysates were incubated overnight at 4°C on the wheel. For PDE4D7 IPs, antibody or IgG was added and the lysates were incubated overnight at 4°C on the wheel. The following morning 20ul protein G-agarose beads were added for 2h. Prior to analysis by SDS-PAGE, the beads were washed thrice with 3T3 lysis buffer and boiled in 20ul 2x sample buffer. Test and control IPs, along with input samples, were loaded onto pre-cast polyacrylamide gels followed by western immunoblotting for the PPI hits.

2.2.5 Expression and Purification of Recombinant Proteins

Full length PDE4D7, N-terminal PDE4D7 and the N-terminal PDE4D7-S42A mutant were expressed as N-terminal GST fusion proteins using pGEX-6P1 (Invitrogen). 10mls of Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin was inoculated with BL21 *E. coli* cells (Invitrogen) containing the plasmids and grown overnight in an orbital shaker at 37°C. The following morning this was added to 500ml of LB + ampicillin, and grown for a further 2-4 hours, until the cell density reached OD₆₀₀ ~0.6-0.7, at which point cell growth was in the logarithmic phase. At this point, 1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures to induce protein expression. Cells were grown for a further 3-4 hours at 30°C and subsequently pelleted by centrifugation at 6000 x g for 10 min at 4°C. The cell pellets were resuspended in 10ml of ice cold GST lysis buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.05% NP-40) supplemented with protease cocktail inhibitor tablet (Roche). Cells were lysed by sonication; 3 x 30 sec, with 30 sec rest in between. Following lysis, cell lysates were centrifuged at 13000 rpm for 15 min at 4°C to collect the soluble fractions and remove cell debris.

Glutathione-sepharose beads (Amersham) were washed thrice with GST lysis buffer and 200ul of slurry was added to 15ml conical tubes. 10ml of the lysed cell supernatant was then added to each 15ml tube and incubated end-over-end overnight at 4°C. Subsequently the beads were pelleted by centrifugation, the supernatant was removed and the beads were washed with GST lysis buffer. The beads were again pelleted and washed with elution buffer (EB) (50mM Tris-HCl pH 8.0). Again the beads were spun down, and most of the supernatant was removed. The beads were resuspended in the remaining supernatant and transferred to a 1.5ml tube on ice; beads from different tubes were combined if they were the same sample. The volume was brought up with more elution buffer, the beads were pelleted and the supernatant was removed. The beads were then resuspended in 500ul EB + glutathione (6.8mg of reduced glutathione (Sigma) per 1ml of EB) and gently mixed by hand. The beads were spun down and the supernatant (eluate) was collected. This was repeated a number of times and the eluate was pooled. Overnight dialysis of the collected eluate was

carried out in dialysis buffer (100mM NaCl, 50mM Tris-HCl; pH 8.0, 5% glycerol, 5mM DTT) in slide-a-lyzer cassettes (Piercenet) at 4°C to remove any detergents or glutathione. Following recovery of the eluates from the cassettes, the protein concentration was determined by a Bradford assay.

2.2.6 Protein Concentration Assay

Bradford assays were carried out to determine the total protein concentration in all lysate samples. BSA was used to make standards, i.e. protein samples of known concentration. A titre of BSA concentrations was used; 0-5ug in dH₂O up to 50ul. The unknown lysate samples were assayed in triplicate at 1/50 dilutions. The Bradford assay is a colourimetric assay whereby addition of Coomassie Brilliant Blue Dye (Bio-Rad) is added to the samples and changes from brown/red to blue upon binding to protein (Bradford 1976). Here the absorbance spectra of the dye shift from 490 to 590, and absorbance at A₅₉₀ is measured for each standard and unknown sample. A standard curve is generated from the absorbance values of the BSA standards, and from this the unknown total protein concentrations are determined.

2.3 Protein Analysis

2.3.1 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis was carried out to separate proteins according to their molecular weight. Briefly, equal concentration of protein samples were denatured and reduced in 5x SDS-PAGE sample buffer (10% SDS, 300 mM Tris-Cl; pH 6.8, 0.05% bromophenol blue, 50% glycerol, 10% β -mercaptoethanol) followed by boiling for 2 min. Protein samples were then resolved on precast polyacrylamide gels (4-12% NuPAGE Novex Bis-Tris gel, Invitrogen) immersed in MOPS SDS running buffer (50mM MOPS, 50mM Tris, 0.1% SDS, 1mM EDTA pH 7.7) (NuPage, Invitrogen). Pre-stained protein marker (Bio-Rad) was loaded to the first well of the gel while the protein

samples were loaded to the subsequent wells. The gel was then run for 1 h 40 min at 200 V.

For IP samples, ~20ul 2x SDS-PAGE sample buffer was added to the washed beads. The beads were boiled for 5 min, followed by brief centrifugation. The supernatant was then loaded onto the gel.

2.3.2 Coomassie staining

For direct protein visualisation, gels were removed from the pre-cast gel cassette and stained with Coomassie blue (1.25 g Coomassie Brilliant Blue, 44% methanol [v/v], 6 % acetic acid [v/v]) and incubated for 20 min at room temperature with gentle agitation. Residual Coomassie background stain was then removed using destain solution (10% methanol [v/v], 10% acetic acid [v/v]) and incubated for 5-6 h at room temperature with gentle agitation to give clearly visible bands. The gel was washed in sterile water and the molecular weight of proteins was estimated by referring to the protein marker.

2.3.3 Western Immunoblotting

For Western blotting, resolved proteins were electrotransferred onto nitrocellulose membranes (0.45 µm pore, Protran, Whatman GmbH) using X-Cell II blotting module (Invitrogen) in transfer buffer (NuPage) containing 20% methanol for 2 h at 30V. Successful transfer was indicated by full transfer of the pre-stained molecular weight markers onto the nitrocellulose membrane. The membrane was then blocked in 5% (w/v) non-fat dry milk (Marvel) or PhosphoBLOCKER blocking reagent (Cell Biolabs, Inc.) in TBST (20 mM Tris-Cl; pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature with gentle agitation. Membranes were then probed with specific primary antibodies (Table 2.1) diluted in 1% milk/TBST solution and incubated overnight at 4°C. The membranes were washed thrice for 10 min each in TBST before adding appropriate horseradish peroxidase (HRP)-conjugated anti-immunoglobulin G

(IgG) secondary antibodies diluted 1:5000, or Alexa Fluor fluorescent secondary antibodies diluted 1:10,000, in 1% milk/TBST solution. After secondary antibody incubation, membranes were washed and detected by enhance chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific) and autoradiography. Chemiluminescent images of immunodetected bands were recorded on blue-light sensitive autoradiography X-ray films (Kodak BioMax MS, Carestream Health, Inc.) which were then developed using the Kodak® X-Omat Model 2000 processor. Immunoblot intensities were quantitatively analysed using Quantity One software (Bio-Rad) and averaged from at least three independent experiments. Alternatively, blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, UK) for fluorescence detection of the secondary antibodies. Fluorescence signal intensity was quantified using the Odyssey application software (LI-COR Biosciences, UK).

Antibody	Supplier	Catalogue #	Host	Dilution
PDE4D7	In house- Altabioscience	n/a	Sheep	1/1000
PDE4D7	In house-Millipore	n/a	Rabbit	1/1000
panPDE4D	In house-Millipore	n/a	Sheep	1/5000
RXXpS (PKA substrate antibody)	Cell Signaling	9621	Rabbit	1/1000
pSer42	In house-AMSBIO	n/a	Rabbit	1/1000
VSV	Sigma	V5507	Mouse	1/5000
FLAG	Sigma	F7425	Mouse	1/5000
ERRFI1	Cell Signaling	2440	Rabbit	1/1000
ENKURIN	Novus Biologicals	H00219670- B01P	Mouse	1/1000
NUAK1	Cell Signaling	4458	Rabbit	1/1000
CXCL5	Sigma	SAB1401356	Mouse	1/1000
MAP3K9	Cell Signaling	5029	Rabbit	1/1000
IRAK4	Cell Signaling	4363	Rabbit	1/1000
CSNK1ε	Sigma	SAB2500278	Goat	1/1000
PPP3CB	Abcam	Ab96573	Rabbit	1/1000
NONO	Millipore	05-950	Mouse	1/5000
NIKRAS2	Sigma	WH0028511M2	Mouse	1/1000
SNAPIN	Sigma	SAB1407227	Mouse	1/1000
ATF3	Millipore	DR1086	Mouse	1/1000
BCAR3	Sigma	WH0008412M1	Mouse	1/1000
NLK	Abcam	AB97642	Rabbit	1/1000
RXRβ	Abcam	AB2815	Mouse	1/1000
CT45A3	Abcam	AB111360	Rabbit	1/1000
GTF3A	Abnova	H00002971-A01	Mouse	1/1000
panPDE4A	In house-Millipore	n/a	Sheep	1/5000
GLYR1	Abcam	AB69277	Mouse	1/1000
ZNF302	Abcam	AB67794	Mouse	1/1000
MCRS1	Abcam	AB67393	Mouse	1/1000
NLK	Abcam	AB97642	Rabbit	1/1000
HSPA8	Abnova	MAB6636	Mouse	1/5000
DHX9	Abcam	AB26271	Rabbit	1/1000
GAPDH	Millipore	MAB374	Mouse	1/10k
V5	Sigma	V8137	Rabbit	1/2000

Table 2.2. List of primary antibodies used.

For ECL and film detection, the secondary HRP-conjugated mouse (Amersham), rabbit (Sigma) and goat (Thermo) antibodies were used. For detection using the Odyssey Infrared Imaging System (LI-COR Biosciences, UK) Alexa Fluor antibodies were employed.

2.4 Mammalian Cell Culture

2.4.1 Culture of Human Cell Lines

All cell culture procedures were performed in class II hoods using aseptic technique with sterile plastics and instruments. Media and all other culture reagents were purchased from Sigma. All plastics were purchased from Corning. The health of the cells and cell density were regularly observed using a phase contrast inverted microscope (Leitz Diavert, Germany).

Cells were maintained at 37°C with 5% CO₂ and 95% air. All cell lines were except DuCaps were purchased from the ATCC, the DuCaP cell line was a gift from the PCMM group at Philips, Eindhoven.

All cell lines were cultured in DMEM medium supplemented with 10% foetal bovine serum (FBS) and 100 units/ml penicillin and streptomycin and 2mM glutamine. VCaP and DuCaP cells were sub-cultured at 90% confluence and re-seeded at around 50% confluence. DU145, PC3 and Hek293 cells were sub-cultured at ~80% confluence and re-seeded at 10-30% confluence.

2.4.1.1 Sub-culture of cells

To passage cells, all growth medium was removed and a few ml (depending on size of culture vessel) of 1% trypsin-EDTA was added to the culture vessel. The cells were returned to the 37°C incubator for approx. 5 minutes. Cells were then recovered in fresh growth medium and added to new culture vessel(s).

2.4.1.2 Cell counting

All cell counting was performed using a haemocytometer.

2.4.1.3 Cryo-storage of cells

For storage, cells were detached with trypsin as described above, recovered in fresh complete medium and pelleted by centrifugation before being re-suspended in medium containing 10% DMSO and stored at -80°C .

2.4.2 Transfection of Cell Lines.

HEK-293 cells were transfected with Polyfect reagent (Qiagen) according to the manufacturer's protocol.

VCaP cells were transfected with both DNA and siRNA using Lipofectamine 2000 (Life Technologies). At approx. 90% confluence the growth medium was removed and replaced with Opti-MEM (Invitrogen) 2-4 hours prior to transfection. This strips away any serum and antibiotics which may interfere with the transfection. The complex formation was carried out according to the manufacturer's instructions. Once added, the cells were kept in Opti-MEM for another 4 hours. After this time the Opti-MEM was removed and normal growth medium was added.

DU145 and PC3 cells were transfected with the Amaxa Nucleofection Kit L and V respectively (Lonza); according to the manufacturer's instructions. Briefly, 1×10^6 cells were counted and centrifuged at $10,000 \times g$ for 3 minutes. All medium was removed and the cells were re-suspended in 100ul of Nucleofection solution plus DNA. This cell suspension was placed in a cuvette and electroporated in the Amaxa machine on the appropriate setting. Cells were then recovered in 500ul of complete medium, and the cell suspension was placed in a new culture vessel containing more complete medium.

2.4.3 siRNA Mediated Gene Knockdown in VCaP Cells

To achieve selective knockdown of the PDE4D subfamily or PDE4D7 specifically, double stranded RNA duplexes were designed to target regions of sequences unique to all PDE4D isoforms or the PDE4D7 N-terminus respectively. All oligonucleotide sequences were screened against the human expressed sequence tag (EST) library data base to ensure specificity of the siRNA. The duplexes were custom made by Thermo. siGENOME Non-Targeting siRNA#2 (Thermo) was used as a negative control.

PDE4D7 siRNA:

Sense strand: 5' AUACCUGUGAUUUGCUUUC 3'

Antisense strand: 5' GAAAGCAAUACACAGGUAU 3'

panPDE4D siRNA:

Sense strand: 5' AAGAACUUGCCUUGAUGUACA 3'

Antisense strand: 5' UGUACAUCAAGGCAAGUUCUU 3'

The cells were transfected with the siRNA using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

2.4.3.1 Treatment of Cells

2.4.3.1.1 5-aza-2'-deoxycytidine treatment of PC3 cells

Cells were seeded in 10cm cell culture dishes in complete medium. 10nM 5-aza-2'-deoxycytidine (5-aza-2-dC) was added in three pulses post seeding; at 24h, 48h and 72h, in fresh complete medium. DMSO was added to the control cells. On day 4 cells were harvested as described below.

2.4.3.1.2 Forskolin and KT5720 treatment of cells

Cells were seeded in 10cm cell culture dishes or 6 well plates. Treatment was carried out 1 week post-seeding of VCaP and DuCaP cells, to allow for expression of endogenous PDE4D7. Cells were pre-treated with 4uM KT5720 (Enzo Life Sciences) for 20min prior to 100mM forskolin or DMSO treatment.

2.4.3.2 Protein Lysate Harvesting

For IP and co-IP assays and western blot analysis, cells were harvested in 3T3-lysis buffer (25mM HEPES, 10% w/v glycerol, 50mM NaCl, 1% w/v Triton x100, 50mM NaF, 30mM NaPP, 5mM EDTA, pH7.4) containing Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche). For phosphorylation studies, PhosStop Phosphatase Inhibitor Cocktail Tablets (Roche) were also added. For PDE assays, cells were harvested in KHEM buffer (50mM KCl, 50mM HEPES, 10mM EGTA, 1.92mM MgCl₂, pH7.4), vortexed (due to lack of detergent) and snap frozen. Harvested lysate was centrifuged at 2000xg for 10 minutes to remove any insoluble material. The supernatant was transferred to a new Eppendorf tube and either used straight away or frozen at -80°C until required.

2.4.4 xCelligence Measurement of Cell Proliferation and Migration

The xCelligence system allows real time measurement of cell proliferation or migration on the bases that as cells grow over, or migrate through an electrode, they change the resistance of the current flowing through the metal. These changes in electrical impedance are recorded by the xCelligence machine (Acea) and interpreted by the proprietary software RTCA system (Acea). Changes in electrical impedance are represented by changes in cell index (CI); a dimensionless unit of measurement where CI=zero when cells are absent, and increases as cells proliferate or migrate (figure 2.2).

The xCelligence system comprises a gold electrode-plated culture vessel and a docking unit for this vessel within a 37°C incubator (5% CO₂, 95% air), which is connected to the impedance measurement unit outside the incubator. This is connected to a laptop PC on which proprietary RTCA software is run. This software collects the cell index data and generates growth or migration curves for the cells in the docked vessel. For proliferation assays the vessel used is an Eplate, while for migration assays a CIM plate is employed (figure 2.2).

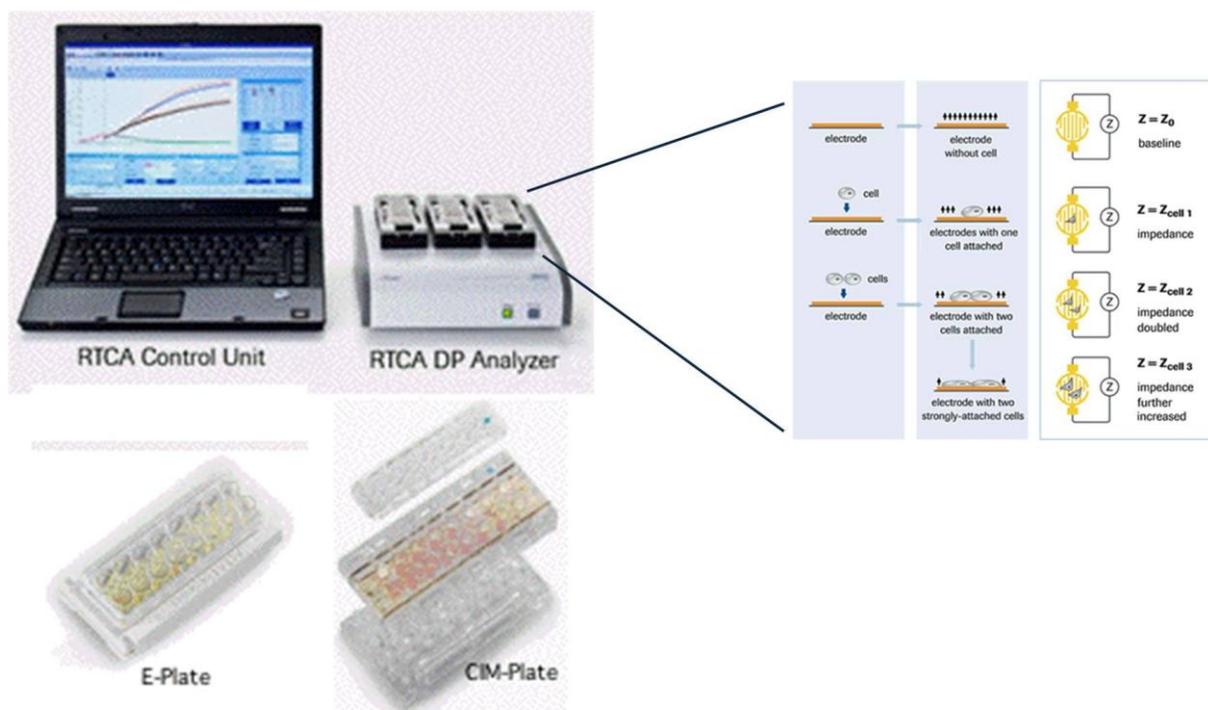


Figure 2.2. The xCelligence system for real time cell proliferation and migration measurement.

Changes in electrical impedance are represented as changes in CI values, corresponding to increasing number of cells on the Eplate surface, or migrating through the electrode membrane of top chamber of the CIM plate. Z =electrical impedance.

2.4.4.1 Cell proliferation assays

A cell number titration for each of the cell lines used was initially carried out to find the optimum number of cells required to produce a good growth curve, dependant on adherence properties and doubling times. 30,000 VCaP cells, 10,000 DU145 cells and 10,000 PC-3 cells were used in all experiments. This discrepancy is due to the vastly different adherence properties between the two cell lines as well as the significantly longer doubling time of the VCaP cell line. The volume of cell suspension required for 30,000 or 10,000 cells was subtracted from 200 μ l. The resulting figure was the volume of medium alone that was then

added to each well in the Eplate. With this medium the plate was inserted into the docking station and 'blanked', i.e. the impedance reading for medium alone was recorded. Following this, the cells were added to each well, to give a total of 200 μ l/well. The Eplate was returned to the docking station and the electrical impedance was measured every 10 minutes for ~4 hours and then every 15 minutes for 4-5 days.

siRNA transfection of VCaP cells was carried out the following day.

DNA transfection of DU145 and PC3 cells was carried out prior to seeding of the cells.

2.4.4.2 Cell Migration Assays

A cell number titration was initially carried out, and 40,000 DU145 cells was found to be optimum for the migration curve. The CIM plate contains two compartments (figure 2.2). 165 μ l complete medium was added to the wells in the lower compartment; the serum acted as the chemoattractant. (Serum-free medium was added to 2-3 wells; these served as migration control wells). The volume of cell suspension required for 40,000 cells was subtracted from 200 μ l. The resulting figure was the volume of complete medium alone that was then added to all the wells in the top compartment. The CIM plate was then inserted into the docking station and 'blanked', i.e. the impedance reading for medium alone was recorded. Cells were transfected as described above and 40,000 were added to each well in the top compartment, giving a total volume of 200 μ l. The CIM plate was returned to the docking station and the electrical impedance was measured every 10 minutes for ~18 hours, prior to any proliferative events.

2.5 PLA Probe Staining and Confocal Microscopy

The Duolink Proximity Ligation Assay (PLA) (Olink Bioscience) is designed primarily to look at co-localisation of interacting proteins *in situ*, when two different antibodies are used. In this case, the assay was used to detect PDE4D7 alone, on the premise that signal amplification would allow detection of endogenous protein. Cells were seeded in multichamber slides (Nunc Lab-Tek II Chamber Slide System-Thermo). Cells were firstly rinsed in TBS and then fixed in 4% paraformaldehyde at RT for 10min. Cells were then washed twice with TBS and permeabilised with 0.1% Triton-X100 in TBS at RT for 15 min. Blocking was then carried out using the Duolink blocking solution for 30 minutes. Following this cells were incubated with two primary antibodies at a dilution of 1:500 for 1 hour 45 minutes-2 hours. The antibodies were from two different host animals. After this incubation, cells were washed with Duolink wash buffer A (0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20, pH 7.4). Cells were then incubated for 1 hour with PLA probes, which, like secondary antibodies, recognise the primary antibodies, but are conjugated to oligonucleotides. Following washing, a solution is added for 30 minutes to bring about hybridisation of the PLA probes. Again samples are washed, and a ligation reaction is carried out for 1 hour 40 minutes which brings about a rolling circle amplification (RCA) reaction. This amplifies the signal of the detected protein. All incubations were carried out in a humidity chamber, which was fabricated by placing a beaker of water into a 37°C incubator. Finally, the slides were mounted with coverslips using Duolink mounting medium and observed using a Zeiss Pascal laser-scanning confocal microscope (LSM) 510 Meta and an Axiovert 100 microscope (Carl Zeiss, UK) equipped with an oil immersion objective (63x/1.4 NA plan apochromat lens). Images were captured and processed on Zeiss LSM Image Examiner and analysed using the NIH Image J software (ImageJ, <http://rsb.info.nih.gov/ij/>). The detected protein showed up as discrete red spots.

2.6 Statistical Analysis

Statistical significances between groups were determined by the use of Student's *t*-test or ANOVA, as indicated. Values were considered significant if $p < 0.05$. Where representative immunoblots or microscopy images were shown, similar data were obtained ≥ 3 times. Microsoft Excel was used to calculate the student's *t*-test; Graphpad Prism (Graphpad software) was used for the ANOVA test.

3 PDE4D7 Signalling is Altered as Prostate Cancer Progresses

3.1 Introduction

The role of cAMP in PC progression has been well studied, as first noted by the differential expression of adenylyl cyclases in normal and tumour prostate tissue (Shima, Kawashima et al. 1976). Since then, a plethora of studies suggest that alterations in cAMP signalling cascades have an active role in PC progression. Increased expression of GPCRs (Nelson, Bagnato et al. 2003; Weng, Wang et al. 2005) and promiscuous G_s signalling (Kasbohm, Guo et al. 2005) can lead to AR signalling in the absence of androgen, thereby driving the progression to AI PC. PKA isotype switching also promotes the AI phenotype (Kvissel, Ramberg et al. 2007; Merkle and Hoffmann 2011). As discussed, cross talk of cAMP effector proteins with the AR is also an important factor in androgen-independent AR signalling and PC progression. However, given the importance of cAMP in this process, little is known on the role of cAMP PDEs in PC.

As cAMP is a ubiquitous second messenger, specificity of action within the cAMP signalling system is maintained by compartmentalisation of proteins that act to produce, are affected by and degrade the cyclic nucleotide. As a consequence, the roles of individual PDEs are often directed by their differential tissue and cell expression. Uckert et al. determined the PDE profile in a zone-specific manner in the normal prostate, and found high PDE4 activity (Uckert, Kuthe et al. 2001). They later found PDE4 protein to be highly expressed in glandular and stromal cells, suggesting a role for this enzyme in proliferation (Uckert, Oelke et al. 2006; Waldkirch, Uckert et al. 2010). PDE4 has also been suggested to be involved in PC progression, although with varying observations (Rahrmann, Collier et al. 2009; Sarwar, Sandberg et al. 2013).

Due to the high level of PDE4 expression noted by Uckert and colleagues and the suggested implication of PDE4 in PC, our group set out to determine the PDE4

expression profile in PC samples, with the aim of identifying a potential PDE PC-specific biomarker (Byrne, Henderson et al. 2014).

This aims of this chapter are to discuss firstly the earlier findings by our group on the changing PDE mRNA expression profile as PC progresses and to expand on these findings by investigating the levels of PDE4D7 protein within AS and AI cell lines. Secondly, to investigate the effect of PDE4D7-mediated cAMP hydrolysis on PC cell proliferation and migration. Thirdly, I present a hypothesis on why PDE4D7 expression changes as PC moves towards androgen insensitivity.

3.2 Results

3.2.1 Phosphodiesterase 4D7 is downregulated as prostate cancer becomes androgen-insensitive

3.2.1.1 PDE4 mRNA expression profiling

PDE4 isoforms are differentially expressed in a tissue and cell-specific manner (Houslay 2010). To establish which, if any, PDE4 isoforms are associated with PC, our group carried out a PDE expression profile screen on 19 prostate cancer xenografts and cell lines representing both AS and AI cancer by means of qPCR (Henderson, Byrne, in review). The xenografts and cell lines used are shown in table 3.1. PDE4A, PDE4B and PDE4D enzymes were detected. PDE4D was the most highly expressed subfamily and interestingly, showed a downregulation between the AS and AI samples. PDE4D7 was observed to be the most abundantly expressed isoform in the AS samples, and to show the most profound downregulation in all AI samples. We observed a 40-fold reduction in PDE4D7 transcript expression in the AI samples, with no correlation between xenograft or cell line (figure 3.1). The samples used were derived from both primary and metastatic tumours, and the differential PDE4D7 expression was observed between all AS and AI samples, with no correlation to tumour origin.

Sample Description	Cell Line	Xenograft	Origin	Androgen Sensitivity	AR Expression	PSA Expression	AR Sequence	TMPRSS2: ERG Fusion
VCaP	X		Vertebra	AS	YES	YES	Wt	YES
DuCaP	X		Dura Mater	AS	YES	YES	Wt	YES
LNCaP	X		Lymph Node	AS	YES	YES	T877A	ETV1
PC346C	x		Primary	AS	YES	YES	Wt	NO
PC346P		X	Primary	AS	YES	YES	Wt	NO
PC346B		X	Primary	AS	YES	YES	Wt	NO
PC82		X	Primary	AS	YES	YES	Wt	YES
PC310		X	Primary	AS	YES	YES	Wt	YES
PC295		X	Lymph Node	AS	YES	YES	Wt	YES
PC-EW		X	Lymph Node	AS	YES	YES	Wt	YES
PC346Flu2		X	Primary	AS	YES	YES	T877A	NO
PC3	X		Bone	AI	NO	NO	N/A	NO
DU145	X		Brain	AI	NO	NO	N/A	NO
PC324	X		Primary	AI	NO	NO	N/A	NO
PC346DCC	X		Primary	AI	Low	Low	K311R	NO
PC346Flu1	x		Primary	AI	YES	YES	Wt	NO
PC135		X	Primary	AI	NO	NO	N/A	NO
PC374		X	Skin	AI	NO	NO	N/A	ETV1
PC133		X	Bone	AI	NO	NO	N/A	ETV4

Table 3.1. The 19 xenograft and cell line samples used in the PDE profiling of PC.

The PC samples used represented both AS and AI carcinoma, and both primary and metastatic disease. The presence of AR and PSA, and the TMPRSS2:ERG gene fusion are indicated.

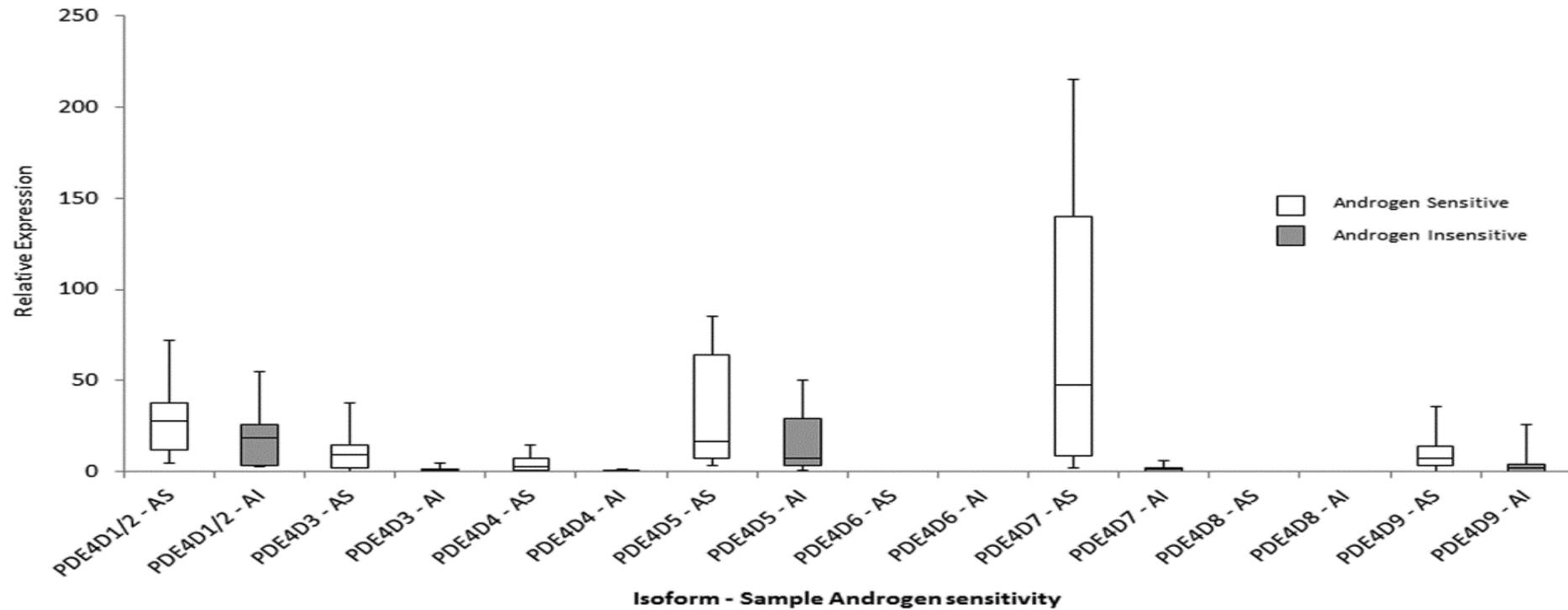


Figure 3.1. Differential Expression of PDE4D isoforms in AS and AI PC.

PDE4D7 is the most abundant isoform in AS samples, with significant downregulation into AI disease (Byrne, Henderson et al. 2014).

This screen provided a model of the changing PDE4D mRNA profile during disease progression. With the dramatic change in PDE4D7, we wished to further study this enzyme in the context of PC. Firstly, we wished to validate the protein levels of PDE4D7 in AS versus AI PC cell lines. As discussed, the transcript level of PDEs does not always match that of the protein, due to post-translational modifications (Rhodes, Yu et al. 2004; Richter, Jin et al. 2005; Rahrmann, Collier et al. 2009). Also, the presence of transcript does not given any indication of the presence of a functional enzyme.

The cell lines used in this study to investigate the role of PDE4D7 in PC include; the AS VCaP and DuCaP cell lines, and the AI PC3 and DU145 cell lines.

DuCaP and VCaP cells phenotypically represent clinical AS prostate carcinoma. They were isolated from metastatic sites in the dura mater and lumbar vertebrae, respectively, from patients with castrate resistant prostate cancer (CRPC) (as occurs following hormonal therapy, discussed later) post-mortem. The samples were xenografted into immune-deficient SCID mice from which immortalised cell lines were established. They express AS markers such as the AR and PSA (Korenchuk, Lehr et al. 2001; Lee, Korenchuk et al. 2001).

The PC3 and DU145 cell lines are classical cell models of AI PC, and have been extensively used to study PC over the last few decades. They are derived from bone and brain metastases, respectively, and represent late stage PC with no expression of AR or PSA, and are not responsive to androgens (Stone, Mickey et al. 1978; Kaighn, Narayan et al. 1979).

3.2.2 Validation of PDE4D7 protein expression in AS and AI PC cell lines

3.2.2.1 PLA probes confirm differential PDE4D7 protein expression between AS and AI PC

For the most of this project, a specific PDE4D7 antibody that could detect endogenous levels of protein was unavailable. PDE4 isoform-specific antibodies are often difficult to raise due to the differential immunogenic properties of the various unique N-termini (unpublished observations in our laboratory). A specific antibody that could detect only overexpressed PDE4D7 was available, but was of no use for western blot analysis of endogenous protein. In order to study the level of endogenous PDE4D7, the Duolink PLA system (Olink) was employed (figure 3.2). The principle behind the Duolink system is that of a co-IP, in which antibodies against two proteins believed to interact are used for probing. These antibodies must be raised in different host animals. Following incubation with these primary antibodies, secondary antibodies conjugated to oligonucleotides (PLA probes) are added to the reaction. One probe must be minus, one plus; e.g., goat plus and rabbit minus. A ligation step is then performed using more oligonucleotides and ligase. The ligase catalyses binding of these oligonucleotides with the PLA probes which then join to form a closed circle if the proteins are in close enough proximity (<30-40nm), i.e. if they are interacting. Lastly an amplification reaction is carried out where more nucleotides and fluorescently labelled oligonucleotides are added to the reaction. The oligonucleotide arm of one PLA probe acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as template, thereby generating a concatemeric product. The fluorescently labelled oligonucleotides will hybridise to the RCA product which creates a signal that is detected by fluorescent microscopy. The signal is seen as distinct red spots and each spot represents one molecular interaction, but not all molecules are detected. It is essentially like PCR within the cell. Here, the PLA probes were used to detect PDE4D7, rather than an interacting pair. A panPDE4D antibody, which detects all PDE4D isoforms, was used with the aforementioned PDE4D7 antibody that detects only overexpressed levels of protein in the hope that both

would bind PDE4D7 and signal amplification would allow detection of endogenous protein in the VCaP cell line.

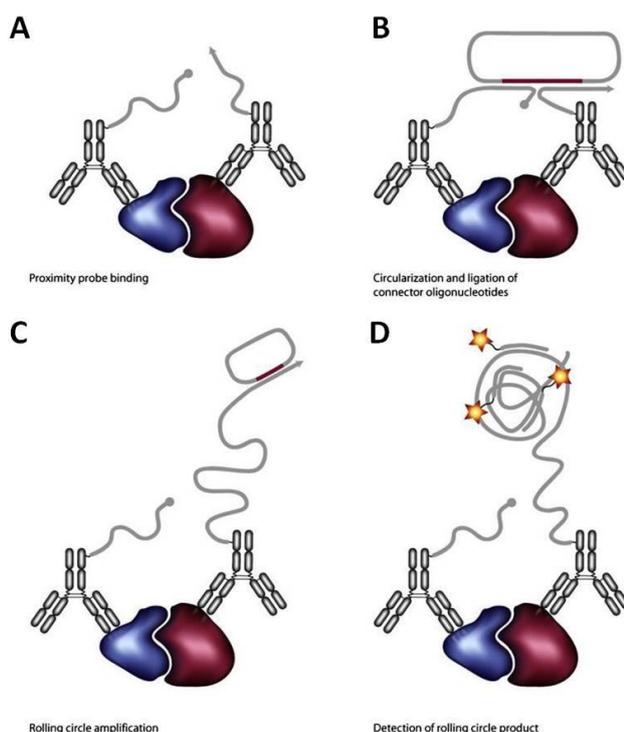


Figure 3.2. Schematic representing the in situ PLA probe mechanism.

A; cells are incubated with specific primary antibodies from different hosts. The pair of PLA probes (plus and minus) which have oligonucleotides attached are added and they bind the respective antibody. **B;** ligase catalyses the binding of the PLA oligonucleotides to additional oligos and a circle is formed. **C;** additional fluorescently labelled oligonucleotides are added, and the oligonucleotides arm of one PLA probe acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as template, thereby generating a concatemeric product. **D;** the fluorescently labelled oligonucleotides hybridise to the RCA product which creates a signal that is detected by confocal fluorescent microscopy.

Employing confocal microscopy, spots were indeed detected in VCaP cells (figure 3.3), which became abundant following overexpression of wtPDE4D7 (figure 3.3 A&B), or a dominant-negative construct (figure 3.3 C), which displaces endogenous PDE4D7 protein (dnPDE4D7). Gratifyingly, control experiments gave no positive results as no spots were detected in VCaP cells when PDE4D7 and panPDE4B antibodies were used in combination (figure 3.3 F), nor with co-detection of PDE4B2 and panPDE4D (figure 3.3 G). No spots were detected in the AI PC3 (figure 3.3 H) and DU145 cells (figure 3.3 I), confirming specific downregulation of the PDE4D7 enzyme into AI PC.

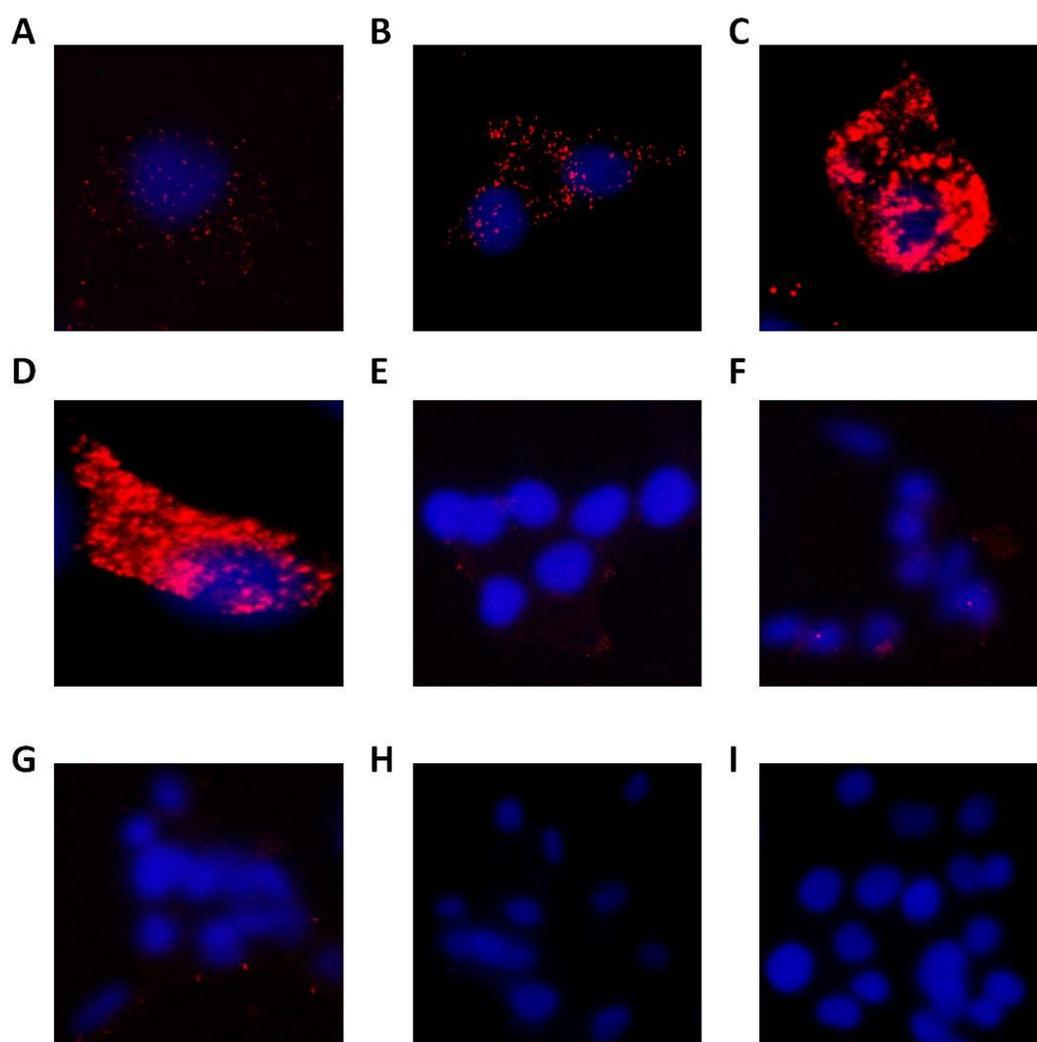


Figure 3.3. PLA probes verify PDE4D7 is differentially expressed in AS and AI PC cells at the protein level.

PLA signal (red) is only visible where PDE4D7 is expressed. DAPI staining (blue) was used to mark nuclei. **A&B**; VCaP cells were probed with panPDE4D antibody and PDE4D7 antibody. **C**; VCaP cells overexpressing PDE4D7 were probed with panPDE4D antibody and PDE4D7 antibody. **D**; VCaP cells transfected with dnPDE4D7 were probed with panPDE4D antibody and PDE4D7 antibody. **E**; secondary antibody only, **F**; VCaP cells were probed with panPDE4B antibody and PDE4D7 antibody. **G**; VCaP cells were probed with panPDE4D antibody and PDE4B2 antibody. **H**; PC3 cells were probed with panPDE4D antibody and PDE4D7 antibody. **I**; DU145 cells were probed with panPDE4D antibody and PDE4D7 antibody. Images shown are representative of three separate experiments on different cell preparations.

3.2.2.2 **Generation and Characterisation of a Novel PDE4D7-Specific Antibody: Confirmation of Differential PDE4D7 Protein Expression between AS and AI PC**

Due to the fact that the PDE4D7 antibody described above could only be used to detect over-expressed PDE4D7 or used in PLA studies, I decided to raise a new PDE4D7-specific antibody with the hope that it could be used to confirm our novel findings regarding PDE4D7 protein levels in the different stages of PC. To this end, specific anti-sera were generated against the complete PDE4D7 unique N-terminal region. This proved quite difficult due to the short N-terminus of this isoform, and in light of the fact that previous attempts using unique PDE4D7 N-terminal peptides as antigens had failed. However, using a GST-fusion of the whole N-terminal region as an antigen proved successful due to the increased immunogenic nature of the fusion protein. The serum from the final bleed of the inoculated sheep was purified by affinity chromatography and the purified antibody was validated for specificity by means of peptide array technology and western blot analysis.

Peptide array technology entails the production of immobilised peptide libraries of a full-length protein of interest. The protein is spotted onto the array in overlapping 25mer peptides which are conjugated to a nitrocellulose membrane using an amino-bond. Each peptide overlaps the next by 20 amino acids, so each is shifted along by 5 amino acids, until the full length protein is spotted. At any one time a batch of arrays is made. The peptide arrays can then be used to map residues of protein-protein interactions, by incubating with a purified protein of interest, or indeed to detect the epitope to which an antibody binds and validate its specificity. A PDE4D7 array was Coomassie blue stained to ensure all residues were indeed spotted (figure 3.4 A). Another PDE4D7 array from the same batch was probed with the novel antibody and signal was detected by chemiluminescence. Dark spots represent areas of cross-reactivity within the unique N-terminus (figure 3.4 B). As expected, the novel PDE4D7 antibody only recognised peptides within the unique N-terminal region, demonstrating the specificity of interaction. The PDE4D7 antibody was also tested for specificity

using protein standards of other long forms PDE4Ds, all of which migrate on SDS PAGE at ~100kDa (figure 3.4 C). Again, only PDE4D7 was recognised.

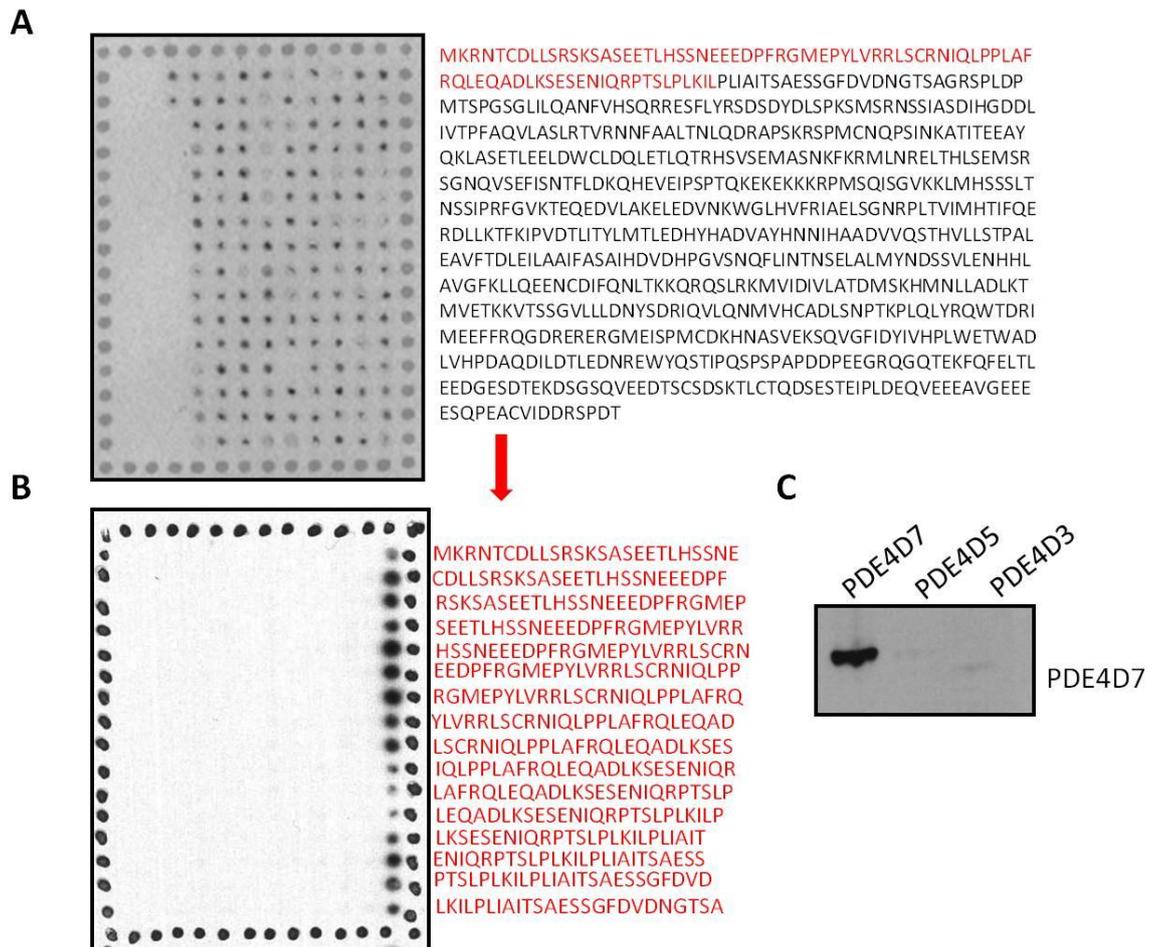


Figure 3.4. Peptide array technology verifies a novel PDE4D7 antibody.

A; a full length PDE4D7 peptide array stained with Coomassie Brilliant Blue. The 4D7 sequence is shown beside the array, with the unique N-terminal region highlighted in red. **B**; a full length PDE4D7 peptide array was probed with the novel PDE4D7 antibody. Control arrays with no primary antibody and pre-immune serum were also used, on which no spots were detected. **C**; PDE4D7, PDE4D5 and PDE4D3 protein standards were blotted with the novel PDE4D7 antibody. N=3.

Following the successful raising and characterisation of a highly-specific PDE4D7 specific antibody, the protein expression of the isozyme could be verified in PC cell lines by means of western blot and immunoprecipitation (IP). Using this novel antibody, it was again determined that PDE4D7 protein expression is downregulated as the cancer becomes androgen independent (figure 3.5 A), as PDE4D7 could only be detected in DuCaP and VCaP cell lines but not PC3 and DU145s. This result was confirmed using IP analysis, where endogenous PDE4D7 was pulled down with the new antibody and detected with the pan-PDE4D antibody (figure 3.5 B). Overall, the protein expression studies carried out with the new antibody verified the PLA work (figure 3.3) and supported the findings from the PCR study (figure 3.1).

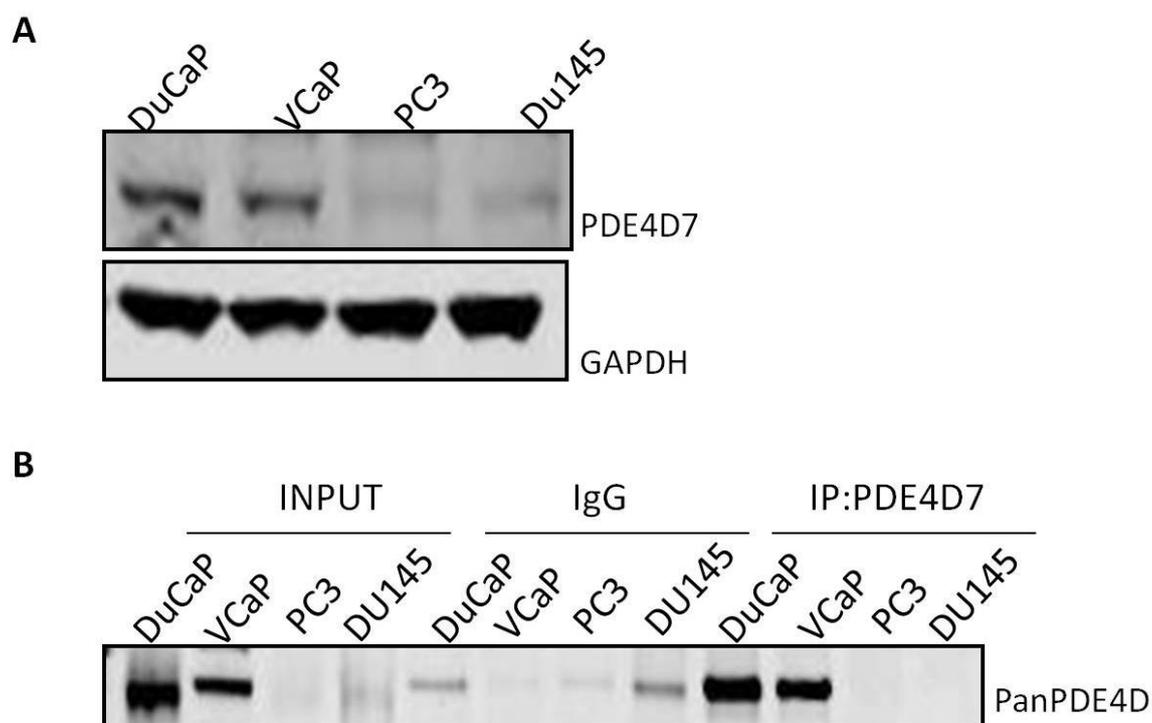


Figure 3.5. A novel PDE4D7-specific antibody validates PDE4D7 downregulation in AI PC cell lines.

A; whole cell lysates of AS (DuCaP and VCaP) and AI (PC3 and DU145) PC cell lines were subjected to SDS-PAGE and western blot analysis, followed by probing with the novel PDE4D7-specific antibody. The predicted weight of PDE4D7 on SDS PAGE is ~100kDa, at which a band is detected in DuCaP and VCaP cells only. N=3.

B; immunoprecipitation (IP) was carried out in AS (DuCaP and VCaP) and AI (PC3 and DU145) cell lines using the novel PDE4D7 antibody. The western blot was probed with panPDE4D. Normal sheep IgG served as a negative control. Input is the whole cell lysate from which the IP was carried out. N=3.

3.2.3 PDE4D7 Mediates Prostate Cancer Cell Proliferation

Immunocytochemistry and FRET analyses have shown that the majority of PDE4D7 localises to the sub-plasma membrane region where it plays a major role in regulating cAMP levels (Byrne, Henderson et al. 2014). Taken together with the qPCR screen data, these results suggest that PDE4D7 is the main cAMP hydrolysing PDE in AS PC cells and acts at the sub-plasma membrane region. Following validation of the downregulation of PDE4D7 as PC becomes AI, I wished to assign a function to this enzyme and to determine if this event was physiologically relevant. Uckert et al. suggested that PDE4 is involved in proliferation of normal prostate epithelium (Uckert, Oelke et al. 2006). With this in mind, proliferation assays were carried out to study the effect altering PDE4D7 levels may have on the phenotype of PC cells.

3.2.3.1 xCelligence Measurement of Real Time Cell Proliferation and Migration

The xCelligence method (Acea) of measuring cell growth relies on the detection of small changes in electrical impedance evoked by cells growing in specially modified culture plates. This technique avoids the use of invasive “end-point” techniques and labels. Additionally, monitoring can be done in real time, allowing kinetic analysis. Cells were seeded into an Eplate (proliferation assay) or a CIMplate (migration assay). Eplates are coated on the underside with embedded gold electrodes. CIM plates comprise two compartments, separated by a gold electrode-plated membrane. As cells grow over, or move through the electrodes, they cause a change in the electrical impedance, which is measured by the xCelligence machine and recorded as changes in cell index (CI). This is a dimensionless unit of measurement representing the measurement of zero impedance when cells are absent, and increasing impedance when cells begin adhering to the plate and proliferate, or move through the electrodes as they migrate. The advantage of using this system is that cell growth or migration is measured in real time, thus transient effects caused by any treatment can be measured, rather than just end-point analysis.

Firstly, a dominant-negative PDE4D7 approach was employed to investigate how displacement of endogenous PDE4D7 and accumulation of cAMP at the plasma membrane would affect AS cell proliferation. Our group was the first to use a novel dominant-negative approach to study the unique intracellular roles of sequestered PDEs (McCahill, McSorley et al. 2005). Dominant-negative (dn) constructs contain a single nucleotide substitution within the active site cAMP binding pocket, so as to render the enzyme catalytically inactive but without affecting its tertiary or quaternary structure. The dn construct is overexpressed in cells of interest, such that it causes displacement of the cognate endogenous wild-type (wt) protein from its functionally relevant intracellular location. This inhibits hydrolysis of cyclic nucleotide at this region, and renders any nearby PKA hyper-sensitive to activation. These constructs do not affect global cAMP gradients or total cellular PDE activity. The mutation in PDE4D7 that renders it catalytically inactive is D559A. Transfection of both dn- and wt- PDE4D7 constructs resulted in a measurable over-expression of both proteins compared with mock (empty vector) transfected cells (figure 3.6 C). Resulting displacement of PDE4D7 in VCaP cells following dnPDE4D7, over-expression led to a significant increase in the growth rate of the cells compared to control ($p=0.02$, students t-test), as determined by the slope of the log phase of the growth curve (figure 3.6 A,B). This increase was in contrast to a corresponding decrease caused by overexpression of wtPDE4D7. These results strongly implicate PDE4D7 in PC cells as having a role in growth control.

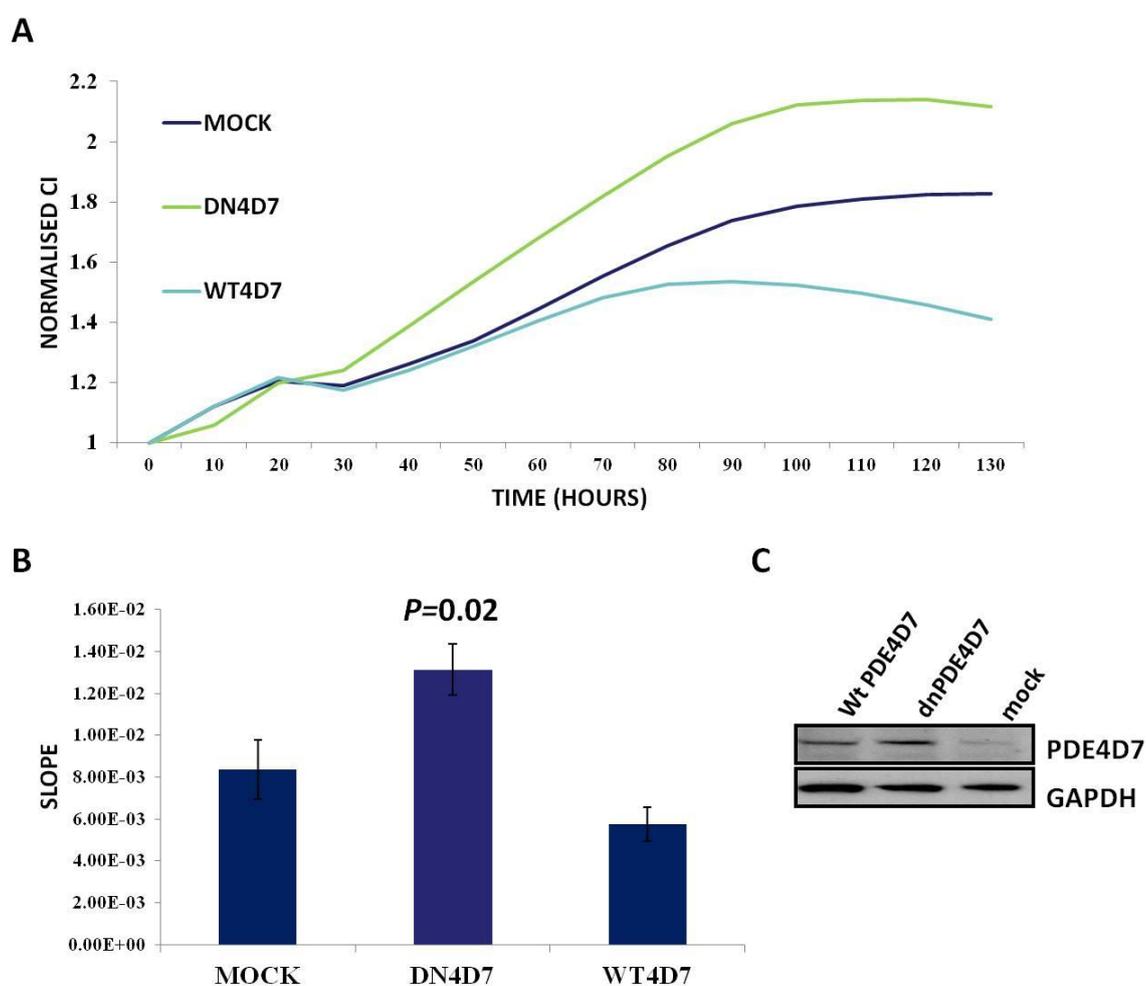


Figure 3.6. Dominant-negative displacement of PDE4D7 increases the proliferation of AS VCaP cells.

A; the electrical impedance-based xCELLigence system was used to measure the proliferation of VCaP cells transfected with wild type (wt) PDE4D7 or the dominant negative, catalytically inactive mutant (dn) or a control construct (mock), as shown by a growth curve. **B;** the slope of the growth curve was calculated by linear regression analysis using the cell indices (CI) from each proliferation assay. **C;** wt- and dn-PDE4D7 constructs were expressed at equal levels as assessed by western blot analysis using a PDE4D7 antibody. GAPDH was used as a protein loading control. N=6.

As displacement of PDE4D7 increased AS proliferation, I set out to see if knockdown of PDE4D7 by means of siRNA would also cause such an increase. siRNA takes advantage of the host cell's immune response to double stranded RNA, such as when under viral attack. Once inside the cell, the siRNA forms a complex with the RNAi-induced silencing complex (RISC) which directs the siRNA to the target mRNA. The siRNA then brings about degradation of the target mRNA sequence by means of endo and exo-nucleases.

siRNA targeted against the N-terminus of PDE4D7 (siPDE4D7) was compared to siRNA targeted to the common C-terminal region of all PDE4D isoforms (siPDE4D), along with a scrambled non-targeting control siRNA (siControl). As expected, both siPDE4D7 and siPDE4D evoked reductions of the protein levels of PDE4D (figure 3.7 C, D). It should be noted that a number of different PDE4D isoforms exhibit similar molecular weights and the increased silencing of the siPDE4D vs siPDE4D7 was expected. Knockdown of PDE4D7 led to a marked increase in VCaP proliferation ($p=0.06$, students t-test) compared to global PDE4D knockdown (figure 3.7 A, B), further indicating a specific role for this enzyme in cAMP regulation in PC.

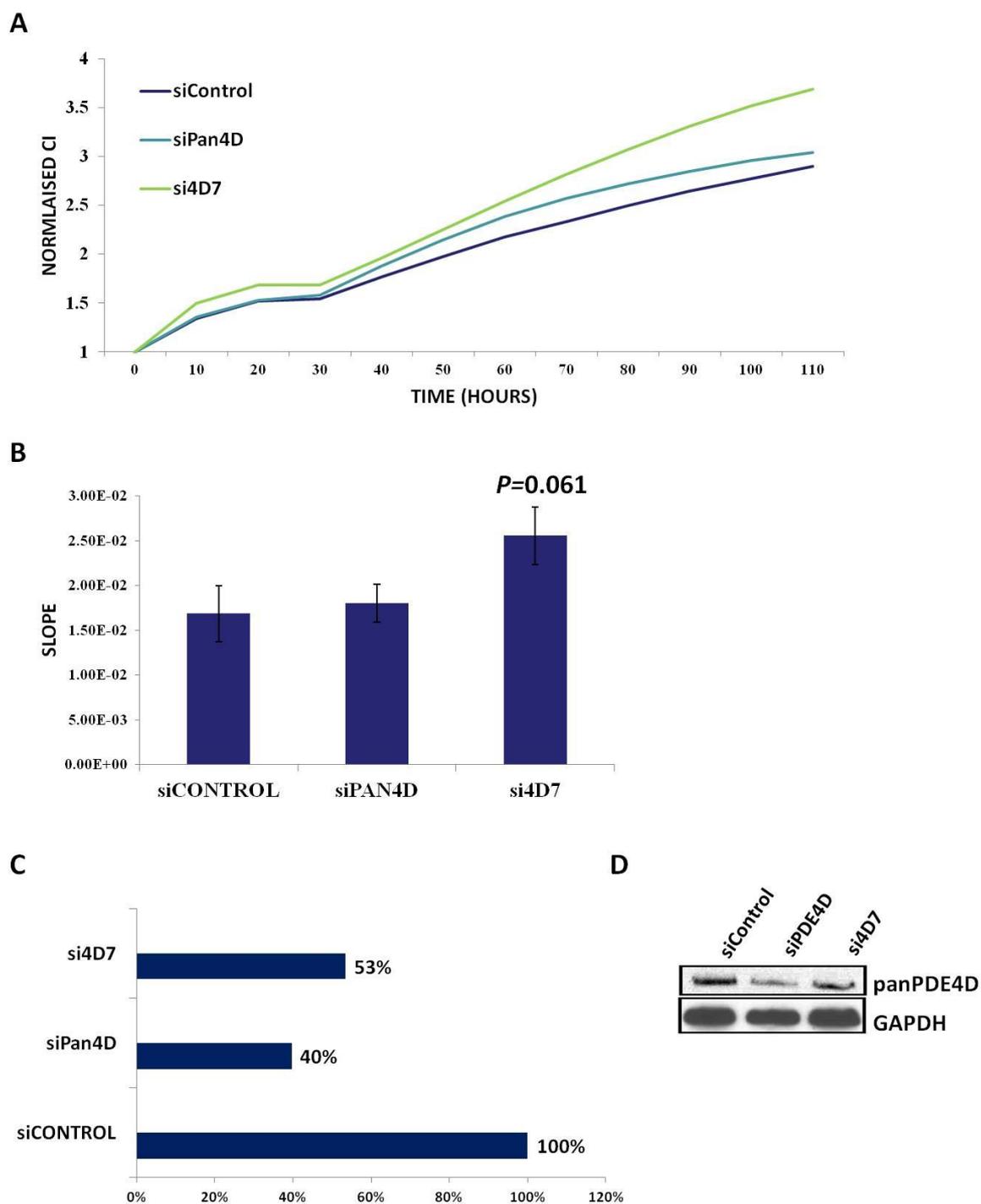


Figure 3.7. siRNA mediated knockdown of PDE4D7 specifically increases the proliferation of AS VCaP cells.

A; the electrical impedance-based xCELLigence system was used to measure the proliferation of VCaP cells transfected with siRNA targeted against PDE4D7 (si4D7) or total PDE4D (Pan4D) or a non-targeting control (siControl), as shown

by a growth curve. **B**; the slope of the growth curve was calculated by linear regression analysis using the cell indices (CI) from each proliferation assay. **C**; expression of PDE4D7 and total PDE4D as a percentage of scrambled control, as calculated from western blot analyses of protein knockdown (**D**), which shows that siRNA knockdown decreased the abundance of endogenous PDE4D and PDE4D7 expression. N=5.

With the observed increase in proliferation of AS cells when PDE4D7 is displaced or downregulated and the corresponding decrease when it is over-expressed, the logical next-step was to investigate whether ‘re-expression’ of the enzyme into PDE4D7-deficient AI cells would retard cell proliferation. Indeed, over-expression of wtPDE4D7 (figure 3.8 C) led to a significant decrease in the proliferation of DU145 ($p=0.002$ students t-test) (figure 3.8 A and B), and PC3 cells ($p=0.00003$, students t-test) (figure 3.9, A and B), suggesting that PDE4D7-mediated cAMP hydrolysis causes a reduction in proliferative signalling events in PC cells.

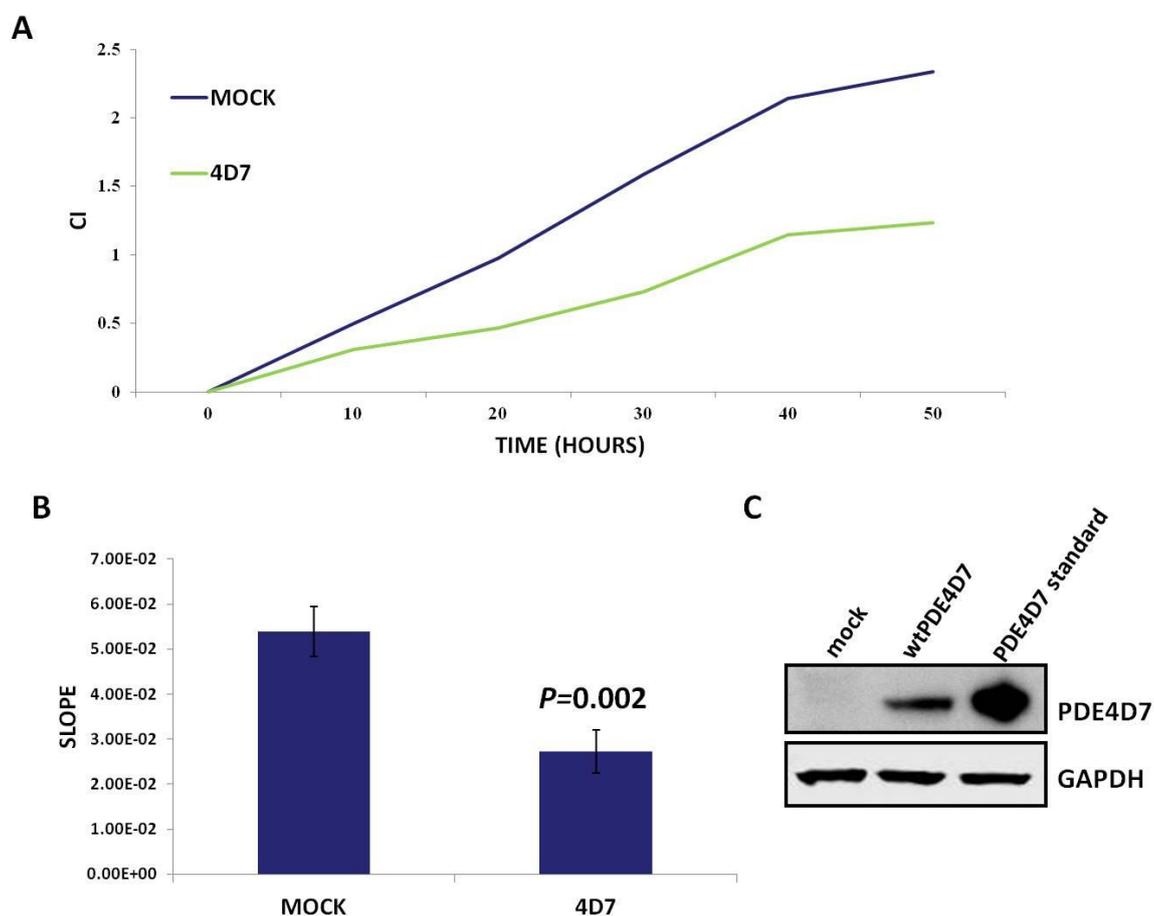


Figure 3.8. PDE4D7-mediated cAMP hydrolysis reduces the proliferation rate of DU145 cells.

A; the electrical impedance-based xCELLigence system was used to measure the proliferation of Du145 cells transfected with wild type (wt) PDE4D7 or a control construct (mock), as shown by a growth curve. **B;** the slope of the growth curve was calculated by linear regression analysis using the cell indices (CI) from each proliferation assay. **C;** western blot analysis of transfection. N=3.

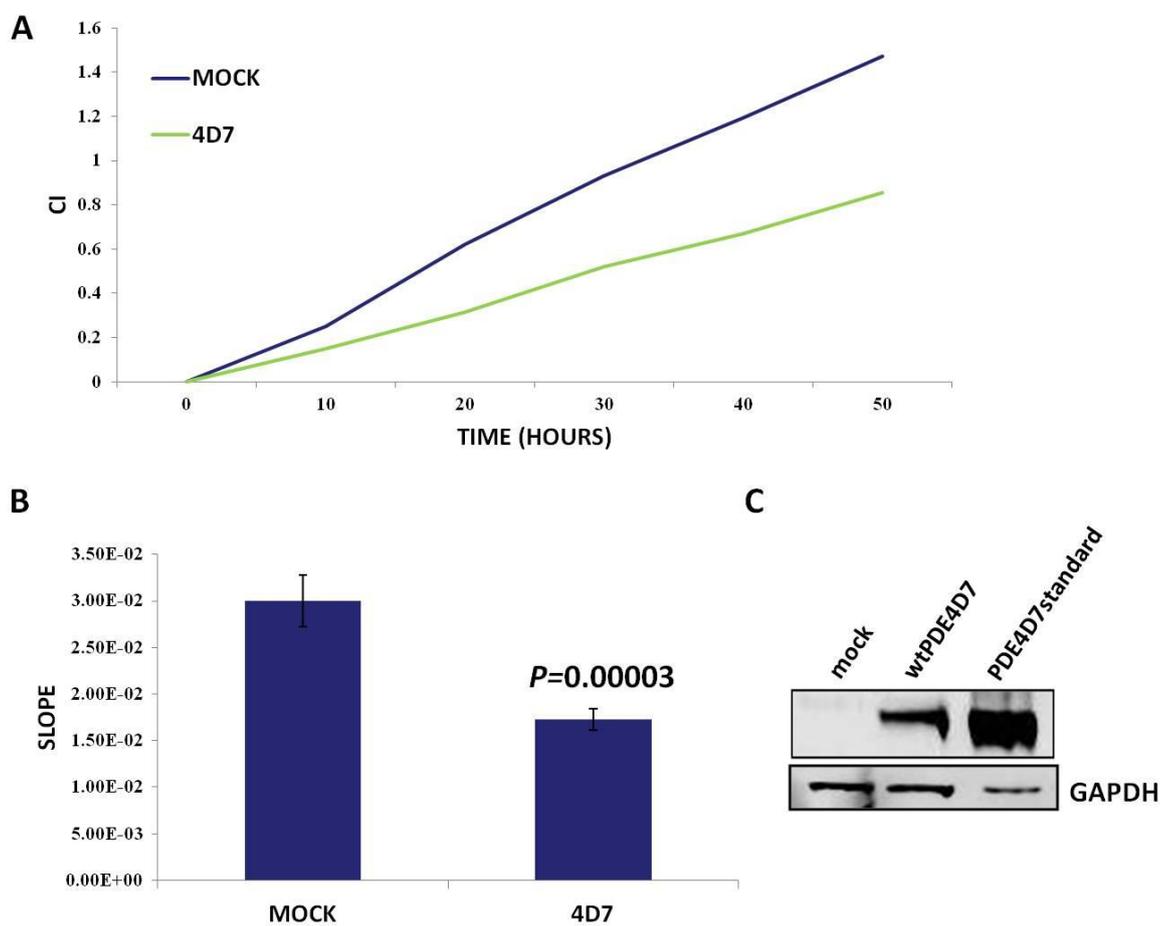


Figure 3.9. PDE4D7-mediated cAMP hydrolysis reduces the proliferation rate of PC3 cells. **A**; the electrical impedance-based xCELLigence system was used to measure the proliferation of PC3 cells transfected with wild type (wt) PDE4D7 or a control construct (mock), as shown by a growth curve. **B**; the slope of the growth curve was calculated by linear regression analysis using the cell indices (CI) from each proliferation assay. **C**; western blot analysis of transfection. N=3.

3.2.4 PDE4D7 may Mediate Prostate Cancer Cell Migration.

The role of cAMP in cancer cell migration is controversial, but it has long been known that PKA is involved in cell migration (Howe, Baldor et al. 2005; Howe 2011). It has been suggested that cAMP can inhibit the migration of invasive breast cancer cells via both PKA and EPAC-dependent pathways involving downregulation of $\beta 3$ integrin (Spina, Di Maiolo et al. 2012). It has also been suggested that cAMP/PKA signalling can inhibit the migration of bladder cancer cells (Ou, Zheng et al. 2013) and pancreatic cancer cells (Zimmerman, Roy et al. 2013). However, a lot of evidence suggests cAMP positively regulates cancer cell migration, for example in breast cancer (Chioni, Shao et al. 2010), and ovarian cancer (McKenzie, Campbell et al. 2011). In these cases, migration was mediated via PKA acting in the vicinity of the plasma membrane, and PKA was upregulated at the leading cell edge. Plasma membrane voltage-gated sodium channels (VGSCs) are often expressed on epithelial-derived cancer cells including breast and prostate cancer cells, and expression correlates with more aggressive metastatic cell lines (Diss, Archer et al. 2001). Here they do not serve a conductance function, but may act as cell adhesion molecules and mediate migration to potentiate the metastatic potential of cancer cells (Brackenbury, Djamgoz et al. 2008). VGSC activity stimulates PKA, which in turn, increases VGSC expression and externalisation (Chioni, Shao et al. 2010). Thus, the spatial dynamics of cAMP and the resulting differential activation of effector proteins may be an important factor in how the cAMP-signalling pathway regulates cancer cell migration. Indeed, the Zimmerman study showed that cell migration was retarded by increases in cAMP resulting from inhibition of total PDE3, and forskolin-induced increases in global cAMP. This suggests that the effect of cAMP increase seen by Zimmerman et al. is not specific, and specific effects may depend on local, region-specific increases in cAMP.

Given the evidence that cAMP is involved in cancer cell migration and thus metastatic potential, the effect of PDE4D7-mediated cAMP hydrolysis on the migration of PC cells was investigated. If a loss of PDE4D7 is associated with an increase in proliferation, perhaps it also effects PC cell migration. The VCaP cell line which represents AS, non-aggressive cancer shows very low migratory

potential compared to the AI highly aggressive DU145 cell line (figure 3.10 A). Although these cell lines differ biochemically in many ways, such as AR expression, the effect of increasing cAMP hydrolysis at the plasma membrane in DU145 cells was investigated. When PDE4D7 was overexpressed (figure 3.10 D), DU145 cells showed a significantly lower migration rate (figure 3.10 B, C). Cells were assayed for 18 hours, prior to any proliferative events and the cell index is proportional to the number of cells that have migrated at a given time.

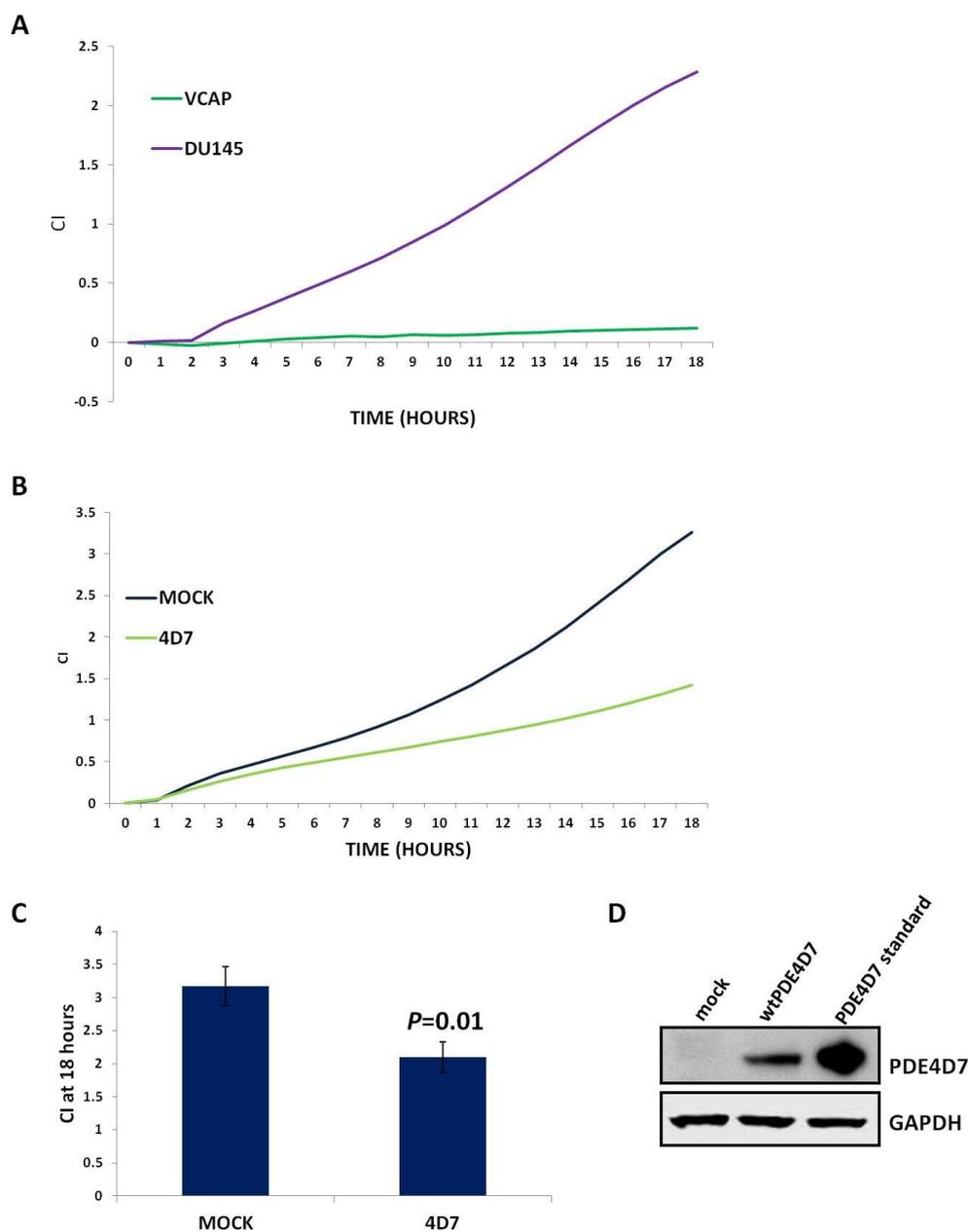


Figure 3.10. Increasing PDE4D7 cAMP hydrolysis reduces the migratory potential of DU145 cells.

A; the electrical impedance-based xCELLigence system was used to compare the migration rates of AS VCaP cells to AI DU145 cells. **B**; the system was used to measure the migration rate of DU145 cells transfected with wild type (wt) PDE4D7 or a control construct (mock), as shown by a migration curve. **C**; the migration of DU145 cells transfected with wt PDE4D7 or mock after 18 hours, as represented by cell index (CI). **D**; western blot analysis of transfection for **B** and **C**. N=3.

3.2.5 Loss of PDE4D7 during the transition from AS to AI PC may be due to an Altered Epigenome

The evidence presented here suggests that PDE4D7 expression is lost as PC progresses, and that this loss may contribute to increased cell proliferation and migratory potential due to aberrant cAMP signalling at the plasma membrane. I wanted to investigate why PDE4D7 undergoes differential transcriptional regulation as PC progresses.

It is known that a disrupted epigenome is associated with prostate carcinogenesis, with altered DNA methylation being the best characterised event (Jeronimo, Bastian et al. 2011). Hypomethylation acts to 'loosen' chromatin leading to increased gene transcription and commonly occurs with proto-oncogenes, whereas hypermethylation 'tightens' the chromatin, repressing gene transcription and commonly occurs with tumour suppressor genes. DNA methylation is the addition of a methyl(-CH₃) group to a cytosine residue adjacent to a guanine (CpG) in gene promoter regions (Dobosy, Roberts et al. 2007). DNA methyltransferases (DMTs) carry out the reaction, using S-adenosyl-L-methionine (SAM) as the methyl donor (Cheng 1995). DNA methylation occurs in stretches of DNA that are rich in CpGs, called CpG islands (Goldberg, Allis et al. 2007). ~60% of mammalian genes contain CpG islands within their promoter regions. A number of PC-specific hypermethylated genes are known, and their detection in body fluids is showing promise as disease-specific biomarkers (Dobosy, Roberts et al. 2007). It has been found that noncoding RNA (ncRNA) found upstream of a promoter region can regulate DNA methylation by cooperating with histone-modifying machinery (Goldberg, Allis et al. 2007).

There is little information on the epigenetics of PDEs. It has been shown that the PDE4D4 promoter contains a CpG island which becomes hypomethylated due to gestational exposure to environmental oestrogens and is associated with PC (Ho, Tang et al. 2006), although we observed a decrease in PDE4D4 in AI PC (Byrne, Henderson et al., in review). A CpG island is also predicted to be located 150bps upstream of the PDE4D7 5' UTR (Henderson D., thesis 2011) and it overlaps with

a ncRNA called prostate androgen-regulated transcript 1 (PART1) (Gretarsdottir, Thorleifsson et al. 2003) (figure 3.11). PART1 is an androgen-regulated non-coding transcript (Lin, White et al. 2000). The presence of the CpG island and the ncRNA suggest that PDE4D7 transcription may be regulated by DNA methylation, and I hypothesised that hypermethylation of this region is responsible for PDE4D7 downregulation into AI PC.

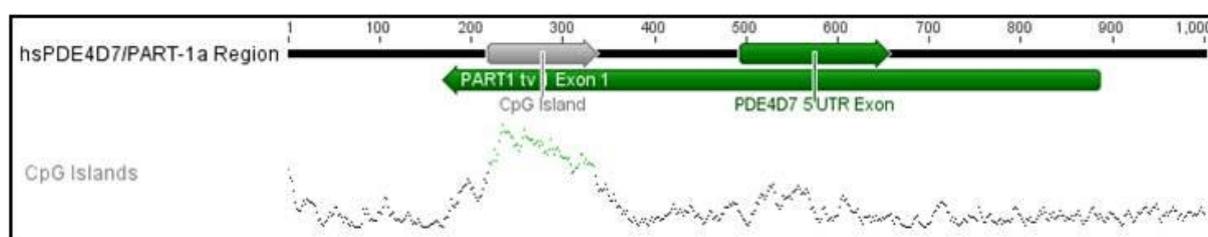


Figure 3.11. A CpG island is predicted upstream of PDE4D7 (Henderson D., thesis 2011).

5-aza-2'-deoxycytidine (5-aza-2-dC) is a cytosine analogue in which the 5 carbon atom is replaced by a nitrogen (Christman, Schneiderman et al. 1985) (figure 3.12). It is used as an inhibitor of DNA methylation (Christman 2002; Garcia, Jain et al. 2010). 5-aza-2-dC is a prodrug that must be activated by phosphorylation to 5-aza-2'-deoxycytidine-triphosphate once inside the cell. Following this, it binds irreversibly to cytosine residues found within CpG islands. By tightly binding the cytosine, 5-aza-2-dC acts to inhibit its methylation by DMTs thereby allowing chromatin opening and gene transcription. It is a current treatment for myelodysplastic syndromes (MDS), a group of bone marrow neoplasms and blood disorders, and has been trialled for use in cancer therapy to allow re-transcription of tumour suppressor genes (Christman 2002; Garcia, Jain et al. 2010).

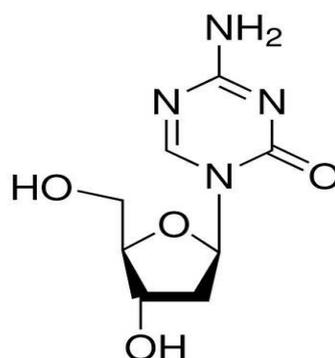


Figure 3.12. The structure of 5-aza-2'-deoxycytidine.

5-aza-2-dC treatment of cells is commonly used to study gene repression. It is often followed by bisulphite sequencing to identify specific methylation sites. Bisulphite treatment deaminates unmethylated cytosines which then convert to uracil, and 5-methylcytosines are unaffected. PCR and DNA sequencing then allow for detection and identification of any methylated cytosines in a given DNA sequence (Darst, Pardo et al. 2010). I hypothesised that the loss of PDE4D7 expression as PC progresses may be due to hypermethylation of its upstream CpG island. In order to investigate this, PC3 cells were treated with 5-aza-2-dC over 3 days, followed by quantitative real time PCR (qPCR) analysis to determine the total PDE4D, and PDE4D7 expression following treatment. The expression of PDE4D5 was also tested as a control.

To analyse the qPCR, the Relative Quantification of Gene Expression approach was used as described by Livak and Schmittgen (Livak and Schmittgen 2001). This method compares the expression of the gene of interest (GOI) against an internal reference gene (RG), i.e. the expression of GOI relative to that of the RG. It allows the quantification of differential expression of a GOI between different samples. The data produced is the fold change in the expression of a GOI. The expression of the internal RG should not vary between samples nor be affected by any experimental treatment. Housekeeping genes such as

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin are often used as reference genes, and despite controversy surrounding the use of a single reference gene (Vandesompele, De Preter et al. 2002; Barber, Harmer et al. 2005), the use of either has been shown to be reliable when studying PC samples (Mori, Wang et al. 2008).

The data output gives Ct values for the GOI and GAPDH, which represents their expression level. For analysis, the comparative Ct approach was used which compares the Ct values of the GOI in the test sample to a control sample, and gives a $\Delta\Delta\text{Ct}$ value.

Firstly, the Ct values of the GOI in all samples are normalised to that of GAPDH;

e.g.; test Ct-GAPDH Ct = ΔCt test

Then; ΔCt test - ΔCt control = $\Delta\Delta\text{Ct}$.

The Taqman® approach was used to determine the relative expression of PDE4D7, PDE4D5 and panPDE4D in the PC3 samples. Taqman® probes provide high specificity which is required to detect and amplify the short unique N-terminal sequences of specific PDE isoforms.

Interestingly, there was a marked increase of almost 6 fold in PDE4D7 expression (figure 3.13 A), and this was almost identical to total PDE4D fold increase (figure 3.13 B). As a control, expression of another PDE4 long form, PDE4D5, was also evaluated. In this case, there was a negligible increase in expression (figure 3.13 C). This suggests that PDE4D7 may be specifically hypermethylated in late stage PC. However, due to time constraints this experiment was carried out only once, and verification is needed before steadfast conclusions can be made. Bisulphide treatment and DNA sequencing would also be necessary, to confirm specific PDE4D7 CpG island methylation.

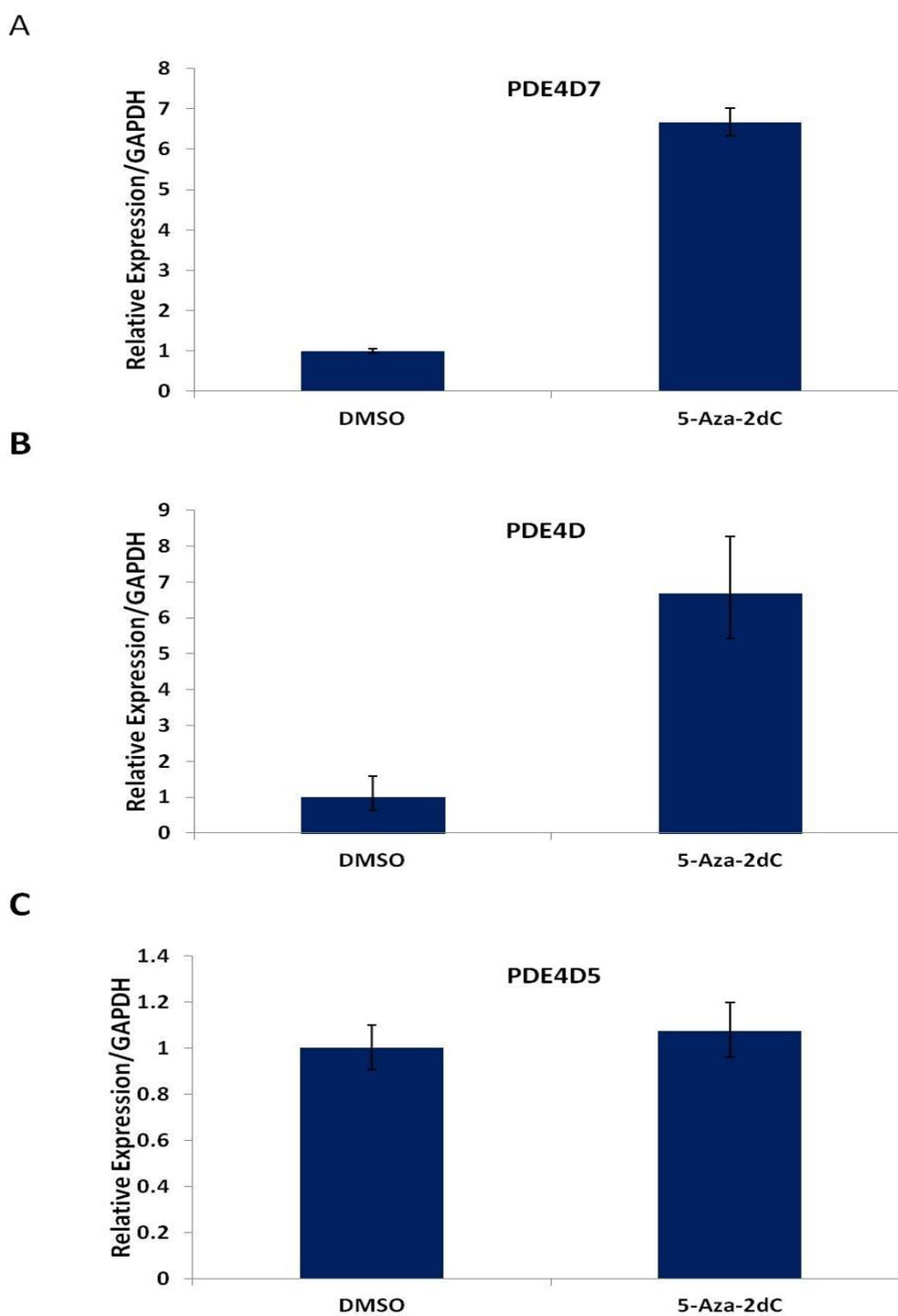


Figure 3.13. Treatment of PC3 cells with the DNA methylation inhibitor 5-aza-2'-deoxycytidine leads to an increase in PDE4D7 expression.

PC3 cells were treated with 5-aza-2'-deoxycytidine (5-Aza-2dC) or vehicle control (DMSO) over 3 days. The fold changes in **A**) PDE4D7 expression, **B**) total PDE4D expression or **C**) PDE4D5 expression were determined by qPCR.

3.3 Discussion

The original aim of the PDE expression profiling of PC samples was to identify a potential novel biomarker, and was done in collaboration with Philips Research and the Prostate Cancer and Molecular Medicine (PCMM) group, both in the Netherlands. As discussed, the current biomarker PSA is far from ideal. Although highly tissue specific, it is not disease-specific, and its use as a PC biomarker has led to much overdiagnosis and over treatment of otherwise non-life threatening cancers (Draisma, Etzioni et al. 2009). We were interested in the PDEs that modulate cAMP signalling because of the growing body of evidence showing that cAMP signalling is perturbed as PC progresses. Such studies have shown that the cAMP effector proteins PKA and EPAC are important in the androgen-independent progression of PC (Sadar 1999; Misra and Pizzo 2013). PKA subunit switching also plays a role in PC progression (Kvissel, Ramberg et al. 2007; Merkle and Hoffmann 2011). Although the molecular mechanisms are ill understood, it is known that cAMP is necessary for NED, another important event in advanced PC (Bang, Pirnia et al. 1994; Pandit, Forman et al. 2009). Aberrant GPCR and G protein expression and signalling events are also implicated in advancing PC (Nelson, Bagnato et al. 2003; Kasbohm, Guo et al. 2005; Weng, Wang et al. 2005). Despite the vast evidence for the role of cAMP in PC progression into androgen-independence, little work has been done on the expression and roles of cAMP PDEs in PC. Uckert et al investigated PDE family expression within the various zones of the prostate (Uckert, Oelke et al. 2006), and other studies have looked more closely at the PDE4 family in PC (Rahrmann, Collier et al. 2009; Sarwar, Sandberg et al. 2013), but no studies have really investigated the role of PDE4 splice variants in PC. The unique N-termini of PDE4 isozymes confer highly specific roles and subcellular localisation to the enzymes, such that they are non-redundant. Isozyme-specific information needs to be gathered to better understand their role in PC. This is important as PDEs are the sole means of cAMP degradation and thus underpin compartmentalised cAMP signalling, with PDE4 isoforms being of particular pertinence in a disease context (Houslay 2010).

PDE4D7 certainly shows promise as a disease-specific biomarker. Our group previously showed that PDE4D7 mRNA is downregulated during the AS to AI PC transition, and whether the protein followed suit was unclear due to lack of competent anti-sera against PDE4D7. Here, using AS and AI PC cell lines I verify that PDE4D7 protein, and thus active enzyme, is also downregulated as PC progresses. PDE4D7 therefore may be a successful biomarker to determine disease stage, which would better allow the patient and clinician to decide if treatment is necessary at that time. Continual monitoring of PDE4D7 levels in a patient may help to identify if the cancer is progressing to an AI state. Our collaborators in the Netherlands have carried out clinical research and found that PDE4D7 mRNA is initially upregulated in AS cancer, and its expression successfully discriminates between non-tumour and tumour tissue. They also investigated the level of PDE4D7 mRNA in PC samples where 20 years longitudinal follow up was available, and found that primary tumours possessing a high relative expression level of PDE4D7 mRNA positively correlated with patient outcome, and prostate cancer-specific survival (PCSS). These data are currently unpublished. This suggests that PDE4D7 expression can predict disease outcome, thereby acting as a prognostic biomarker. Our collaborators in the Netherlands are currently testing the ease with which PDE4D7 can be used as a biomarker; ideally we want to be able to detect it in a urine sample, and such detection is showing promise.

PDE4D7 showed the highest expression of all PDE4 isoforms in all AS PC samples screened. This suggests a possible protective role for this enzyme in early stage PC, where its expression correlates with slow growing, non-invasive PC cells, and its downregulation correlates with faster growing invasive cell types. The finding by our collaborators that PDE4D7 is upregulated in primary prostate tumours compared to normal tissue, may suggest a compensatory mechanism for an increase in cAMP signalling during tumourigenesis. Or, the increase may just be due to hyperplasia of PDE4D7-expressing tumour epithelia. Indeed PNT1 cells, isolated from normal prostate epithelium, also express high levels of PDE4D7 (Henderson D. thesis 2011).

Indeed, the data from the proliferation and migration assays suggest that PDE4D7 mediates AS PC cell growth. The marked increase in cell proliferation upon displacement or reduction in PDE4D7 expression suggests that PDE4D7-mediated cAMP hydrolysis maintains normal proliferation signalling. Downregulation of global PDE4D did not affect cell proliferation, suggesting a specific role for PDE4D7 in cAMP-mediated AS cell proliferation. The observations that 're-expressing' PDE4D7 in AI cells leads to a marked reduction in proliferation rate and migratory potential strengthens this argument. Being the most abundantly expressed PDE4D isoform in AS PC, PDE4D7 must play a pivotal role in the cellular desensitisation system to cAMP in early stage PC. Its downregulation or loss as PC becomes AI would result in a large cAMP increase in the PDE4D7 locale; the sub-plasma membrane region, which may lead to promiscuous signalling and aberrant crosstalk with the AR via PKA and EPAC effector proteins. Several studies highlight the role of increased cAMP concentrations in non-genomic androgen signalling-mediated PC progression. Aberrant PKA phosphorylation of the AR is associated with the transition to AI PC (Nazareth and Weigel 1996; Gioeli, Ficarro et al. 2002), as is an increase in CBP expression (Comuzzi, Nemes et al. 2004). The evidence that GPCRs are over expressed in advancing PC (Nelson, Bagnato et al. 2003; Weng, Wang et al. 2005), coupled with increasing cAMP levels due to loss of PDE4D7 is one way in which certain pro-proliferative pathways would become chronically stimulated. Loss of PDE4D7 therefore may play an important role in the switch from androgenic signalling to autocrine and paracrine signalling as PC moves into androgen insensitivity.

Loss of PDE4D7-mediated cAMP hydrolysis may also play a role in the development of NED, which occurs in some AS PC models. The molecular mechanisms underlying NED are not well understood, but an increase in cAMP is certainly involved (Merkle and Hoffmann 2011). Following androgen withdrawal, NED is accompanied by a huge increase in intracellular cAMP (Burchardt, Burchardt et al. 1999). NE cells secrete neuropeptides and cytokines that can bring about an increase in proliferation of neighbouring non-NE cells, and as PC progresses into androgen insensitivity the NE cell population increases (Gkonos, Krongrad et al. 1995; Jongsma, Oomen et al. 2000; Juarranz, Bolanos et al.

2001; Li, Chen et al. 2013). If PDE4D7 is one of the main regulators of cAMP in AS PC, then loss of its expression and thus activity could, over time, lead to NED.

These results put into question the use of PDE4 inhibitors in the treatment of LUTS associated with BPH (Andersson, Uckert et al. 2007; Uckert and Oelke 2011). It would be interesting to investigate the expression levels of PDE4D7 in BPH samples, to see if it is upregulated as was observed in primary prostate tumour tissue. If indeed it is, its inhibition could possibly potentiate PC development. However, most evidence suggest BPH is not a precursor of PC, and both originate from different prostatic zones (Lee and Peehl 2004).

The *Sleeping Beauty* (SB) transposon study discussed earlier described PDE4D as a PC gene and found its expression to be upregulated in all stages of PC compared to BPH (Rahrmann, Collier et al. 2009). As discussed, our collaborators also found PDE4D to be upregulated in primary tumour tissue compared to normal prostate tissue, however we hypothesise that this may be a protective event, and we show PDE4D to be down regulated as PC progresses. Rahrmann and colleagues suggest that PDE4D is upregulated in both non-invasive and invasive PC, and that its expression is pro-proliferative and PDE4D inhibition is of therapeutic value. They found PDE4D5 to be the predominant isoform expressed, and that global PDE4D knockdown in AI DU145 and PC3 cells reduced their proliferation rate. Little is known about the transcriptional regulation of PDEs, however, PDE4D5 has been shown to be upregulated in response to cAMP due to a cAMP responsive promoter (Le Jeune, Shepherd et al. 2002). This is very interesting in light of the results presented here. A loss of PDE4D7 expression as PC moves into androgen insensitivity would lead to an increase in cAMP and thus aberrant signalling, possibly including an upregulation of PDE4D5 resulting in dysregulated cAMP hydrolysis at the PDE4D5 locale. This in turn would result in perturbed PDE4D5 signalling which could potentiate PC progression. Saying this, our group actually found a decrease in PDE4D5 expression between AS and AI PC samples (figure 3.1), however, it was not significant and also PDE4D5 showed higher expression than other isoforms in the AI samples.

Since PDE4D7 was first described, little work has been done to investigate the signalling networks within which this isoform operates (Wang, Deng et al. 2003).

However, it has been linked to ischemic stroke. The PDE4D locus was found to be a stroke susceptibility gene, due to its role in atherosclerosis. The stroke-associated haplotype was found to extend over the PDE4D7 promoter region and 5' unique exon, and correlated with PDE4D7 mRNA, suggesting it perturbs transcription of this isoform (Gretarsdottir, Thorleifsson et al. 2003). Interestingly, this haplotype region includes the PDE4D7 overlapping region of PART1, a seemingly non-coding transcript which is highly expressed in cancers, including PC (Lin, White et al. 2000). Gretarsdottir et al. suggest the stroke haplotype results in a decrease in PDE4D7 expression, and therefore an increase in cAMP.

The linkage study by Gretarsdottir and colleagues was carried out in an Icelandic cohort, and subsequent independent studies in other population cohorts have given conflicting results on the association of PDE4D and stroke (Staton, Sayer et al. 2006; Bevan, Dichgans et al. 2008; Munshi and Kaul 2008). It is likely that racial and ethnic differences may account for the discrepancies between studies. Saying that, a very recent study confirms the association in a Chinese cohort (Liu, Zhu et al. 2013). The most recent meta-analysis on data collected since the original study, which includes more studies than earlier meta-analyses, also confirms the linkage between PDE4D and stroke (Yoon, Park et al. 2011).

The association of stroke and inflammation is well documented (Huang, Upadhyay et al. 2006; Chiba and Umegaki 2013), as is that of cAMP and inflammation (Moore and Willoughby 1995). It is therefore unsurprising that a cAMP PDE be implicated in stroke. There is also much evidence suggesting a role for inflammation in the development of both BPH and PC, such as a perturbed intra-prostatic immune system cytokine complement (Bouraoui, Ricote et al. 2008; Drake 2010). PC often occurs with comorbidities, and mortality in PC patients with pre-existing stroke is higher than in those without (Jespersen, Norgaard et al. 2011)

Finally, I wished to investigate if the downregulation/loss of PDE4D7 in AI PC was due to aberrant transcriptional regulation, namely hypermethylation. Aberrant DNA methylation is known to play a role in PC development (Jeronimo, Bastian

et al. 2011). A CpG island is predicted upstream of PDE4D7 (Henderson D. thesis 2011), meaning that epigenetic regulation of this isoform is highly plausible. Interestingly, the 5' exon of PDE4D7 which encodes part of its unique N-terminal region overlaps with the non-coding PART1 gene (Gretarsdottir, Thorleifsson et al. 2003). The two genes overlap in a cis-natural (cis-NAT) antisense manner (Henderson, thesis), meaning they are transcribed from opposite DNA strands. Natural antisense transcripts commonly include a protein coding gene with a non-coding antisense RNA partner, and the list of such pairs is growing and is suggested to comprise 15-25% of the mammalian genome (Werner and Berdal 2005; Faghihi and Wahlestedt 2009). Cis-NATs are a type of regulatory RNA, in which the antisense partner can exert regulatory functions over the transcription of the coding gene, and antisense knockout studies result in aberrant expression of the sense partner (Werner and Berdal 2005). The role of such non-coding RNAs (ncRNAs) in the epigenetic regulation of coding genes is gaining much interest (Su, Xiong et al. 2010). Cis-NATs have been shown to both positively and negatively regulate the expression of genes (Faghihi and Wahlestedt 2009; Magistri, Faghihi et al. 2012), and the long term silencing of tumour suppressor genes is mediated by cis-NATs (Malecova and Morris 2010). Cis-NATs likely regulate transcription by recruiting histone-modifying enzymes to their sense strand partner to bring about chromatin remodelling (Magistri, Faghihi et al. 2012).

Although carried out only once, the upregulation of PDE4D7 following treatment with the DNA methylation inhibitor 5-aza-2dC suggests that hypermethylation may be responsible for the loss of expression in AI PC. Total PDE4D expression is also upregulated to same extent, suggesting total upregulation represents that of PDE4D7, and thus PDE4D7 is specifically hypermethylated. This is also suggested by the observation that PDE4D5 is not upregulated following treatment. I hypothesise that PART1 exerts transcriptional control over PDE4D7. PART1 is an androgen-regulated, non-coding transcript highly expressed in the prostate, placenta, thymus and salivary gland, and is upregulated in malignant compared to normal prostate samples (Lin, White et al. 2000; Sidiropoulos, Chang et al. 2001). The authors here do not describe whether the malignant samples are AS or AI, but a later study describes the upregulation of PART1 in AS

LNCaP following DHT treatment, but not in DU145 or PC3 cells (Yu, Blackburn et al. 2003). Together, this suggests that PART1 may positively regulate PDE4D7 expression in normal prostate tissue and early stage AS prostate carcinoma. As PC progresses, and signalling moves away from androgenic pathways, transcription of PART1 may be inhibited. This would then allow hypermethylation of the PDE4D7 promoter region and subsequent loss of expression. It must be noted that PDE4D7 does not appear to be under androgenic regulation (Henderson D. thesis 2011). This is an area that requires much further work; Firstly, the upregulation of PDE4D7 following DNA methyltransferase inhibition needs to be confirmed. If PDE4D7 is repeatedly specifically upregulated, it would be interesting to assay the proliferation and migration of AI PC cells following treatment, with the expectation that growth rate and migratory potential would be decreased. Also, bisulfite treatment and DNA sequencing would be necessary to confirm specific hypermethylation of the PDE4D7 CpG island.

3.4 Chapter Summary

- 4) PDE4D7 is differentially expressed between AS and AI PC cell lines and is a potential diagnostic biomarker of disease stage.
- 5) PDE4D7-mediated cAMP hydrolysis at the plasma membrane plays a role in the regulation of PC cell proliferation and migration.
- 6) Loss of PDE4D7 expression in AI PC may be due to hypermethylation.
- 7) I conclude that loss of PDE4D7-specific cAMP hydrolysis plays a role in the development of the AI phenotype (figure 3.15).

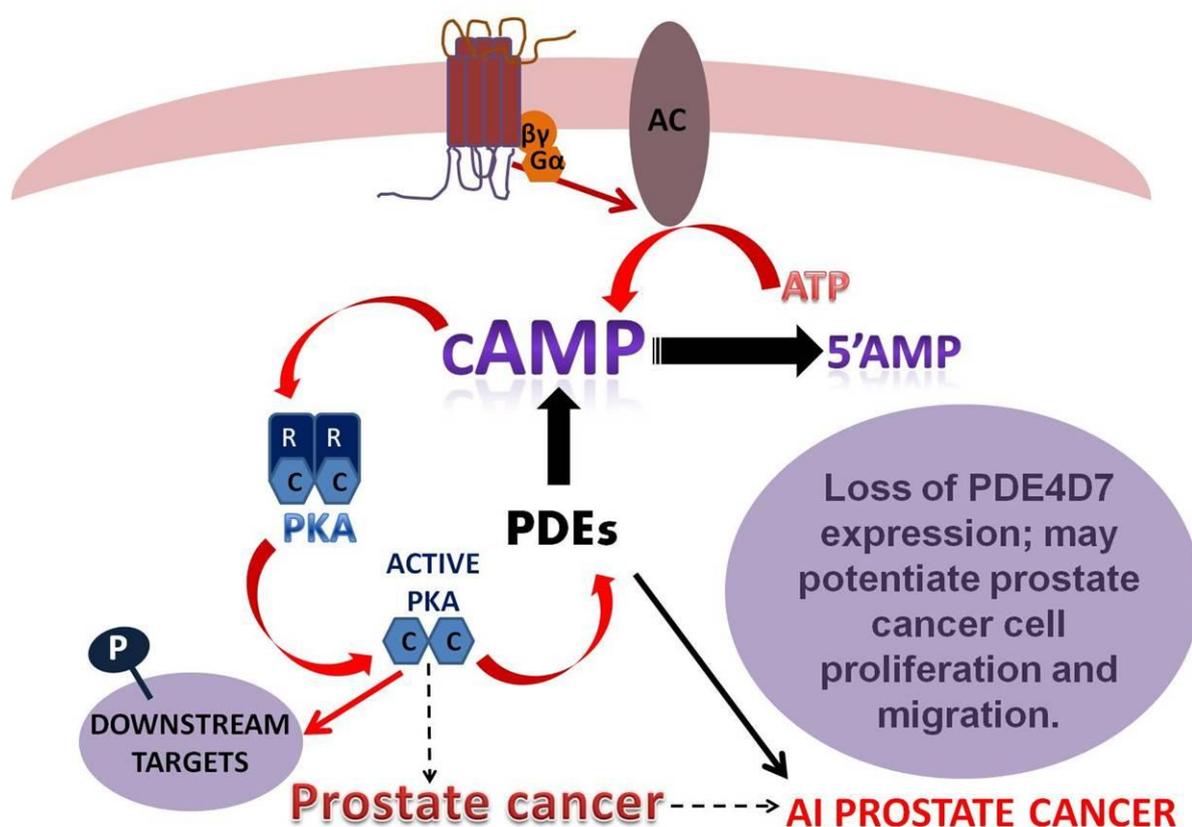


Figure 3.14. cAMP signalling dysregulation plays an important role in PC progression and loss of PDE4D7 is likely a contributing factor.

4 Unique N-terminal Phosphorylation of PDE4D7

4.1 Introduction

PDEs are subject to a hierarchy of regulation. Primarily, regulation comes at the level of their tissue (Cole, Soutar et al. 2008) and cell-specific expression (Houslay 2010). Brain region-specific splice variant expression provides an elegant example of such a paradigm and underscores the non-redundant roles afforded to PDE isoforms (D'Sa, Eisch et al. 2005). Secondly, they are regulated by the possession of family specific domains such as GAFs, PAS', Ca²⁺/Cams, and UCRs which act to transduce regulatory signals from the signalling network within which a PDE is functioning (Bender and Beavo 2006; Omori and Kotera 2007). PDEs are then further regulated by post-translational modifications (PTMs) such as phosphorylation, SUMOylation and ubiquitination (MacKenzie, Baillie et al. 2002; Li, Baillie et al. 2009; Li, Vadrevu et al. 2010). Over the past decade or so, PTMs of proteins have been recognised as an elegant way to manage protein regulation, and therefore fine-tune the functional outcome of signals within a cell. Aberrant PTMs are associated with cancer development and show potential both as disease biomarkers and targets for therapeutic intervention (Krueger and Srivastava 2006).

All long form PDE4 isoforms are subject to phosphorylation by PKA within the conserved RRESF motif in their UCR1 region, and this underpins their activation (MacKenzie, Baillie et al. 2002). It is believed that in the inactive state, the UCR2 domain acts as an internal inhibitory unit by straddling the active site. It may be that the UCR1 and UCR2 domains interact via electrostatic interactions (Beard, Olsen et al. 2000). PKA phosphorylation of UCR1 causes a conformational change which relieves this inhibitory constraint by dissociation of UCR2 from the active site (Lim, Pahlke et al. 1999; Burgin, Magnusson et al. 2010) and possibly from UCR1 (figure 4.1).

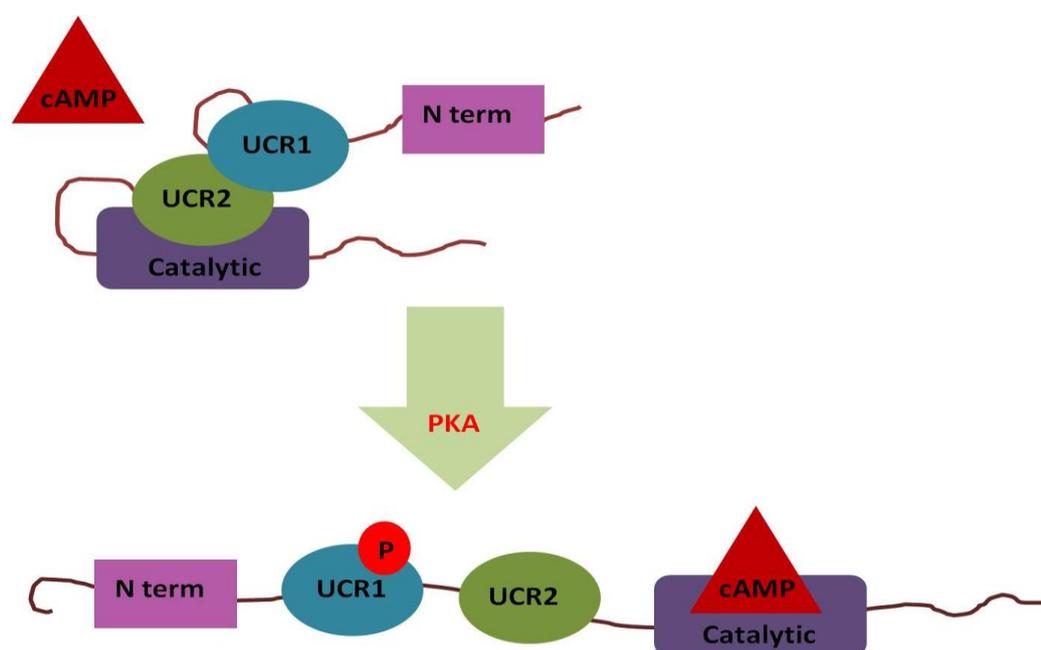


Figure 4.1. A simplistic schematic representing the proposed model for the activation of long PDE4 isoforms.

cAMP cannot access the active site due to hindrance by UCR2. Following an increase in active PKA, UCR1 is phosphorylated leading to a conformational change around the active site and activation of the PDE.

Aside from UCR1 phosphorylation, the long isoform PDE4D3 undergoes PKA phosphorylation within its unique N-terminus, on serine13 (Sette and Conti 1996). To date, this is the only known case of a long PDE isoform being phosphorylated by PKA other than within its UCR1 domain. This Ser13 falls within a region involved in regulating a host of protein-protein interactions with the scaffolding proteins mAKAP, Nudel, Lis1, EPAC1, as well as members of the MAPK signalling pathway and protein phosphatase 2A (PP2A) (Carlisle Michel, Dodge et al. 2004; Dodge-Kafka, Soughayer et al. 2005; Dodge-Kafka and Kapiloff 2006; Collins, Murdoch et al. 2008; Dodge-Kafka, Bauman et al. 2010; Murdoch, Vadrevu et al. 2011). Phosphorylation of ser13 increases the affinity of PDE4D3 for mAKAP (Carlisle Michel, Dodge et al. 2004) while causing dissociation from

Ndel1 (Collins, Murdoch et al. 2008). This complex and dynamic signalosome acts to tightly and elegantly regulate cAMP signalling within the heart, and underscores the complexity and intricacy of the cAMP signalling axes.

The aims of this chapter are to present evidence for the previously undiscovered PKA-mediated phosphorylation of PDE4D7 within its unique N-terminus (on serine 42), to show that this event likely occurs *in vivo*, and that it acts to regulate PDE4D7 activity.

4.2 Results

4.2.1 The Unique N-terminal Region of PDE4D7 Contains a PKA Consensus Site

PDE4D7 was first described by Wang and colleagues. The unique N-terminal region is encoded by two exons, PDE4D7a and PDE4D7b, rather than a single exon, as in all other known PDE4D isoforms (Wang, Deng et al. 2003). The unique N-terminal region of PDE4D7 is 90 amino acids long, encoded by 270 base pairs (figure 4.2).

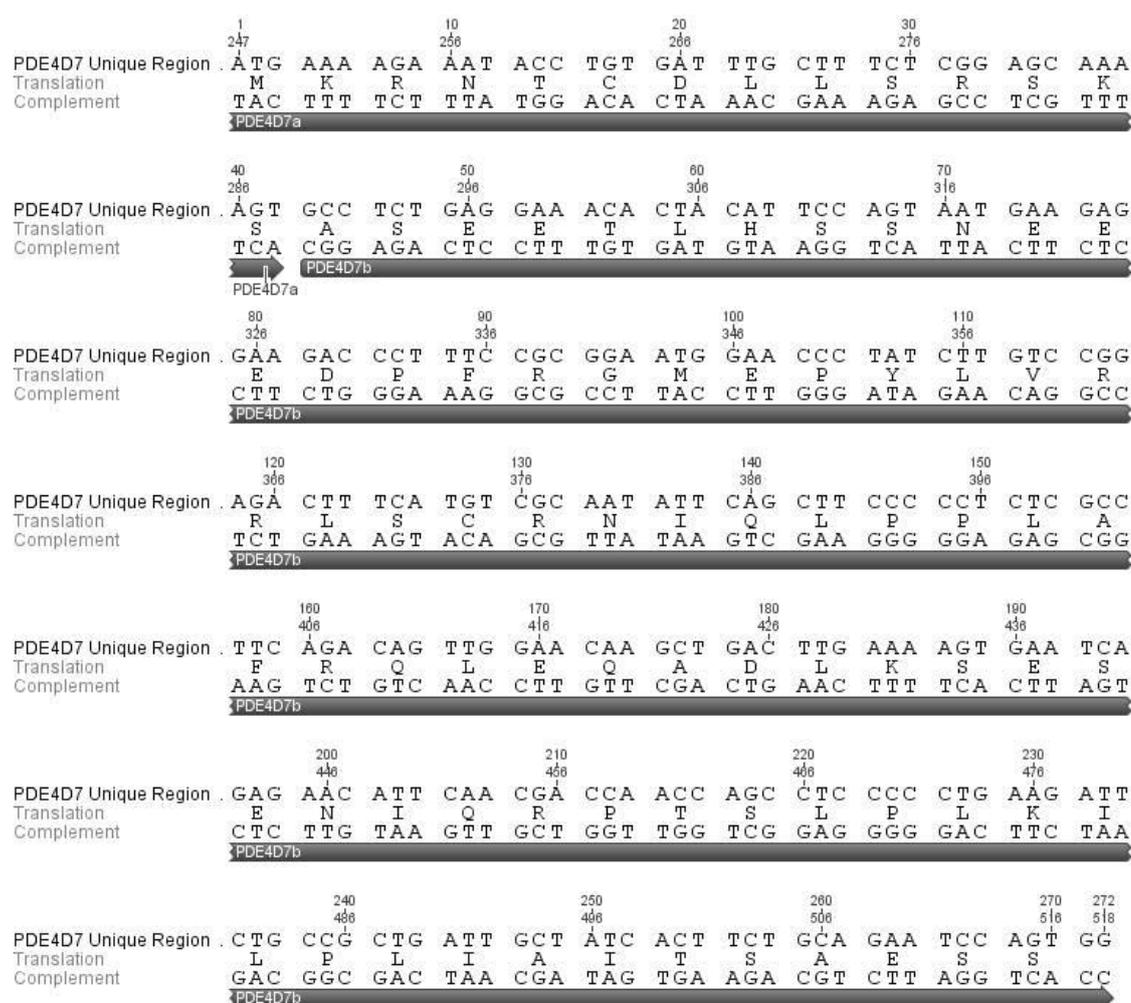


Figure 4.2. The unique N-terminal region of PDE4D7 (AF536976.1) comprises exon a and exon b.

Interestingly, within the PDE4D7 unique N-terminus there is a consensus PKA site of ³⁷LVRRLSCR⁴⁴ (figure 4.3).

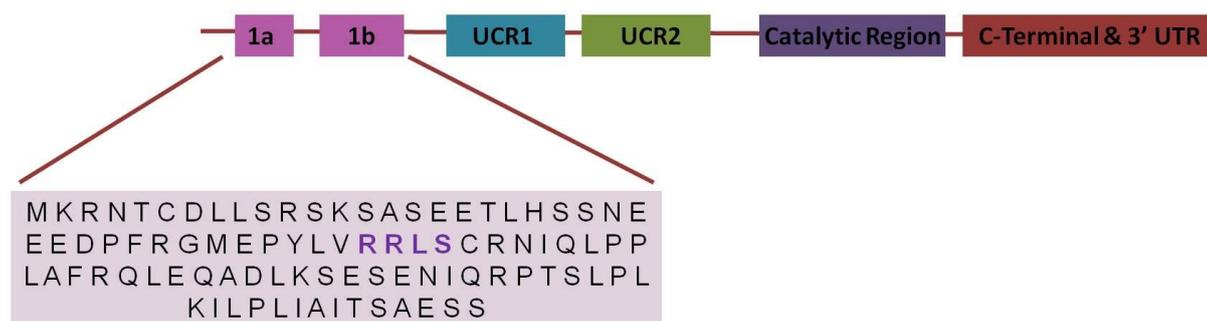


Figure 4.3. The unique N-terminus of PDE4D7 contains a PKA phosphorylation consensus site; RRLS.

The consensus PKA phosphorylation motif is RxxS(h) or RRxS(h) where x is any amino acid, S is the target serine for the phospho moiety, and (h) represents a preferred hydrophobic residue. The residue in the (h) position of the PKA consensus motif within the N-terminus of PDE4D7 is a cysteine (C43). This is not a hydrophobic residue and so renders this a suboptimal PKA consensus site and this is the reason why it has not been investigated before now. However, I thought it quite plausible that this site, serine42 (Ser42) is indeed subject to PKA phosphorylation, and the following results confirm this.

4.2.2 PDE4D7-Ser42 is Phosphorylated by PKA *in vitro*

4.2.2.1 Peptide Array Technology and a Novel Phospho-Specific Antibody Confirm that Serine 42 is a PKA Phospho-site.

As described in Chapter 3, peptide array technology was used to create PDE4D7 arrays. These arrays were used to investigate whether the N-terminus of PDE4D7 can be phosphorylated by PKA. Peptide array technology is a relatively novel method of investigating the PTMs of proteins. It has been used by our group to successfully identify ubiquitination and SUMOylation sites on PDE4D5 (Li, Baillie et al. 2009; Li, Vadrevu et al. 2010), and PKA sites on PI3K and DNA-PK (Huston, Lynch et al. 2008; Perino, Ghigo et al. 2011).

In order to investigate PDE4D7 N-terminal phosphorylation, PKA assays were carried out using PDE4D7 arrays. Firstly, an array was incubated with 100units of purified PKA catalytic subunit in a phospho-buffer containing Mg^{2+} and ATP. A control assay which did not contain active PKA was also carried out. Following this, the arrays were probed with a PKA-substrate antibody. This antibody detects the motif RXXpS, where X is any residue; i.e. this antibody detects any serine phosphorylated by PKA. Following incubation with HRP-conjugated secondary antibody and chemiluminescent detection, dark spots representing phosphorylated peptides were observed on the test array. The peptides phosphorylated were those encompassing the UCR1 region of PDE4D7, as expected, and also those containing the RRLS motif within the N-terminus (figure 4.4).

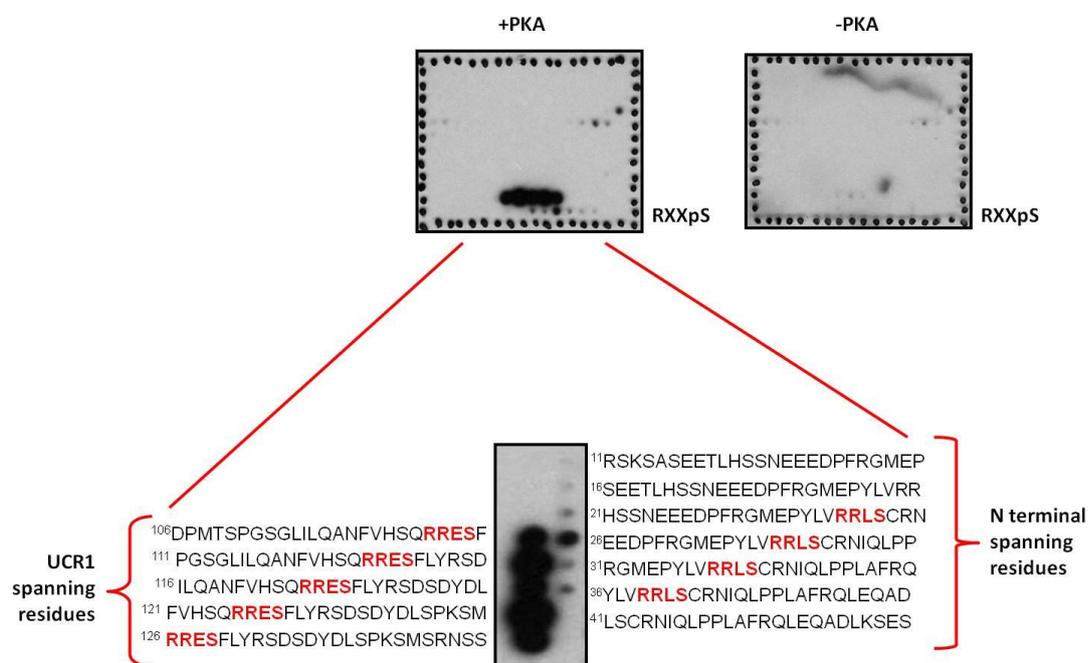


Figure 4.4. Peptide array technology confirms PDE4D7-Ser42 is phosphorylated by PKA. PDE4D7 peptide arrays were subjected to PKA assays; arrays were incubated in phospho-buffer with (+PKA) or without (-PKA) active PKA catalytic subunit. A PKA substrate antibody (RXXpS) detects phosphorylation (dark spots) of the UCR1 PKA consensus site RRES, and the potential novel PKA substrate site, RRLS, on the test (+PKA) array. No spots were observed on the control array (-PKA). N=3.

The spots representing the UCR1 region are much darker than those of the RRLS motif. This may be due to the lack of an optimal hydrophobic residue immediately after the target serine at position 42. However, the detection of phosphorylation at Ser42 led me to investigate further this possible event. In order to determine if Ser42 is phosphorylated *in vivo*, a phospho-specific antibody was raised. This antibody, pSer42, would detect only PKA phosphorylated serine 42 of PDE4D7. The sequence CEPYLVRRLL(p)SCRN was used

to create an antigen peptide that was injected into rabbits to raise the antibody. Following collection of serum, the antibody was purified. The purified pSer42 antibody was then validated by means of peptide array. PKA assays were carried out on PDE4D7 arrays, including a control without active PKA. The arrays were then probed with the pSer42 antibody, or pre-immune serum from the host rabbit which acts as a negative control, to ensure detection is due to the antibody raised against the inoculated antigen and not some artefact e.g. non-specific binding (figure 4.5).

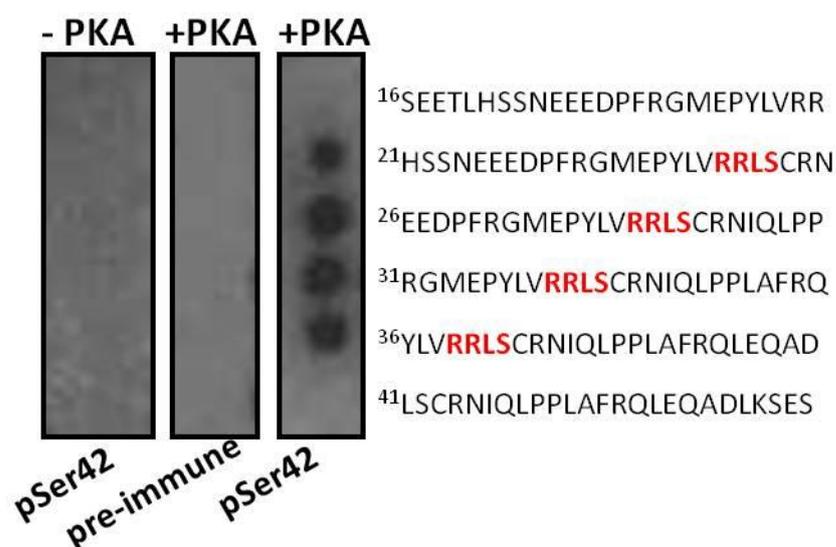


Figure 4.5. Peptide array technology verifies specificity of the novel phospho-Ser42 antibody, and confirms that this site can be PKA phosphorylated.

Again, PDE4D7 arrays were incubated in phospho-buffer, with (+PKA) or without (-PKA) active PKA catalytic subunit. The novel pSer42 antibody successfully and specifically detected phosphorylation (dark spots) of the RRLS motif in PDE4D7. The -PKA assay and pre-immune serum acted as negative controls. N=3.

The PDE4D7 peptide arrays served two purposes here; firstly they verified that the novel pSer42 antibody successfully detects PKA phosphorylation of this site, and does so specifically. Secondly, they validated the results from the first PKA assay experiment (figure 4.4), and gave credence to the idea that PDE4D7 is indeed phosphorylated within its N-terminal region.

To further support the notion that a unique phosphorylation event can occur at Ser42, a phosphoresistant mutant was created. Site-directed mutagenesis (SDM) was carried out to change the Ser42 residue to a phospho-null/resistant alanine (S42A). The mutant protein (N-terminal only) was cloned and purified as a GST recombinant protein, as was the wild type (wt) protein (N-terminal only). Both proteins encompass the entire N-terminal region of PDE4D7 only. Henceforth they will be called S42A-Nt and wt-Nt respectively, to represent the mutation or wt.

Firstly, expression and purification of the proteins were verified by SDS-PAGE and Coomassie staining (figure 4. 6). The N-terminal region of PDE4D7 weighs ~10kDa, and with a GST tag weighs ~46kDa. Purified GST was also loaded on the gel as a control.

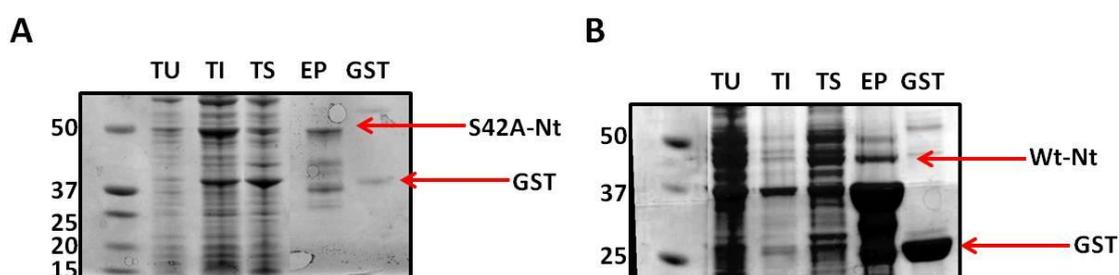


Figure 4.6. Verification of protein purification by SDS PAGE and Coomassie staining.

GST recombinant S42A-Nt (A) and Wt-Nt (B) purified proteins (along with purified GST) were detected by Coomassie staining. S42A-Nt and Wt-Nt run at ~46kDa, GST runs at ~26kDa. Degradation can be seen, as is normal with purified proteins. TU = total uninduced protein, TI = total induced protein, TS = total soluble protein, EP = eluted protein.

PKA assays were then carried out with the purified proteins. 2ug of each protein was incubated with or without 25 units of active catalytic PKA subunit in phospho-buffer containing Mg^{2+} and ATP. The proteins were then tested for phosphorylation by SDS-PAGE and western blot analysis. Probing of the blots with both the RXXpS and the pSer42 antibodies detected phosphorylation of only wt-Nt following incubation with PKA (figure 4.7). These results again verify that PDE4D7 is phosphorylated on S42, and confirm specificity of the pS42 antibody.

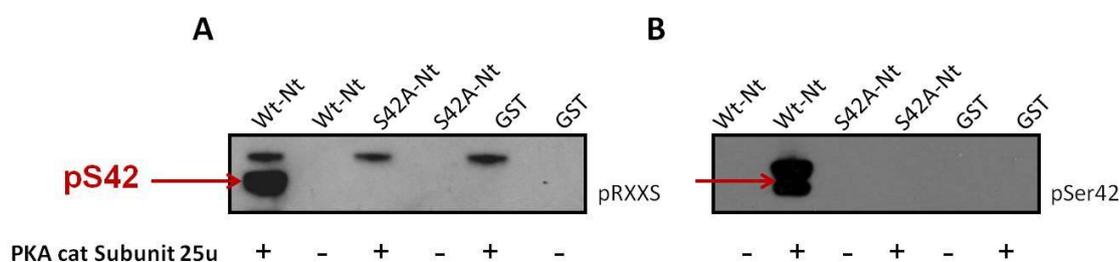


Figure 4.7. Purified wt and S42A proteins were assayed for phosphorylation by PKA.

The GST fusion proteins were subjected to PKA assays. Incubation with PKA catalytic (cat) subunit brings about phosphorylation of the wt protein only (indicated by arrows), as detected by a PKA substrate antibody (RxxpS) (A) or the phospho-ser42 specific antibody (pS42) (B).

4.2.3 PDE4D7 Ser42 is Phosphorylated by PKA *ex vivo*-Overexpression System

The next step was to investigate if this phosphorylation event occurs in cells, in response to an increase in intracellular cAMP. This employed full length wild type PDE4D7 and mutant PDE4D7 VSV-tagged constructs. A phosphoresistant alanine mutant (S42A), as described above, was cloned along with a phosphomimetic mutant (S42D). The phosphomimetic mutant has an aspartate substitution at the Ser42 position which bestows a negative charge to mimic the stoichiometry of PKA phosphorylation, ensuring the enzyme remains constitutively 'active' (Hoffmann, Wilkinson et al. 1998; MacKenzie, Baillie et al. 2002). These VSV-tagged constructs were overexpressed in Hek293 cells, which were then treated with DMSO, forskolin or KT5720 (PKA inhibitor) and forskolin. Forskolin activates adenylyl cyclases and therefore raises cAMP levels. KT5720 is an ester derivative and a protein kinase inhibitor with specificity for PKA (Kase, Iwahashi et al. 1987). Cells were treated with 4uM of KT5720, 20 minutes prior to treatment with 100uM forskolin for 5 minutes. Cells treated with forskolin alone were also challenged with 100uM for 5 minutes. Forskolin and KT5720 were dissolved in DMSO, therefore this was used as a vehicle control.

Interestingly, basal Ser42 phosphorylation was observed in the control, wt transfected cells. The band representing phosphorylated Ser42 (phospho band) is increased in intensity following forskolin treatment, and reduced to basal levels with pre-treatment of KT5720 (figure 4.8 A). This confirms that phosphorylation of PDE4D7-Ser42 is PKA-dependent. No such phosphorylation is observed with the S42A and S42D mutants, again confirming specificity of the phospho-ser42 antibody. These results indicate that PDE4D7 is indeed phosphorylated within its N-terminal region at Ser42 in cells, and that this event is mediated by PKA. A forskolin time course was carried out (figure 4.8 B) to investigate if PDE4D7-Ser42 phosphorylation is maintained with a sustained increase in PKA levels. Phosphorylation remains above that of basal level for the duration of the timecourse (20 minutes), but is maximal at 5-10 minutes following forskolin challenge. A reduction in the phospho band is then observed, which stands to reason, as the UCR1 region becomes phosphorylated leading to an increase in

PDE activity and a corresponding decrease in active PKA in the vicinity of the enzyme. A forskolin timecourse was then carried out in cells expressing the dominant-negative PDE4D7 construct (figure 4.8 C). This construct is catalytically inactive while possessing an intact N-terminal region. This showed a marked and continual increase in Ser42 phosphorylation compared to wtPDE4D7. This finding suggests that phosphorylation at Ser42 is protected by the catalytic activity of the enzyme, possibly to prevent hyper-phosphorylation of this site. Forskolin-treated lysates were also probed with the PKA substrate antibody RXXpS, to ensure that forskolin challenge was indeed increasing PKA mediated phosphorylation of multiple unidentified substrates (figure 4.8 D). Hek293 cells transfected with a Control construct were also challenged with forskolin and compared to wtPDE4D7 transfected cells, to ensure the pSer42 antibody was not detecting any non-specific band in the lysate (figure 4.8 E); indeed our group have previously confirmed absence of PDE4D7 expression in Hek293 cells.

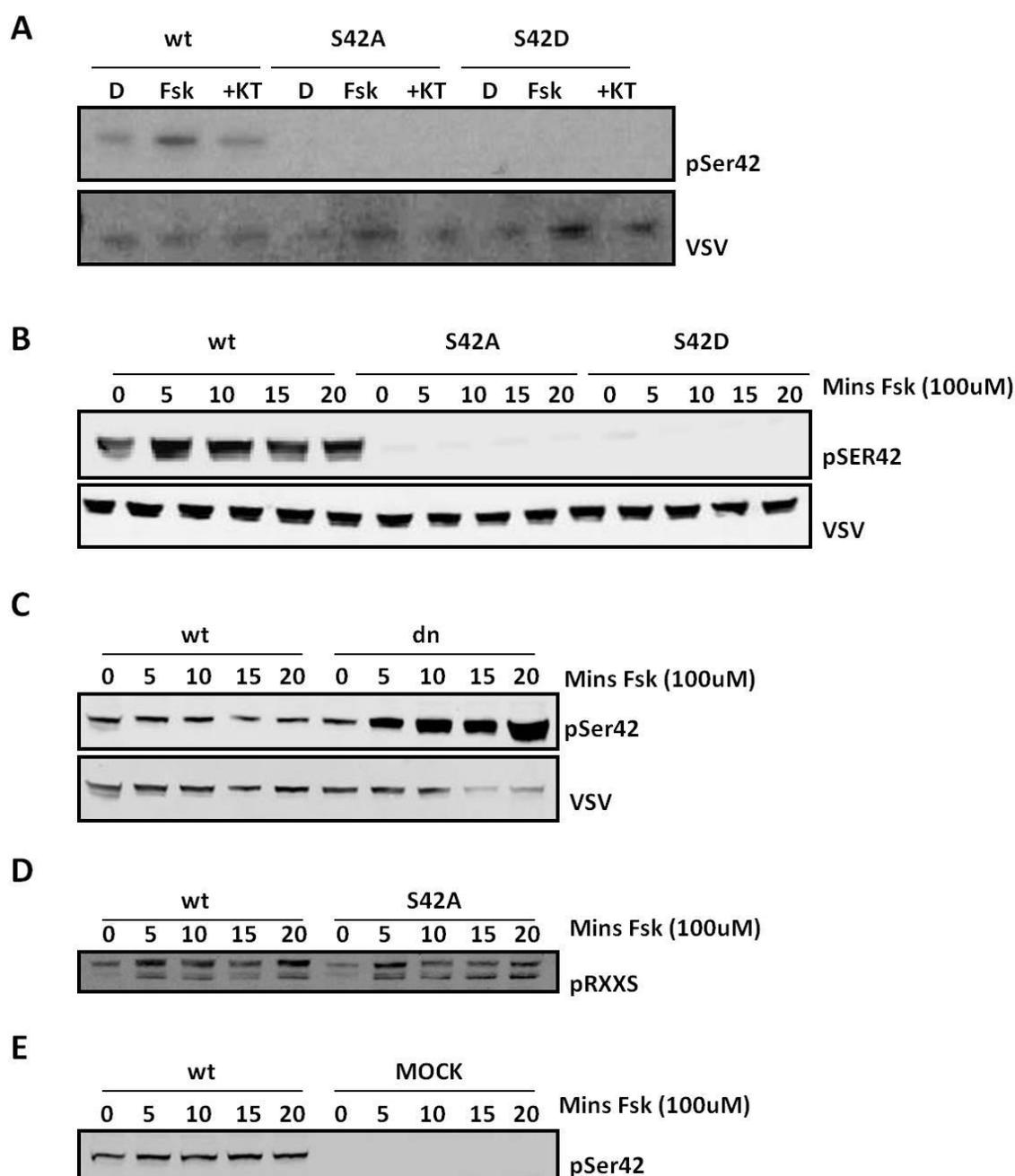


Figure 4.8. The unique N-terminus of PDE4D7 is phosphorylated by PKA *ex vivo*.

A; Hek293 cells were transfected with VSV-tagged wild type PDE4D7 (wt) or the S42A or S42D mutants and treated with forskolin (Fsk) or forskolin after a pre-treatment with the PKA inhibitor KT5720 (+KT). DMSO served as a vehicle control (D). Cellular lysates were blotted with serine 42 specific antibody or VSV antibody. N=3. **B;** Hek293 cells were transfected with VSV-tagged wild type PDE4D7 (wt) or the S42A or S42D mutants and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with serine 42 specific antibody or VSV

antibody. N=3. C; Hek293 cells were transfected with wt PDE4D7 or the dominant negative, catalytically inactive mutant (dn) and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with a serine 42 specific antibody or VSV antibody. N=3. D; Hek293 cells were transfected with wt PDE4D7 or the S42A mutant and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with a PKA-substrate antibody (RXXpS). N=3. E; Hek293 cells were transfected with wt PDE4D7 or a control construct (mock) and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with a serine 42 specific antibody. N=1.

4.2.4 PDE4D7 Ser42 Phosphorylation Negatively Regulates Enzyme Activity

As discussed, all long form PDE4 isozymes are activated following PKA phosphorylation of their UCR1 domains (MacKenzie, Baillie et al. 2002). PDE4D3 is also known to be phosphorylated by PKA within its N-terminus, but this has no effect on enzyme activity (Sette and Conti 1996). I wished to investigate if the phosphorylation of Ser42 had any effect on PDE4D7-mediated cAMP hydrolysis. To do this, a PDE activity assay was performed on lysates of Hek293 cells that had been transfected with wt, S42A or S42D PDE4D7 VSV-tagged constructs. The C terminal VSV tag allowed for specific detection of the expression of each enzyme, and has previously been used in such experiments without interfering with enzyme activity (Hoffmann, Wilkinson et al. 1998). The cells were left untreated (NT) or treated with 100uM forskolin for 5, 10, 15 or 20 minutes. Since maximal phosphorylation of Ser42 was found to occur after 5 minutes (figure 4.8 B) the NT and 5 minute forskolin-treated cells were analysed for activity. Along with the PDE assay, the lysates were analysed for expression of each construct by means of western blot analysis and a VSV antibody. This was so densitometry could be used to calculate the expression of each construct, treated and untreated, compared to that of GAPDH. The expression of each was then taken as a percentage of NT wtPDE4D7, such that wtPDE4D7 basal activity equals 100%. The PDE assay values were then normalised against construct expression to give

an accurate activity reading. The picomoles of cAMP hydrolysed per minute per μg protein by the wt and mutant enzymes under basal and forskolin-challenged conditions are shown in figure 4.9 A. The activity of each enzyme is represented as a percentage of wtPDE4D7 NT in figure 4.9 B. Our group have previously determined that Hek293 cells exhibit negligible PDE4 activity ($< 5\%$ of transfected amounts), thus any activity measured is due to the overexpressed enzyme.

Following forskolin treatment and thus a rise in cAMP and PKA activity, wt PDE4D7 showed ~ 2 fold (95%) increase in cAMP hydrolysis activity, as was expected due to UCR1 phosphorylation (Sette and Conti 1996). Interestingly, the phosphoresistant S42A mutant showed increased basal activity compared to wtPDE4D7 NT, of $>170\%$ ($p=0.03$, ANOVA); such that it was hyper-active and forskolin challenge did not further increase its activity. The phospho mimetic S42D mutant showed a small non significant increase in basal activity compared to wtPDE4D7 NT (36%), which was increased following forskolin challenge to a similar degree as wtPDE4D7 with forskolin (80%).

The hyperactivity observed with the S42A mutant suggests that phosphorylation of this site provides a mode of negative regulation on PDE4D7, ablation of which leads to activation. The increase in basal activity observed with the S42D enzyme is not significant; suggesting the negative charge afforded by the aspartate residue confers activity similar to that of basal wtPDE4D7 activity.

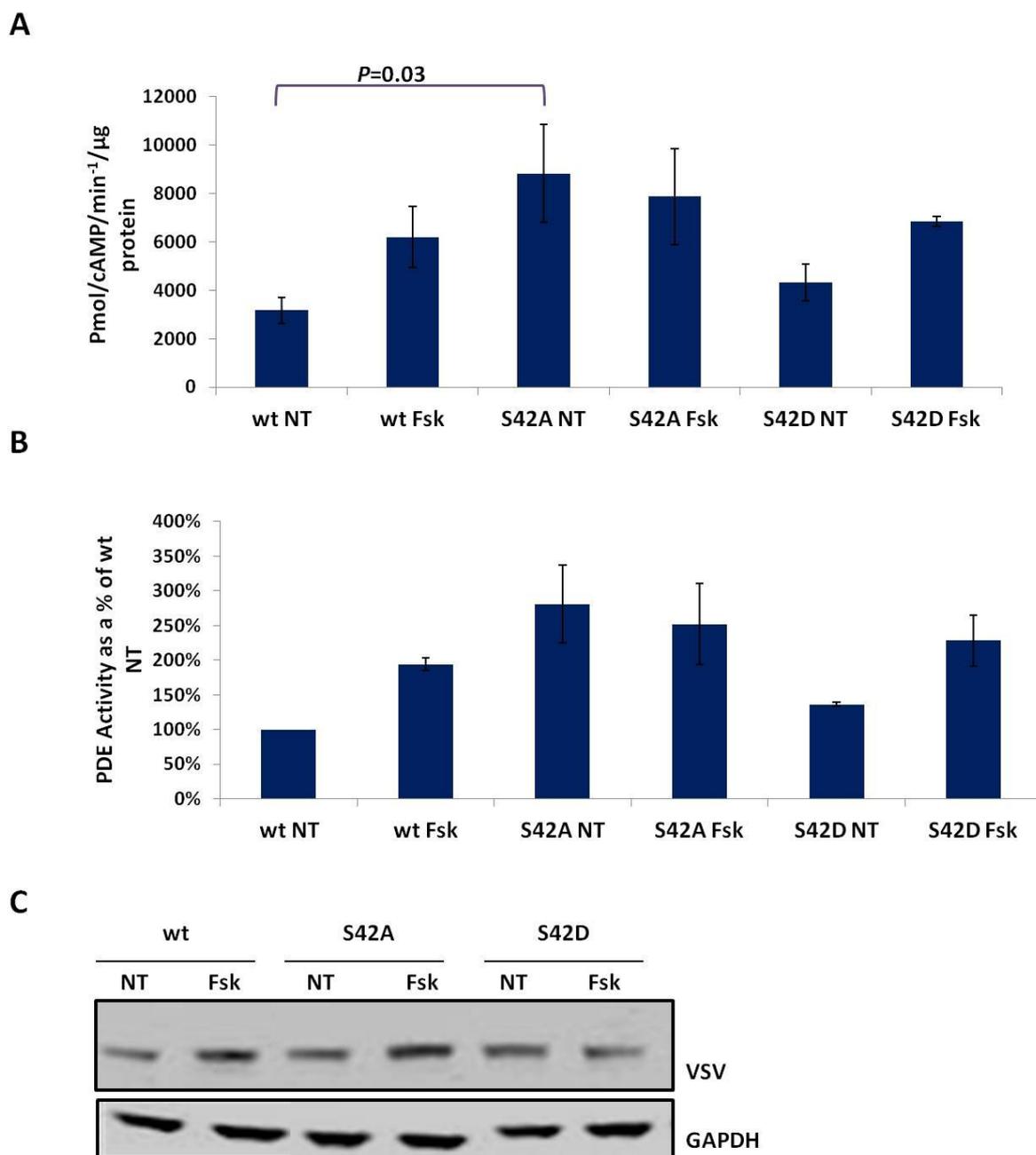


Figure 4.9. Phospho-null mutation of the serine 42 -site renders PDE4D7 hyper-active.

Hek293 cells were transfected with VSV-tagged wt PDE4D7 (wt) or the S42A or S42D mutants and treated with forskolin (Fsk) for 5 minutes, or left untreated (NT). The PDE activity of cellular lysates was determined (A) and expressed as a percentage of wild type control (wt NT) (B). ANOVA was used to determine significance of change. C; the cell lysates used for the activity assays were analysed for construct expression by means of western blots. N=3.

Since ablation of Ser42 phosphorylation led to a hyperactive enzyme, it was assumed that this mutation would result in a hyper-phosphorylated UCR1 region. To investigate this, the lysates prepared for the activity assays were analysed by SDS PAGE and western blotting, followed by immunoblotting for phospho-UCR1 compared to phospho-Ser42 and VSV. Interestingly, the S42A mutation resulted in a marked reduction in phospho-UCR1, whereas the S42D mutation seemed to have no effect on UCR1 phosphorylation compared with wtPDE4D7 (figure 4.10 A). These results again suggest that the phosphorylation of Ser42 acts to negatively regulate PDE4D7 activity; ablation of which leads to a hyper-active enzyme which so rapidly hydrolyses any available cAMP that little or no active PKA becomes available to phosphorylate the UCR1 domain. Indeed, the UCR1 region is hyperphosphorylated in the dominant-negative (dn) construct, which is catalytically inactive (figure 4.10 B).

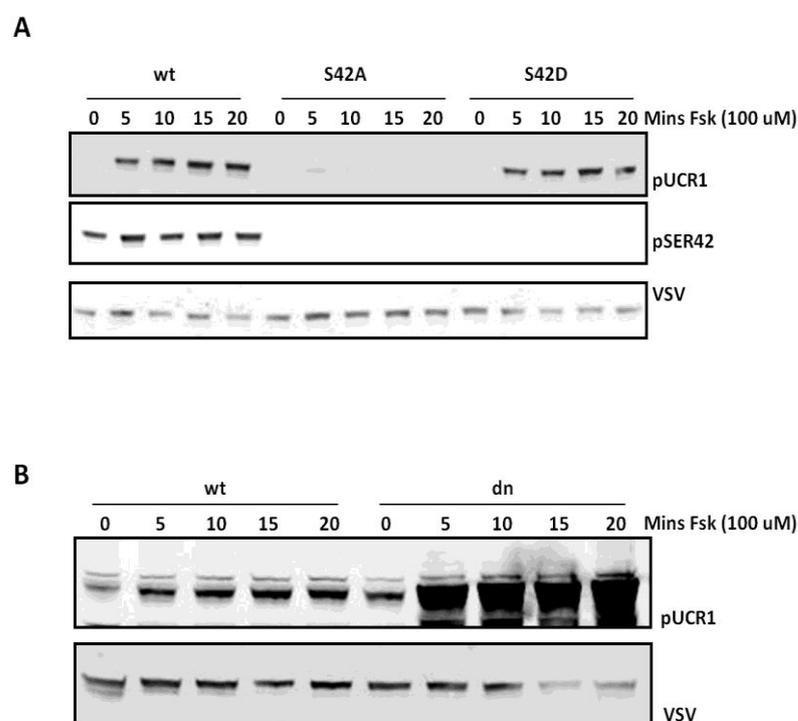


Figure 4.10. Ablation of phospho-serine42 in PDE4D7 inhibits UCR1 phosphorylation due to hyper-hydrolysis of cAMP.

A; Hek293 cells were transfected with wt PDE4D7 or the S42A or S42D mutants and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with a serine 42 phospho-specific antibody or a VSV antibody or a phospho-UCR1 antibody. **B;** Hek293 cells were transfected with wt PDE4D7 or the dominant negative, catalytically inactive mutant (dn) and treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with a phospho-UCR1 antibody or a VSV antibody. N=3.

4.2.5 PDE4D7 Ser42 is Phosphorylated by PKA *ex vivo*-Endogenous Prostate Cancer Cell System.

The next step was to investigate if endogenous PDE4D7 is phosphorylated on Ser42. The AS DuCaP and VCaP cell lines were treated with DMSO, forskolin or forskolin following pre-treatment with KT5720. In both cell lines, forskolin challenge resulted in an increase in pSer42 which was reduced when a KT5720 pre-treatment was carried out (figure 4.11 A and B). Forskolin treatment timecourses show an increasing pSer42 band intensity over 20 minutes, compared with total PDE4D7 (figure 4.11 C and D). Interestingly, there is basal phosphorylation of PDE4D7 N-terminal in these endogenous systems also.

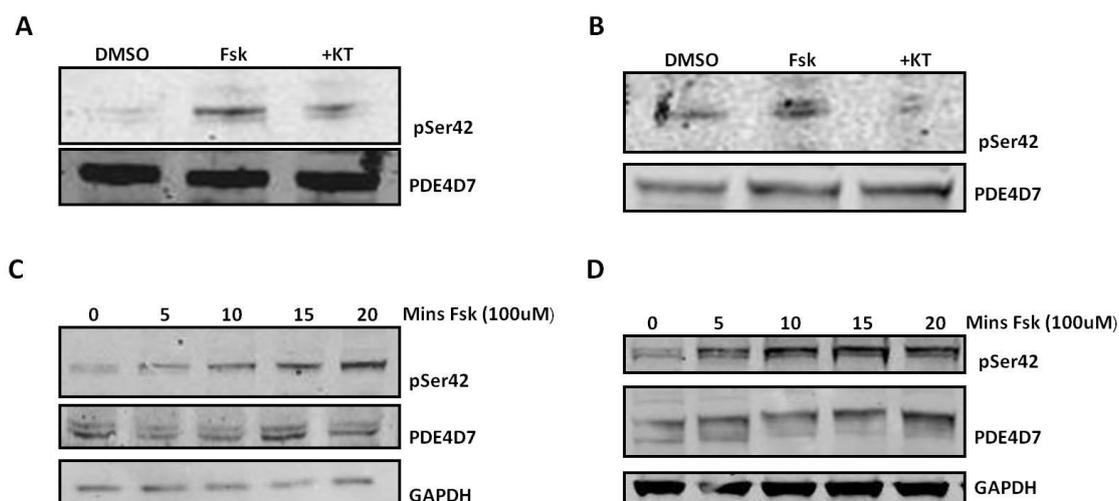


Figure 4.11. Endogenous PDE4D7 Ser42 is phosphorylated by PKA.

A; DuCaP cells were treated with forskolin (Fsk) or forskolin following a pre-treatment with the PKA inhibitor KT5720 (+KT). DMSO served as a vehicle control. Cellular lysates were blotted with serine 42 phospho-specific antibody or PDE4D7 antibody. **B;** VCaP cells were treated with forskolin (Fsk) or forskolin following a pre-treatment with the PKA inhibitor KT5720 (+KT). DMSO served as a vehicle control. Cellular lysates were blotted with serine 42 phospho-specific

antibody or PDE4D7 antibody. C; DuCaP cells were treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with serine 42 phospho-specific antibody or PDE4D7 antibody or GAPDH antibody. D; VCaP cells were treated with forskolin (Fsk) over 20 minutes. Cellular lysates were blotted with serine 42 phospho-specific antibody or PDE4D7 antibody or GAPDH antibody.

These results suggest that PDE4D7 N-terminal PKA dependant phosphorylation *occurs* in endogenously expressed proteins within cultured cells. An interesting observation is that endogenous Ser42 shows basal phosphorylation. Taken together with the results of the PDE activity assay, these data suggest that phosphorylation of Ser42 negatively regulates PDE4D7 activity, and may allow for basal cAMP signalling. It could be that PDE4D7 is constitutively phosphorylated on Ser42 under such conditions, or certainly a sub-population of PDE4D7. Usually phospho-sites are subject to rapid dephosphorylation by phosphatases, but Ser42 may be resistant to such dephosphorylation, particularly when cAMP is at basal levels.

4.2.6 Mutation of PDE4D7 N-terminal Phospho-site Confirms Loss of PDE4D7-mediated cAMP Hydrolysis is Important for the AI Phenotype.

In chapter 3, I presented evidence that PDE4D7-mediated cAMP hydrolysis reduced the proliferation rate of AI PC cells. If an abundance of cAMP at the plasma membrane is an important factor in the aberrant proliferation of AI PC, then it would be expected that overexpression of the hyperactive S42A mutant would substantially reduce the growth rate of such cells. Indeed, DU145 cells transfected with this mutant or wtPDE4D7 displayed significantly reduced proliferation (figure 4.12 A and B). Both proteins brought about such a reduction, as previously reported for wtPDE4D7, but S42A retarded growth by a further 65% (figure 4.12 C). The expression of the mutant construct was 15% that of wtPDE4D7 (figure 4.12 D), and so cell index and slope values were corrected for this by multiplying the values by 15%. The decrease in proliferation rate

caused by the hyperactive mutant supports the hypothesis that loss of PDE4D7-mediated cAMP hydrolysis is an important factor in the development of the AI phenotype.

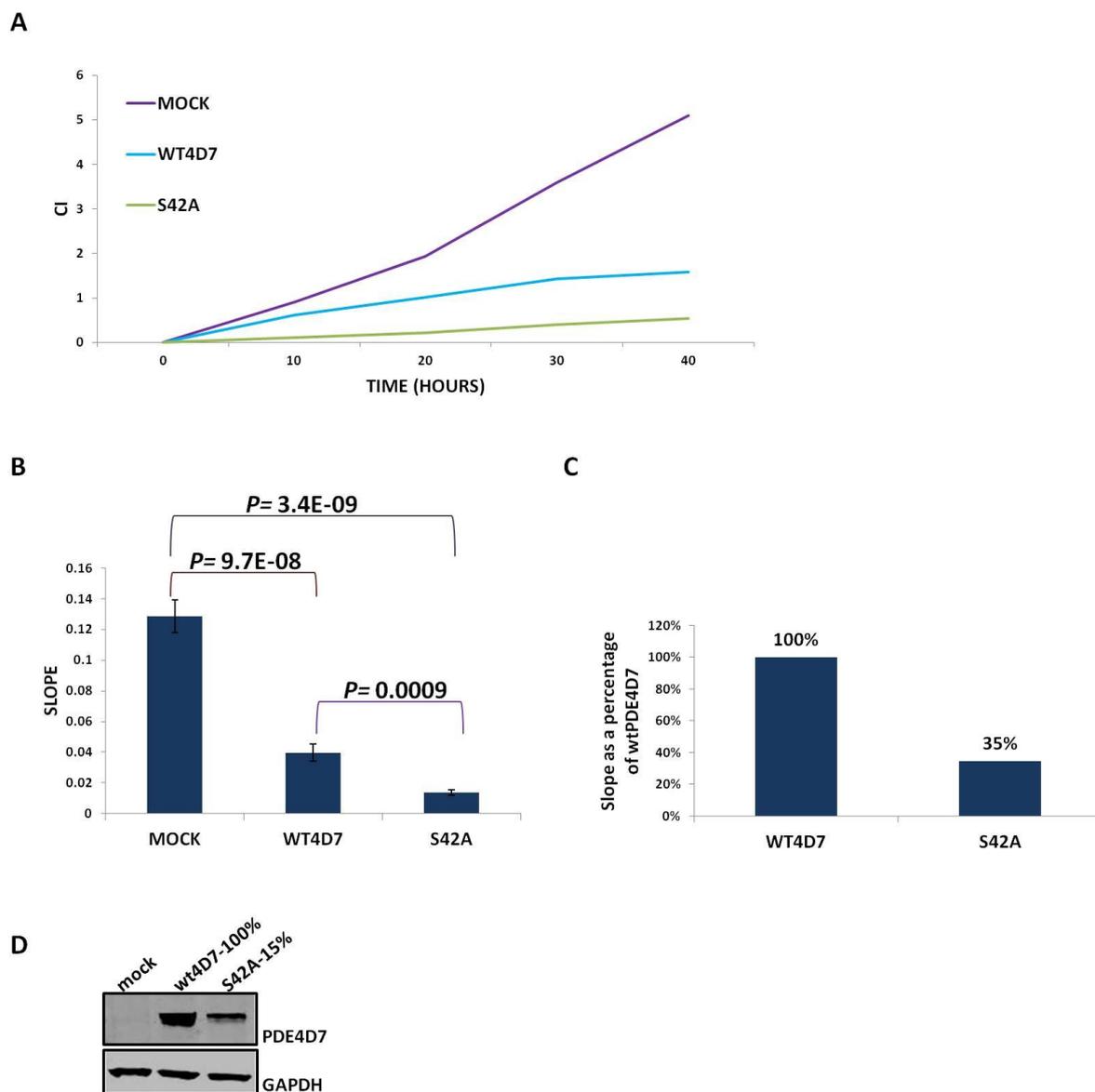


Figure 4.12. The S42A-PDE4D7 mutant significantly reduces AI cell growth compared to wtPDE4D7.

A; the electrical impedance-based xCELLigence system was used to measure the proliferation of DU145 cells transfected with VSV-tagged wild type PDE4D7 (wt)

or the S42A mutant or a control construct (mock), as shown by a growth curve. **B**; the slope of the growth curve was calculated by linear regression analysis using the cell indices (CI) from each proliferation assay. **C**; the slope of DU145 cells transfected with S42A-PDE4D7 as a % of wtPDE4D7. **D**; western blot analysis of transfection, S42A construct expressed at 15% that of wt, the cell indices and slope values were adjusted for this.

4.3 Discussion

PDE splice variants are subject to various levels of regulation; differential tissue expression, subcellular localisation and protein-protein interactions, with further regulation achieved through PTMs that regulate their enzymatic activity and association with binding partners (Bender and Beavo 2006; Omori and Kotera 2007). Tight control of these regulatory mechanisms is paramount to maintaining compartmentalised cyclic nucleotide signalling and thus cellular homeostasis (Houslay 2010).

In the last decade or so, PTMs have gained much interest as markers of disease and as potential therapeutic targets (Krueger and Srivastava 2006). As discussed, hyperphosphorylation is an important event in diseases such as AD (Cole, Soutar et al. 2008). Inappropriate PTMs of signalling pathway intermediates is also an important factor in the repression or activation of anti- and pro-oncogenic pathways. Among them, aberrant phosphorylation is a key event in carcinogenesis and occurs with the tumour suppressor PTEN and members of the MAPK signalling cascade (Krueger and Srivastava 2006). The tumour suppressors Rb and p53, are also both hyperphosphorylated during tumourigenesis, due to inhibition of phosphatase activity (Yatsunami, Komori et al. 1993). In breast cancer, the Akt substrate of 160kDa (AS160), is hyperphosphorylated on a single site; T642, compared to normal breast tissue (Jiang, Sun et al. 2010). The phosphoproteome of a tumour may predict the efficacy of a given anti-cancer drug, and its elucidation is becoming easier thanks to the ever increasing

number of phospho-specific antibodies being designed (Krueger and Srivastava 2006).

Phosphorylation plays an important role in the regulation of PDE4 isoforms. PKA phosphorylation on the UCR1 region of long form PDE4s is fundamental to their activation, enabling hydrolysis of local cAMP (Sette and Conti 1996; MacKenzie, Baillie et al. 2002). It is believed that a conformational change involving the active site and UCR2 underpins the mechanism of long form PDE4 activation. PDE4D3 was rendered constitutively active following cleavage of its UCR2 domain (Lim, Pahlke et al. 1999), and the crystal structure of the PDE4 UCR2 domain showed that it exhibited good shape complementarity with the active site, its affinity for which was increased by PDE4 inhibitors (Burgin, Magnusson et al. 2010). It has also been proposed that the UCR1 and UCR2 domains interact, via electrostatic interactions in the inactive conformation, and PKA phosphorylation of UCR1 disrupts this interaction (Beard, Olsen et al. 2000). Perhaps UCR2 is bound to both UCR1 and the active site and dissociation of both interactions occurs following PKA phosphorylation of UCR1, as depicted in figure 4.1.

While PKA activates long PDE4 isoforms, ERK phosphorylation acts to inhibit their activity (Hoffmann, Baillie et al. 1999). All PDE4 isoforms possess two ERK docking sites around the catalytic region; FQF and KIM, which are C and N-terminal to the target serine respectively (MacKenzie, Baillie et al. 2000). ERK phosphorylation at this site inhibits long and super short PDE4 isoforms, but activates short PDE4 isoforms (Baillie, MacKenzie et al. 2000; MacKenzie, Baillie et al. 2000). The PDE profile within a given cell will therefore determine the result of ERK signalling within that cell. ERK inhibition of long PDE4 isozymes allows a transient increase in local cAMP and PKA activity, which permits downstream signalling cascades. PKA then phosphorylates the PDE, overriding ERK inhibition and bringing about cAMP hydrolysis and cessation of signal via feedback control.

Phosphorylation of PDE4 isoforms may not only act to regulate enzyme activity, but may be important for protein-protein interactions. Indeed, the long PDE4D3

isoform is phosphorylated by PKA within its unique N-terminus on Ser13, an event distinct from UCR1 phosphorylation (Sette and Conti 1996). Phosphorylation on Ser13 mediates interactions with scaffolding proteins such as MAKAP, as well as PKA, MAPKs and PP2A (Carlisle Michel, Dodge et al. 2004; Dodge-Kafka, Soughayer et al. 2005; Dodge-Kafka and Kapiloff 2006; Collins, Murdoch et al. 2008; Dodge-Kafka, Bauman et al. 2010; Murdoch, Vadrevu et al. 2011). This phosphorylation event acts to sequester PDE4D3 in the vicinity of local cAMP, and enables a dynamic ‘swapping’ of binding partners mechanism, thereby spatially and temporally sculpting the signalling cascade in which the aforementioned scaffolds and enzymes are involved.

PDE4D3 is the only known case to date where such N-terminal phosphorylation occurs within a PDE4 isoform. However, here I present evidence for similar PKA mediated phosphorylation of PDE4D7 within its unique N-terminal region. The use of peptide array technology and purified GST recombinant proteins verified that Ser42 is a PKA substrate. This was further validated with the successful cloning of phospho-site mutants and the use of a novel phospho-site specific antibody. The successful raising of this antibody was pivotal to the success of these experiments, and necessary for any *ex vivo* endogenous, and potential future *in vivo* studies.

It must be noted there is a concern with PKA assays carried out using purified PKA catalytic subunit, as they can produce artifactual results. It is known that phospho-proteins can be phosphorylated by a greater number of kinases *in vitro* compared to *in vivo* because isolated catalytic units of kinases used *in vitro* are promiscuous with regard to substrates, possibly due to lack of regulation such as that conferred by the regulatory domains of PKA (Ge Wu 2010, Assay Development: Fundamentals and Practices). However, I show that the phosphorylation of Ser42 occurs *ex vivo*; both in overexpressing Hek293 cells, and in the DuCaP and VCaP endogenous systems. The latter results especially give much credence to the notion that this event occurs *in vivo*.

What is interesting from the results presented here is the level of basal Ser42 phosphorylation, as opposed to UCR1 phosphorylation, which occurs only after

forskolin challenge. Basal (pertaining to cells which have not been stimulated) phosphorylation of PKA substrate proteins has been previously reported (Steinberg and Kiss 1985). Steinberg and colleagues found that many PKA substrates show basal phosphorylation which is dependent on active PKA catalytic subunits and the extent of phosphorylation is mediated by basal AC activity. They suggest that PKA can be partially activated under basal cAMP conditions. Interestingly, they reported that such basal phosphorylation was not transient, but stable and prolonged. Steinberg and colleagues also looked at vimentin which has two cAMP regulated phospho sites, but they could not detect dual-phosphorylated vimentin even under activating PKA conditions, suggesting that cAMP can bring about both phosphorylation and dephosphorylation by independent mechanisms, or phosphorylation of one site may inhibit that of another. Basal phosphorylation of hormone sensitive lipase (HPL), involved in adipose tissue lipolysis, has also been reported (Garton, Campbell et al. 1989; Garton and Yeaman 1990). Here, under basal conditions, phosphorylation of site 2 (termed the basal site) inhibits subsequent phosphorylation of site 1. Stimulation of the adipocytes causes a net increase in phosphorylation of site 1 resulting in activation of HPL. Such mutually exclusive phosphorylation of two sites also occurs on acetyl-CoA carboxylase (Munday, Carling et al. 1988). These data suggest that enzyme activity can be subject to a complex balancing act of other protein kinases and phosphatases, and that such basal phosphorylation events are likely important for cellular homeostasis.

The observations by Steinberg and colleagues are very interesting in light of the results presented here. Phosphorylation of PDE4D7 on Ser42 under basal conditions may retain the enzyme in an inactive state to allow basal cAMP signalling, thus acting in a positive feedback loop. Following ligand stimulation of the Gs-coupled GPCR associated with PDE4D7 signalling, the rise in cAMP and thus PKA activity may lead to phosphorylation of UCR1 in concurrence with dephosphorylation of pSer42, such as suggested by Steinberg and colleagues in their system. Alternatively, phosphorylation of UCR1 may simply override the effect of pSer42, while both sites remain phosphorylated. The latter hypothesis seems more plausible, as Ser42 remains phosphorylated alongside UCR1 phosphorylation following forskolin challenge (figure 4.10 A).

However, PKA phosphorylation of UCR1 is sufficient to activate PDE4D7, and loss of this phosphorylation following a drop in intracellular cAMP is sufficient to inactivate PDE4D7 again. So why then have this phosphorylation event within the N-terminus? It could be that basal cAMP signals in cells expressing PDE4D7 are important for cellular homeostasis and a reduction in the intrinsic activity of the PDE4 complement is required to allow these signals to propagate. Nevertheless, it certainly seems necessary, as inhibition of this event leads to hyperactivity as observed with the PDE4 activity assays, and results in a significantly higher rate of cAMP hydrolysis as suggested by the proliferation assays. I hypothesise that PDE4D7 N-terminal phosphorylation may be involved in keeping PDE4D7 in an inactive conformation. The UCR1 and UCR2 domains are suggested to form an interacting module via non-covalent interactions in the inactive state, which is disrupted upon UCR1 phosphorylation (Beard, Olsen et al. 2000). Perhaps in the inactive state the phosphorylated N-terminal region of PDE4D7 interacts with the UCR domains in a similar manner, possibly acting to stabilise the UCR1-UCR2 module/catalytic site interaction. Following stimulation of the cell and a rise in cAMP, UCR1 becomes phosphorylated which overrides the effect of N-terminal phosphorylation and allows the enzyme to take on a looser conformation via release of the UCR1/UCR2/catalytic domain association. Such a mechanism would prevent enzyme activity under basal levels of cAMP, thereby allowing basal signalling to occur. ERK phosphorylation and/or UCR1 dephosphorylation may then result in reformation of the tighter inactive enzyme conformation (figure 4.13).

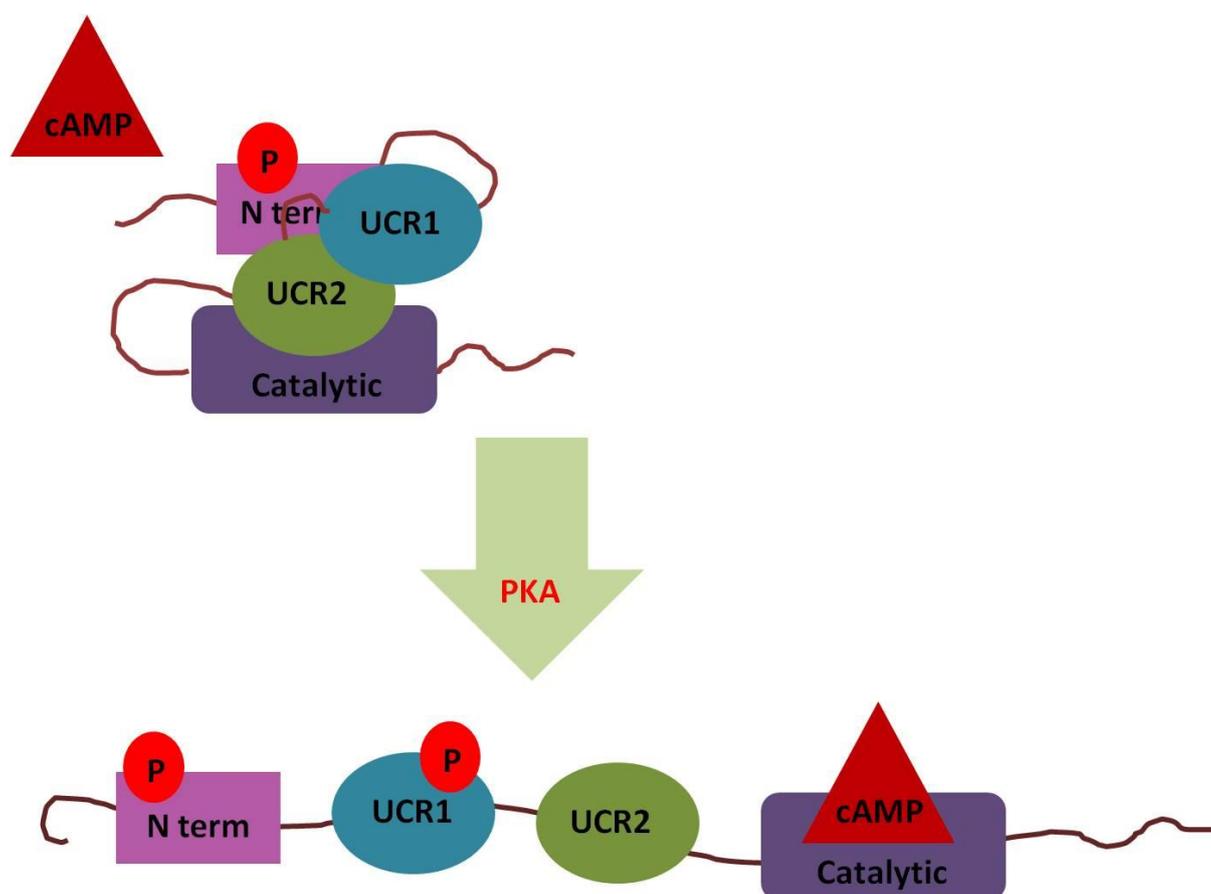


Figure 4.13. Possible mechanism of Ser42 phosphorylation regulation.

Under basal conditions, the enzyme is in a tight inactive conformation where Ser42 is phosphorylated by PKA and is associated with UCR1/UCR2 via electrostatic interactions. A rise in cAMP and PKA brings about UCR1 phosphorylation leading to dissociation of the N-terminal/UCR1/UCR2/active site conformation. Enzyme inhibition due to ERK phosphorylation and/or dephosphorylation of UCR1 causes the enzyme to adopt the tight inactive conformation again.

It may be that the N-terminal region of PDE4D7 is intrinsically disordered, which would allow transient association with the UCR1/UCR2 module in a kinetically favourable manner. Disordered protein domains have gained much interest in recent years. Modular proteins such as PDEs often possess both structured and

disordered domains (Dyson and Wright 2005), the latter being linker regions between the UCR domains of PDE4 isoforms, and possibly the N-termini. Eukaryotes possess the highest degree of protein disorder, as disorder permitted rapid evolution to quickly become multi-cellular organisms (Mittag, Kay et al. 2010). Alternatively spliced exons have been found to commonly code for regions of intrinsic disorder in the resultant protein product, which prevents any structural damage to rest of the protein (Romero, Zaidi et al. 2006). Intrinsic disorder has also been shown to be strongly associated with protein regulation and signalling, with disordered regions often possessing phosphorylation sites, and being involved in auto-inhibition of enzyme function (Romero, Zaidi et al. 2006). Charged residues within a disordered protein region also give rise to the 'polyelectrostatic' effect, where multiple charges influence its binding affinity for other proteins via long-range electrostatic interactions, which give rise to a net charge (Borg, Mittag et al. 2007). The charges are often given by phosphorylation of multiple sites; however, in the case of PDE4D7, Ser42 phosphorylation may confer an electrostatic effect that increases the affinity of the N-terminus for the UCR2/UCR1 module. The change in net charge upon UCR1 phosphorylation may bring about the conformational change necessary for activation. Given that the UCR1/UCR2 electrostatic interaction is ablated by UCR1 phosphorylation or mutation of the target serine to aspartate (Beard, Olsen et al. 2000), it seems likely that the change in net charge conferred by the negatively charged phosphate group on UCR1 induces conformational change. Perhaps phosphorylation of UCR1 causes a conformational change of the whole UCR1/UCR2/N-terminal module, resulting in a more linear enzyme in which cAMP can access the active site. Conversely, phosphorylation of Ser42 alone provides PDE4D7 with a net charge that supports a UCR1/UCR2/N-terminal module, which associates with the catalytic domain to ensure enzyme inactivity. Indeed, when Ser42 is mutated to aspartate, enzyme activity is negligibly altered (figure 4.9), giving credence to the idea that the phosphorylation of Ser42 provides a negative charge necessary for the inactive conformation. Such electrostatic interactions are known to play important roles in stabilising protein structures and phosphate groups provide enhanced electrostatic interactions not available to unmodified amino acids (Johnson and Barford 1994).

Another advantage of disorder is that such proteins or protein domains can bind other proteins in ways difficult for ordered proteins by wrapping around their binding partner, and thus increasing the specificity of the interaction by surface area (Mittag, Kay et al. 2010). Perhaps the PDE4D7 N-terminus wraps around the UCR1/UCR2/catalytic site module. Intrinsic disorder also allows proteins to interact with high specificity, but low affinity. This is desirable where such interactions need to be quickly 'turned on and off' (Mittag, Kay et al. 2010). Yet another advantage of disorder is that it allows proteins to bind various partners while retaining specificity (Wright and Dyson 1999), this may be the case with the PDE4D3 N-terminal domain, where phosphorylation of Ser13 is important for association with many other proteins, and indeed may also be the case for PDE4D7 Ser42. It is plausible to suggest that PDE4D7 may act in such a signalosome at the sub-plasma membrane region of PC cells. Like PDE4D3, it may associate with an AKAP and other proteins. The search for the PDE4D7 interactome is discussed in Chapter 5.

As discussed, PDE4 isoforms are said to exist in either the high affinity rolipram binding site (HARBS) or the low affinity rolipram binding site (LARBS) depending on tissue/cell expression, as different affinities for rolipram have been observed with the same isoform in different tissues and cellular fractions. (Souness and Rao 1997). Perhaps PDE4D7 N-terminal phosphorylation influences the 'choice' of conformer.

Phosphorylation can also bring about a change in protein localisation, such as with the AR (Gioeli, Ficarro et al. 2002). It would be interesting to investigate if this occurred with PDE4D7 following a rise in cAMP. Due to time constraints the phospho-Ser42 specific antibody was not optimised for immunocytochemistry, but this is something we wish to pursue. Our collaborators in the Netherlands possess tissue slices from prostate cancer patients with varying stages of disease. It would be very interesting to use this antibody to investigate if there is a change in the population of N-terminally phosphorylated PDE4D7 as PC progresses from AS towards AI, until PDE4D7 expression is eventually lost. Perhaps the phospho-status of PDE4D7 would give an indication of disease stage.

As discussed, aberrant phosphorylation is often associated with disease, including cancer. Hyperphosphorylation of tumour suppressor proteins is a common occurrence in various cancers (Yatsunami, Komori et al. 1993; Krueger and Srivastava 2006; Jiang, Sun et al. 2010). It is quite conceivable to suggest that PDE4D7 becomes hyperphosphorylated on Ser42 during prostate carcinogenesis. A shift in the equilibrium towards a larger population of Ser42 phosphorylated PDE4D7 enzymes would result in inappropriate levels of cAMP and thus PKA at the plasma membrane. This in turn would perpetuate the situation, increasing PDE4D7 N-terminal hyperphosphorylation. A large population of inactive PDE4D7 would lead to the abundance of cAMP at the plasma membrane we see in AI PC (Chapter 3). It is known that PKA isotype switching occurs during PC progression (Merkle and Hoffmann 2011), perhaps this would lead to aberrant PDE4D7 N-terminal phosphorylation. However, PDE4D7 expression is lost in AI PC, it is not just inactivated. Perhaps, as cAMP is ever increasing, inappropriate and promiscuous downstream signalling may be important in the shift away from androgenic signalling, which, as hypothesised in Chapter 3, would result in transcriptional repression of PART1 and consequently PDE4D7.

4.4 Chapter Summary

- 1) PDE4D7 is phosphorylated by PKA within its N-terminal region.
- 2) PDE4D7 N-terminal phosphorylation likely occurs in endogenously expressed proteins from cultured cells.
- 3) PDE4D7 N-terminal phosphorylation acts to negatively regulate enzyme activity.
- 4) Expression of the hyper-active S42A PDE4D7 mutant decreased AI PC cell growth, backing up the notion that increased cAMP within the PDE4D7 locale is a contributing factor to the AI PC phenotype.

5 The Search for the PDE4D7 Interactome

5.1 Introduction

The end of the last century saw science move from the genomic era into the proteomic era, with protein interaction networks, or interactomes, becoming a main area of research in the cell biology field (De Las Rivas and Fontanillo 2010). Proteins rarely work alone, in fact >80% are said to work in complexes (Berggard, Linse et al. 2007), thus protein-protein interactions (PPIs) are important for any given protein to exert its biological functions. PPIs can be binary (direct) or functional (indirect), they may be defined as *specific contacts between proteins that occur by selective molecular docking in a particular biological context* (De Las Rivas and Fontanillo 2010), *and are usually part of a larger subcellular PPI network, or signalling hub* (Berggard, Linse et al. 2007). Elucidating how the proteome of a given cell is organised into these higher-order networks can be achieved by the daunting task of mapping all constitutive and transient PPIs, and large scale high-throughput proteomics experiments are ever increasing our knowledge of PPIs, with many online repositories now available (Drewes and Bouwmeester 2003; De Las Rivas and Fontanillo 2010). However, the hunt for PPIs is far more difficult than DNA sequencing, as these interactions are dynamic, ‘moving targets’, which often depend on cell type, cell cycle stage, PTMs, environmental factors and the presence of other interactors; and despite the vast amount of research and the technological advances in the methods used for such investigations, our knowledge of the complex protein networks within cells is still developing (Berggard, Linse et al. 2007; Blow 2009; De Las Rivas and Fontanillo 2010; Ngounou Wetie, Sokolowska et al. 2013). Expounding PPIs gives us insight in the workings of cell signalling pathways and thus the pathophysiology of disease. Knowledge of PPIs is essential, especially when a protein is considered as a promising therapeutic target. In such a case, any up/downstream proteins or events that would be affected by intervention should be identified.

A good deal is known about PDE4 interactions, particularly with scaffolds such as AKAPs and β -arrestins. As discussed, the long isoform PDE4D3 interacts with various proteins in different signalling cascades depending on subcellular localisation, such as with AKAP79 to mediate relaxin signalling (Halls and Cooper 2010), and MAKAP to regulate local cAMP in the heart (Carlisle Michel, Dodge et al. 2004). PDE4D3 functions in a highly complex signalosome within the heart, where its phosphorylation status mediates its interactions with binding partners in a dynamic manner (Collins, Murdoch et al. 2008). PDE4D3 is also a member of the cardiac ryanodine receptor (RyR2)/ calcium-release-channel macromolecular complex which plays a role in excitation coupling in the heart (Lehnart, Wehrens et al. 2005). Thus, this isoform interacts with a range of other proteins to tightly control cAMP gradients within the heart. Interestingly, PDE4D3 can also act as a scaffold where it interacts with LIS1 to regulate neuronal cell migration (Murdoch, Vadrevu et al. 2011).

Dynamic PPIs also play an important role in PDE4D5-mediated cAMP regulation at the β 2-AR in cardiac cells, where it interacts with RACK1 or β -arrestin (Bolger, Baillie et al. 2006). Indeed, PDE4D5 is also suggested to function as a scaffold in the heart by sequestering EPAC1 (Berthouze-Duquesnes, Lucas et al. 2013).

Recognition of certain protein domains is also important for PDE4 PPIs. PDE4A5 and PDE4D4 interact with other proteins via SH3 domains, and such interactions regulate their subcellular localisation (Beard, O'Connell et al. 1999; McPhee, Yarwood et al. 1999; Beard, Huston et al. 2002).

I have described how PDE4D7 protein expression is absent in AI PC cells and that PDE4D7-mediated cAMP signalling regulates AS PC cell proliferation. I have also presented evidence to suggest PDE4D7 is N-terminally phosphorylated by PKA, which may indeed mediate PPIs and this may be perturbed as PC progresses. However, no PDE4D7 interacting partners are known, and so I sought to identify such protein(s) in order to gain information on the signalling pathway(s) in which PDE4D7 is involved. If a pathway could be identified, the expression and/or activity of other signalling intermediates within that pathway could be compared

between AS and AI PC cells, perhaps providing a further point for potential therapeutic intervention.

The aims of this chapter are to present the three proteomics approaches employed to investigate the PDE4D7 interactome; IP coupled with mass spectrometry (IP/MS); a yeast-two-hybrid (Y2H) screen; and ProtoArray technology.

5.2 Results

5.2.1 Immunoprecipitation Coupled with Mass Spectrometry

Immunoprecipitation coupled with mass spectrometry (IP/MS) is akin to affinity purification/MS, a common biochemical method for investigating PPIs (Ngounou Wetie, Sokolowska et al. 2013). The protein of interest, or bait, is overexpressed as a tagged fusion protein in cell system. Following an appropriate expression time, lysis of the cells and immunoprecipitation of the bait protein is carried out using an antibody against the tag, which increases specificity of the IP. This is a co-complex approach, so that any interacting partners, either direct or functional, are pulled down with the bait. Gel bands representing these interacting proteins are excised, digested and analysed by MS to determine their identity.

Mass spectrometry (MS) is an analytical tool that has been used for decades to measure the molecular mass of organic compounds such as proteins/peptides, lipids, carbohydrates, nucleosides and environmental compounds (Biemann 1963). Technological advances in MS over the last 20 years or so have made the identification of PPIs a relatively simple task, even allowing for the purification and analysis of large macromolecular complexes (Berggard, Linse et al. 2007). MS can determine the peptide and protein composition of very pure to more crude starting samples, and there are now a number of different instruments and types of analyses to give the desired information based on the sample used. There are two methods commonly used for protein analyses; Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption Ionisation (MALDI) (Trauger, Webb et al. 2002). ESI is a popular choice for identification of large proteins within a complex sample, particularly when coupled to nano-scale liquid chromatography and tandem MS (nLC-ESI-MS/MS) (Yang, Zhang et al. 2007). Firstly, proteins in the sample are digested into smaller fragments by proteolysis, usually by trypsin. The fragments are then separated and purified by nano-capillary HPLC, analysing each peptide mass as they elute from the HPLC column. The peptide fragments are then fed into the mass spectrometer via an inlet and ionisation, such as ESI, is carried out to allow separation based on mass

to charge (M/Z) ratio. If ESI is being used, charged droplets are produced when the solubilised sample is passed through a high voltage needle at atmospheric pressure (Wysocki, Resing et al. 2005). A high vacuum then allows the ions to easily travel from one end of the instrument to the other (An Introduction to Mass Spectrometry, Scott E. Van Bramer, 1998).

Tandem MS (MS/MS) instruments allow the detection of specific ions in a given sample. Ions are then fragmented and the m/z of the fragment ions is evaluated. (Wysocki, Resing et al. 2005). Tandem MS usually comprises two mass spectrometers connected by a chamber, in which ions are selected in the first spectrometer according to weight, activated by collision with argon in the chamber, and the ion fragments are then analysed by a mass analyser in the second spectrometer. Analysis can be performed by a choice of mass analysers, which resolve the m/z by different methods, to give data output of a mass spectrum; a graph showing each specific molecule/amino acid by mass and how much of each molecule/amino acid is present in the sample. From analysis, the protein composition of the original sample can be determined by comparing sequence information to the databases, as the masses of all 20 amino acids are known.

To identify PDE4D7 PPIs, VSV-tagged PDE4D7 was overexpressed in Hek293 cells. This was because at the time of this experiment, the new highly specific PDE4D7 antibody had not yet been raised. The cells were lysed and immunoprecipitation undertaken with agarose beads conjugated to a VSV antibody. An IP was also carried out with mock transfected lysates. Following SDS-PAGE, the gel was Coomassie stained to identify any bands co-purifying with the PDE4D7-VSV. Any bands observed to repeatedly pull down ($n=3$), were excised from the gel, along with the corresponding gel band in the control lane. These bands were then subjected to nLC-ESI-MS/MS using quadrupole time of flight (Q-TOF). Q-TOF employs an electric field to accelerate the ions and the time it takes them to reach a detector at a known distance is measured. The velocity of an ion depends on its m/z , and heavier ions travel more slowly. The use of nLC-ESI-MS/MS coupled with Q-TOF is commonly used due to enhanced sensitivity and

thus improved identification of amino acids (Lacorte and Fernandez-Alba 2006). Figure 5.1 illustrates the IP/MS workflow.

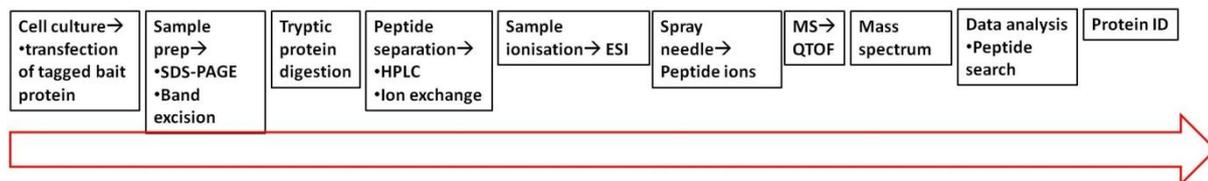


Figure 5.1. nLC-ESI-MS/MS with Q-TOF workflow.

The bands excised for MS analysis are shown in figure 5.2. These bands could only be seen in the PDE4D7 VSV IP lane, and not in any other lane. Bands present in all lanes, or solely in any control lane were not excised. Since they could only be seen with the naked eye on the actual gel and did not scan well, the bands excised are indicated by red lines. Bands 5 and 7 represent two bands that were too close to excise individually, and so were analysed together. The corresponding areas in the mock-transfected VSV IP lane were also excised and analysed. IPs were also carried out using agarose beads conjugated to a FLAG antibody as a negative control. Along with the test bands, the band representing PDE4D7 was analysed to confirm identification.

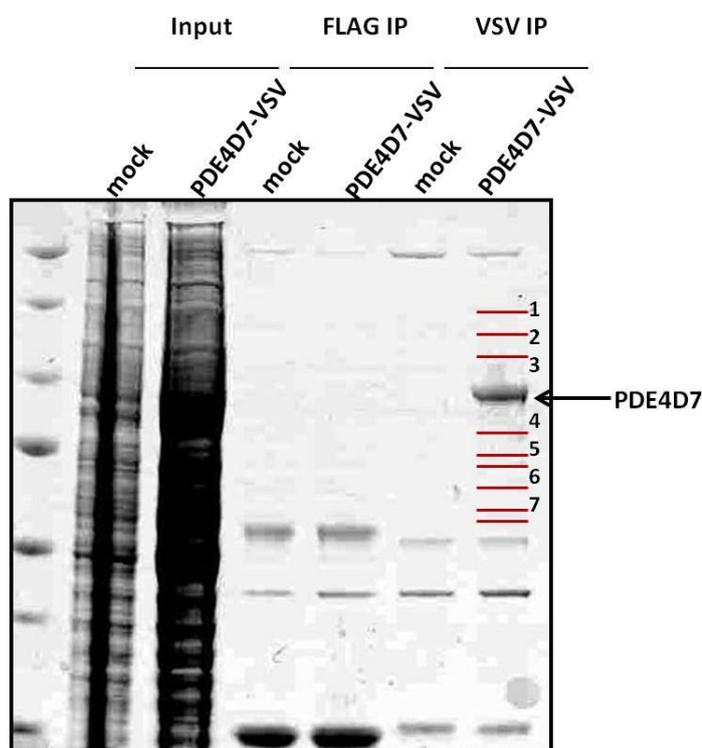


Figure 5.2. A PDE4D7 pull down assay produces a number of possibly interacting proteins. Hek293 cells were transfected with VSV-tagged PDE4D7 or a control construct (mock) and cellular lysates were immunoprecipitated (IP) with agarose beads conjugated to VSV antibody or FLAG antibody. The samples were analysed by SDS-PAGE and Coomassie Blue staining. Inputs are the whole cell lysates from which the IPs were carried out. Red bands represent samples 1-7 that were excised for MS analysis, along with the corresponding region in the mock VSV IP lane. N=3.

5.2.1.1 IP/MS Identified 5 potential PDE4D7 Interacting Partners

IP/MS identified 20 proteins in the samples, 5 of which were in the PDE4D7 samples only (table 5.1), along with identification of PDE4D7. The putative function of the interactors was obtained using Uniprot (<http://www.uniprot.org/uniprot>).

Identified Protein	Accession Number	Putative Function
Heat shock cognate 71 kDa protein (HSPA8)	gi 123647	Repressor of transcriptional activation. Chaperone. Involved in the spliceosome and activating pre-mRNA splicing.
PREDICTED: similar to heat shock protein 70 [Equus caballus]	gi 149732056	Unknown.
Octamer-binding protein (NONO)	gi 1083440	Binds DNA and RNA. Pre-mRNA splicing. Defective RNA retention. DNA repair. Necessary for cAMP dependant activation of CREB target genes. . It has also been shown to act as a transcriptional coactivator of the AR activation function 1 (AF-1) domain, and conflictingly, to repress AR transactivation. It is activated in response to stress.

ATP-dependent RNA helicase A (DHX9).	gi 100913206	A member of the DEAH/DEAD box containing family of RNA helicases. It catalyses ATP-dependant unwinding of dsRNA and DNA-RNA complexes. It shuttles between nucleus and cytosol and functions as a transcriptional regulator.
PREDICTED: protein phosphatase 1 regulatory subunit 12A isoform 4	gi 109097899	Regulates myosin phosphatase activity in the cytoplasm.

Table 5.1. MS analysis identified 5 potential PDE4D7 interacting proteins.

Those in bold were chosen for PPI verification, based on association with cAMP signalling and cancer, as found in the literature. Verification analysis by secondary co-immunoprecipitation is reported below in figure 5.12.

5.2.2 Yeast Two-Hybrid (Y2H) Screen

The Y2H method was first described in the late 1980's (Fields and Song 1989) and has become the method of choice when looking for novel interactions. It takes advantage of eukaryotic transcription factors which have discrete and separable DNA binding and Activation Domains (DBD/AD) (Causier and Davies 2002). It takes place in genetically-modified yeast strains, into which the protein of interest and potential binding partners are co-transformed as fusion vectors, attached to the DNA DBD and AD of a transcription factor respectively, usually Gal4. The protein of interest is called the 'bait', and any interaction with 'prey' proteins brings about the reconstitution of the functional transcription factor, recruitment of RNA polymerase and transcription of a reporter gene that allows the yeast to grow on selective media, or cause a colour change. The reporter gene may code for an essential amino acid, such that only cells containing a bait-prey interacting pair may grow in broth deficient in amino acids. *E.coli* are

often employed to amplify the plasmids quickly, prior to a second yeast cell selection. The amplified plasmids are co-transformed back into yeast and positive clones are selected for by means of a β galactosidase assay. Yeast cells are grown on agar containing the lactose analog X-gal, which is broken down by β galactosidase to produce a blue colour. This colour represents colonies positive for a bait-prey interaction. A schematic representing the Y2H method is shown in figure 5.3. Y2H screens can be high-throughput to study protein interactions on a large scale, and many PPIs in the literature have been identified by this method (Bruckner, Polge et al. 2009).

Commonly, a cDNA-library Y2H screen is carried out (Bruckner, Polge et al. 2009). This investigates pair-wise interactions between a bait and a defined set of preys present in a given cDNA library. Libraries may contain DNA fragments or full length ORFs to cover a whole transcriptome. This method involves colony PCR analysis and sequencing to identify interacting partners.

Y2H screens are generally preferred over IP/MS, mainly due to accessibility, as MS requires expensive instrumentation. Y2H is also an *in vivo* approach, whereas IP/MS employs *ex vivo* and *in vitro* techniques. Another advantage of Y2H concerns its binary nature, as this can give information about the dynamics of a PPI, such as dissociation rates (Blow 2009). Y2H also has the ability to detect weak PPIs (Causier and Davies 2002). However, a Y2H screen cannot detect interactions if they are dependent on a PTM (Bruckner, Polge et al. 2009). Since its introduction by Fields and Song, the Y2H has been modified to improve its use for identifying PPIs, and also interactions between proteins and nucleic acids (Causier and Davies 2002).

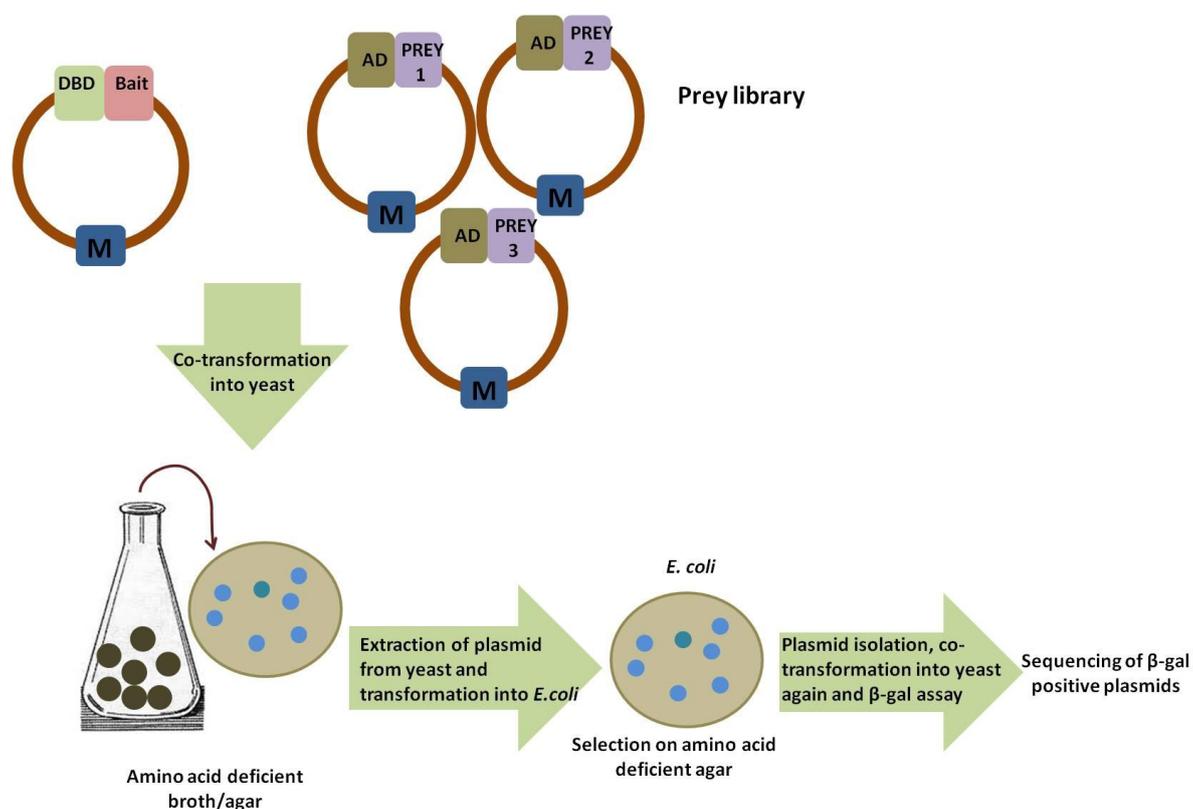


Figure 5.3. A yeast two hybrid screen.

Plasmids engineered to express genes encoding fusion bait and prey proteins are transformed into yeast cells which are grown in selective medium, allowing only transformed cells to grow. *M*=marker gene, such as an essential amino acid. Colonies are then selected on amino acid deficient agar followed by isolation of the plasmids which are co-transformed into *E. coli* for amplification. Plasmids are again isolated, and co-transformed into yeast for confirmation of interactions and selection of cells expressing a bait-prey interacting pair by a β galactosidase assay. These positive plasmids are then sequenced for identification of the bait/prey pair.

5.2.2.1 A Yeast Two-Hybrid Screen Identified 13 Potential PDE4D7 Interacting Partners

The PDE4D7 Y2H screen was carried out by Mr. Bangze Jin of Shanghai Genomics Inc. PDE4D7-VSV (pcDNA3.1-PDE4D7) was used as template for the bait fusion vector; pGB-PDE4D7. Following cloning into yeast Y190 strains, and sequencing of pGB-PDE4D7, a self-activation test was performed which is imperative to ensure the bait-DBD fusion does not autonomously activate transcription of the reporter gene in the absence of the prey. The prey library available to us was a human foetal brain cDNA library. The Y2H screen involved 4 phases;

Phase I: Subcloning; PDE4D7 was cloned into the pGB vector, and the construct was verified by DNA gel electrophoresis and sequencing (figure 5.4 A), followed by purification of the bait plasmid.

Phase II: Toxicity and self activation test; yeast were transformed with the bait plasmid and auto-activation was tested for by a β galactosidase assay. pGB-PDE4D7 did not induce toxicity in yeast, or self activation (figure 5.4 B-D).

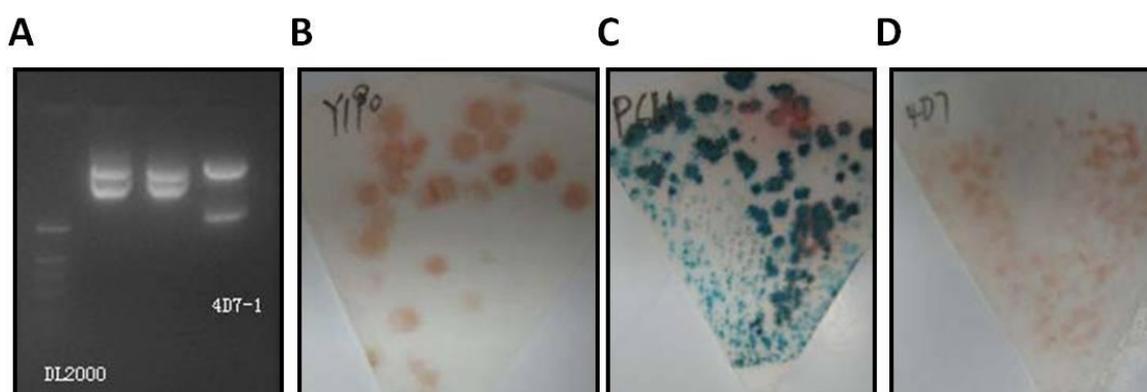


Figure 5.4. pGB-PDE4D7 was successfully cloned and did not induce self-activation in yeast.

A; verification of the cloning of PDE4D7 into the pGB vector. **B;** a negative β galactosidase control test shows brown colonies. **C;** a positive β galactosidase

control test shows blue colonies. **D**; a β galactosidase assay for the bait plasmid, pGB-PDE4D7. Brown colonies confirm lack of self activation.

Phase III and IV: Y2H screening of cDNA libraries and phenotypic validation (colour change). Yeast cells were co-transformed with bait plasmid and prey library. Cells were firstly transformed with the bait plasmid, cultured, and then transformed with prey plasmids from the library. 69 colonies grew on the amino acid-deficient agar plates and so were validated by a β galactosidase assay. Of these, 45 proved positive for β galactosidase (figure 5.5 A), the plasmids from which were isolated and transformed into *E.coli* for amplification. The amplified plasmids were then isolated and co-transformed into yeast for further validation by β galactosidase assays. From this, 15 positive colonies grew (figure 5.5 B). Sequencing of the 15 clones verified 13 potential PDE4D7 interactors (table 5.2). The putative function of the interactors was obtained using UniProt (<http://www.uniprot.org/uniprot>).

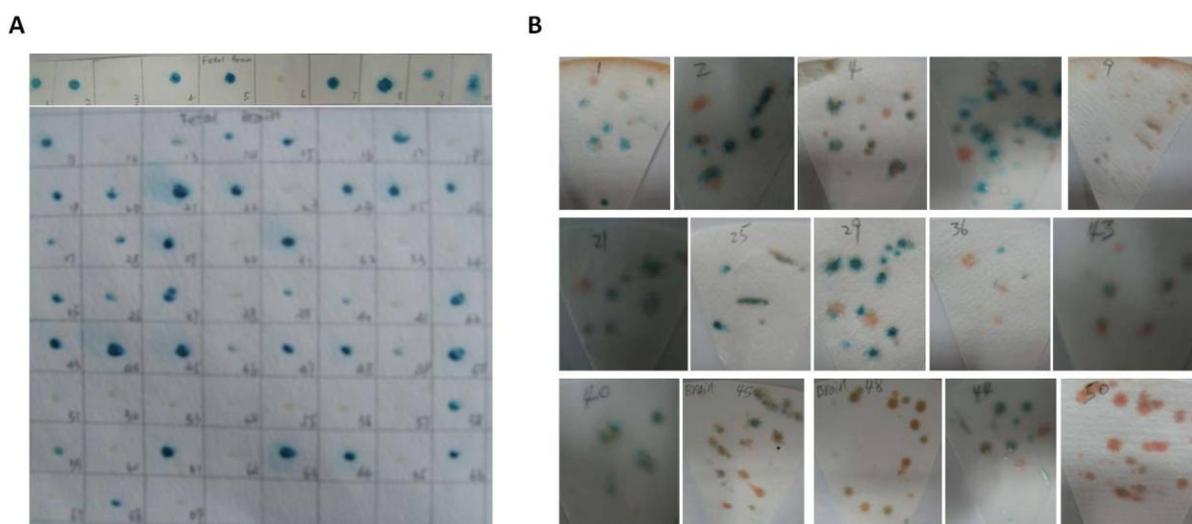


Figure 5.5. Results of the yeast the hybrid screen.

A; yeast cells were co-transformed with the bait plasmid (pGB-PDE4D7) and prey library. 45 of the yeast colonies that successfully grew on selective agar plates gave a positive result (blue colour) for the β galactosidase assay. **B**; these 45

positive plasmids were isolated and amplified in bacteria before being transformed back into yeast cells, and 15 colonies produced positive results (blue colour) for the β galactosidase assay. Varying degrees of blue colour change can be seen, representing different binding affinities of the bait-prey pairs.

Identified Protein	Accession Number	Putative Function (Uniprot)
Microspherule Protein 1/ MSP58 (MCRS1)	NM_006337.3	Putative regulatory component of the chromatin remodelling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair.
SNAP-associated protein (SNAPIN)	NM_012437.4	a component of the SNARE complex of proteins that is required for synaptic vesicle docking and fusion.
ENKURIN/ C10ORF63	NM_145010.2	Adapter that functions to localize a calcium-sensitive signal transduction machinery in sperm to a calcium-permeable ion channel.
Protein phosphatase 3 catalytic subunit beta isozyme (PPP3CB)	NM_021132.2	Calcium-dependent, calmodulin-stimulated protein phosphatase.
Retinoid X receptor beta (RXRB)	NM_021976.3	a member of the retinoid X receptor (RXR) family of nuclear receptors which are involved in mediating the effects of retinoic acid (RA).
PHD finger protein 19 (PHF19)	NM_001009936.1	Transcriptional repressor.
BTB (POZ) Domain Containing 2 (BTBD2)	NM_017797.3	Involved in several cellular processes including proliferation, apoptosis and transcription regulation.

UPF3 Regulator Of Nonsense Transcripts Homolog (UPF3B)	NM_080632.2	Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons.
Zinc finger protein 302 (ZNF302)	NM_001012320.1	May be involved in transcriptional regulation
Ribosomal protein L12 (RPL12)	NM_000976.3	Ribosomal protein that is a component of the 60S subunit.
General Transcription Factor IIIA (GTF3A)	NM_002097	Functions as an RNA polymerase III transcription factor to induce transcription of the 5S rRNA genes.
Glyoxylate Reductase 1 Homolog (GLYR1)	NM_032569.3	Regulates p38 MAP kinase activity by mediating stress activation of p38alpha/MAPK14 and specifically regulating MAPK14 signalling.
PDE4A	NM_001111307.1	Phosphodiesterase 4A family

Table 5.2. A Y2H screen identified 13 potential PDE4D7 interacting proteins. Those in bold were chosen for PPI verification, based on association with cAMP signalling and cancer as found in the literature. Verification analysis by co-immunoprecipitation is reported below in figure 5.12.

5.2.3 ProtoArray Technology

For years the yeast two hybrid screen was the only available genome-wide method for studying protein interactions and it is associated with a number of limitations; false positives, particularly from proteins that function as transcriptional activators, incorrect protein folding, inappropriate PTMs, and difficulty controlling environmental conditions (MacBeath and Schreiber 2000). The subsequent advent and advancement of protein microarrays has allowed rapid and robust screening of interactions in a high-throughput, miniaturised and parallel manner. Protein-protein, protein-nucleic acid, protein-small molecule, protein-lipid, and enzyme-substrate interactions can be identified, as well as antibody specificity and autoantibody characterisation (Schweitzer, Predki et al. 2003; Predki 2004). Protein microarrays have been successfully used to identify differentially expressed proteins in ovarian cancer (Hudson, Pozdnyakova et al. 2007), and to identify novel intermediates of the NF κ B signalling pathway (Fenner, Scannell et al. 2010). The commercial introduction of functional protein microarrays has led to huge potential for the discovery of novel protein interactions and drug development (Predki 2004).

The ProtoArray human protein microarray-based platform (Invitrogen) was employed to investigate PDE4D7 PPIs. These microarrays contain >9,500 proteins, highly purified from Sf9 insect cells as N-terminal GST fusion proteins which are immobilised at spatially addressable positions on nitro-cellulose-coated glass slides. Expression in insect cells allows for correct mammalian protein folding and PTMs (Bouvier, Menard et al. 1998). A bait protein that is overlaid on the array may interact with the immobilised proteins, which are spotted in duplicate and extend from the array surface via the epitope tags, allowing them to be spatially accessible to the purified bait. Each ProtoArray has a barcode that can be used to download the Protein Content List for that array. The arrays also contain a number of control proteins which allow for correct orientation of the array and verification of probing and detection methods, some of which are listed in table 5.3. Following probing of the array with bait and antibody detection, the array is scanned using a suitable microarray scanner and

images of the array are analysed for any interactions (figure 5.6). Binding of the protein of interest to any protein on the array manifests as a fluorescent spot, and both duplicate protein spots on the array must be bound before a positive result is recorded (see figure 5.7). Any spots other than the positive controls represent a possible interaction. The scanned images of the arrays are analysed by a software programme that gives the spots a confidence score and a flag value of A-E, where A indicates strongest fluorescence/binding, and the duplicate pair must both score A or B to be considered interactors of the bait protein. The test array scores are cross-referenced with those from control arrays, and any hits with a score of A/B on the test array only, are considered for further investigation. The protein identities (Ids) can then be used to identify any interactor in the Protein Content List.

Protein	Function
Alexa Fluor® Antibody(Rabbit anti-mouse IgG Antibody labelled with Alexa Fluor® 647, Alexa Fluor® 555, and Alexa Fluor® 488).	Serves as a positive control for fluorescence scanning and for orientation of the microarray image.
Bovine Serum Albumin (BSA)	A negative control for non-specific protein interactions.
GST Protein Gradient	Serves as a negative control and signals are used by ProtoArray® Prospector software for background and statistical significance calculations
Alignment Control Kinase (PKCeta)	Kinases autophosphorylate and produce signals which are used for orientation of the microarray image; also serves as a positive control for the radiolabel and assay conditions.
BioEase™ V5 Control Protein (biotinylated, V5-tagged control protein)	A positive control for detection with the Anti-V5-Alexa Fluor® 647 Antibody, used with the ProtoArray Control Protein Microarray (described below).
Anti-biotin Antibody (mouse anti-biotin antibody)	Detects biotin labeled protein probes and serves as a control for anti-mouse antibody detection reagent.
Yeast calmodulin (Cmd1p) or human calmodulin (CALM2)	A positive control for protein-protein interaction application and interacts with the Array Control Protein.

Table 5.3. ProtoArray slides contain a number of control proteins for background, orientation of the array, and verification of detection conditions.

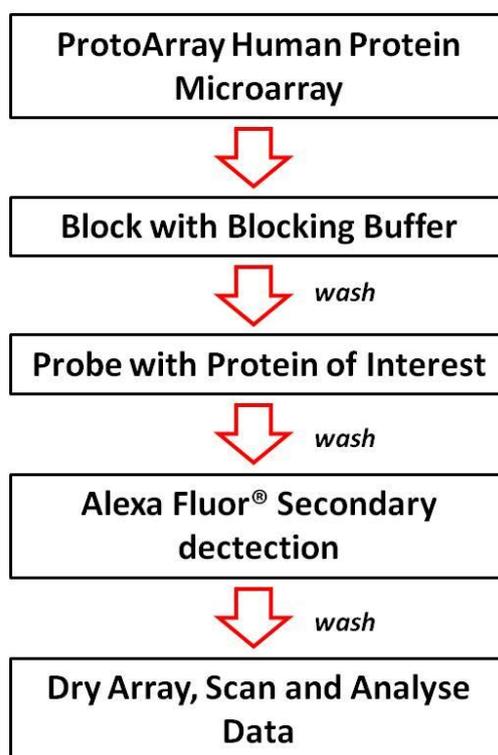


Figure 5.6. The ProtoArray experimental workflow

5.2.3.1 Optimisation of ProtoArray Conditions

Due to the expense of the ProtoArray slides, the experimental conditions were optimised prior to the PPI experiment, using a ProtoArray Control Protein Microarray with an Array Control Protein (Invitrogen). The control ProtoArray chips contain only controls (table 5.3) which allow validation of probing and scanning procedures. The control protein is a recombinant BioEase™-V5-tagged yeast calmodulin kinase (Cmk1p) and probing with an anti-V5 antibody verified the experimental procedure and conditions as seen by detection of the Alexa Fluor™ and biotin antibodies, the BioEase™V5 control protein and calmodulin (figure 5.7).

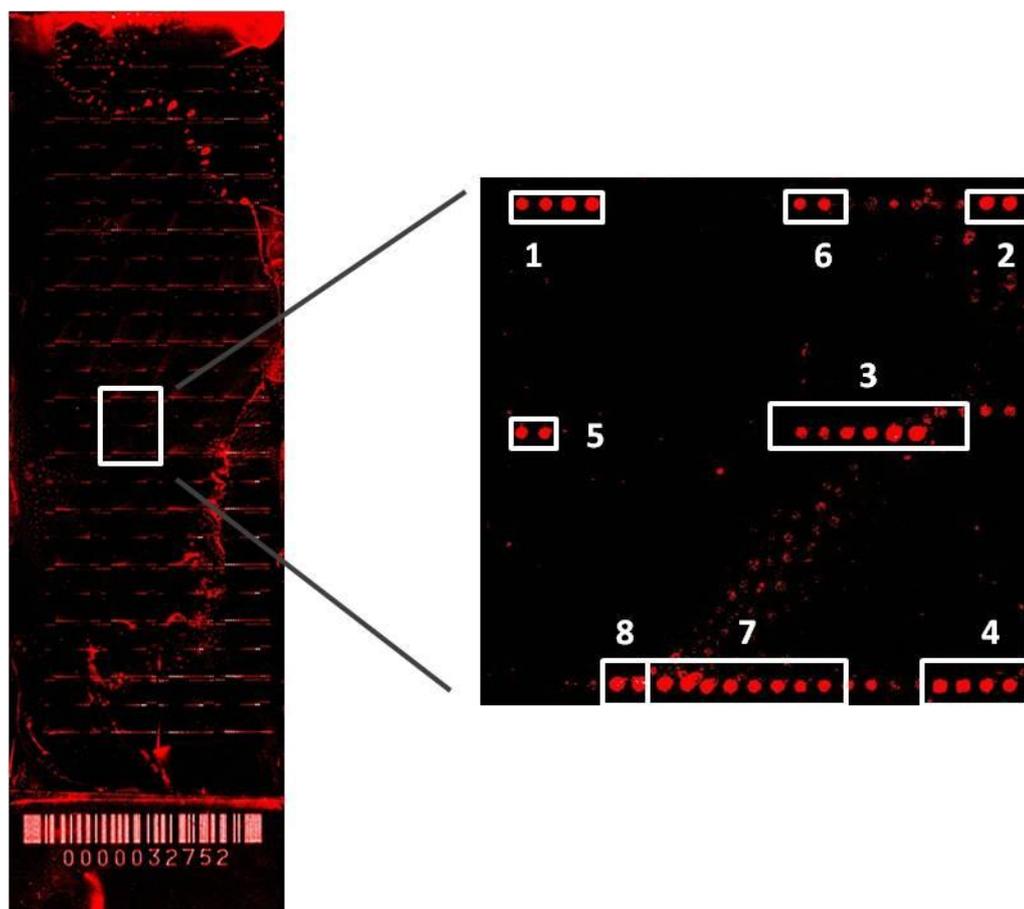


Figure 5.7. A ProtoArray Control Protein Microarray verifies experimental conditions.

A ProtoArray Control Protein Microarray was incubated with Array Control Protein. The array was then probed with anti-V5 primary antibody and Alexa Fluor® secondary antibody, and scanned. The bound Array Control Protein (spots 7), biotin (spots 6) and calmodulin (spots 8,) were detected on the ProtoArray Control Protein Microarray; validating the experimental procedure and conditions such as wash times, buffers etc. Spots 1-5 are Alexa Fluor® antibodies immobilised on the ProtoArray Control Protein Microarray which serve as positive controls for fluorescent scanning and allow correct orientation of the array. N=1.

5.2.3.2 Validation of Purified PDE4D7

A full length recombinant GST-fusion of PDE4D7, purified from a baculovirus SF9 expression system, was purchased. Prior to carrying out the ProtoArray, the protein and the ability of a PDE4D7 antibody to detect the protein were verified by means of a spot blot and western blot analysis. The PDE4D7 specific antibody discussed in Chapter 3 that detects only overexpressed protein was used, as the novel highly specific antibody was not available at the time of this experiment. The PDE4D7-GST fusion protein was successfully detected using a GST antibody (figure 5.8 A) and the PDE4D7 antibody (figure 5.8 B); verifying both the protein and the antibody. Spot blot analysis was also carried out to verify that the PDE4D7 antibody could detect the native protein (figure 5.8 C).

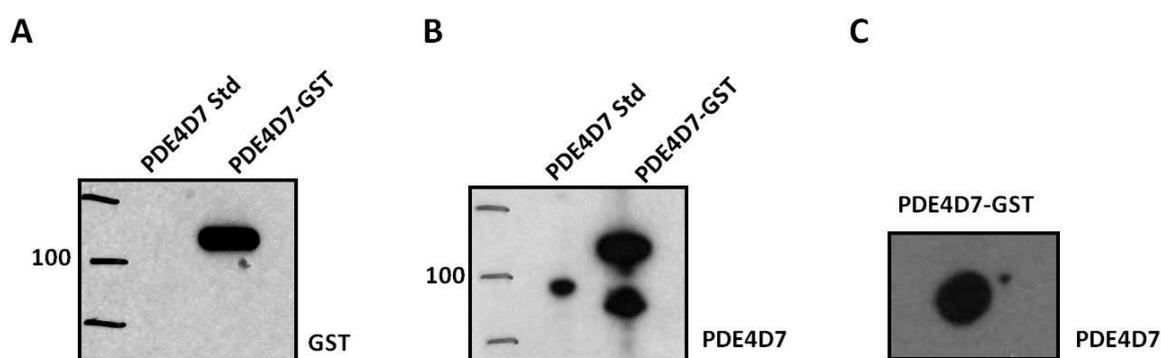


Figure 5.8. Validation of PDE4D7-GST for ProtoArray.

The GST-PDE4D7 fusion protein was validated by blotting with **A)** GST antibody and **B)** PDE4D7 antibody. **C)** spot blot analysis confirms the PDE4D7 antibody can detect native PDE4D7-GST. N=1.

5.2.3.3 ProtoArray Technology Identifies 22 Potential PDE4D7 Interactors

Following the positive control experiment, a PDE4D7 ProtoArray along with two negative control ProtoArrays was investigated. Since the ProtoArray proteins and PDE4D7 were GST fusions, one negative control employed incubation with purified GST, the second was an antibody-only control. All three ProtoArrays contained the same protein library. Scanning the ProtoArrays detected positive control spots such as the Alexa Fluor® antibodies, again verifying the probing/detection/scanning protocols and allowing for orientation of the arrays. Both control arrays displayed minimal spots other than the controls, whereas there were a number of fluorescent spots on the PDE4D7 array (figure 5.9).

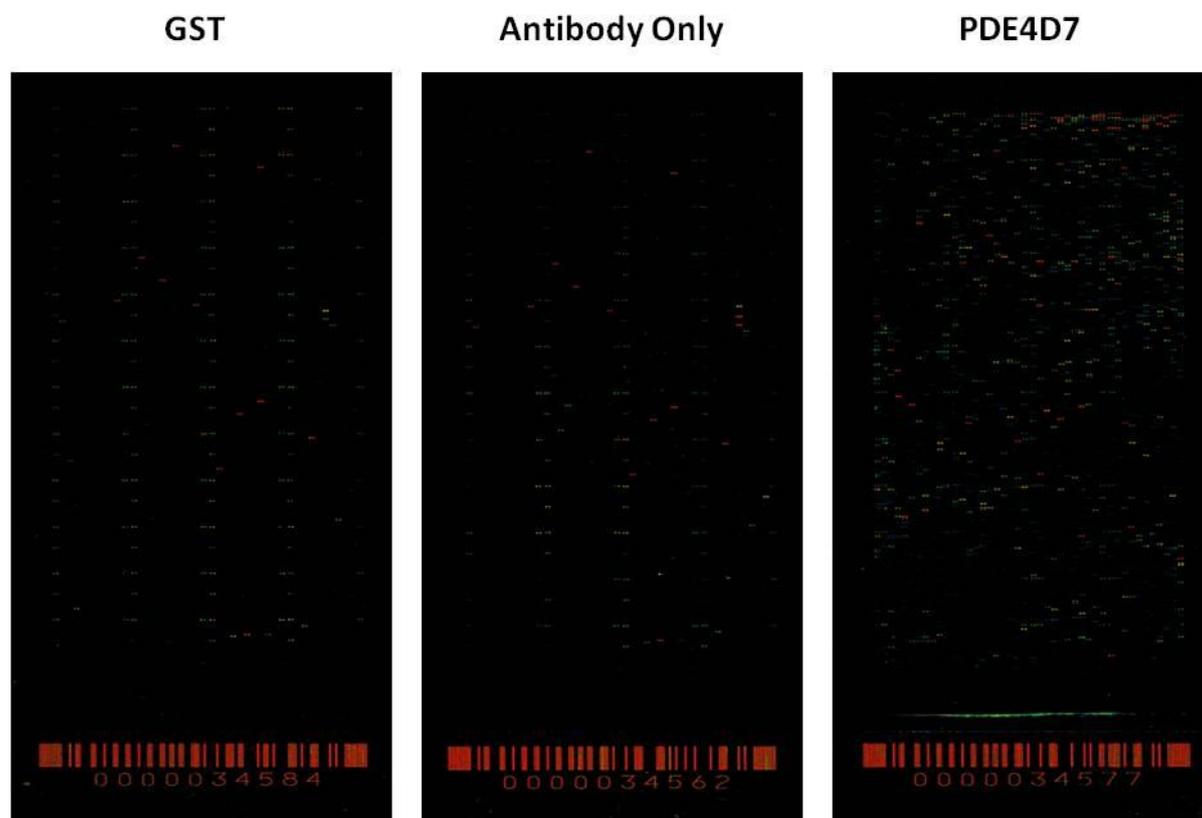


Figure 5.9. ProtoArray technology detects a number of potential PDE4D7 interactors, compared to control arrays.

ProtoArray Human Protein Microarrays containing identical protein libraries were incubated with purified GST-PDE4D7 fusion protein (PDE4D7) or purified GST or secondary antibody only. The arrays were then probed with PDE4D7 primary antibody followed by Alexa Fluor secondary antibody, and a number of potential protein interactors were detected on the GST-PDE4D7 array only. N=1.

Following scanning of the arrays and acquisition of the images (figure 5.9), BlueFuse (BlueGenome) microarray processing software was used to analyse and interpret the results. BlueFuse gave an excel output listing the IDs of all the proteins on each array. The IDs were then used to find the corresponding protein accession numbers in the Protein Content List, which were entered into the excel files (figure 5.10). The scores for all proteins from the PDE4D7 array were cross referenced with those of the negative controls. Any high scoring proteins

that scored A-C in the controls were disregarded, until a shortlist of proteins with a flag score of A/B for PDE4D7, but D/E for the controls, was obtained. The shortlist of hits was then checked against the scanned image of the PDE4D7 ProtoArray, to ensure the corresponding pair of fluorescent spots were prominent, and thus not a false positive, in which case it would be disregarded. The final list of potential PDE4D7 interactors is shown in table 5.4. The putative function of the interactors was obtained using UniProt (<http://www.uniprot.org/uniprot>).

ROW	COL	SUBGRIDROW	SUBGRIDCOL	SPOTNUM	BLOCK	NAME	ID	CONFIDENCE	FLAG
50	1	1	1	21	21	1 AlexaAntiMouseAb~N/A	HA20273~B01R01C21	0.96	A
51	1	1	1	22	22	1 AlexaAntiMouseAb~N/A	HA20273~B01R01C22	0.85	B
52	1	1	2	1	23	1 Buffer	HA20273~B01R02C01	0.18	D
53	1	1	2	2	24	1 Buffer	HA20273~B01R02C02	0.24	D
54	1	1	2	3	25	1 HumanIgA2~N/A	HA20273~B01R02C03	0.64	C
55	1	1	2	4	26	1 HumanIgA2~N/A	HA20273~B01R02C04	0.28	D
56	1	1	2	5	27	1 HumanIgA1~N/A	HA20273~B01R02C05	0.9	A
57	1	1	2	6	28	1 HumanIgA1~N/A	HA20273~B01R02C06	0.18	D
58	1	1	2	7	29	1 Buffer	HA20273~B01R02C07	0.26	D
59	1	1	2	8	30	1 Buffer	HA20273~B01R02C08	0.21	D
60	1	1	2	9	31	1 BSA~N/A	HA20273~B01R02C09	0.6	C
61	1	1	2	10	32	1 BSA~N/A	HA20273~B01R02C10	0.11	D
62	1	1	2	11	33	1 Hs~Ref:NM_024548.2~uORF:HA20273~B01R02C11		0.64	C
63	1	1	2	12	34	1 Hs~Ref:NM_024548.2~uORF:HA20273~B01R02C12		0.78	B
64	1	1	2	13	35	1 Hs~MGC:BC011414.1~uORF:HA20273~B01R02C13		1	A
65	1	1	2	14	36	1 Hs~MGC:BC011414.1~uORF:HA20273~B01R02C14		0.9	A
66	1	1	2	15	37	1 Hs~MGC:BC004932.1~uORF:HA20273~B01R02C15		0.67	C
67	1	1	2	16	38	1 Hs~MGC:BC004932.1~uORF:HA20273~B01R02C16		0.79	B
68	1	1	2	17	39	1 Hs~MGC:BC037854.1~uORF:HA20273~B01R02C17		1	A
69	1	1	2	18	40	1 Hs~MGC:BC037854.1~uORF:HA20273~B01R02C18		1	A

Figure 5.10. An example of the BlueFuse results for PDE4D7 PPIs.

A; an Alexa Fluor® positive control pair scores a high confidence and Flag score. B; a BSA negative control pair scores a low confidence and Flag score. C and D; potential PDE4D7 interactors score high confidence and flag scores.

Identified Protein	Accession Number	Putative Function
AMPK-related protein kinase 5	NM_014840	Involved in tolerance to glucose starvation.
Activating transcription factor 3 (ATF3)	NM_001674.1	Member of the mammalian (CREB) protein family of transcription factors.
Regulatory factor X, 5 (RFX5)	NM_000449.2	Influences HLA class II expression.
Atlastin GTPase 3 (ATL3)	NM_015459.3	Involved in endoplasmic reticulum tubular network biogenesis.
Mitogen-activated protein kinase kinase kinase 9 (MAP3K9/MLK1)	NM_033141.2	Phosphorylates a number of transcription factors in response to activation by environmental stress and pro-inflammatory cytokines.
Chemokine (C-X-C motif) ligand 5 (CXCL5)	NM_002994.2	Proposed to bind the G-protein coupled receptor chemokine (C-X-C motif) receptor 2 to recruit neutrophils, to promote angiogenesis and to remodel connective tissues. Thought to play a role in cancer cell proliferation, migration, and invasion.
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (UCHL1)	NM_004181.2	specifically expressed in the neurons and in cells of the diffuse neuroendocrine system. Mutations in this gene may be associated with Parkinson disease.
Phosphatidic acid phosphatase type 2B (PPAP2B)	NM_003713.3	Hydrolyses extracellular lysophosphatidic acid and short-chain phosphatidic acid.
Leukocyte specific transcript 1 (LST1)	NM_205840.1	Possible role in modulating immune responses; a membrane protein that can inhibit the proliferation of lymphocytes
CHK2 checkpoint homolog (CHEK2)	NM_001005735	A cell cycle checkpoint regulator and putative tumour suppressor.
Lipolysis stimulated lipoprotein receptor (LSR), transcript variant 2	NM_205834.2	Unknown
Peroxisomal biogenesis factor 16 (PEX16), transcript	NM_004813.1	Peroxisome rganisation and biogenesis.
Mal, T-cell differentiation protein-like (MALL),	NM_005434.3	Raft-mediated trafficking in endothelial cells
Cancer/testis antigen family 45, member A3,	BC028711.2	Unknown. Expressed in cancer cell lines.

V-raf murine sarcoma 3611 viral oncogene homolog (A-RAF)	NM_001654.1	This proto-oncogene belongs to the RAF subfamily of the Ser/Thr protein kinase family, and maybe involved in cell growth and development.
Breast Cancer Anti-Estrogen Resistance 3 (BCAR3)	NM_001261409.1	Component of intracellular signal transduction that causes estrogen-independent proliferation in human breast cancer cells.
Interleukin-1 receptor-associated kinase 4 (IRAK4)	NM_016123	Activates NF-kappaB in both the Toll-like receptor (TLR) and T-cell receptor (TCR) signalling pathways
Nemo-like kinase (NLK),	NM_016231.2	MAPK and Wnt signalling. Inhibits NFkB.
Casein kinase 1, epsilon (CSNK1E).	NM_001894	Implicated in the control of cytoplasmic and nuclear processes, including DNA replication and repair. Component of the circadian clock.
ERBB receptor feedback inhibitor 1 (ERRFI1/MIG6)	NM_018948.2	Negatively regulates EGFR signalling.
Homo sapiens NUAKE family, SNF1-like kinase, (NUAK1).	NM_014840	Serine/threonine-protein kinase involved in various processes such as cell adhesion, regulation of cell ploidy and senescence, cell proliferation and tumour progression.
NFKB inhibitor interacting Ras-like 2 (NKIRAS2)	NM_017595.2	A potent regulator of NFkB activity by preventing the degradation of NF-kappa-B inhibitor beta (NFKBIB).

Table 5.4. ProtoArray hits of potential PDE4D7 interactors.

Those in bold were chosen for PPI verification, based on association with cAMP signalling and cancer as found in the literature.

Verification analysis by secondary co-immunoprecipitation is reported below in figure 5.12.

5.2.4 Verification of Potential PDE4D7 Interactors

From the three proteomics approaches employed, the following 23 proteins were chosen for verification of PDE4D7 PPIs. The reasons behind the prioritisation of these candidates are discussed below:

1) Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors and is involved in responses to various stress signals (Thompson, Xu et al. 2009). It is implicated in various cancers and a number of studies suggest it regulates PC cell growth (Liu, Gao et al. 2013; Wang and Yang 2013). Interestingly, ATF3 is androgen-regulated (Pelzer, Bektic et al. 2006) and can bind the AR, inhibiting AR-mediated transcription (Wang, Jiang et al. 2012).

2) Breast Cancer Anti-Estrogen Resistance 3 (BCAR3) is a ubiquitously expressed protein involved in breast cancer cell proliferation and overexpression confers resistance to anti-oestrogens (van Agthoven, van Agthoven et al. 1998).

3) Casein kinase 1, epsilon (CSNK1 ϵ) regulates circadian rhythm and is suggested to be involved in cancer by phosphorylating p53, and may be a potential target for cancer therapy (Knippschild, Milne et al. 1997; Yang and Stockwell 2008). More recently CSNK1 ϵ has been implicated in beta-catenin active cancers (Kim, Dunn et al. 2010), and beta-catenin signalling is often dysregulated in PC (Lee, Madar et al. 2013).

4) Nemo-like kinase (NLK) is a MAPK-like kinase involved in wnt signalling, it has been shown to be downregulated in metastatic prostate tumours compared to primary tumours and normal tissue, and to inhibit AR-mediated transcription thereby inducing apoptosis (Emami, Brown et al. 2009).

5) Glyoxylate Reductase 1 Homolog (GLYR1) regulates stress induced MAPK/p38 signalling (Fu, Yang et al. 2006). It has been shown to be a highly mutated driver gene in colorectal cancer (CRC), with loss of function in a subset of tumours

(Alhopuro, Sammalkorpi et al. 2012), and the fusion protein *GLYR1-SLC9A8* which also results in *GLYR1* loss of function has recently been implicated in PC progression (Wang, Liu et al. 2014). CRC has been shown to predispose younger men to PC, and there may be common genetic factors (Moot, Polglase et al. 2003).

6) Octamer-binding protein NONO/p54nrb mediates cAMP-regulated transcription by tethering transducers of regulated CREB (TORCs) to RNA polymerase II at CRE loci and has been found to regulate AR transcriptional activity (Ishitani, Yoshida et al. 2003; Amelio, Miraglia et al. 2007; Dong, Sweet et al. 2007).

7) ENKURIN is an adaptor protein that can bind SH3 domains, with high affinity for the p85 regulatory subunit of PI3K (Sutton, Jungnickel et al. 2004) which mediates breast cancer tumour activity (Folgiro, Di Carlo et al. 2012), and AS PC cell growth upon PKA phosphorylation (Feola, Cimini et al. 2013).

8) Cancer/testis antigen family 45, member A3 is member of the cancer/testis (C/T) gene family that is normally expressed exclusively in the testes but is expressed elsewhere in a wide variety of malignant tumours (Costa, Le Blanc et al. 2007). CT45 is a recently identified isoform, expression of which is correlated to cancer progression and poor prognosis (Koop, Sellami et al. 2013).

9) Protein phosphatase 3 catalytic subunit beta isozyme (PPP3CB) is upregulated in response to cAMP signalling (Consales, Volpicelli et al. 2007). Long non-coding RNA (lncRNA) within the PPP3CB locus has been associated with cancer (Tahira, Kubrusly et al. 2011).

10) General Transcription Factor IIIA (GTF3A) is involved in cAMP signalling by interacting with cAMP-responsive element modulator (CREM) (De Cesare, Fimia et al. 2003).

11) Interleukin-1 receptor-associated kinase 4 (IRAK4) is a critical component of the Toll-like receptor (TLR) signalling pathway and SNPs in this gene may be associated with PC (Sun, Wiklund et al. 2006; Rogers, Jones et al. 2013).

12) ERBB receptor feedback inhibitor 1 (ERF1) is a negative regulator of EGFR signalling, which is associated with many cancers including PC (Siu, Virtanen et al. 2011).

13) NFκB inhibitor interacting Ras-like 2 (NKRAS2) is an NFκB inhibitor (Huxford and Ghosh 2006). NFκB has been shown to work with CREB to induce tumour cell migration and invasion during hypoxia, and PKA/ NFκB signalling mediates AI PC cell growth (Vinnall, Mahaffey et al. 2011; Nakayama 2013).

14) ATP-dependent RNA helicase A (DHX9) has been shown to be necessary for human embryonic stem cell (hESC) renewal and cancer cell proliferation (Narva, Rahkonen et al. 2012). It has also been implicated in multi-drug resistance in cancer (Zhong and Safa 2004), and interestingly maps to a major PC susceptibility locus (Lee, Eki et al. 1999).

15) Homo sapiens NIAK family, SNF1-like kinase, (NIAK1) is a member of the stress-activated AMP protein kinase (AMPK) family which has been found to mediate tumourgenesis, particularly invasion and angiogenesis (Kusakai, Suzuki et al. 2004; Chen, Li et al. 2013).

16) Mitogen-activated protein kinase kinase kinase 9 (MAP3K9) is a serine/threonine kinase within the ERK/MAPK signal transduction cascade and is associated with melanoma, lung cancer and CRC (Slattery, Lundgreen et al. 2012; Stark, Woods et al. 2012; Fawdar, Trotter et al. 2013).

17) Chemokine (C-X-C motif) ligand 5 (CXCL5) is part of the intraprostatic immune system and has been associated with PC progression by mediating proliferation, migration, metastasis, angiogenesis and epithelial to mesenchymal transition (EMT) (Begley, Kasina et al. 2008; Begley, Kasina et al. 2008; Eyman, Damodarasamy et al. 2009; Kuo, Chen et al. 2011; Karagiannis, Saraon et al. 2013).

18) Retinoid X receptor beta (RXRB) is a steroid hormone receptor involved in transcriptional regulation of target genes, loss of which is implicated in some

cancers (Cheung, Yan et al. 2003). Conversely, RXRB mRNA has been shown to correlate negatively with breast cancer associated patient survival (Liu, Graham et al. 2012).

19) SNAP-associated protein (SNAPIN) has been shown to be phosphorylated by PKA (Chheda, Ashery et al. 2001), and it has been shown to be associated with hepatocellular carcinoma (HCC) (Inagaki, Yasui et al. 2008) and PC (Quintero, Herrala et al. 2013).

20) Microspherule Protein 1 (MCRS1) has recently has been assigned oncogenic status (Okumura, Zhao et al. 2005) and is associated with colorectal cancer (CRC) (Shi, Chen et al. 2009).

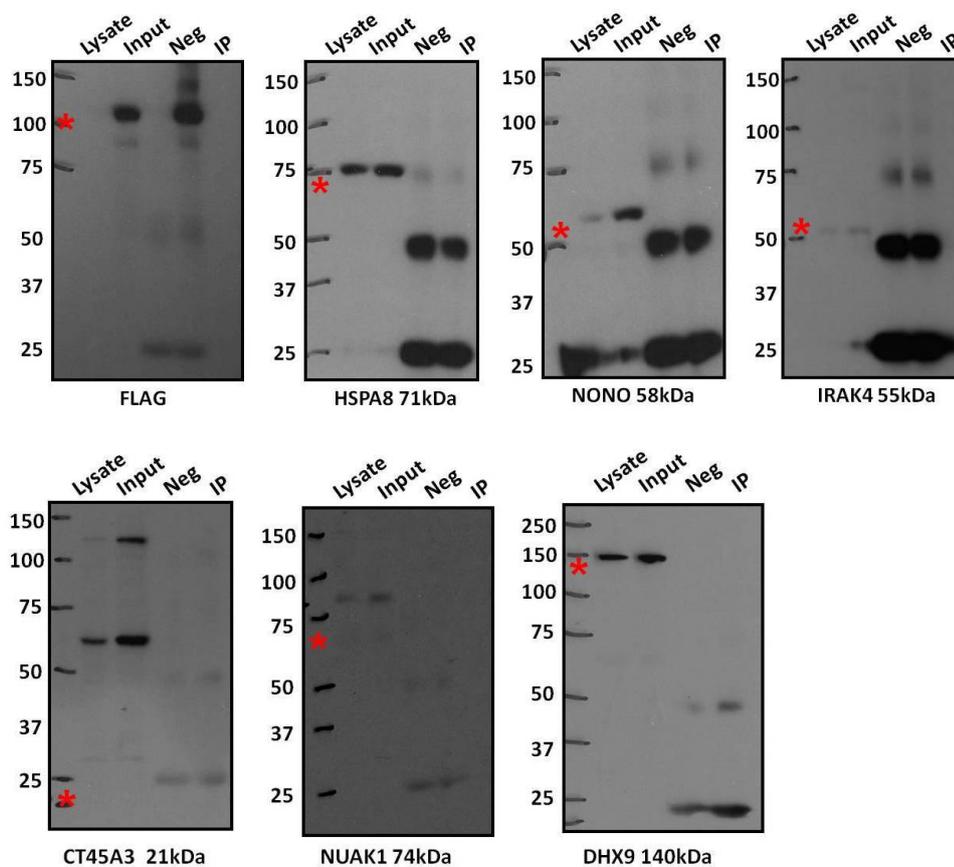
21) Heat shock cognate 71 kDa protein (HSPA8) is a member of the HSP70 family of heat shock chaperones which is expressed in various tumours and may mediate cell proliferation and survival (Rohde, Daugaard et al. 2005; Jose-Eneriz, Roman-Gomez et al. 2008).

22) Zinc finger protein 302 (ZNF302) is located on chromosome 19q13, SNPs in which are associated with PC (Schaid, Stanford et al. 2007; Hsu, Sun et al. 2009).

23) PDE4A; the phosphodiesterase 4A subfamily; it would be very interesting to find an interaction between two different PDE4 isoforms.

Antibodies against the above proteins were used to investigate PPIs by means of co-IPs, which is a commonly used method to verify PPIs that have been identified using the approaches described here (Berggard, Linse et al. 2007). Initially, VCaP and Hek293 cells were transfected with VSV-tagged or FLAG tagged PDE4D7, followed by a VSV/FLAG IP and immunoblotting for a number of the PPI hits. Detection was carried out using HRP-conjugated secondary antibody and chemiluminescence. PDE4D7 was successfully immunoprecipitated; however, none of the above PPIs could be verified (figure 5.11).

A



B

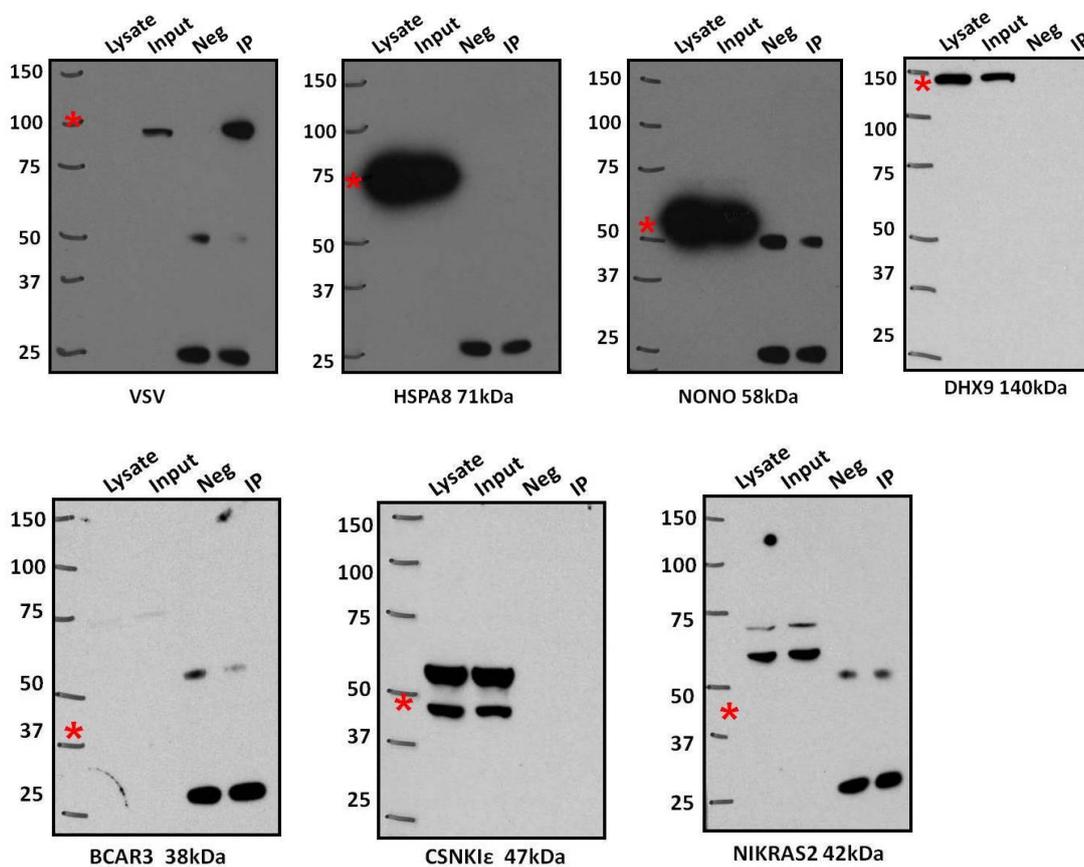


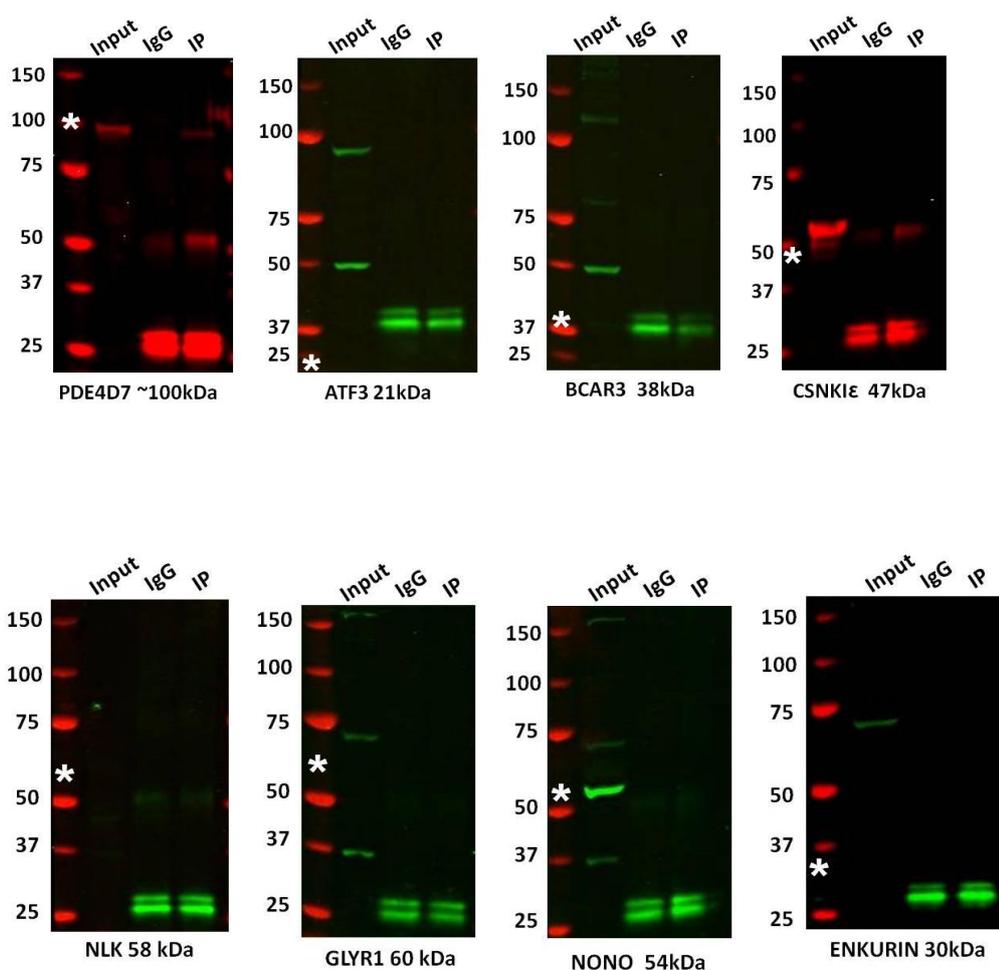
Figure 5.11. Initial verification of a number of PDE4D7 PPIs by co-immunoprecipitation of overexpressed PDE4D7-VSV and immunoblotting for PPI hits. **A;** HeK293 cells were transfected with FLAG tagged PDE4D7, or were untransfected. Cellular lysates were immunoprecipitated (IP) with agarose beads conjugated to FLAG antibody. Lysate=untransfected lysate from which a FLAG IP was carried out (Neg). Input=transfected lysate from which a FLAG IP (IP) was carried out. N=3. **B;** VCaP cells were transfected with VSV tagged PDE4D7, or were untransfected. Cellular lysates were immunoprecipitated (IP) with agarose beads conjugated to VSV antibody. Lysate=untransfected lysate from which a VSV IP was carried out (Neg). Input=transfected lysate from which a VSV IP (IP) was carried out. The approximate weight at which the proteins should run is specified under each western blot, and denoted by red asterisks. N=3.

PDE4D7 was successfully immunoprecipitated in both HeK293 (figure 5.11 A, top left blot) and VCaP (figure 5.11 B, top left blot) cells, thus, any interactors present would have been co-purified. The proteins HSPA8, NONO, DHX9, CSNK1 ϵ and IRAK4 are present in the cell lysates, suggesting these proteins indeed do not interact with PDE4D7. CT45A3 and NUAK1 do not seem to be present in HeK293 lysate, as bands observed are not at their respective predicted weights; thus these are non-specific bands. For this reason it cannot be concluded that they are not PDE4D7 interactors. BCAR3 and NIKRAS2 are not present in VCaP cell lysate, but VCaP cells endogenously express PDE4D7, and so this may suggest that indeed these proteins do not interact with PDE4D7, at least not in early stage PC.

Although VCaP cells express PDE4D7 endogenously and therefore any physiological PDE4D7 interactors, the overexpression of PDE4D7 to enable immunoprecipitation would have resulted in non-physiological levels of this enzyme, and which can inhibit interaction complex formation (Berggard, Linse et al. 2007). Following the generation of the novel highly specific PDE4D7 antibody, antibodies against the rest of the chosen PPI hits were purchased and all 23 were investigated using endogenously expressed proteins. It was also at this stage in the project that the DuCaP cell line became available. IPs of endogenous PDE4D7

were accordingly carried out using VCaP (figure 5.12 A) and DuCaP cells (figure 5.12 B), followed by probing for all 23 possible interacting partners. IPs of endogenous PDE4D7 has the advantage of physiologically-relevant interacting partners being pulled down. Here, detection was carried out using the Odyssey (LiCor) imaging system, which is more sensitive than chemiluminescence. PDE4D7 was successfully immunoprecipitated; however still none of the PPIs could be verified.

A



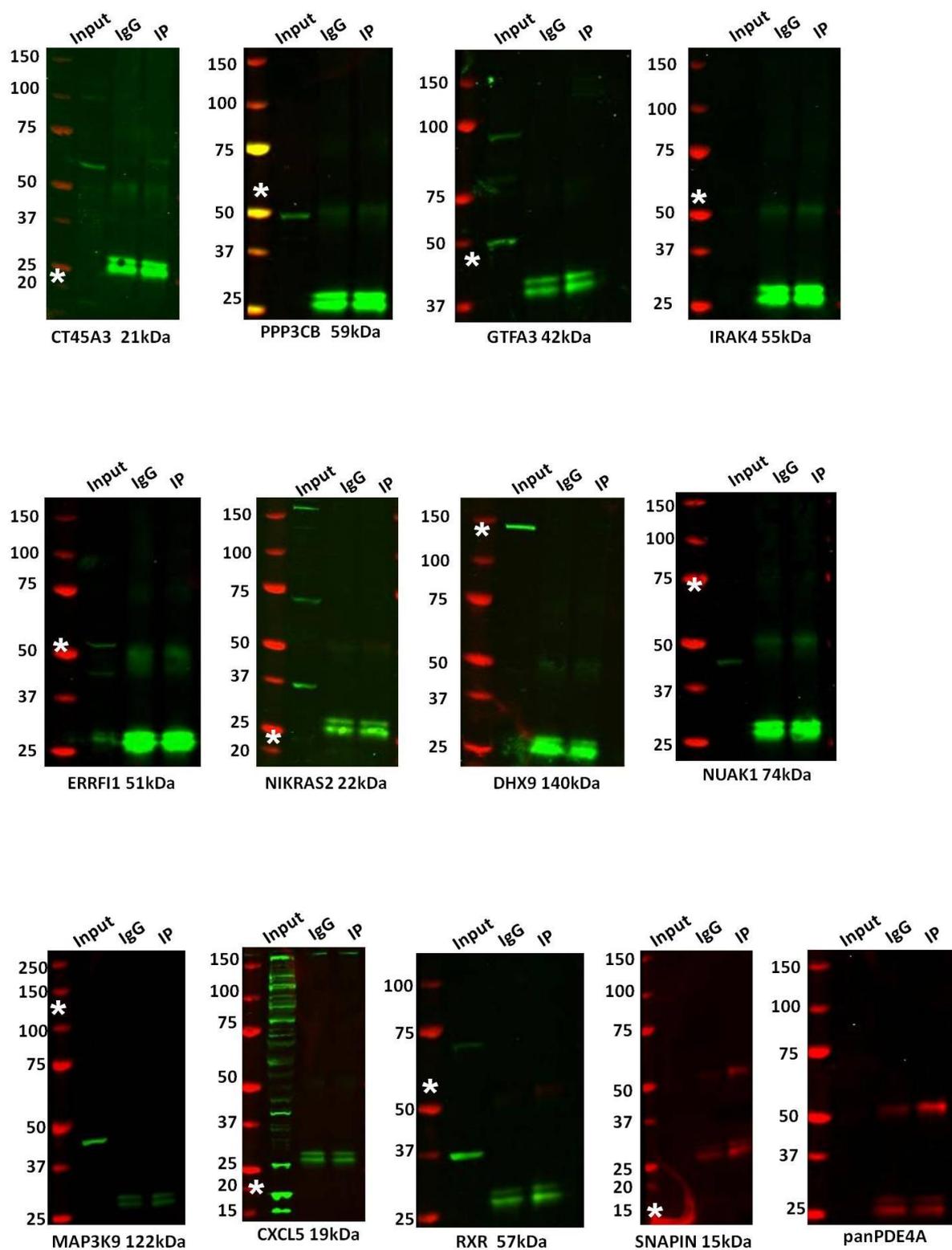


Figure 5.12 A

B

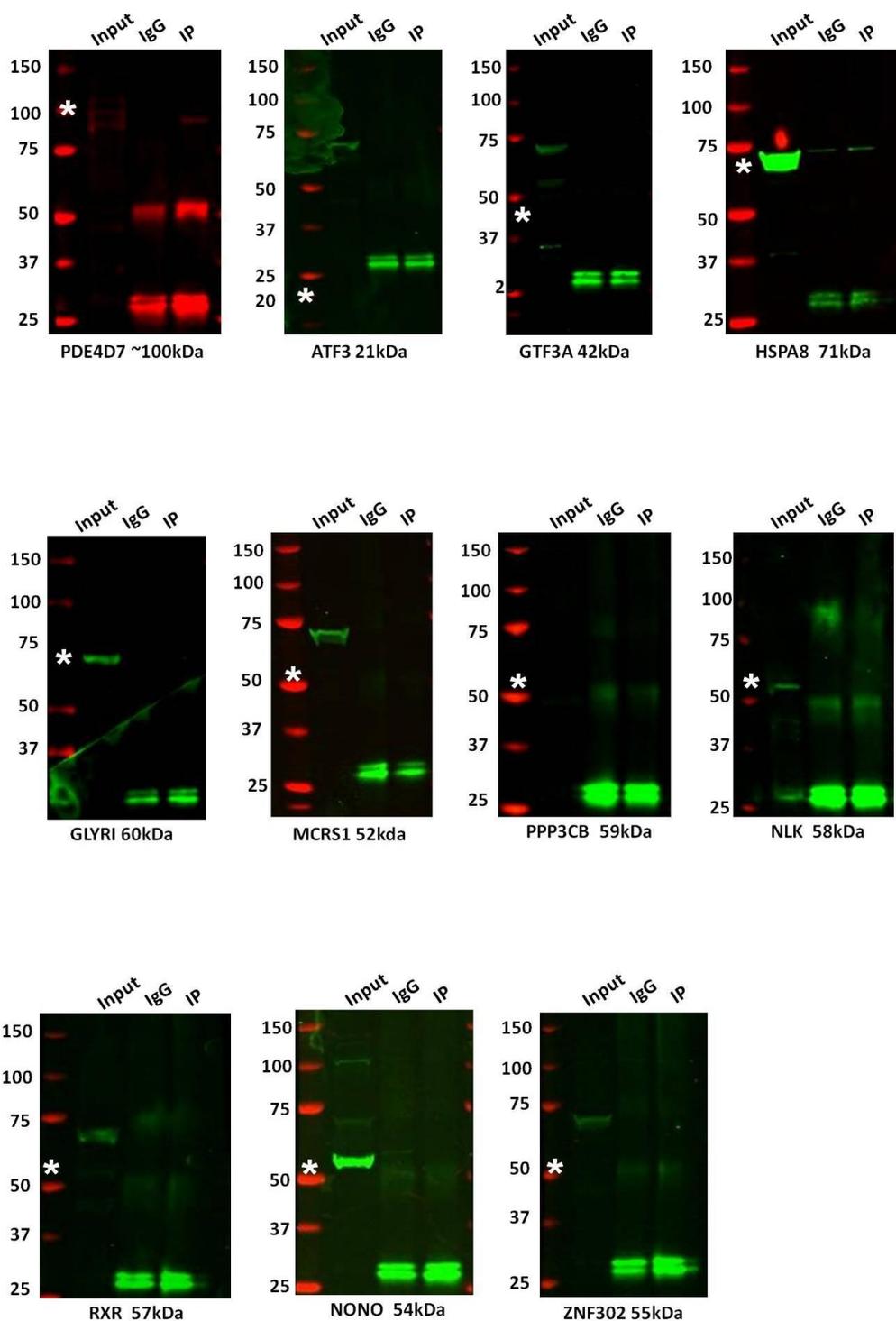


Figure 5.12. Verification of PDE4D7 PPIs by co-immunoprecipitation of endogenous PDE4D7 and immunoblotting for PPI hits.

A; endogenous PDE4D7 was immunoprecipitated from VCaP cells using the novel PDE4D7 antibody. Cellular lysates were blotted with antibodies targeted against

the potential interacting proteins. Input=lysate from which the control (IgG) and PDE4D7 IPs (IP) were carried out. N=3. **B**; endogenous PDE4D7 was immunoprecipitated from DuCaP cells using the novel PDE4D7 antibody. Cellular lysates were blotted with antibodies targeted against the potential interacting proteins. Input=lysate from which the control (IgG) and PDE4D7 IPs (IP) were carried out. The approximate weight at which the proteins should run is specified under each western blot, and denoted by white asterisks. N=3.

PDE4D7 was successfully immunoprecipitated in both VCaP (figure 5.10 A top left blot) and DuCaP (figure 5.10 B top left blot) cell lines, thus any interacting partners would have been pulled down. None of the possible 23 interactors were pulled down in the IP lane alone, suggesting these proteins do not interact with PDE4D7 in a cellular context. However, aside from the panPDE4A antibody which has been well characterised in our laboratory, the antibodies were not verified for affinity and specificity prior to the Co-IPs. A number of the proteins are not detected in the inputs suggesting they are not expressed in PC cells, or perhaps the antibodies failed to detect a low level of those proteins. ATF3, NLK, GLYR1, ENKURIN, CT45A3, NUAK1, MAP3K9, RXR and SNAPIN are not present in the VCaP input lanes, or certainly not at the predicted weights. It seems the antibodies against these proteins are detecting some non-specific bands, as is quite common. PPP3CB and GTF3A may be expressed in VCaP cells, but are running slightly different to their predicted weights. BCAR3 which weighs 38kDa seems to be expressed in VCaP cells but this weight is obscured by the light chains of the antibodies in the IgG and IP lanes, and so it is difficult to determine whether or not a band is co-purifying. ATF3, GTF3A, MCRS1 and ZNF302 appear not to be expressed in DuCaP cells.

Lack of an interaction with a PDE4A isoform was not unexpected as we previously determined very low PDE4A mRNA expression in these cell lines (Henderson D. thesis 2011). For the other proteins investigated, the use of positive controls such as purified protein or standards from overexpressing cell lysates would confirm their specificity, and if these proteins are indeed not part of the PDE4D7 interactome. However, to employ such controls would require

much cloning and a great deal of time, and would not be reasonable for a first verification experiment.

5.3 Discussion

Intracellular signalling systems depend on direct and functional PPIs to tightly regulate signal transduction. Spatial and temporal regulation of cAMP signalling is paramount to cell homeostasis, and is mediated by the sequestration of specific PDE isoforms within the vicinity of the cAMP pool in order to shape appropriate cAMP gradients. Targeting of the PDE to this pool is arbitrated by protein-protein interactions, such as the anchoring of PDE4D3 by MAKAP to the nuclear membrane in cardiomyocytes (Dodge, Khouangsathiene et al. 2001) or sequestration of PDE4D5 by β -arrestin (Bolger, McCahill et al. 2003) or RACK1 (Yarwood, Steele et al. 1999). These specific interactions act to maintain appropriate cAMP signalling cascades. PPIs are often dynamic and may be influenced by PTMS; an elegant example of both is the PDE4D3 'switching of partners' which is induced by phosphorylation within its N-terminus, thereby modulating the affinity of PDE4D3 for different interactors (Carlisle Michel, Dodge et al. 2004; Collins, Murdoch et al. 2008).

The function of a novel protein is often elucidated by determining its interactome, as function can be inferred from identifying annotated binding partners; the 'guilt by association' model. By identifying such binding partners, biologists can gain insight into the hierarchical organisation of the proteome of a given tissue (Drewes and Bouwmeester 2003). A number of methods have been developed to investigate PPIs, three of which were employed in the search for the PDE4D7 interactome. It has been demonstrated that different approaches used to identify PPIs often produce very little overlap, and so analysis of PPIs by more than one method is important (von Mering, Krause et al. 2002; Ngounou Wetie, Sokolowska et al. 2013). The three approaches reported here, have a unique set of advantages and drawbacks.

IP/ MS is a desirable method of detecting PPIs because it involves the *ex vivo* analysis of protein complexes made up of direct and functional (non-direct) interactions that have occurred in mammalian cells, and not *in vitro* (Drewes and Bouwmeester 2003). This allows the overexpressed bait protein to undergo PTMs, which may be necessary for increased affinity towards a binding partner (Berggard, Linse et al. 2007). IP/ MS has been successfully used to identify many PPIs, and technological advances have allowed the ultra-sensitive detection of peptides in the femtomolar range (Drewes and Bouwmeester 2003), enabling determination of whole macromolecular complexes (Ho, Gruhler et al. 2002) and the mapping of whole signalling networks (Bouwmeester, Bauch et al. 2004).

However this approach has a number of limitations. The IP favours proteins of high abundance and PPIs with high affinity, as transient or weak interactions may dissociate upon cell lysis (Berggard, Linse et al. 2007). Transient overexpression of the bait protein can often lead to a high bait:prey ratio, limiting the detection of less abundant interactors, and leading to the isolation of chaperones and HSPs that are likely interacting with misfolded overexpressed protein. Also, the epitope recognised by the antibody used for affinity purification may be within a protein-binding domain and thus inhibit any PPIs (Gingras, Aebersold et al. 2005). Although a major part of its appeal, the high sensitivity of MS can result in the identification of large numbers of contaminant proteins, thus producing false-positives (Berggard, Linse et al. 2007). MS is also quite inaccessible and very costly, being usually carried out only in specialised laboratories.

None of the MS hits were verified as PDE4D7 interactors. This could be the result of a variety of confounding factors. Overexpression of the tagged-PDE4D7 was carried out in Hek293 cells. On retrospective reflection, this may not have been a good choice as our group has found this cell line to express little PDE4D7. Thus, these cells may not express PDE4D7 interacting proteins, nor the scaffolds required for correct subcellular localisation. VCaP cells which express PDE4D7 and thus any interacting partners would have been a better expression system for this experiment; however, these cells are incredibly difficult to transfect and due to time constraints Hek293 cells were employed. Now that a PDE4D7-

specific antibody capable of detecting endogenous protein is available, this experiment could be repeated, with a greater chance of isolating valid PDE4D7 interactors. Immunoprecipitation of endogenous PDE4D7 would eliminate the problems associated with overexpressed bait, such as misfolding and thus isolation of chaperones, as is what likely occurred here, with the identification of HSPA8.

The Y2H technique is one of the most widely used for the detection of PPIs, and has been instrumental in the mapping of the human proteome and interactome, essential for identifying protein networks responsible for disease (Gandhi, Zhong et al. 2006; Berggard, Linse et al. 2007). It is relatively cheap, simple to set up and the PPIs under investigation occur *in vivo*. The Y2H system allows for the detection of weak interactions due to significant signal amplification by the genetic reporter system. It also enables discrimination between weakly and strongly interacting pairs due to differential activation of reporter genes, leading to weak or strong penetrance of the interacting phenotype, i.e. the colour change (Estojak, Brent et al. 1995).

However, this method also possesses a number of shortcomings. Interactions are not always detected in both orientations, some have been identified when one partner is bait and the other prey, but not when the reciprocal screen is performed (Estojak, Brent et al. 1995). Unlike IP/ MS, the Y2H method can only detect binary or direct physical interactions (De Las Rivas and Fontanillo 2010). Y2H screens also give many false positives, a big problem when these are listed in the PPI databases and repositories. Such discrepancies can be seen with the well studied *Saccharomyces cerevisiae* (*S. cerevisiae*), where mapping its interactome by four independent high throughput Y2H screens produced very little overlap (Deane, Salwinski et al. 2002; De Las Rivas and Fontanillo 2010). Such problems may arise from the use of different yeast strains, vectors, experimental design, and whether the proteins are C- or N-terminally fused to the transcription factor domains (Caufield, Sakhawalkar et al. 2012). Another problem that may occur during a Y2H screen is the incorrect folding and post-translational modification of mammalian proteins, upon which an interaction may rely. However, the development of mammalian two-hybrid systems, which

employ the same method of bait and prey fusion proteins bringing about transcription of a reporter gene, has helped to alleviate this problem (He and Li 2008).

The above limitations may be why a bona fide PDE4D7 PPI was not identified using the Y2H system. The unique N-terminal region of PDE4D7 should be responsible for specific PPIs, and incorrect folding within yeast cells may inhibit accessibility of the region to any interactors. If N-terminal phosphorylation is necessary to bring about an interaction, or vice versa, then this too would be thwarted. Although PDE4D7 is expressed in brain (Wang, Deng et al. 2003), the use of a foetal brain library may not allow for the detection of interactions occurring in prostate cells, or indeed PC cells. However, this prey library was all that was available at the time of this experiment.

DNA microarrays have been hugely successful in gene expression profiling, but, as discussed, protein levels are not always correlated with mRNA abundance, and so the development of protein microarrays was the next logical step in the endeavour to understand complex cellular molecular networks (Zhu and Qian 2012). Protein microarrays allow the immobilisation of all annotated proteins of a given organism onto a chip, thereby comprising a whole proteome. The largest proteome chip to date was recently developed, containing >17,000 human proteins (Jeong, Jiang et al. 2012). This en masse analyses of PPIs and the miniaturised size of the microarrays means very low quantities of purified bait proteins and antibodies are required. Protein microarrays have successfully identified PPIs as well as a host of interactions between proteins and other biomolecules (Schweitzer, Predki et al. 2003; Predki 2004; Zhu and Qian 2012). Unlike the Y2H system, the proteins on the ProtoArray chips are expressed in insect cells which allows correct folding and PTMs to occur (Bouvier, Menard et al. 1998). They have been used to successfully identify proteins involved in ovarian cancer progression and to elucidate many new components of the NF κ B signalling network (Hudson, Pozdnyakova et al. 2007; Fenner, Scannell et al. 2010).

Although much progress has been made with protein microarrays, the expression, purification and immobilisation of such large numbers of

biochemically diverse proteins in a functional state remains costly and labour intensive (Braun 2012). Therefore, ProtoArray chips are expensive and not accessible to many laboratories. Also, this approach identifies only binary interactions and it is likely to produce a large number of false positives due to the detection of proteins that can physically interact, but under physiological conditions may never be in close enough proximity, or expressed at the same cell cycle stage (Berrade, Garcia et al. 2011). The detection of weak or transient interactions may also be inhibited due to the rigorous washing steps involved in the procedure.

The conditions of the ProtoArray are not physiological, which may be the reason why no real PDE4D7 PPIs were identified using this approach. Another issue may lie with the GST tag, which is fused to the N-terminus of PDE4D7. Although commonly used in the study of PPIs, it has been demonstrated that GST tags can induce misfolding of the tagged protein domain (Wissmueller, Font et al. 2011). Perhaps this large tag disrupted the folding of the N-terminal domain, which of course is crucial for PDE4D7 specific interactions. Such misfolding may inhibit bona fide interactions, and promote false positives. Saying that, the PDE4D7 specific antibody which is targeted against the N-terminus, successfully detected the fusion protein in both native and denatured states. Additionally, PDE4D7 PTMs, such as N-terminal phosphorylation, may be essential for PPIs *in vivo*.

Unfortunately, no light has been shed on the PDE4D7 interactome. The three approaches used to identify interacting partners have been well validated and successful in determining PPIs, as seen in the wealth of literature, but they all present a number of limitations, and are far removed from a physiological setting. However, the negative findings for the putative PDE4D7 PPIs reported here cannot be accepted with confidence just yet, and a number of further steps should be taken. In order to verify that the Co-IP results are not false negatives, positive controls for the antibodies that did not detect protein in the cell inputs should be used to ensure that indeed they are not expressed in the AS PC cells used. Also, reciprocal Co-IPs should be carried out; where each of the potential interactors is immunoprecipitated followed by immunoblotting for PDE4D7, as interactions can sometime occur in one direction only (Elion 2007). Co-IP is the

commonest method to verify PPIs identified by the three approaches reported here, and has the advantage of retaining all proteins in their native states and concentrations when examining endogenously expressed proteins (Berggard, Linse et al. 2007; Dwane and Kiely 2011). However, it also presents some limitations, notably it may not allow detection of weak or transient interactions due to the number of washing steps involved and the time taken to carry out the experiment, respectively (Lee, Ryu et al. 2013). Thus, a number of other approaches could be employed, particularly if the antibodies fail to detect positive controls. Peptide array technology could be used to create full length arrays of each of the 23 potential interactors, which could then be overlaid with purified PDE4D7 protein. If PDE4D7 were to interact with any of the proteins, this method would also identify the epitope to which it binds. The reciprocal experiment could then also be carried out. Another technique used to verify molecular interactions identified by a previous approach is surface plasmon resonance (SPR) (Berggard, Linse et al. 2007). SPR involves the immobilisation of the bait protein on a chip via an affinity tag, such as a GST tag. Prey proteins are then added to the chip in solution. SPR biosensors measure the change in refractive index of the solvent near the surface that occurs following complex association and disassociation, thereby not only confirming the PPI, but giving quantitative information on the kinetic and thermodynamic parameters of the interaction in a label-free manner (Lalonde, Ehrhardt et al. 2008). Of course, this would require a large number of purified proteins. Should a bona fide PDE4D7 PPI be validated, a number of subsequent experiments could be carried out; for example the Duolink PLA probe system discussed in Chapter 3 would further verify any interaction via co-localisation. The novel phospho-specific antibody described in Chapter 4 could be employed to investigate the effect of N-terminal phosphorylation on the PDE4D7 PPI. In any case, using the novel PDE4D7 antibody now available to us, IP/MS with PC cell lines may be considered, and would undoubtedly yield more likely PDE4D7 interactors.

6 Final Discussion

6.1 PDE4D7 is a Promising Novel Biomarker for Prostate Cancer

In collaboration with Philips Research and the PCMM, we set out to identify a novel PC biomarker. This is currently a major endeavour in PC research, as the current biomarker, PSA, is not disease-specific and thus often leads to over diagnosis and over treatment of non-life threatening indolent cancers, or BPH (Draisma, Etzioni et al. 2009). Due to the wealth of literature implicating cAMP signalling in this disease (Bang, Pirnia et al. 1994; Sadar 1999; Nelson, Bagnato et al. 2003; Kasbohm, Guo et al. 2005; Weng, Wang et al. 2005; Kvissel, Ramberg et al. 2007; Merkle and Hoffmann 2011; Misra and Pizzo 2013; Sarwar, Sandberg et al. 2013), we chose to investigate the expression of cAMP PDEs as PC progresses, in the hope that changes in cAMP signalling would translate into potential novel biomarkers. Indeed, we identified differential expression of PDE4D isoforms at the mRNA level between AS and AI PC cell lines and xenografts (Byrne, Henderson et al., under review), with PDE4D7 showing the most acute change such that its expression successfully discriminates between tumour and non-tumour tissue, and correlates with prostate cancer-specific survival (PCSS) (unpublished data, PCMM). By means of urine detection, our collaborators have also found, in an initial study using a small sample size, that PDE4D7 downregulation correlates with diagnosed metastasis of PC, thus PDE4D7 may be indicative of not only PC progression, but also of metastatic potential. Although PDEs are intracellular and not excreted, urinary sediments contain dislodged prostatic cells from which PDE4D7 mRNA abundance was measured. This is an exciting discovery, as a novel biomarker we can non-invasively detect in urine, with both diagnostic and prognostic value, and disease-specificity could be a huge asset in the fight against the disease. The correlation of PDE4D7 with disease stage suggests that this is indeed a very promising biomarker, which would allow clinicians and patients to make more informed decisions about whether or not to undergo cancer treatment. Furthermore, PDE4D7 is not under androgenic regulation (Byrne, Henderson et al., under review); therefore

measurement of its expression level would not merely reflect that of other AS PC biomarkers.

Despite the growing body of literature on the role of cAMP in PC, this was the first investigation of the expression of an individual cAMP PDE isoform in PC samples. Uckert and colleagues described the expression of PDE isoforms in the different prostatic zones, but not within the different cell types (Uckert, Oelke et al. 2006). Other studies have looked at the PDE4 family in relation to PC, with a downregulation of the PDE4B subfamily in AI PC being reported (Kashiwagi, Shiota et al. 2012; Sarwar, Sandberg et al. 2013), but with no investigation into the expression of splice variants. The PDE4 family is of particular relevance to disease, and is the main cAMP hydrolysing family (Houslay 2010). PDE4 splice variants have highly-specific, non-redundant roles due to their unique N-termini, hence Isozyme-specific knowledge is crucial to understanding their roles in diseases such as PC.

6.2 A Novel PDE4D7 Specific Antibody may be of Clinical Value

Studying the expression of PDE splice variant proteins is not easy as raising highly specific antibodies is made difficult due to the disparity in immunogenicity of their unique N-termini. During my PhD experimental work, I managed to successfully generate a highly specific PDE4D7 antibody by fusing GST to the unique N-terminal region to create a highly-immunogenic antigen. This antibody, along with PLA probe detection, verified that PDE4D7 protein is downregulated in AI PC cell lines, thus reducing PDE4 activity at a discrete intracellular location. Our group have determined that the major population of PDE4D7 localises to the sub-plasma membrane region (Byrne, Henderson et al. 2014), therefore its downregulation/loss as PC transitions from an AS to AI disease would result in perturbed cAMP signalling at this locale. The availability of this antibody is also exciting with regards to PDE4D7 detection in urine samples. Protein analysis by means of western blot or ELISA is far simpler and

less technically challenging than mRNA analysis, which can be subject to much loss and contamination during the handling process. It has recently been reported that measuring urinary protein levels may be superior to measuring mRNA levels in the detection of disease, though the measurement of both is best (Hricik, Nickerson et al. 2013). Thus, if this novel PDE4D7 antibody can detect protein in urine sediments from PC patients, which our collaborators will investigate, it may be a very useful tool in the clinic. Indeed, this year Mohan and colleagues reported the detection of prostate specific membrane antigen (PSMA) in urine using a bioaffinity matrix (Mohan, Donovan et al. 2013), which they reported in the media as a potential home 'pregnancy' test for prostate cancer. In short, a 'pee-on-a-stick' device which would allow men to purchase a home testing kit from the pharmacy. However, PSMA is also present in urothelial and bladder carcinomas (Ristau, O'Keefe et al. 2013), which may lead to non-specific results when using a urine test. What's more, PSMA expression has been found to be dependent on the TMPRSS2:ERG gene fusion event (Yin, Rao et al. 2011), which is present in 70% of primary prostate adenocarcinomas (Mehra, Tomlins et al. 2007), meaning the PSMA test may not work for some subsets of PC cases. On the other hand, our group found the differential expression of PDE4D7 to be consistent between AS and AI cells lines and xenografts, with and without the TMPRSS2:ERG fusion, and with no correlation to tumour origin. The device fabricated by Mohan and colleagues employed bacteriophages; however, an even simpler test would be akin to a pregnancy test, which contains immobilised anti-hCG antibodies, and if present in the urine, bound hCG brings about a colour change. With the availability of the high affinity and highly specific PDE4D7 antibody, it is plausible to suggest that such a device could be fabricated to detect the presence of PDE4D7 protein in urine samples. Such a test could help determine if the primary tumour has become androgen-insensitive/castration-resistant following ADT. On the other hand, if PDE4D7 is indeed upregulated from normal tissue to early stage AS tissue, as observed by our collaborators, then such a device would allow for the detection of early stage PC, for which there is currently no biomarker (Cernei, Heger et al. 2013). Such early stage detection would of course require normal PDE4D7 levels to be known, and a threshold for PC detection set. It is also becoming clear that the use of a single biomarker for PC detection is not sufficient (Laxman, Morris et al.

2008), and so perhaps such a device that can measure more than one biomarker in this way, such as PDE4D7 along with PSMA, and perhaps together with PSA testing, would be of greatest clinical value.

6.3 PDE4D7 Mediates Androgen-Sensitive Prostate Cancer Cell Proliferation

The observation that PDE4D7 showed the highest relative expression of all PDE4 isoforms in AS PC cell lines and xenografts, strongly suggests that this isoform plays a pivotal role in cAMP signalling in early stage PC. But what is the physiological relevance of the dramatic downregulation of this enzyme into androgen insensitivity? Given that Uckert et al. had previously suggested a role for PDE4 in the proliferation of normal prostate epithelium (Uckert, Oelke et al. 2006), it seemed prudent to investigate the role of PDE4D7 in this respect. Indeed, evidence presented here suggests that PDE4D7-specific-mediated cAMP hydrolysis at the sub-plasma membrane region acts to maintain normal proliferative signalling in AS PC cells. Loss of PDE4D7, via displacement of active enzyme or gene knockdown, resulted in a marked increase in AS PC cell proliferation. This increase was not observed following knockdown of global PDE4D, which convincingly suggests a specific role for PDE4D7 in cAMP mediated AS cell growth. The marked decrease in proliferation and migration rate following the re-expression of PDE4D7 in AI cell lines backs up this hypothesis. This is further substantiated by the observed decrease in AI cell proliferation following over-expression of the hyper-active PDE4D7 mutant. The importance of increasing cAMP in the progression of PC is well reported, particularly in the shift away from androgenic signalling. Cross talk between the cAMP and AR signalling networks plays an important role in such steroid independent PC progression (Merkle and Hoffmann 2011), including aberrant PKA phosphorylation of the AR (Nazareth and Weigel 1996; Gioeli, Ficarro et al. 2002) and over expression of GPCRs (Nelson, Bagnato et al. 2003; Weng, Wang et al. 2005). An increase in the number of ligand-bound GPCRs would lead to aberrant synthesis of cAMP, which, when coupled with loss of PDE4 isoforms such as PDE4D7, would

potentiate PC progression into androgen-insensitivity. Increasing levels of cAMP also results in NED, another pivotal event in the progression of PC (Burchardt, Burchardt et al. 1999; Merkle and Hoffmann 2011). If PDE4D7 is the main “gatekeeper” of cAMP signalling in early stage AS PC, then loss of this isoform may be a major contributing factor in the shift towards non-genomic androgen signalling and in the development of NED, and thus loss of PDE4D7 expression may play a role in the development of the AI phenotype, and PC progression.

The results presented here conflict with the study by Rahrman et al. who suggest that PDE4D is pro-tumourigenic, and its inhibition would be of therapeutic value for the treatment of PC (Rahrman, Collier et al. 2009). They found that knockdown of global PDE4D reduced the proliferation rate and migratory potential of PC cells. Interestingly, the cells used were the AI DU145 and PC3 lines, which our group found to express very little PDE4D. They studied the proliferation of these cells using end point assays, whereas, here, I performed real time proliferation and migration assays, which provide constant quality control for the cells under investigation. Given the non-redundant roles of PDE isoforms, it is naive to suggest inhibiting all PDE4D splice variants in the treatment of PC, or any disease, and doing so may well be detrimental to PC patients. However, Rahrman and colleagues do pinpoint the majority of PDE4D expression in PC to that of PDE4D5. Our group also found PDE4D5 to have the highest relative expression in the AI samples, and this part of their findings may be in agreement with the results presented here. PDE4D5 transcription is cAMP-responsive (Le Jeune, Shepherd et al. 2002), so as PDE4D7 is downregulated during the AS to AI transition, the resulting increase in cAMP may bring about aberrant PDE4D5 expression, which in turn could potentiate PC progression due to inappropriate cAMP hydrolysis at the PDE4D5 locale. This hypothesis, along with my observation that global PDE4D knockdown has little effect on AS cell growth, suggests that the effect of cAMP on PC progression is varied, and depends on the subcellular localisation of the cAMP pool and the PDEs involved.

6.4 Perturbed Transcriptional Regulation of PDE4D7

The transcriptional regulation of PDE4D7 is not currently understood, however it does not appear to be under the control of androgens (Byrne, Henderson et al., under review). The reason for PDE4D7 downregulation as PC becomes AI is unknown, but as presented here, it is plausible to suggest DNA hypermethylation is involved, as a perturbed epigenome has been shown to play a role in the development of PC (Jeronimo, Bastian et al. 2011). The predicted CpG island upstream of PDE4D7 (Henderson D. thesis 2011), and the specific upregulation of PDE4D7 following inhibition of DNA methylation backs up hypothesis. This is further supported by the cis-NAT genetic structure of PDE4D7, with the non-coding transcript PART1. NcRNAs can both positively and negatively regulate the transcription of their coding partner by recruiting chromatin remodelling machinery (Faghihi and Wahlestedt 2009; Su, Xiong et al. 2010; Magistri, Faghihi et al. 2012). PART1 is androgen-regulated (Lin, White et al. 2000), and in response to DHT can be upregulated in AS cells, but not in AI PC cells (Yu, Blackburn et al. 2003). Perhaps PART1 positively regulates PDE4D7 transcription in normal prostate cells and AS PC. As PC progresses and signalling moves towards non-steroidal pathways, PART1 expression would be inhibited, resulting in the loss of positive PDE4D7 transcriptional regulation, and thus loss of expression. The loss of PDE4D7-mediated cAMP hydrolysis might then potentiate the inhibition of PART1 transcription, and thus PDE4D7 expression; a 'vicious circle' of feedback. However, this area requires further work before any firm conclusions can be drawn.

6.5 A Novel Mode of PDE4D7 Regulation

Most proteins are subject to PTMS that alter their activity, subcellular localisation or molecular interactions, and such modifications are a main area of cancer research (Krueger and Srivastava 2006). Protein phosphorylation is perhaps the commonest and certainly the best studied PTM. Reversible protein phosphorylation is paramount to a host of cell signalling events, with >2% of the genome encoding kinases and phosphatases (Hunter and Pawson 2012). To date, PDE4D3 is the only PDE4 isoform known to undergo unique N-terminal phosphorylation (Sette and Conti 1996). Here, I presented evidence to suggest that PDE4D7 is also subject to PKA-dependent phosphorylation within its unique N-terminus (on serine 42), that this event occurs under basal conditions, and that it provides a novel, isoform-specific mode of regulation.

The hyper-activity imparted on PDE4D7 following ablation of this phospho-site, coupled with inhibition of UCR1 phosphorylation, suggest that Ser42 phosphorylation retains the enzyme in an inactive state to allow basal cAMP signalling. Stimulation of the PDE4D7-associated Gs-coupled GPCR would elicit the rise in cAMP and active PKA necessary to bring about UCR1 phosphorylation and relieve this constraint. I hypothesise that the conformation of PDE4D7, whilst in the inactive state, comprises a tightly folding UCR1/UCR2/catalytic site module, enveloped and stabilised by a disordered phosphorylated N-terminal region. Disorder of the N-terminus would allow for dynamic interaction with this module. Indeed, modular proteins such as PDE4 isoforms often possess disordered regions (Dyson and Wright 2005). The notion of a disordered PDE4D7 N-terminus is further supported by the fact that alternatively spliced exons often encode disordered protein domains to prevent structural impairment to the rest of the protein (Romero, Zaidi et al. 2006). It is then likely that all PDE4 long isoform unique N-terminal regions are disordered, which would enable a common C-terminal domain to be fused to a number of different N-termini. Furthermore, disordered regions are often subject to phosphorylation, the charge conferred by which influences associations with other proteins via electrostatic interactions (Romero, Zaidi et al. 2006; Borg, Mittag et al. 2007).

Phosphorylation on Ser42 may increase the affinity of the N-terminal region for the UCR1/UCR2/catalytic site module, which would be ablated by the change in net charge following UCR1 phosphorylation, leading to a looser, active enzyme conformation which is accessible to cAMP. Indeed, it has been shown that UCR1 and UCR2 interact via electrostatic interactions, and dissociate upon UCR1 phosphorylation or mutation of the target serine to aspartate (Beard, Olsen et al. 2000), suggesting that it is the net charge responsible for dissociation of the module and conformational change to the active state. Also, as presented here, mutation of PDE4D7-Ser42 to aspartate had negligible effect on enzyme activity, giving credence to the idea that the negative charge bestowed on this site by phosphorylation acts to retain PDE4D7 in an inactive conformation via electrostatic interactions.

The increasing and sustained phosphorylation of endogenous PDE4D7-Ser42 in the AS cell lines, following activation of PKA, strongly suggests that PDE4D7-Ser42 is phosphorylated *in vivo*. Perhaps, at any one time a proportion of the PDE4D7 protein population is phosphorylated on Ser42 to allow for basal cAMP signalling, which may be necessary for cellular homeostasis. Indeed, stable and prolonged basal phosphorylation has previously been described for a number of PKA substrates (Steinberg and Kiss 1985), suggesting such basal phosphorylation events are likely important for cellular homeostasis. But what is the implication of this event in PC? Hyperphosphorylation of tumour suppressor genes is an important event in carcinogenesis (Yatsunami, Komori et al. 1993; Krueger and Srivastava 2006; Jiang, Sun et al. 2010). The evidence presented in Chapter 3 ardently suggests that loss of PDE4D7 expression and activity is pro-tumourigenic. Perhaps, aberrant Ser42 phosphorylation plays a role in the downregulation of PDE4D7. The normal population of PDE4D7 proteins would likely comprise both Ser42 phosphorylated and unphosphorylated pools, as the stoichiometry of any phospho-substrate population ranges from completely unphosphorylated to completely phosphorylated molecular states (Prabakaran, Lippens et al. 2012). As PC progresses, this stoichiometry may be perturbed, with a shift in the equilibrium towards the phospho-PDE4D7. A larger proportion of the PDE4D7 population phosphorylated on Ser42 would result in the increase of cAMP levels at the sub-plasma membrane region. This inappropriate level of

cAMP could play a role in the shift away from androgenic signalling, which as hypothesised, may lead to transcriptional inhibition of PART1 and PDE4D7, thus self-perpetuating inhibition. If indeed PDE4D7 is initially upregulated in early stage PC, cAMP at the PDE4D7 locale would be subject to inappropriate hydrolysis, and hyperphosphorylation may provide molecular protection against the increase in expression of active enzyme.

In light of the results presented here, it is interesting to think of PDE4D7 as a tumour suppressor as its expression correlates with slow growing AS PC cells, which show no/little migration. This is in contrast to a loss of PDE4D7 expression, which is correlated with fast growing, advanced AI PC cells with developed migratory potential. Intriguingly, a tumour suppressor gene has been proposed to reside on the long arm of chromosome 5, with linkage to PC between 5q11 and 5q12 (Ozen, Navone et al. 1998; Xu, Dimitrov et al. 2005), and PDE4D maps to 5q12.1 (Szpirer, Szpirer et al. 1995).

6.6 A Novel PDE4D7 Phospho-Specific Antibody may be of Clinical Value

Since a changing phospho-proteome is implicated in many cancers, the study of phosphorylation-based signalling pathways is crucial to understanding cancer biology and to the diagnosis and treatment of this disease (Krueger and Srivastava 2006). Labelling with radioactive phosphate is a widely used method to identify phospho-substrates, but it gives no spatio-temporal information on these proteins (Teraishi and Miura 2009). The advent of phospho-site specific antibodies has allowed for such investigations. These antibodies are invaluable tools in uncovering the phospho-proteome in health and disease and are often used in the detection of disease biomarkers (Teraishi and Miura 2009; Lothrop, Torres et al. 2013). A major advance in the study of the phospho-proteome is phosphoflow cytometry (Rogne and Tasken 2013). This is a combination of flow cytometry with phospho-specific antibodies against various signal phospho-proteins to analyse intracellular signalling networks *ex vivo*, and it is also being

used to investigate the phospho-signalling events in a number of diseases, such as cancer. However, this assay is limited by the availability of phospho-antibodies, and the raising of antibodies that recognise biologically and pathologically important PTMs is now a growing area of research (Lothrop, Torres et al. 2013). If indeed PDE4D7 N-terminal hyperphosphorylation occurs as PC progresses, the novel phospho-site specific antibody reported here may be of clinical value. PDE4D7 hyperphosphorylation would be a result of aberrant PKA signalling, due to an increase in cAMP as discussed early, and/or due to changes in the expression of PKA regulatory subunits, which occurs during PC progression (Cho, Lee et al. 2000). If the latter is the case, and the antibody could detect PDE4D7-pSer42 in patient samples (for example by means of phosphoflow cytometry), it would indicate inappropriate PKA activity which may then be targeted. Indeed, targeted inhibition of RI α has been shown to inhibit prostate tumour growth *in vivo* (Cho, Kim et al. 2002). If PDE4D7 N-terminal hyperphosphorylation does indeed occur, and if it is an early event in PC, then it would be another potential biomarker for the detection of early stage PC, for which none currently exist (Cernei, Heger et al. 2013). However, a great deal more work must be done in this area, not least investigating the specificity of the pSer42 antibody in immunocytochemistry/immunohistochemistry. If the antibody proves successful in these applications, it could be used to investigate the PDE4D7 N-terminal phospho-status as PC progresses, using patient tissue samples of various stages of disease.

6.7 Conclusions

The findings presented within this thesis:

- 1) Support both the previous findings of the qPCR PDE screen to show that PDE4D7 is significantly downregulated as PC progresses into androgen-insensitivity, and, the notion that PDE4D7 is a promising novel biomarker for PC and disease stage.
- 2) Suggest that the novel PDE4D7 antibody shows promise as a valuable diagnostic tool for the detection of early stage PC, or to determine disease stage.
- 3) Suggest that loss of PDE4D7-mediated cAMP hydrolysis is pro-proliferative and pro-migratory, and plays a role in the development of the AI PC cell phenotype.
- 4) Suggest that PDE4D7 N-terminal PKA mediated phosphorylation provides a novel molecular mode of regulation on enzyme activity.
- 5) Suggest that the novel phospho-specific antibody may also be a valuable diagnostic tool in the detection of PC, should we find that the N-terminal phospho-status of PDE4D7 changes as PC progresses. Perhaps employing both of the novel antibodies may provide a dual diagnosis tool by detecting concurrent decreasing levels of PDE4D7 with increasing levels of phospho-PDE4D7.
- 6) Call for further research on both PDE4D7 epigenetic regulation and N-terminal phosphorylation, as both may provide novel points for the therapeutic manipulation of PC.

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