The Role of Defective in Cullin Neddylation 1 Domain Containing 1 (DCUN1D1) in prostate cancer

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Declaration

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Abstract

Defective in Cullin Neddylation 1 Domain Containing 1 (DCUN1D1) is an E3 ligase for the post translational process, neddylation. Neddylation is similar and runs in parallel to the ubiquitin proteasome pathway. Although established as an oncogene in various squamous cell carcinomas, the role of DCUN1D1 in prostate cancer has not been explored and it could be a novel drug target for prostate cancer treatment. We investigated the role of DCUN1D1 in prostate cancer. The expression of DCUN1D1was evaluated in prostate cancer cell lines and human tissue samples and its effect on tumourigenesis was tested using proliferation, migration, apoptosis and *in vivo* tumour growth assays. Microarray analysis and the connectivity map database were used to determine the signalling pathways responsible for its mechanism of action and to identify compounds specific for DCUN1D1 to inhibit prostate cancer growth. DCUN1D1 expression was upregulated in multiple prostate cancer cell lines, particularly androgen independent prostate cancer cells. Its expression was also upregulated by 42% in separate cohorts of human tissue samples. Blockage of DCUN1D1 expression led to a significant reduction in proliferation and migration of prostate cancer cells and significantly, it inhibited xenograft formation in MF1 nude mice. Microarray analysis identified deregulation of key gene expression, cellular growth and proliferation, developmental, cell death and cancer pathways as the possible mechanisms by which DCUN1D1 mediates its activated. We tested 30 drugs identified using the connectivity map database as having gene expression signatures positively correlated with DCUN1D1 knockdown. Monensin and podophyllotoxin were demonstrated to inhibit prostate cancer proliferation and induce apoptosis in prostate cancer cells through DCUN1D1-dependent activity. Therefore, DCUN1D1 plays a critical role in prostate cancer growth through the deregulation of crucial cellular pathways. In addition, the connectivity map database was successfully employed to identify small molecule inhibitors of DCUN1D1 that reduce prostate cancer growth. This could lead to an innovative approach for prostate cancer treatment.

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Contents

Declaration ii

Abstract iii

Acknowledgement iv

List of Tables vii

List of Figures vii

List of Abbreviations ix

Chapter 1: Introduction 1

1.1 Background and Context of Research 1

1.1.1 Prostate gland 1

1.1.2 Prostate cancer 2

1.1.3 Prostate cancer staging 3

1.1.4 Detection and diagnosis of prostate cancer 4

1.1.5 Treatment of prostate cancer 7

1.1.6 Prostate cancer signalling pathways 8

1.1.7 The role of ubiquitination under physiological and cancer conditions 12

1.1.8 Neddylation: a description of an emerging pathway in cancer treatment 15

1.1.9 DCUN1D1 and cancer 19

1.1.10 The use of bioinformatics tools in drug discovery: the connectivity map database 21

1.2 Rationale of the study 23

1.3 Aims and Objectives 23

Chapter 2: Materials and Methods 24

2.1 Determination of DCUN1D1 expression level in prostate cancer cell lines and human tissue 24

2.1.1 Cell lines and maintenance of the cells 24

2.1.2 Evaluation of DCUN1D1 expression in human tissue samples 25

2.1.3 Quantitative real time PCR (qRT-PCR) 26

2.1.4 Western blot analysis 27

2.2 Determination of the relevance of DCUN1D1 for: proliferation, migration, apoptosis, and tumour formation *in vivo* 28

2.2.1 Generation of DCUN1D1 knockdown cell lines 28

2.2.2 MTT Proliferation Assay 29

2.2.3 Quantification of cell migration using transwell chambers 29

2.2.4 Apoptosis Assay 29

2.2.5 Subcutaneous implantation of MF1 nude mice 30

2.3 Determination of DCUN1D1 signalling pathways in microarray analysis. 30

2.4 Connectivity map database analysis 31
2.4.1 Identification of drugs for *in vitro* analysis 31
2.4.2 Drugs 32
2.4.3 Calculating IC50s using GraphPad Prism version 5.01 32
2.4.4 Isobologram 32
2.4.5 Statistical analysis: Paired t-test 33
Chapter 3: Results 34
3.1 Differential expression of DCUN1D1 in prostate cancer. 34
3.1.1 DCUN1D1 is upregulated in human prostate cancer cell lines. 34
3.1.2 DCUN1D1 is upregulated in human prostate cancer tissue. 34
3.2 Blockage of DCUN1D1 inhibits proliferation and migration of prostate cancer cells. 36
3.3 Inhibition of DCUN1D1 leads to the induction of apoptosis in prostate cancer cells. 38
3.4 DCUN1D1 is essential for *in vivo* prostate cancer tumour growth. 39
3.5 Microarray analysis of DCUN1D1 knockdown in prostate cancer cells. 40
3.6 Screening of drugs for DCUN1D1 specific anti-prostate cancer activity. 42
3.6.1 Analysis of high scoring drug activity on prostate cancer cell proliferation. 48
3.6.2 Analysis of the induction of apoptosis in response to drug treatment. 49
3.6.3 Analysis of high scoring drug activity on DCUN1D1 mRNA and protein expression. 50
3.6.4 Combinatorial therapy studies 52
Chapter 4: Discussion 56
Chapter 5: Conclusion 62
Chapter 6: Areas of further research 63
Chapter 7: References 64
Chapter 8: Appendix 91
8.1 Table A1: Top 50 genes upregulated following DCUN1D1 knockdown in DU145 cell lines in descending order. 91
8.2 Table A2: Top 50 genes downregulated following DCUN1D1 knockdown in DU145 cell lines in descending order. 92
8.3 Table A3: Comparison of pathway enrichment between the current study and recent studies 93
8.4 Table A4: Comparison of functions altered in the current study and following wide spread gene expression analysis 94

List of Tables

Table 1: Real time PCR analysis of DCUN1D1 gene expression in human prostate cancer tissue..... 35
Table 2: List of top up and downregulated genes after inhibition of DCUN1D1..... 42
Table 3: 30 drugs identified through the connectivity map database using p-values and scores..... 44
Table 4: List of drugs identified through the connectivity map database using p-values and scores..... 46
Table 5: Summary of drug activity in DU145 cells..... 52

List of Figures

Figure 1: Schematic representation of the human prostate gland and zonal distribution..... 1
Figure 2: Prostate cancer staging..... 4
Figure 3: Schematic diagram of the ubiquitination, neddylation pathway and the downstream 26S proteasome..... 12
Figure 4: Schematic representation of the 26S proteasome, the proteolytic 20S component of the 26S proteosome and the proteasome inhibitor bortezomib (PS-341)..... 15
Figure 5: Aligned amino acid sequences of human, mouse and rat NEDD8 and human ubiquitin..... 16
Figure 6: Structural images of the NAE inhibitor MLN4924 and adenosine 5' –monophosphate (AMP)..... 19
Figure 7: Regulation status of DCUN1D1 in prostate cancer cell lines..... 35
Figure 8: DCUN1D1 upregulation occurs in human prostate cancer tissue..... 36
Figure 9: Blockage of DCUN1D1 in DU145 and PC3 prostate cancer cells lines..... 37
Figure 10: Blockage of DCUN1D1 expression inhibits proliferation and migration in prostate cancer cell lines..... 38
Figure 11: Blockage of DCUN1D1 expression induces apoptosis in prostate cancer cell lines..... 39
Figure 12: Inhibition of DCUN1D1 reduces tumour formation in MF1 nude mice..... 40
Figure 13: Bioinformatics analysis of the top 10 functions deregulated following DCUN1D1 knockdown in DU145 prostate cancer cells..... 41
Figure 14: Bioinformatics analysis of the top 10 pathways deregulated following DCUN1D1 knockdown in DU145 prostate cancer cells..... 41
Figure 15: Schematic diagram showing our approach to drug screening..... 45
Figure 16: Proliferation assay in DU145 prostate cancer cell lines using drugs at cmap concentrations..... 48
Figure 17: Proliferation assay in DU145 prostate cancer and DU145 DCUN1D1 knockdown..... 49
Figure 18: Induction of apoptosis after treatment with the high scoring drugs..... 50
Figure 19: DCUN1D1 expression in DU145 cells 24 hours post treatment with high scoring drugs..... 51
Figure 20: Dose dependent response of DU145 prostate cancer cells to monensin treatment..... 53
Figure 21: Dose dependent response of DU145 prostate cancer cells to podophyllotoxin treatment..... 54

Figure 22: Combinatorial effect of monensin and podophyllotoxin on DU145 prostate cancer cell line proliferation..... 54
Figure 23: Isobologram analysis of the combinatorial effect of monensin and podophyllotoxin..... 55

List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADT	Androgen deprivation therapy
AMP	Adenosine monophosphate
AppBp1	Amyloid precursor protein binding protein 1
APS	Ammonium persulfate
AR	Androgen receptor
ATCC	American type culture collection
ATG12	Autophagy-related protein 12
ATG8	Autophagy-related protein 8
ATP	Adenosine triphosphate
BCA	Breast cancer associated
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer 1, early onset
CAND1	Cullin-associated and neddylation-dissociated
Cdk1	Cyclin-dependent kinase 1
Cdk2	Cyclin-dependent kinase 2
Cdk4	Cyclin-dependent kinase 4
Cdt-1	Chromatin licensing and DBA replication factor 1
CRL	Cullin RING Ligase
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBD	DNA binding domain
DCN-1	Defective in cullin neddylation 1 *(C. elegans)*
Dcn1p	Defective in cullin neddylation 1 *(S. cerevisiae)*
DCUN1D1	Defective in cullin neddylation 1 domain containing 1
DEN1/NEDP1/SENP8	Deneddylase 1/NEDD8-specific protease 1/Sentrin-specific protease 8
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleotide nucleic acid
dNTPs	Deoxynucleotide triphosphates
DRE	Digital rectal examination
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EPCA	Early prostate cancer antigen 2
FAT10	HLA-F adjacent transcript 10
FBS	Fetal bovine serum
FGF8	Fibroblast growth factor 8
FGFR2	Fibroblast growth factor receptor 2
FSH	Follicle-stimulating hormone
FUB1	Fub1p/YCR076C
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GO	Gene Ontology
HECT	Homologous to E6-AP carboxy terminus

HIF1α	Hypoxia-inducible factor 1-alpha
HIFU	High Intensity Focused Ultrasound
HRP	Horseradish peroxidise
Hsp90	Heat shock protein 90
IKK	IκB kinase
IL-6	Interleukin 6
IPA	Ingenuity Pathways Analysis
ISG15	Interferon induced
IκB	NF-kappa-B inhibitor alpha
JNK	c-Jun N-terminal kinase
KLF 4	Kruppel-like 4
KLK15	Kruppel-like 15
LBD	Ligand binding domain
LH	Luteinising hormone
LHRH	Luteinising hormone-releasing-hormone
MAPK	Mitogen-activated protein kinase
Mdm2	Mouse double minute 2 homolog
MEI-1	Meiosis inhibitor 1
MIT	Massachusetts Institute of Technlogy
MMP2	Matrix metalloproteases 2
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
MTT	Thiazoyl blue tetrazolium bromide
NEDD8	Neural precursor cell expressed developmentally downregulated 8
NF-κB	Nuclear factor kappa B
NKX3.1	NK3 homeobox 1
NLS	Nuclear localization signal
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NTD	N terminal domain
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCA3	Prostate Cancer Antigen 3
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PINK1	PTEN induced putative kinase 1
PIP2	Phophorylate phosphatidylinositol (4,5) bisphosphate
PIP3	Phosphatidylinositol (3,4,5) trisphosphate
PMEPA1	Prostate transmembrane protein androgen induced 1
PreEBM	Prostate Epithelial Cell Basal Medium
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
pVHL	Von Hippel-Lindau tumour suppressor
RANK	Receptor activator of NF-κB
RBX1/2	Ring box protein 1/2
RING	Really Interesting New Gene

RMA	Robust Multi-array Average
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640
RTK	Receptor Tyrosine Kinase
SCC	Squamous cell carcinomas
SCCRO	Squamous cell carcinoma related oncogene
SCF	Skp1-Cul1-F-box
shRNA	Short hairpin RNA
SIRT1	Sirtuin 1
SMC2	Structural maintenance of chromosome protein 2
SUMO-1	Small ubiquitin-related modifier 1
TAp73	Transactivating isoform of tumour protein 73
TEMED-N,N,N',N'	Tetramethylethylenediamine
TGFβ	Transforming growth factor beta 1
TMPRSS2	Transmembrane protease, serine 2
TNF-α	Tumour necrosis factor alpha
TRUS	Trans-rectal ultrasound-guided needle biopsy
TURP	Transurethral resection of the prostate
UAE	Ubiquitin activating enzyme
Ub	Ubiquitin
UBA3	Ubiquitin-like modifier activating enzyme 3
UBC	Ubiquitin conjugating enzyme
UBC12/UBE2M	Ubiquitin conjugating enzyme E2 M
UBE2F	Ubiquitin-conjugating enzyme E2 F
Ufm1	Ubiquitin-fold modifier 1
Urm1	Ubiquitin-related modifier-1
UPP	Ubiquitin proteasome pathway
VEGF-A	Vascular endothelial growth factor A

Chapter 1: Introduction

1.1 Background and Context of Research

1.1.1 Prostate gland

The prostate gland is a small gland (3cm long, 4cm wide and 2cm deep) that is situated at the base of the bladder, in front of the rectum and surrounds the urethra (Kumar & Majumder, 1995). It is divided into the central, transitional, fibromuscular and peripheral zones (Figure 1) (McNeal, 1981); the latter of which is the area in which majority of adenocarcinomas of prostate cancer (PCa) arise (McNeal, 1988).

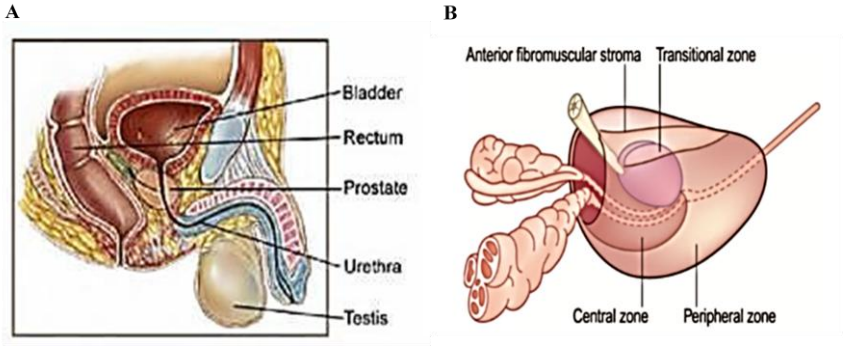

Figure 1. Schematic representation of the human prostate gland and zonal distribution. A) Diagram showing the prostate gland situated below the bladder, in front of the rectum. B) Schematic model of prostate gland zones namely the central, transitional, anterior fibromuscular and the peripheral zone. http://mesotheliomacancer1.info/prostate-cancer-diagram. (Harvey et al, 2012).

The function of the prostate is to secret an alkaline milky fluid that liquefies semen and helps to neutralize the acidic conditions of the vaginal tract (Kumar & Majumder, 1995). However, it is the ability of the prostate to grow in response to various androgens that helps to explain its propensity for tumour development. Maturation of the prostate gland is initiated at puberty where increased secretion of testosterone, results in the differentiation of prostate gland cells, among other activities (Gupta et al., 1977). The process begins through binding of the luteinising hormone-releasing-hormone (LHRH) to the anterior pituitary gland (Schally et al., 1970). It is the luteinising hormone (LH) secreted by the anterior pituitary gland which then targets the Leydig cells of the testes to produce testosterone (Neaves, 1975). Testosterone and its metabolite (dihydrotestosterone) are then recognized by androgen receptors (ARs) expressed in prostate epithelial cells to promote prostate cell differentiation and to mediate the transcription of genes necessary for prostate gland function. As such, the AR and its related pathways have been studied extensively, especially their roles in PCa development and progression. We describe the AR and its function in detail later in the study and begin by describing the current knowledge on PCa.

1.1.2 Prostate cancer

Prostate cancer (PCa) is the second leading cause of cancer related deaths in the world (Globocan 2008) and according to the South African National Cancer registry 2004 it is the most common cancer in South African males. In the United States of America, where cancer surveillance is rigorously undertaken, an estimated 233000 new cases of PCa will be diagnosed in 2014 and 29480 deaths will occur from it in the same period (Siegel et al., 2014). The incidence is highest in men over the age of 60 years but can occur in men younger than this age (Wingo et al., 1998; Siegel, 2013; Siegel et al., 2014). Risk factors associated with PCa development include age, family history of PCa, race, hormones, oxidative stress, dietary intake, environmental agents and occupation (Meikle et al., 1985; Steinberg et al., 1990; Giovannucci et al., 1993; Lesko et al., 1996; Giovannucci et al., 1998; Wingo et al., 1998; Sharma-Wagner et al., 2000; Merrill & Morris, 2002; Chen et al., 2003; Wu et al., 2009). Many of these risk factors have been identified due to a direct correlation with PCa development or because of their protective ability, in that a low level of these factors increases PCa risk. However, some of the data around these risk factors, such as occupation, are inconsistent. In addition, risk factors such as race where African American men show a high incidence and mortality rate (Jemal et al., 2009; Siegel, 2013), could be affected by other aspects such as access to healthcare, attitude to seeking medical advice and following up on appointments which include digital rectal examinations (Merrill & Morris, 2002). If PCa is suspected, the symptoms associated with it are difficulty urinating, pain with ejaculation, blood or semen in the urine and back pain.

In the next sections, we will discuss the classification/staging of PCa, the various methods used to screen for PCa with a brief summary of the current biomarkers used in PCa diagnosis. We will also discuss the treatments options that are available, with an in depth analysis of chemotherapy and androgen deprivation therapy because we identify our protein of interest, DCUN1D1, as a novel target which could be used as an alternative to these types of treatment. We also review the signalling pathways that have been associated with PCa development and progression focusing mainly on the ubiquitination and neddylation pathways and the two drugs that have emerged as chemotherapeutics agent through mechanisms of action dependent on these pathways. We then summarize current literature on DCUN1D1 and its link to ubiquitination and neddylation. Lastly, we explore gene expression profiles as bioinformatics tools to drug discovery in order to understand the context of this study and the connectivity map database we used to identify inhibitors of DCUN1D1.

1.1.3 Prostate cancer staging

Prostate cancer staging involves evaluation of the extent of spread of the disease and prediction of patient prognosis. It consists of clinical and pathological staging which require analysis of clinical findings such PSA levels, digital rectal examinations, imaging as well as pathological analysis of tissue samples using the Gleason score (Gleason, 1974; Edge et al., 2010). The Gleason score is used to grade the tumour based on the level of differentiation of the cells, with well differentiated cells having a low grade and poorly differentiated tumours having a high grade and being aggressive (Gleason, 1992). It provides a score for each tumour sample based on the sum of the grades of the most prominent (primary) and second most prominent (secondary) tumour grades and ranges between 2 and 10 (Gleason, 1992). These grades are then used in PCa diagnosis and staging. In addition to grading, PCa can also broadly be classified as localized, locally advanced and metastatic but pathological staging provides distinct classifications by applying the TNM system.

The currently used TNM system was developed by the American Joint Committee on Cancer and the International Union Against Cancer and describes the extent of the primary tumour (T), the involvement of the lymph nodes (N) and the presence of distant metastatic disease (M) (Edge et al., 2010). The extent of the tumour is divided into the T1, T2, T3 and T4 stages and each of these classifications has a further subclassification denoted as a, b or c (Figure 2). These classifications describe the spread of the tumour within the prostate gland, extension into the prostatic capsule, seminal vesicles and surrounding organs such as the rectum or the bladder. The N classification then describes the involvement of the lymph nodes in terms of presence/absence of nodal invasion. The M classification describes surrounding and distant organ involvement including the lymph nodes, bone or other sites. These descriptions also assist in assessing the risk of each patient for cancer progression. In this regard, descriptions of tumour localization, T1-T4 pathological staging, Gleason score analysis and pathological analysis of tumour cores occur. This allows for grouping of patients into very low risk, low risk, intermediate risk, high risk and very high risk (Edge et al., 2010).

However, prior to the establishment of the stage of the disease, initial prostate cancer diagnosis is required which involves testing for known biomarkers of PCa, digital rectal examinations and prostate tissue biopsy. The prostate specific antigen (PSA) is the most widely used biomarker for PCa development however inefficiencies in its use are leading to increased research into alternative molecules to track PCa growth. The following section discusses PSA and the main points in the detection and diagnosis of PCa.

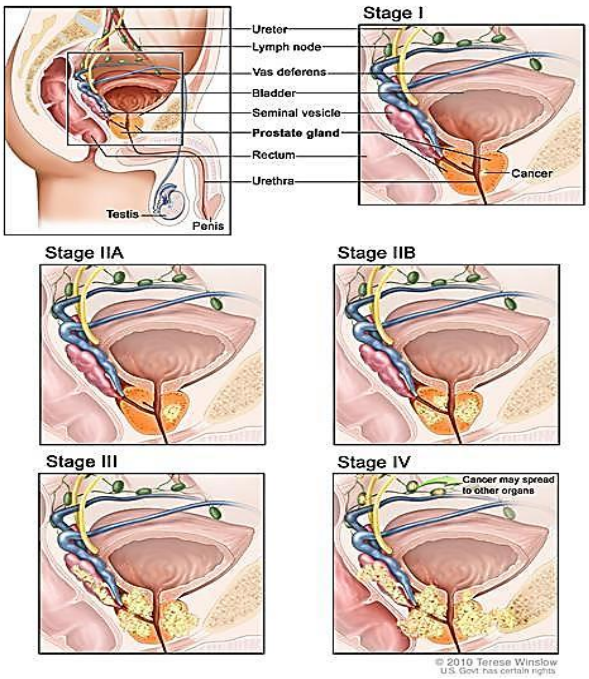

Figure 2. Prostate cancer staging. Schematic diagram showing the extent of primary tumour growth over different stages according to the TNM system. Stage 1: non-palpable tumour that is likely not visible through imaging. Stage IIA: organ-confined tumour that is palpable on digital rectal examination and is visible through imaging. Stage IIB: indicates spread of PCa over both lobes. Stage III: tumour that has grown into prostatic capsule and describes the involvement of the seminal vesicles. Stage IV: indicating the invasion of surrounding organs such as the external sphincter, rectum, bladder, levator muscles and/or pelvic wall. http://www.cancer.gov/PublishedContent/MediaLinks/680623.html

1.1.4 Detection and diagnosis of prostate cancer

Establishing the presence of PCa in an individual can be achieved through various assessments but it is complicated mainly by the architecture and positioning of the prostate gland. As described previously, the prostate gland is a small gland that is situated among large organs in the male lower abdomen. This makes it difficult to access and difficult to treat. Currently, PCa detection is performed by testing for serum prostate-specific antigen (PSA) levels, digital rectal examinations and where indicated prostate biopsies (Edge et al., 2010). Each of these tests is useful in trying to determine the presence or absence of PCa, however a combination of these tests is recommended for accurate diagnosis.

The prostate specific antigen (PSA) and biomarkers in prostate cancer

Minimally invasive tests are done on urine and blood samples to detect molecules that are differentially expressed in the body due to the development of PCa. Theoretically, these tests allow one to determine whether an individual has PCa and the stage of the development. Prostatic acid

phosphatase (PAP) was the first molecule to be used as a biomarker for PCa progression and marker to response to androgen deprivation therapy in metastatic PCa and since then several molecules have been evaluated as PCa biomarkers. Genetic and protein biomarkers have all been investigated however PSA, PCA3 and TMPRSS2-ETS have been the most promising. PSA has been the biomarker most extensively used to detect, screen and diagnose PCa. As such, PSA testing has evolved to the point of analysis of PSA isoforms, PSA density, PSA velocity and age-specific testing of PSA levels but this has also been due to the inefficiencies which have been identified in using PSA as a PCa biomarker. We briefly review PSA, PCA3 and TMPRSS2-ETS and their use in PCa detection below.

PSA is a glycoprotein that is secreted by the epithelial cells of the prostate gland (Papsidero et al., 1980; Watt et al., 1986). Upon ejaculation semen coagulates and prostatic secretions within the semen, PSA in particular, help dissolve the semen to allow for sperm motility (Huggins & Neal, 1942; Lee et al., 1989). PSA is a member of the kallikrein-related serine proteases and cleaves the semenogelin and fibronectin proteins which are responsible for the coagulation of semen (Syner & Moghissi, 1972; Suominen et al., 1974; Watt et al., 1986; Lilja et al., 1987). Other than its basic function in prostatic fluid, serum PSA testing has been used for PCa detection. Elevated PSA levels were thought to be associated with PCa development (Stamey et al., 1987) because areas of the prostate containing the tumour have disruptions in their basement membranes which facilitates increased secretion of PSA to the bloodstream (Webber et al., 1995). However, PSA can be elevated by different conditions which affect prostate gland homeostasis. Benign prostate hyperplasia (BPH), inflammation of the prostate, urinary tract infections, prostatic and non-prostatic trauma (catherisation) also increase serum PSA and as such, other modifications have been suggested to improve the predictive value of this test (Stamey et al., 1987; Dalton, 1989; Neal et al., 1992; Catalona et al., 1998; Ulleryd et al., 1999). The normal PSA cutoff is 4.0ng/ml and evaluation of PSA molecular isoforms, PSA density (total PSA/ prostate volume), PSA velocity (rate of change of PSA over time) and age-specific reference ranges are also used to detect PCa (Babaian et al., 1990; Benson et al., 1992; Carter et al., 1992; Dalkin et al., 1993; Oesterling et al., 1993; Smith, 1994; Björk et al., 1996; Nixon et al., 1997; Catalona et al., 1998; Kalish, 1999; Gann et al., 2002). Although these tests assist in improving the use of PSA, the increased frequency in PSA testing is leading to overdiagnosis and overtreatment of patients. Therefore, even with value of the serum PSA test and the existence of modifications to the test, it is necessary to identify more specific and accurate biomarkers of PCa. It is also why many investigators and institutions are calling for serum PSA to stop being used as a PCa biomarker.

PCA3 and TMPRSS2-ETS are two of the many biomarkers being investigated for use as alternatives to PSA and they are very promising. PCA3 is noncoding RNA that is produced only in the prostate gland and is highly expressed in >95% PCa biopsies relative to healthy individuals and individuals

with BPH (Bussemakers et al., 1999; Hessels et al., 2003). PCA3 as a urine-based diagnostics molecule was also more sensitive and specific for detection of PCa than PSA (Hessels et al., 2003; van Gils et al., 2007) which could be attributed to the fact that testing for PCA3 involves initial massaging of the prostate. TMPRSS2-ETS on the other hand is a gene fusion between the TMPRSS2 transmembrane protease and one of ETS transcription factors (Tomlins et al., 2005). Chromosomal rearrangements in PCa result in the TMPRSS2-ETS fusion but most commonly TMPRSS2-ERG with ERG now being an androgen receptor responsive gene. This rearrangement has been reported to be essential throughout PCa developed from prostate neoplasia to metastatic and hormone refractory PCa (Iljin et al. 2006; Wang et al. 2006; Nam et al. 2007). It is observed in 40-80% of PCa cases, it has been suggested as a predictor of prognosis and has recently been investigated as a urine-based biomarker for PCa diagnosis (Laxman et al., 2006; Soller et al., 2006; Yoshimoto et al., 2006; Nam et al., 2007).

Therefore, although there have been failures with using PSA for PCa detection, TMPRSS2-ETS fusions and PCA3 testing (which involves a digital massage of the prostate) are highly specific biomarker tests that are being investigated. However, testing for serum or urine biomarkers alone is not efficient for PCa detection at this point. Digital rectal examination in combination with biomarker testing and biopsies is recommended for accurate PCa diagnosis.

Digital rectal examination (DRE) and Biopsies

Digital rectal examination involves feeling the prostate gland for nodules, irregularities in the outline or anatomy of the prostate as well as gland fixation (Bickley, 2012). Prostate tissue biopsying is the sampling of prostate gland tissue for evaluation for malignancies that is performed after either or both serum PSA tests and DRE are abnormal. It involves the use of trans-rectal ultrasound-guided needle biopsy (TRUS) or the transurethral resection of the prostate (TURP) for tissue sampling in different areas of the prostate (Sfakianos et al., 2011; Harvey et al., 2012). A sextant biopsy involving the resection of 6 cores or an extended biopsy scheme using 12 or more cores is used, with additional cores obtained when necessary. Pathological analysis of the tissue cores then occurs where a Gleason score is awarded to each of the cores and each tissue sample is examined for the presence and percentage of its volume that is occupied by cancerous tissue. This then allows for determinations to be made on the presence/absence of PCa. Normal PSA levels have been set at 4.0ng/mL and measurements above or at this point would likely be followed by DRE and biopsies. However, some investigators have argued for these examinations to be performed at PSA levels ranging between 2.5ng/mL - 4.0ng/mL since they detected PCa in 22-27% of men with PSA levels between these ranges (Catalona et al., 2000; Okihara et al., 2001). However, lower PSA cutoff levels have also been observed to reduce the usefulness of DRE and have raised concerns of increased number of unnecessary biopsies performed and detection of clinically insignificant tumours (Chen et al., 1999).

Although, the usefulness of both techniques is undeniable, there are some disadvantages associated with each of them including lack of specificity and complications following biopsies.

Improvements can be made to both techniques such as using experienced examiners to perform both techniques and optimizing the number and location of the cores to be taken during the performance of biopsies. However, it is the application of all of these techniques including the modifications to the serum PSA test that provides more accuracy in PCa diagnosis and improves the predictive value of these techniques also in eliminating the possibility of PCa. Ultimately, identifying a new, more specific and sensitive biomarker of PCa could also assist in this regard. However, following accurate diagnosis of PCa, effective treatment is required and although progress has been made, treatment of PCa is still challenging. In the next section we describe the current treatment methods available for PCa and as mentioned previously we focus on chemotherapy and androgen deprivation therapy.

1.1.5 Treatment of prostate cancer

Despite the high mortality rate reported, it is possible to treat PCa but it is complicated by several factors. The architecture and location of the prostate gland makes it difficult to access, tumours within a gland can be heterogeneous (as evidenced by a tumour having different grades) and PCa development involves multiple stages which include hormone independent growth. In addition, PCa tumour growth can be a slow process with individuals likely to die from diseases other than PCa (Wingo et al., 1998). As described previously, although the incidence of PCa is high, mortality in terms of PCa related deaths is lower than the incidence in some regions but PCa still remains the second leading cause of cancer-related deaths globally (Siegel, 2013). Therefore, physicians are often faced with the dilemma of balancing patient education and disease treatment. According to the American Cancer Society, commencement of treatment usually takes into account the extent of the disease (tumour grades and stage), the patient's age, life-expectancy, co-morbidities, the feelings of the patient and doctor as well as whether treatment would be curative. Treatment of PCa can then involve: active surveillance (applied in low-intermediate risk patients), radical prostatectomy, interstitial prostatic brachytherapy, external beam therapy, cryotherapy, High Intensity Focused Ultrasound (HIFU) therapy, androgen deprivation therapy (ADT)/hormonal therapy and chemotherapy (Chodak et al., 1994; D'Amico et al., 1998; Blana et al., 2004; Walsh, 2011; Taneja, 2013; Zamboglou et al., 2013). For the purposes of this project, we will be focusing on ADT and chemotherapy.

Chemotherapeutic agents currently used in PCa treatment are mainly anti-neoplastic agents that kill cancer through microtubule inhibition, inhibition of DNA synthesis and function, and target the rapidly dividing nature of cancer cells. Docetaxel, estramustine, mitoxantrone are some of the chemotherapeutic agents used in the treatment of PCa with the aforementioned mechanisms of

activity (Ho et al., 1991; Dahllöf et al., 1993; Díaz & Andreu, 1993). Docetaxel is also the standard frontline treatment for metastatic PCa, with patients showing improvements in survival rates, however adverse side effects are observed with its use and several clinical trials have occurred to try to improve its efficacy (Petrylak et al., 1999; Tannock et al., 2004; de Bono et al., 2010). Nonetheless, although anti-neoplastic agents have shown marked usefulness in the treatment of PCa, hormonal or ADT has been the primary target for chemical PCa treatment. The basic principle of this approach has been the inhibition of androgen induced prostate cell growth, with studies targeting the relationship between the androgen and the AR responsible for its signal transduction. This has been a largely successful method of PCa treatment but patients undergoing ADT have suffered deregulation in male sexual activity and often relapse with androgen-independent tumours. This leads to patients having hormone refractory or castration resistant PCa and studies have shown that following the initiation of ADT for advanced PCa, clonal selection, AR gene amplification, mutations in AR genes and increased sensitivity of AR to androgens due to increased stability and nuclear localization, have led to the emergence of androgen-independent tumours (Koivisto et al., 1997; Holzbeierlein et al., 2004; Mizokami et al., 2004). Even though no link has been found between AR signalling and our protein of interest as yet, AR signalling plays a critical role in PCa and is essential in understanding the context of PCa development and progression and current approaches to chemical PCa treatment. Although AR signalling is still targeted, protein targets other than direct inhibitors of AR have also been investigated for the treatment of PCa. Our view is that targeting alternative proteins and protein pathways may be the key to advancing PCa treatment. Protein degradation is one of the pathways that have increasingly been identified as a target for anti-cancer therapy. We perform a brief review of the signalling pathways that have been identified as playing a key role in PCa in order to demonstrate the current understanding of PCa signalling pathways and where protein degradation pathways fit in this scheme. We also perform a detailed description of the ubiquitination and neddylation pathways which are involved in protein degradation and to which our protein of interest, DCUN1D1 is linked.

1.1.6 Prostate cancer signalling pathways

There are many intricate signalling pathways that are simultaneously active within normal and cancer cells. This is the case also in PCa. Although many have been described, some of the pathways demonstrated to play a key role in PCa include the AR, NF-κB, PI3K/AKT and MAPK signalling pathways. These pathways include some of the key transcription factors regulating prostate cell tumourigenesis but also PCa survival pathways. We have chosen to review these pathways not only because they play a key role in PCa but also because they encompass the survival pathways or the pathways that are linked to DNA or protein synthesis. They are therefore in contrast to the approach taken in this study but are the pathways also affected by current approaches to chemical PCa treatment. We explore these signalling pathways below.

Androgen receptor signalling and prostate cancer

As mentioned previously, the androgen receptor (AR) plays a key role in PCa due its effect on prostate cell growth. It is a steroid hormone receptor of the nuclear receptor family. It recognizes testosterone and its metabolite dihydrotestosterone, dimerizes and translocates to the nucleus (Wilson & French, 1976). There are four functional domains through which it mediates its activity including the N-terminal domain (NTD) with the transcriptional activation function 1 (AF-1) region, the DNA binding domain (DBD), the hinge domain with a nuclear localization signal (NLS) and the ligand binding domain (LBD) with the transcriptional activation function 2 (AF-2) region (Tilley et al., 1989; Jenster et al., 1995; Haelens et al., 2007). Although it remains in an inactive conformation in the cytoplasm through Hsp90 binding (Fang et al., 1996), upon ligand binding it dimerizes and translocates to the nucleus to mediate transcription of several genes including PSA, NKX3.1, VEGF-A, GATA 2, Myc, KLF 4, FGF8 , Cdk1, Cdk2, Cdk4, PMEPA1 and TMPRSS2 (Cleutjens et al., 1996; Joseph et al., 1997; Lu et al., 1997; Gregory et al., 1998; Gregory et al., 2001; Gnanapragasam et al., 2002; Böhm et al., 2009; Thomas et al., 2010; Gao et al., 2013). As a result, ARs play a key role in various crucial cellular activities including cell proliferation, development, transcription, the cell cycle, signal transduction, etc. and disruptions in AR expression and signalling can often lead to tumourigenesis. Amplification of AR gene expression has been implicated in PCa. It is observed in majority of hormone refractory PCas, with several studies demonstrating that resumption of AR-dependent activity is a key mechanism of recurrence of PCa following androgen deprivation therapy (Koivisto et al., 1997; Linja et al., 2001; Brown et al., 2002). In addition, AR mutations which increased AR activity in response to nonadrogenic molecules and to different transcription factors as well as the expression of AR splice variants have all been implicated in the role of AR in PCa (Newmark et al., 1992; Marcelli et al., 2000; Watson et al., 2010; Harada et al., 2012; Hay & McEwan, 2012; Gao et al., 2013; Schultz et al., 2014; Sun et al., 2014). The consequence of all these alterations in AR expression is the upregulation of AR signalling pathways which has been correlated with PCa development and progression. Therefore, this pathway has extensively been targeting in chemical PCa treatment as indicated in the previous section.

NF-κB signalling in prostate cancer

Nuclear factor kappa B (NF-κB) is another transcription factor that has been associated with PCa (Suh et al., 2002; Jin et al., 2008). The NF-κB family is composed of five members namely p65 (RelA), p100/p52, p105/p50, c-Rel and RelB which homo and heterodimerize in order to bind κB enhancer sites on specific DNA sequences (Scott et al., 1993; Dobrzanski et al., 1994; Héron et al., 1995; Martin & Fresno, 2000; Coope et al., 2002). They are induced in response to a wide spectrum of stimuli leading to the activation of canonical (IKK activation, IκB phosphorylation) and non-canonical NF-κB signalling pathways (Osborn et al., 1989; DiDonato et al., 1997; Xiao & Harhaj,

2001; Coope et al., 2002). Some of the downstream target genes of these pathways include the AR, IL-6, PSA and TNF-α which have been associated with PCa development and progression (Libermann & Baltimore, 1990; Sawyers, 2002; Gupta et al., 2005; Jin et al., 2008). These genes are all upregulated in PCa and have been shown to be associated with androgen dependent or androgen independent PCa as well as PCa prognosis.

PI3K/AKT signalling in prostate cancer

PI3K/AKT are key signal transduction molecules which have been implicated in several crucial cellular activities. PI3K has been shown to phosphorylate phosphatidylinositol (4,5) bisphosphate (PIP2) into phosphatidylinositol (3,4,5) trisphosphate (PIP3) and this leads to the recruitment and activation of AKT (Carpenter et al., 1993; Franke et al., 1995). PI3K can bind to several different receptors including RTKs (receptor tyrosine kinases) and GPCRs (G protein coupled receptors) while AKT activates a wide spectrum of proteins including mTOR (Franke et al., 1995; Phillips-Mason et al., 2000; Seasholtz et al., 2001; Murga et al., 2002; Hahn-Windgassen et al., 2005; Várnai et al., 2005). As such, PI3K/AKT plays a role in signal transduction activity related to cell survival, proliferation and differentiation. PCa appears to rely heavily on PI3K/AKT activity with the PI3K/AKT/mTOR signalling pathway upregulated in PCa primarily due to loss of expression of the negative regulator PTEN (Di Cristofano et al., 1998; Liao et al., 2003; Ghosh et al., 2005). As such, AKT inhibitors are currently being evaluated in phase II clinical trials for PCa treatment (Chee et al., 2007).

MAPK signalling and prostate cancer

The MAPK signalling pathway is one of the most essential signalling pathways in cells with its three major endpoints ERK, JNK and p38 MAPK being the effectors of proliferative and cellular stress responses (Fuchs et al., 1998; Zhang et al., 2002; Faust et al., 2012). The MAPK pathway is activated in PCa but its role in PCa is highlighted because this pathway mediates the activity of several growth factors including EGF and TGF-α which are often overexpressed in PCa (Abreu-Martin et al., 1999; Gioeli et al., 1999a; Seth et al., 1999; Kinkade et al., 2008). In addition, it has also been implicated in early prostate gland development through the upstream activity of FGFR2, a fibroblast growth factor receptor and MAPK pathway inducer, which has been linked to prostate gland development during embryogenesis (Marker, et al., 2003).

Although there many signalling pathways and transcription factors deregulated in PCa, a lot of their activity converges on the above mentioned pathways. Most importantly however, even within these pathways which largely promote DNA and protein synthesis; protein degradation is at play. For example the transcription factor NF-κB is directly regulated by protein degradation in that its

activation relies on IκB phosphorylation, ubiquitination and proteasomal degradation (Chen et al., 1995).

Protein degradation is a highly regulated process and it is as essential to regulating normal cellular activity as protein synthesis. The ubiquitin-proteasome pathway is one of the pathways that regulate protein degradation and it is responsible for majority of the degradation of intracellular proteins (Rock et al., 1994). It consists of an enzymatic cascade that conjugates ubiquitin molecules to specific protein substrates and a multi-subunit 26S proteasome which degrades the ubiquitin-tagged proteins (Figure 3). Protein degradation and the ubiquitin-proteasome pathway have been described; however ubiquitin-like molecules and related pathways such as neddylation are not well understood and their role in cancer development is still under investigation. Additionally, even though proteasome inhibitors such as bortezomib and inhibitors of neddylation such as the neddylation activating enzyme inhibitor MLN 4924 have been developed, efficacy can be improved upon and adverse effects are still observed (Hideshima et al., 2001; Hideshima et al., 2003; Soucy et al., 2009). We review the ubiquitination, neddylation pathways and our protein interest DCUN1D1 in detail below.

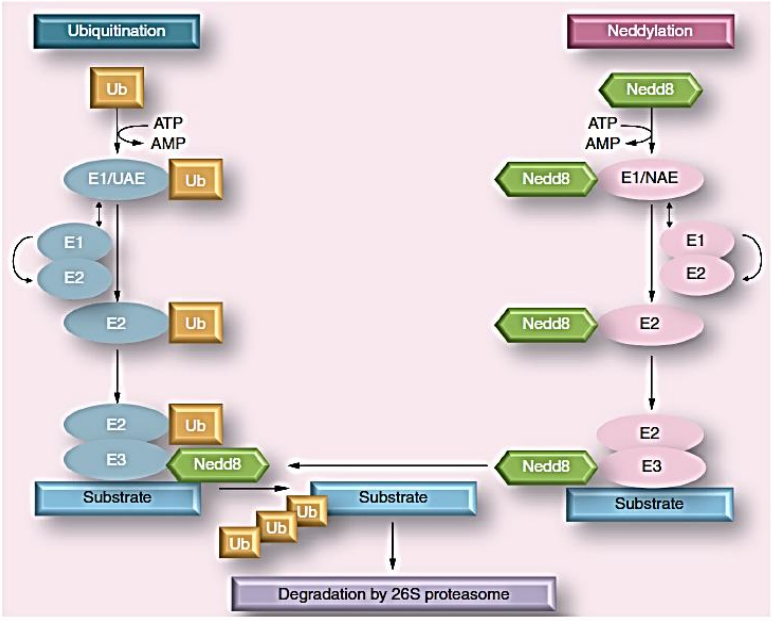

Figure 3. Schematic diagram of the ubiquitination, neddylation pathway and the downstream 26S proteasome. *Left*: the ubiquitination pathway begins with activation of the ubiquitin (Ub) by the E1 or ubiquitin activating enzyme (UAE) using ATP. This is then followed by the transfer of Ub from the E1 to the E2 (ubiquitin conjugating enzyme) which transfer Ub to the E3 ligase. The E3 ligase then transfers Ub to specific target proteins called substrates. This can occur for several cycles resulting in polyubiquitination of substrate proteins which tags the proteins for degradation by the 26S proteasome. *Right*: the neddylation pathway is similar, in that it also begins with the activation of NEDD8 in an ATP-dependent manner by an E1 named the neddylation activating enzyme (NAE). It is followed by transfer of NEDD8 to the E2 enzyme which transfers NEDD8 to the E3 ligase. The E3 ligase then mediates the transfer of NEDD8 to specific substrate proteins. If the substrate proteins are the cullin family of the proteins, the substrate with bound NEDD8 can then form part of the E3 ligase of the cullin RING ligase family to enhance ubiquitination. (Duncan et al. 2012)

1.1.7 The role of ubiquitination under physiological and cancer conditions

The rate of degradation of a protein is a process that is essential to cellular activity. Protein turnover rates determine when and at what level a protein is available to perform its function and the ubiquitin-proteasome pathway plays a crucial role in this aspect. It is a selective protein degradation pathway that employs a repeated three step enzymatic cascade to add multiple 8.5 kDa ubiquitin (Ub) molecules to specific target proteins, tagging them for degradation by the 26S proteasome (Figure 3). The E1 is the first enzyme in the cascade and its function is to activate ubiquitin using ATP (Ciechanover et al., 1981; Ciechanover et al., 1982). The ubiquitin activating enzyme (UAE) was the only E1 to be characterised initially, however, Uba6 has recently been identified as having ubiquitin activating activity (Groettrup et al., 2008). This is in contrast to the second enzyme in the cascade, the E2 ubiquitin conjugating enzyme (UBC), where 37 UBCs have been identified in humans which

recognize activated ubiquitin and catalyse it's transfer to specific E3 ligases (Pickart & Rose, 1985; Jentsch et al., 1990; Michelle et al., 2009). The E3 ligase is the last enzyme in the cascade and it catalyzes the transfer of Ub first to the lysine residue of a specific substrate then to the lysine residue of Ub molecules attached to the substrate (Hershko et al., 1986; Reiss et al., 1989; Peng et al., 2003; Li et al., 2007). This can be mediated by the E2 enzyme in complex with the E3 ligase or by the E3 ligase directly (Ciechanover et al., 1980). There are ~617 genes encoding for putative human E3 ligases that have been identified in humans therefore, even though the increased number of E2s relative to the E1s contributes to the specificity of this pathway, E3 ligases are the main specificity factors in ubiquitination (Myung et al., 2008). As a result of the large number of E3 ligases and the importance of their activity, they have been studied extensively.

Firstly, this large group of enzymes is classified into three classes based on conserved domains found in their core proteins. The HECT (homologous to E6-AP carboxy terminus) domain proteins, the RING (Really Interesting New Gene) and the U-box domain proteins (Freemont & Hanson, 1991; Beaudenon, 1995; Hatakeyama et al., 2001). Majority of the E3 ligases that have been described thus far are the RING finger domain E3 ligases. These enzymes can be further divided into two subclasses namely the monomeric simple RING ligases or multi-subunit cullin RING ligase (CRL) complexes. Of particular interest to this project are the multi-subunit cullin RING ligases. They consist of a core scaffold protein from the cullin family of proteins (cullin 1, 2, 3, 4A, 4B, 5 and 7), a RING finger domain-containing protein RBX1/2, a substrate binding protein and an adaptor protein (Skowyra et al., 1997; Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999; Chen et al., 2001; Zheng et al., 2002). The multi-component CRL and the different combinations of proteins that can be used in these complexes allow for recognition of a variety of protein substrates with distinct specificity. These substrates in turn are implicated in crucial functions in the cell such as cell cycle regulation, cell growth, proliferation, intracellular signalling, DNA repair, pro and anti-apoptotic signalling and inflammation (Chen et al., 1995; Fang, 2000; Clifford et al., 2001; Fukuchi et al., 2003). This indicates the importance of ubiquitination in normal cellular activity and why it, together with other protein degradation pathways is often deregulated in cancer.

There is a very close relationship between ubiquitination and cancer development with polyubiquitination and subsequent proteasome degradation either leading to the stabilization of oncoproteins or destabilization of tumour suppressor proteins. For example, the tumour suppressor p53 is heavily regulated by ubiquitination through targeted degradation by the Mdm2 E3 ligase (Fang, 2000). P53 is normally inactivated in many cancers and dysregulations in Mdm2 expression or activity can alter p53 degradation, making ubiquitination a key regulator of p53 and cancer. The BRCA1 gene which plays a crucial role in breast cancer development is a ubiquitin E3 ligase which is implicated in cell cycle regulation, DNA repair and apoptosis (Thangaraju et al., 2000; Hashizume et al., 2001; Greenberg et al., 2006). These are just two examples where ubiquitination plays a role in

cancer, either through the regulation of degradation of a tumour suppressor protein or where one of the components of the pathway is itself a tumour suppressor protein. This has been illustrated further by the role of the 26S proteasome in cancer, which has been identified as a target for cancer treatment through the development of proteasome inhibitors.

The 26S proteasome is responsible for the degradation of ubiquitinated proteins. It is made up of a central barrel-shaped 20S proteasome that has a hollow cylinder shape together with 19S regulatory particles on either or both ends of the 20S proteasome (Baumeister et al., 1988; Glickman et al., 1998). Due to importance of the 26S proteasome as demonstrated in its in role in the UPP and the functions affected as a result of the degradation of its target protein substrates and its subsequent dysregulation in multiple different diseases (including cancer); the 26S proteasome has become a unique target for disease treatment. Several inhibitors of the proteasome have been developed over time that block degradation of polyubiquitinated proteins and reverse dysregulation of proteins observed under disease conditions. Proteasome inhibitors employ a novel mechanism of anti-cancer activity. Instead of targeting the inhibition of DNA and protein synthesis to kill cancer cells as done by previous chemotherapeutic agents, they target the inhibition of protein degradation and the approach has proven quite successful. We briefly describe one the most successful proteasome inhibitors bortezomib. Bortezomib (PS-341) (Millenium Pharmaceuticals) is a small molecular weight dipeptide boronate which directly inhibits the chymotryptic site of the proteolytic 20S catalytic core of the 26S proteasome (Adams et al., 1998; Adams, 2004). It is a stable, potent, selective but reversible inhibitor of the 26S proteasome which has been shown to have anti-cancer activity against several different cancers including ovarian, PCa and Lewis lung carcinoma (Herrmann et al., 1998; Adams et al., 1999; Teicher et al., 1999; Frankel et al., 2000). It has also successfully deterred the characteristic uncontrollably growth of tumour cells in multiple myeloma (MM) cancer (Adams, 2002; Orlowski et al., 2002; Hideshima et al., 2003; Richardson et al., 2003; Singhal et al., 2003). However, trials undertaken identified adverse effects such as gastrointestinal symptoms, fatigue, thrombocytopenia, and sensory neuropathy in some patients (Orlowski et al., 2002; Singhal et al., 2003). This indicated that as much as proteasome inhibitors are a very promising class of anti-cancer drugs, the knowledge obtained in the success of these drugs should be used in identifying more efficacious and safe anti-cancer drugs

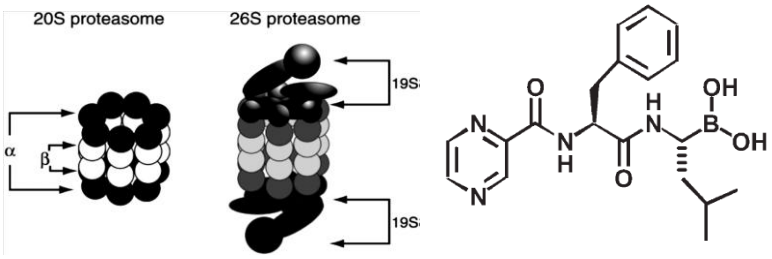

Figure 4. Schematic representation of the 26S proteasome, the proteolytic 20S component of the 26S proteosome and the proteasome inhibitor bortezomib (PS-341). *Far left:* central barrel-shaped 20S proteasome showing the two outer α rings and the two inner β rings. It consists of four stacked hollow rings made up of seven distinct subunits. *Middle:* 26S proteosme consisting of the 19S regulatory subunits and the 20S proteasome. *Right:* the structure of bortezomib (PS-341) a small molecular weight dipeptide boronate which directly inhibits the chymotryptic site of the proteolytic 20S catalytic core of the 26S proteasome. (Adams, 2004)

The identification of proteasome inhibitors as viable targets for anti-cancer drugs has further emphasized the importance of the ubiquitin-proteasome pathway in cellular activity and the inefficiencies observed in their use in the treatment of multiple myeloma cancer has highlighted a potential gap in the market. Therefore our view is that ubiquitin-like molecules and related pathways such as NEDD8 are potentially new drug targets for cancer treatments. Unlike ubiquitin, ubiquitin-like molecules have narrower ranges of protein substrates but their substrates are involved in crucial cellular activity. Therefore it is likely that if used in anti-cancer activity there is the potential to obtain the same effect but through fewer protein targets and perhaps fewer side effects. NEDD8 in particular, is also involved in protein degradation due to its link to cullin-mediated ubiquitination but it has more specific substrate proteins than the 26S proteasome and therefore could allow for a more specific, targeted treatment for PCa, in this instance.

1.1.8 Neddylation: a description of an emerging pathway in cancer treatment

We have highlighted ubiquitin, the ubiquitin-proteasome pathway and their importance in cell activity. However, there are several molecules that are similar either in sequence, structure or cascade activity to ubiquitin that have also been identified in prokaryotic and eukaryotic cells. These molecules are appropriately called ubiquitin-like proteins (UBL) and include: NEDD8, SUMO-1, ATG8, ATG12, ISG15, Urm1, Ufm1, FAT10 and FUB1 (Ciechanover et al., 1980; Haas et al., 1987; Loeb, 1992; Michiels et al., 1993; Fan et al., 1996; Matunis et al., 1996; Kamitani, 1997; Mahajan et al., 1997; Mizushima, 1998; Mizushima et al., 1998; Furukawa & Mizushima, 2000; Komatsu et al., 2004). Majority of these molecules conjugate to a wide range of protein substrates and can affect different functions in the cell, however the exact function of some of these molecules is not fully

understood as yet. NEDD8 and the neddylation pathway have been described to some extent already and like ubiquitination have been associated with cancer.

NEDD8, neural precursor cell expressed developmentally downregulated 8, is an 81 amino acid, 9kDa ubiquitin-like molecule. It is highly conserved in mammals and shows 60% identity to the amino acid sequence of ubiquitin (Figure 5) (Kumar & Tomooka, 1992; Kumar & Yoshida, 1993; Kamitani, 1997). It also undergoes activation and transfer to specific substrate proteins relying on a conserved Gly 76 for activation and Lys 48 for substrate recognition (Kamitani, 1997). However, it has its own distinct E1, E2 and E3, it also has a narrower range of target proteins which seem to localize mainly in the nucleus, resulting in a distinct expression pattern in the cell (Kamitani, 1997). The process of addition of NEDD8 to specific substrates or the neddylation pathway has a dedicated E1-activating enzyme known as the neddylation activating enzyme (NAE), an AppBp1/UBA3 heterodimer (Gong & Yeh, 1999). It catalyzes the activation of NEDD8 in an ATP and Mg^{2+}-dependent manner and transfers it through a transthiolation reaction to one of two E2s, UBC12/UBE2M and UBE2F (Liakopoulos et al., 1998; Huang et al., 2009). It has been suggested that neddylation also requires E3 ligase activity for the final transfer to its target proteins, namely the cullin family of proteins. Significantly, our protein of interest DCUN1D1 is one of the few proteins to have been characterised as having NEDD8 E3 ligase activity.

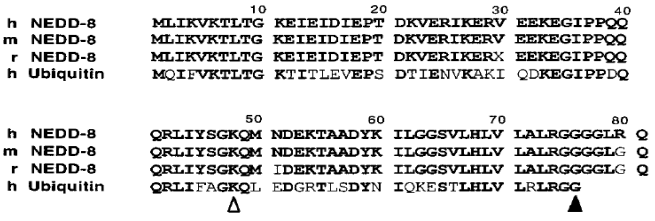

Figure 5. Aligned amino acid sequences of human, mouse and rat NEDD8 and human ubiquitin. Alignment shows high sequence similarity among the sequences with the identical amino acid typed in bold. The closed triangle shows Gly76 necessary for NEDD8 and ubiquitin conjugation and the open triangle shows Lys 48 necessary for substrate recognition. (Kamitani, 1997).

The neddylation pathway itself runs parallel to the ubiquitination pathway but it functions independent of ubiquitination (Figure 3). There are various levels of regulation from the recognition of one of the E2s by the E1 NAE, to the subsequent transfer of NEDD8 to its substrate proteins. It also undergoes regulation through the activity of two NEDD8 specific cleavage proteins. DEN1/NEDP1/SENP8 catalyzes the cleavage of NEDD8 from an immature 81 amino acid polypeptide to a 76 amino acid mature product while COP9 signalosome together with DEN1 catalyse deneddylation of neddylated cullin molecules (Mendoza et al., 2003; Pintard et al., 2003; Wu et al., 2003; Xirodimas et al., 2004). However, apart from the components of the neddylation pathway and

the functions of each component, very little is known about which proteins actually get neddylated. Although several proteins have been identified as undergoing NEDD8 conjugation, many of these studies have established only the ability of NEDD8 to interact directly or indirectly with certain proteins but they have not explored the consequences of the conjugation. The most extensively studied substrates of neddylation to date are the cullin family of proteins mentioned previously as components of ubiquitin E3 ligases and these substrates have illustrated the importance of neddylation in cellular activity.

NEDD8 substrates and the importance of neddylation

Neddylation has been associated with many important cellular proteins. Mass spectrometry, *in vitro* and *in vivo* neddylation assays have identified firstly, the cullin family of proteins as NEDD8 substrates but also several CRL components such as ROC1/RBX1, elongin B/C, Skp1 and F-box proteins (Jones et al., 2008). Regulators of neddylation, AppBp1/Uba3, UBC12, p120 ^{CAND1}, DCUN1D1, the 19S proteasomal regulatory subunits and proteins unrelated to neddylation such as DNA polymerase, RNA polymerase, SMC2 were also found to be substrates of NEDD8 (Jones et al., 2008). The cullin family of proteins i.e. cullin 1, 2, 3, 4A, 4B and 5 are the most extensively studied NEDD8 substrates (Osaka et al., 1998; Kamura et al., 1999; Liakopoulos et al., 1999; Morimoto et al., 2000; Querido et al., 2001; Pintard et al., 2003; Meyer-Schaller et al., 2009). As mentioned previously, cullin proteins form scaffolding molecules in CRL complexes and as such play a key role in ubiquitination. They are susceptible to modification by neddylation with the E2 conjugating enzymes, UBC12/UBE2M and UBE2F, targeting cullin 1-4 and cullin 5 respectively for NEDD8 conjugation (Huang et al., 2009). The conjugation then results in conformational changes in CRL complexes, converting them from a closed confirmation to a more open confirmation (Duda et al., 2008). It also allows for optimal binding of ubiquitin within the CRL complex to substrate proteins by accelerating E2-E3 interactions, enhancing the ability of the E2 to bind ubiquitin and creating optimal spatial conditions for ubiquitin transfer to specific substrates by the E2 (Kawakami et al., 2001; Saha, 2008). In addition, neddylation accelerates the removal of CAND1 from Cul1-RBX1 (Duda et al., 2008), which normally inhibits cullin 1 interaction with other components of the E3 and thus negatively regulates ubiquitination. Therefore in this instance, neddylation enhances the ubiquitination of proteins targeted for degradation by some cullin RING E3 ligases and by extension plays a role in the functions regulated by these proteins such as cell cycle regulation, cell proliferation, intracellular signalling and apoptotic signalling.

In addition, studies have occurred which identified EGFR, Mdm2, the tumour suppressor p53, TAp73, pVHL, parkin, PINK1, BCA (Breast Cancer Associated) protein and the ribosomal protein L11 as substrates of NEDD8 but they also established the downstream effects of these alterations (Stickle et al., 2004; Xirodimas et al., 2004; Oved et al., 2006; Watson et al., 2006; Xirodimas et al., 2008; Choo

et al., 2012). EGFR neddylation in response to EGF ligand binding led to endosomal/lysosomal degradation of EGFR, L11 neddylation results in the protection of L11 from 26S proteasomal degradation thus stabilizing its cellular levels. However, functions independent of ubiquitination or stabilization of protein levels are also affected by neddylation. For example, pVHL neddylation is required for fibronectin assembly, BCA3 neddylation decreases NF-κB dependent transcription activation through the recruitment of the SIRT1 deacetylase to NF-κB and parkin-PINK1 neddylation deregulates mitochondrial homeostasis (Stickle et al., 2004; Gao et al., 2006; Choo et al., 2012). Consequently, it can be concluded that a variety of biological processes including transcription, replication, DNA repair, chromatin organization and remodelling, mitochondrial activity and matrix organization are affected by neddylation. Therefore, neddylation as a post translational modification plays a role in tertiary protein interactions where it's binding can enhance the protein's degradation by ubiquitination or endosomal/lysosomal degradation. It can also allow for the recruitment of other proteins, nuclear localization of proteins and can affect protein assembly. However, this does not account for all of the substrates that have been identified by mass spectrometry as NEDD8 substrates. Therefore, a lot is still to be uncovered about neddylation, the biological consequences of its addition to substrate proteins and its contribution to disease phenotypes including cancer.

By virtue of the substrates of NEDD8 identified above, and as a result of some studies that have been performed already, it has been hypothesized that neddylation could play a key role in cancer development and progression. The cullin family of proteins as components of E3 ligases regulate the activity of a variety of proteins already associated with cancer development. The DNA replication factor Cdt-1, pIκBα, NRF2 stress responsive factor, HIF1α, c-Jun, and the cell proliferation regulator c-Myc are CRL substrates of $CRL1^{Skp2}$,$CRL1^{\beta TrCP1}$,$CRL3^{KEAP1}$,$CRL2^{VHL}$, $CRL1^{Fbw7}$ and CRL^{Fbw7} respectively and they have all been associated with various cancers (Winston et al., 1999; Clifford et al., 2001; Kobayashi et al., 2004; Nateri et al., 2004; Yada et al., 2004; Nishitani et al., 2006). In addition, the established NEDD8 substrates EGFR, p53, Mdm2, and pVHL have been reported to play a role either in lung cancer, PCa, glioblastoma and renal cell carcinoma (Reifenberger et al., 1993; Eastham et al., 1995; Clifford et al., 2001; Zhang et al., 2012). Significantly, an inhibitor of the NAE, MLN4924 has been identified which shows anti-cancer activity against colorectal, liver, breast, pancreatic, lung cancer cell lines and inhibits tumour xenograft growth in colorectal and lung cancer cells (Soucy et al., 2009; Lin et al., 2010; Milhollen et al., 2011; Luo et al., 2012; Toth et al., 2012; Wei et al., 2012). It is also currently in a phase 1 trial for use in adult non hematologic malignancies by Millenium Pharmaceuticals. As mentioned previously, NEDD8 activation involves the use of ATP and during the activation process a NEDD8-AMP acyl adenylate intermediate forms which interacts with the active site thiol of the NAE resulting in NEDD8 binding to NAE and AMP being released (Gong & Yeh, 1999). MLN4924 is structurally similar to AMP (Figure 6) and it inhibits specifically NAE by binding to it instead of AMP. It is also specific to the NAE showing no binding to any other

related enzymes such as UAE, SAE (SUMO-1 E1), UBA6 (FAT10 E1) and ATG7 (ATG8 and ATG12 E1) or other ATP-using enzymes (Soucy et al., 2009). The anti-cancer activity of MLN4924 involves the disruption of CRL-mediated protein turnover, leading to the accumulation of several CRL substrates including Cdt-1, pIκBα (Lin et al., 2010; Milhollen et al., 2010). This results in cell cycle arrest, induction of apoptosis and it also induces autophagy in liver cancer cells.

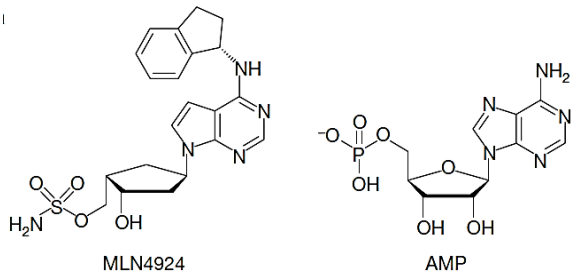

Figure 6. Structural images of the NAE inhibitor MLN4924 and adenosine 5' – monophosphate (AMP). (Soucy et al., 2009)

Therefore, the neddylation pathway has not only been identified as important for normal cellular activity, it is also essential for cancer development. The identification of the NAE inhibitor MLN4924 emphasizes both of these points and further illustrates the importance of protein degradation/ modification pathways for tumourigenesis. For that reason, we have identified neddylation, in particular DCUN1D1 as potentially playing a role in PCa development and we explore the characteristics of DCUN1D1 below.

1.1.9 DCUN1D1 and cancer

The proteins DCN-1 and Dcn1p were originally described in *Caenorhabditis elegans (C. elegans)* and *Saccharomyces cerevisiae (S. cerevisiae)* respectively as conserved proteins essential for cullin neddylation (Kurz et al., 2005). DCN-1 in particular was identified because defects in embryonic cell division of *C. elegans* due to *dcn-1* knockdown were similar to those observed in cells lacking cullin-3 ligase activity and because DCN-1 mediated post-meiotic degradation of MEI-1, a meiotic microtubule degrading protein. Both DCN-1 and Dcn1p have an N-terminal UBA-like domain through which they bind ubiquitin with high affinity but they show specificity for NEDD8 that is independent of the UBA-like domain (Kurz et al., 2005; Kurz et al., 2008). They also show high nuclei expression with lower levels in the cytoplasm, similar to that observed in NEDD8 and COP9 signalosome. Direct binding to NEDD8, Cdc53 (cullin 1 homologue) and RBX1 as well as similarities between defects observed during *dcn-1* knockdown and aberrant neddylation observed in *C. elegans* embryos all indicated that DCN-1/Dcn1p played a key role in the neddylation pathway (Kurz et al., 2005; Yang et al., 2007; Kurz et al., 2008). However, DCN-1 lacks a prototypical RING or HECT

domain and Dcn1p overexpression in *S. cerevisiae* is not able to inhibit Cdc53p deneddylation by COP9 signalosome indicating that they cannot initiate neddylation *in vitro* (Yang et al., 2007; Kurz et al., 2008). Nevertheless, they are essential for normal cellular activity because reduced cullin-3 neddylation in *C. elegans* and *S. cerevisiae* is lethal (Kurz et al., 2005). Similar observations were made in the human DCN-1/Dcn1p homologue known as DCUN1D1/SCCRO, we explore these observations below.

Although initially described in humans due to its association with amplifications along 3q26.3 in various squamous cell carcinomas, DCUN1D1 is an essential component of the E3 complex of neddylation (Sarkaria et al., 2006; Kim et al., 2008). It binds to the core Cul1-RBX1 complex of neddylation, the E2 UBC12/UBE2M and the inhibitory CAND1 proteins (Kim et al., 2008). It is observed to enhance the kinetics of neddylation by the recruitment of NEDD8-bound UBC12/UBE2M and acceleration of CAND1 dissociation from Cul1-RBX1 to promote the formation of the neddylation E3 ligase complex. Significantly, DCUN1D1 is crucial for normal cellular activity *in vivo*, in that DCUN1D1 knockdown in mice results in several defects including high rates of perinatal mortality, runting and male specific infertility (Huang, Kaufman, Ramanathan, & Singh, 2011). Part of its importance stems from the fact that DCUN1D1 mediates nuclear localization of cullin proteins during *in vivo* neddylation. The neddylation E2 UBC12/UBE2M is primarily localized to the nucleus while cullin 1 proteins are localized mainly in the cytoplasm with those lacking NLS not being able to undergo neddylation *in vivo.* In addition, DCUN1D1 is observed to mediate translocation of cullin 1 to the nucleus with DCUN1D1 proteins lacking their NLS not being able to promote cullin 1 neddylation *in vivo*. Therefore, DCUN1D1 does not enhance neddylation but is crucial for its progression since it is required in order translocate the Cul1-RBX1 complex into the nucleus where majority of the UBC12/UBE2M enzymes are located in order to promote E2-E3 interactions for NEDD8 transfer to cullin 1; a function that is not necessary *in vitro*. This activity has proven to be essential also for tumourigensis, as DCUN1D1 has been strongly associated with cancer phenotypes.

Positional cloning of the common 3q amplification led to the discovery of DCUN1D1, a homolog of DCN-1 and Dcn1p with a propensity for amplification in human cancer cells. DCUN1D1 is also known as the squamous cell carcinoma related oncogene (SCCRO) due its prevalence in squamous cell carcinomas (SCCs). It is upregulated in head and neck, lung carcinomas and it plays a role in gliomas and cervical cancer (Sarkaria et al., 2006; Broderick et al., 2010). Cells overexpressing DCUN1D1 were observed to undergo apoptosis upon treatment with DCUN1D1 RNA interference and DCUN1D1 overexpression was shown to result in the malignant transformation of NIH-3T3 fibroblasts and xenograft formation in nude mice (Sarkaria et al., 2010). In addition to promoting cancer cell survival by the inhibition of apoptosis, DCUN1D1 increases cell proliferation, and induces

metastasis, invasion and angiogenesis through MMP2 and VEGF-A related pathways (Sarkaria et al., 2004; O-charoenrat et al., 2008; Broderick et al., 2010). It also binds the promoter of *Gli1*, a component of the hedgehog signalling pathway which is important for embryogenesis and cancer (Sarkaria, et al., 2006).

Therefore, although established as an oncogene in various SCCs, DCUN1D1 is an underexplored potential therapeutic target in PCa and it may be a key new candidate for targeted therapy. We decided to investigate the importance of DCUN1D1 for PCa, assessing DCUN1D1 expression and using molecular biology and genomics approaches to identify DCUN1D1 inhibitors. In particular we chose to use the connectivity map database bioinformatics tool, which uses gene expression signatures to connect genes, diseases and drugs.

1.1.10 The use of bioinformatics tools in drug discovery: the connectivity map database

Since the development of high throughput data generation and analysis, there have been large amounts of gene expression profiles, disease gene profiles as well gene expression profiles of drug activity in cancer that have been generated. Bioinformatics has played an essential in the analysis of this data and in how this data is used in cancer drug discovery. Traditional methods of drug discovery employed a structural approach where virtual screening was used to identify protein inhibitors using the 3D structure of target proteins (Cheng et al., 2007). However, there are limited 3D structures of the multitudes of proteins present in the cell which made this approach difficult. Therefore, computational bioinformatics and systematic medicine have emerged as alternative and successful approaches. These have been applied extensively in identifying potential protein targets to inhibit PCa progression or drug targets in the treatment of PCa (Yi et al., 2009; Yeh et al., 2012; Wen et al., 2013). Microarray analysis, Drugbank, Gene Ontology (GO) and KEGG pathway are some of tools that have been used to generate and analyze large gene expression profiles and to analyze the alterations in gene expression that are observed following the use of drugs in disease treatment (Kanehisa, 2002; Ashburner et al., 2011; Yeh et al., 2012). However, the large size of these data, the complex nature of the profiles revealed and the separation of these sets of data have made it challenging to connect them during drug discovery. The connectivity map database was established to bring them together.

The connectivity map (cmap) database was established in 2006 by *Lamb et al* and is based at the Broad Institute of MIT and Harvard in Cambridge, Massachusetts (Lamb et al., 2006). It is a large public database that uses genomic signatures to find patterns between physiological, disease, chemically-induced or RNA interference-induced biological states. Modern day research has established that each of these states is as a result of multiple different signalling pathways within the cell, composed of multiple different genes. The link between each of these states is not always

understood, which makes drug discovery, disease treatment and basic understanding of molecular biology difficult. The cmap database uses a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules and simple pattern-matching algorithms to enable the discovery of functional connections between drugs, genes and diseases (Lamb, 2007). It contains more than 7,000 expression profiles representing 1,309 compounds and has the potential of helping researchers identify drugs acting through a common mechanism of action e.g. HDAC inhibition, identifying mechanisms of action of specific drugs and identifying compounds for potential use in new therapeutic treatments (Lamb et al., 2006). Significantly, some of the compounds available on the database are U.S. Food and Drug Administration (FDA) approved drugs which means that they are suitable for use in humans. The major appeal of the cmap database is that it allows the researcher to input gene signatures generated from mRNA expression following the treatment of different cultured cell lines, using DNA microarray analysis on Affymetrix plates. These gene signatures are uploaded into the database and comparisons are made between the gene profiles generated following the researcher's experimental conditions and the gene signatures in the database in a manner similar to the comparison of gene sequences by Genbank. Therefore the cmap database allows for simplified connection of gene expression signatures and drug or drug-like molecules to investigate research questions. In this study it is used to identify small molecule inhibitors of PCa that mediate their activity through DCUN1D1.

In summary, the discovery of the PCa biomarker PSA has led to increased detection of PCa, allowing for early detection and treatment in some cases of PCa and a subsequent decline in PCa mortality in developed countries (Siegel, 2013). However, PSA testing has also resulted in the detection of clinically insignificant cancer. In addition, the slow development of PCa means that many individuals die with PCa rather than from PCa (Wingo et al., 1998). In spite of this, there is still a heavy PCa burden in the world even with advances in the detection and treatment of PCa. Therefore, there is a need for effective treatment of all stages of PCa. As a result, we have explored cellular pathways that have emerged as effective targets for cancer treatment, namely the ubiquitination and neddylation pathway. In particular, we investigate the role of the neddylation E3 ligase DCUN1D1 on PCa development and progression and use novel bioinformatics tools to identify potential inhibitors of DCUN1D1 for PCa treatment. We have reviewed the ubiquitination, neddylation pathways and DCUN1D1 and explore their importance to normal cellular activity, their role in tumourigenesis and drugs currently used to target ubiquitination and neddylation. We have highlighted the importance of these pathways and our protein of interest DCUN1D1 and demonstrate their viability as targets for drug development.

1.2 Rationale of the study

PCa is a highly prevalent disease and although treatment options for patients with early stage PCa are high, there are fewer options for individuals with stage III and stage IV disease. The architecture and the location of the prostate gland itself contributes largely to difficulty in PCa but the multi-stage development, heterogeneous nature of tumours within the same gland, androgen dependence and independence of some tumours and the lack of efficient biomarkers; also contribute to the difficulty. Crucial to molecular target based PCa treatment is the identification of specific protein targets for cancer treatment. We have highlighted the emergence of a novel approach to cancer treatment which involves the inhibition of protein degradation in which our protein of interest can potentially play a role. Ubiquitination and the neddylation pathway are essential cellular activities to which DCUN1D1 is linked making it an important cellular protein and potential drug target.

As illustrated above ubiquitination and neddylation are essential pathways for normal cellular activity that can be and are deregulated in cancer. DCUN1D1 is an underexplored oncogene with links to essential cellular activity and could be playing a role in PCa development. In addition, the proteasome inhibitor bortezomib and the NAE inhibitor MLN4924 have indicated that ubiquitination and neddylation are viable emerging targets for anti-cancer therapy but the broad range of proteins targeted by these drugs leads to many side effects that could be avoided with more specific drug targets within these pathways. We propose that DCUN1D1 could be this target.

1.3 Aims and Objectives

Therefore we hypothesize, and aim to establish, that DCUN1D1 may play a role in PCa development and could be a potentially new target for PCa treatment. For this reason, we also explore novel bioinformatics approaches through the connectivity map database in order to identify DCUN1D1 specific inhibitors for PCa.

Specifically, this study has the following aims:

1) To determine the level of expression of DCUN1D1 in a panel of PCa cell lines and human tissue samples.
2) To determine the relevance of DCUN1D1 for: proliferation, migration and apoptosis of PCa cells *in vitro* and tumour formation *in vivo.*
3) Determine signalling pathways in which DCUN1D1 is involved using microarray analysis.
4) Use the connectivity map database to identify drugs with similar effects as DCUN1D1 knockdown and to test their drug activity *in vitro*.

Chapter 2: Materials and Methods

2.1 Determination of DCUN1D1 expression level in prostate cancer cell lines and human tissue

2.1.1 Cell lines and maintenance of the cells

Cell lines and culture

Human PCa cell lines CW22, CW19, LNCaP (CRL-1740), VCaP (CRL-2876), DU145 (HTB-81), DUCaP, PC3 (CRL-1435), CL1 and a normal epithelial prostate cell line, PrEC (PCS-440-010) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). CW22, CW19, VCap, DU145, DUCap, were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, USA). LNCap and CL1 were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich, ST Louis, USA) while PrEC was grown in Prostate Epithelial Cell Basal Medium (PreEBM) (Lonza, Walkersville MD, USA). All of the media was supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin) and 1% penicillin (5000µg/ml)/streptomycin (500µg/ml) (Lonza, Walkersville MD, USA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 until confluent.

Seeding and harvesting of tissue culture cells

All of the cell lines were seeded on tissue culture dishes 100×20mm (BD Falcon, USA). During harvesting, the cells were washed in 1×phosphate buffered saline (PBS) pH 7.4 (137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4{\cdot}7H2O$, 1.4mM KH_2PO_4), detached using 0.05% Trypsin/EDTA (Gibco, Life Technologies, USA) and incubated under humidified conditions for between 1-10 minutes depending on the cell line. Trypsin activity was then inhibited through the addition of DMEM (Gibco, Life Technologies, USA), RPMI 1640 (Sigma-Aldrich, ST Louis, USA) and PreEBM (Lonza, Walkersville MD, USA) containing 10% FBS (Biochrom AG, Berlin) and 1% penicillin (5000µg/ml)/streptomycin (500µg/ml) (Lonza, Walkersville MD, USA). The cells were then resuspended in the media, combined with 0.4% trypan blue stain (Molecular probes, Life Technologies, USA) and quantified using the Countess Automated Cell Counter (Invitrogen, Life Technologies, USA). The volume (µl) of cells to be added was calculated as follows:

$$Volume = \frac{\text{No. of cells required}}{No.\,of cells/ml} \times 1000$$

2.1.2 Evaluation of DCUN1D1 expression in human tissue samples

OriGene TissueScan Prostate Cancer Tissue Array analysis

Real-time PCR analysis of human tissue samples was performed using the Origene TissueScan Prostate Cancer Tissue Array I and II (OriGene Technologies, Rockville, MD, USA) platform. According to the manufacturer's protocol, the samples were evaluated for DCUN1D1 expression and normalized against β-actin. The plate consisted of 48 complementary DNAs (cDNAs) namely: 28 adenocarcinoma of prostate tissue stages I, II and III, 11 benign prostate hyperplasia (BPH), 7 normal prostate tissue and 2 bladder carcinoma tissue samples as other disease controls.

Immunohistochemistry analysis

Sixteen human tissue samples comprising of paired cancer and normal tissue were evaluated for DCUN1D1 expression using immunohistochemistry. The tissue samples were collected and pathologically analysed by Dr Yihong Wang at Brown University, USA as prescribed by their ethics board. Once the tissue was collected, it was fixed in 10% formalin (2ml per 100mg of tissue) and dehydrated using increasing concentrations of ethanol (Merck, Germany). The ethanol was then cleared using xylene (2 times for 1 hour each) and embedded in paraffin (Paraplast X-tra, Sigma-Aldrich, USA) (2× 1 hour incubations) for preservation. Five micrometre thick sections were then sliced from the tissue using a cryostat and mounted onto slides. Although slicing of the sections increased exposure to the epitopes, the paraffin was first cleared using xylene (3 times 5 minutes each) and rehydrated in decreasing concentrations of ethanol. Antibody staining then began with heat-induced antigen retrieval where the slides in 10mM citrate buffer pH 6.0 were boiled at 750W for 10 minutes and at 305W for 20 minutes in a microwave oven. This allowed for the breakdown of the crosslinkages that occur between formalin and tissue proteins in order to increase the probability of binding to antibodies. Prior to antibody incubation, the slides were blocked in 1× Tris buffered saline/0.1% Tween 20 (Sigma-Aldrich, USA) (TBST) and 5% goat serum (Sigma-Aldrich, USA) for 1 hour at 23°C. The slides were then incubated in primary antibody, mouse monoclonal anti-DCUN1D1 (1:200) (Santa Cruz Biotechnology Incorporated) for 16 hours at 4°C in order to probe for the antigen. Washing of the slides then occurred in 1×TBST (3 times for 5 minutes). The slides were then incubated in biotinylated goat anti-mouse (Sigma-Aldrich, USA) for 30 minutes at 23°C, following this; the slides were washed in 1×TBST (3 times for 5 minutes). Using the avidin/biotin method, the slides were incubated in ExtrAvidin-peroxidase/biotin (Sigma-Aldrich, USA) for 30 minutes at 23°C. The solution was then removed through washing in 1×TBST, 3 times for 5 minutes, following which the relevant substrate was added to the slides. The staining was monitored closely and once the sections had developed, the slides were immersed in dH2O. Where necessary, the slides were counter-stained in hematoxylin. The slides were initially washed twice for 5 minutes in dH2O; they were then dehydrated using 95% ethanol (2 times for 10 seconds each) and 100% ethanol (2

times for 10 seconds each). The slides were then cleared using xylene (2 times for 10 seconds each) following which coverslips were mounted on the slides and viewed.

2.1.3 Quantitative real time PCR (qRT-PCR)

Six well tissue culture plates (Cellstar, Greiner Bio-one, Germany) were used to seed 3×10^5 cells/ well. The cells were harvested and counted as described above, incubated and allowed to attach for 16 hours. Total RNA was extracted using the protocols outlined in RNeasy mini kit (Qiagen, Valencia, CA, USA) and using the Qiashredder (Qiagen, Valencia, CA, USA) as previously described (Zerbini et al., 2003). When necessary the extracted RNA was stored at -80°C. Prior to use the RNA was quantified using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) where A260/280 and A260/230 ratios were performed in order to ensure that there was no contamination with protein or organic compounds. Complementary DNAs were then generated from 2µg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). Specifically, 2µg RNA, anchored-oligo(dT)18 primer (2.5µM) and PCR grade water were used to generate the cDNA. The 2720 Thermocycler (Applied Biosystems, Life Technologies, USA) was used to perform the assay. An initial denaturation step at 65°C for 10 minutes was performed, followed by the addition of a reaction buffer mixture. The mixture contained: 5×Transcriptor Reverse Transcriptase Reaction Buffer (8 mM $MgCl_2$), 20U Protector RNase Inhibitor (40U/µl), 1mM Deoxynucleotide Mix (10 mM each) and 10U Transcriptor Reverse Transcriptase (20 U/µl). The last steps of the cDNA synthesis were then performed at 25°C for 10 minutes and 50°C for 60 minutes. The Transcriptor Reverse Transcriptase was inactivated by incubation at 85°C for 5 minutes and the reaction was stopped by incubation at 4°C. The cDNAs were then stored at -20°C until required.

Quantitative real time PCR (qRT-PCR) was performed as described previously (Zerbini et al., 2003). SYBR® fast qPCR master mix (2×) universal (Kapa Biosystems, South Africa), forward and reverse primers and molecular grade water (Sigma-Aldrich, Germany) were used to amplify cDNA. The SYBR® fast qPCR master mix (2×) universal contained SYBR® Green I fluorescent dye, $MgCl_2$, dNTPs, and stabilizers. One microlitre of cDNA in a final volume of 20µl was added to low-profile 96 well cell culture plates (SPL Life Sciences, Korea). The conditions for PCR were: 1 cycle of 5 minutes at 94°C; 45 cycles of 30 seconds at 95°C, 1 cycle of 30 seconds at 56°C, and 1 cycle of 30 seconds at 72°C. Melting curve genotyping: 15 seconds at 95°C, 2 minutes at 65°C, 97°C (continuous acquisition, 5 acquisitions per °C) was then performed. For each run, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize each sample and the primers for GAPDH were also used to quantify the amount of cDNA in a reference sample. A single point from a human GAPDH serial dilution performed previously was used as a reference sample for the absolute quantification of the amplified DNA i.e. gene expression based on a standard curve. All of the samples were run in triplicate and the final presentation of the data occurred as a ratio of gene of

interest to GAPDH. The sequences of the primers used were: DCUN1D1, 5'-TCTGTGATGACCTGGCACTC- 3' (sense) and 5'-GCCATCCATGAACTCCTGTT-3' (anti-sense) (MWG Biotech Incorporated, USA); GAPDH, 5'-GTCTTCACCACCATGGAGAA-3' (sense) and 5'-ATCCACAGTCTTCTGGGTGG-3' (anti-sense) (IDT Technology, USA).

2.1.4 Western blot analysis

Cell lysate preparation

Six well tissue culture plates (Cellstar, Greiner Bio-one, Germany) were used to seed 3×10^5 cells/ well. The cells were harvested and counted as described above, they were then incubated and allowed to attach for 16 hours. Following the necessary treatment with drugs or reagents, the total protein was extracted using 1×cell lysis buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton-X 100, 2.5mM $Na_4P_2O_7$, 1mM beta-glycerphosphare, 1mM Na_3VO_4 and 1µg/ml leupeptin]. The cell lysis buffer was supplemented with protease (Roche, Germany) and phosphatase inhibitors (Roche, Germany) in order to prevent degradation of the proteins following cell lysis. The procedure involved removal of media followed by washing of the cells with 1× PBS pH 7.4, on ice. Whole cell lysates were then collected in sterile 1.5ml tubes following scraping of the attached cells using cell scrapers (Greiner Bio-one, Germany), on ice. The proteins were then separated from the cell debris by centrifugation at 16 000×g for 2 minutes and transferred to sterile 1.5ml tubes.

Protein quantification

Prior to use, the protein level in each sample were quantified using the Bradford reagent protein assay, involving standard curve preparation and extrapolation of protein concentration from the equation of the line. The standard curve was prepared using serial dilutions of bovine serum albumin protein solutions 0.5µg/ml, 0.375µg/ml, 0.25µg/ml, 0.1µg/ml and 0.05µg/ml, where 10µl of each was added to the wells of an EIA-RIA 96 well plate (Costar, Corning Incorporated, USA). Either 1µl or 0.5µl of the cell lysate was then used to determine the concentration of protein in the sample. Since the cell lysate is transparent, 200µl of 1× Protein Assay Dye Reagent Concentrate (Bio-Rad, Germany) was added to each sample so that Coomassie Brilliant Blue G-250 dye within the concentrate could bind the proteins in the samples and the differences in colour changes due to variation in protein concentration could be measured based on their absorbance at 595nm. These absorbance readings were then used to calculate the volume of protein necessary to load 100µg of the protein. The protein was mixed with 1×loading buffer [4× Tris-HCl/SDS pH 6.8, 30% glycerol, 1% SDS, 10% (v/v) β-mercaptoethanol, 0.012% (w/v) bromophenol blue, dH_20] and electrophoresed in a 12% SDS-polyacrylamide gel consisting of 12% separating gel [Acrylamide/Bis-acrylamide (30%/0.8% w/v) (Sigma-Aldrich, USA), 1.5M Tris-HCl pH 8.8, dH_20, 10% (w/v) ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, USA)] and 3.9% acrylamide stacking gel [Acrylamide/Bis-acrylamide (30%/0.8% w/v) (Sigma-Aldrich, USA), 0.5M Tris-HCl pH

6.8, dH_20, 10% (w/v) ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, USA)]. The electrophoreses was performed in 1× running buffer [1% SDS, 0.25M Tris pH 8.3, 1.92M glycine and dH2O]. A protein ladder (Precision plus protein prestained standards, Biorad, USA) was used to keep track of protein separation.

Protein electrophoreses, membrane electroblotting and antibodies

The proteins were electroblotted onto a 0.2µm nitrocellulose membrane (Bio-rad, Germany) using 1× transfer buffer [0.25M Tris pH 8.3, 1.92M glycine, 1% methanol (Kimix Chemical Lab Suppliers, South Africa) and dH2O]. Following this, the membrane was incubated in 5% fat-free milk (Clover, South Africa) dissolved in 1×PBS/0.1%Tween 20 (Sigma-Aldrich, USA) for 1 hour. The membrane was then probed using primary anti-bodies dissolved in 5% fat-free milk (Clover, South Africa) and 1×PBS/0.1%Tween 20 (Sigma-Aldrich, USA) for 16 hours. The membrane was washed with 1×PBS/0.1%Tween 20 (Sigma-Aldrich, USA) (3 times for 10 minutes, with shaking) to remove excess antibodies. It was then incubated with a horseradish peroxidise (HRP) conjugated secondary antibody dissolved in 5% fat-free milk (Clover, South Africa) and 1×PBS/0.1%Tween 20 (Sigma-Aldrich, USA) for 1 hour. Excess secondary antibody was also removed by washing of the membrane in 1×PBS/0.1%Tween 20 (Sigma-Aldrich, USA) (3 times for 10 minutes, with shaking). The bound antibodies were detected by chemiluminescence using chemiluminescence substrate solutions A and B (1:2) (LumiGloReserve, KPL Incorporated, USA) and the membrane was visualized using the UV transilluminator (Biospectrum 500 Imaging System, UVP, UK) and Visionworks LS Acquisition analysis software. The primary antibodies used were mouse monoclonal anti-DCUN1D1 (Santa Cruz Biotechnology Incorporated), rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology Incorporated) and mouse monoclonal anti-β tubulin (Santa Cruz Biotechnology Incorporated). The secondary antibodies used were goat anti-rabbit IgG HRP conjugates (Biorad, USA) and goat anti-mouse IgG HRP conjugates (Biorad, USA).

2.2 Determination of the relevance of DCUN1D1 for: proliferation, migration, apoptosis, and tumour formation *in vivo*

2.2.1 Generation of DCUN1D1 knockdown cell lines

In order to knockdown DCUN1D1 expression in DU145 and PC3 cells, short hairpin RNA (shRNA) was used to express small interfering RNA (siRNA) specific to DCUN1D1 in DU145 (DU145 DCUN1D1-/-) and PC3 (PC3 DCUN1D1-/-) cells. Lentivirus vectors for human DCUN1D1 (TRCN0000134715, Mission Lentiviral Transduction Particles, Sigma-Aldrich, USA) was used and green fluorescent protein (GFP) lentivirus vector was used as a control. DCUN1D1 lentivirus vector (2.3×10^7 MOI) in DMEM (Gibco, Life Technologies, USA) was added to 70-80% confluent DU145 and PC3 cells and allowed to transduce. Twenty four hours post transduction the cells were washed, collected using 0.05% Trypsin/EDTA (Gibco, Life Technologies, USA) and resuspended in complete

media. Cells expressing the DCUN1D1 or GFP lentiviral vector were selected for puromycin (Gibco, Life Technologies, USA) resistant growth in media supplemented with puromycin (Gibco, Life Technologies, USA). The concentration of puromycin used to ensure the selection of the resistant clones was determined to 1μg/ml.

2.2.2 MTT Proliferation Assay

Ninety six well tissue culture plates (SPL Life Sciences, Korea) were used to seed 2×10^3cells/ well. Following drug treatment or reagent addition, 10μl yellow thiazoyl blue tetrazolium bromide (MTT) (5mg/ml) dissolved in sterile 1×PBS pH7.4 was added to the cells and incubated for 4 hours, in order quantify cell proliferation. The yellow MTT solution was converted into dark blue/purple formazan crystals by mitochondrial dehydrogenases of live actively metabolising cells and was solubilised following the 4 hour incubation by the addition of 100μl of solubilisation reagent (10% SLS, 0.01M HCl in dH2O) and incubation for 16 hours. Following this, the absorbance at 595nm was measured using a Multiskan FC microplate photometer.

2.2.3 Quantification of cell migration using transwell chambers

Cell migration was tested using a modified transwell chamber migration assay (8μm pore size membrane, BD Biosciences). DU145 and DU145 DCUN1D1-/- ($2{,}5x10^5$ cells) were seeded in serum-free medium into the upper chamber and allowed to migrate toward 10% FBS as a chemoattractant in the lower chamber for 16 hours. Cells in the upper chamber were carefully removed using cotton swabs, and cells at the bottom of the membrane were fixed with ice-cold methanol and stained with DAPI. Ten different fields were randomly selected and the cells were counted in a fluorescence microscope (Karl Zeiss).

2.2.4 Apoptosis Assay

Twelve or six well tissue culture plates were used to seed 1×10^5cells/ well. Following attachment and 24 hour drug treatment of the cells, 1×10^5 cells were harvested from each sample and transferred to 1.5ml tubes. The cells were then collected following centrifugation at 3000×g for 2 minutes, following which they were incubated in 200μl lysis buffer for 30 minutes at 23°C. The lysate was then centrifuged at 200×g for 10 minutes in order to collect mono and oligonucleosomes enriched in the cytoplasm following apoptosis induction. These were present in the supernatant, from which 20μl was removed and added to the microplate. This was followed by addition of 80μl of immunoreagent [anti-histone-biotin antibody, anti-DNA-POD (anti-DNA-horse radish peroxidase) and incubation buffer)] to the microplate which was covered with adhesive cover foil and incubated with shaking at 100rpm for 2 hours at 23°C. Streptavidin on the microplate binds anti-histone-biotin which binds the exposed DNA-bound histones in the sample. DNA within the histone-nucleosome is then bound by anti-DNA-POD. After the incubation period, the solution was removed by tapping on a water towel

and rinsed carefully, 3 times with 300µl incubation buffer. One hundred microlitres of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution was then added to initiate colour development and quantify apoptosis under the different experimental conditions. The reaction was then inhibited by the addition of 100µl ABTS stop solution. The absorbance at 405nm and the background absorbance at 492nm were measured, where high absorbance rates indicated high apoptosis induction. This procedure was performed as described in the Cell Death Elisa Plus kit (Roche, Germany).

2.2.5 Subcutaneous implantation of MF1 nude mice

Eight week old MF1 beige mice were bred in the University of Cape Town (UCT) animal facility under pathogen free environmental conditions. Immediately prior to implantation of DU145 cells, the cells were infected with lentivirus shRNA against DCUN1D1 and control GFP. They were harvested using 0.05% Trypsin/EDTA (Gibco, Life Technologies, USA) and resuspended in DMEM (Gibco, Life Technologies, USA), containing 10% FBS (Biochrom AG, Berlin) and 1% penicillin (5000µg/ml)/streptomycin (500µg/ml) (Lonza, Walkersville MD, USA). The trypan blue stain 0.4% (Molecular probes, Life Technologies, USA) was used to measure cell viability in order to implant >90% viable cells into the animals. Briefly, DU145 PCa cell lines and DU145 DCUN1D1 knockdown cell lines were implanted through subcutaneous injection into MF1 beige mice on the first day (day 0). The cells ($2x10^6$) were dissolved in 100µl of 1×PBS pH7.4 and injected using a 30 gauge needle over the flank of each animal and proper inoculation of the cell suspension was indicated by blebbing under the skin. All of the procedures were performed under aseptic conditions. Ten animals were used per group and tumour progression was monitored on a weekly basis. The animals were then sacrificed at the completion of the experiment (2 months later) where the tumours were excised, weighted and the animal was examined for possible distal metastasis. All of the procedures with the animals were reviewed and approved by the UCT Faculty of Health Sciences Animal Research Ethics committee. (See Appendix 8.5)

2.3 Determination of DCUN1D1 signalling pathways in microarray analysis.

In order to determine the role of DCUN1D1 on genome wide PCa gene expression we infected DU145 cells with shRNA DCUN1D1 and GFP 16 hours post seeding. The cells were then incubated for 24 hours following which the media was replaced and the cells were allowed to proliferate for 48 hours. Total RNA was then extracted from the cells using Qiashredder (Qiagen, Valencia, CA, USA) and the RNeasy mini kit (Qiagen, Valencia, CA, USA) as described in section 2.1.3 and converted into cRNA as prescribed by the manufacturer (Affymetrix). The experiments were done in duplicates.

The microarray analysis was performed at the Genomics, Proteomics, Bioinformatics and Systems Biology Centre at the Beth Israel Deaconess Medical Centre. The Affymetrix Human Transcriptome Array U133AAofAv2 GeneChips was used for gene transcript profiling of more than 30 000 probe

sets. Following generation of clean, intact RNA, 100ng total RNA was converted into complementary RNA (cRNA) according to the Affymetrix GeneChip WT Plus Reagent kit protocol. This involved conversion to double-stranded cDNA, *in vitro* transcription and labeling to synthesize amplified biotin-labelled antisense mRNA or cRNA. The cRNA, which is the reagent for microarray analysis, was then fragmented into 50-200 base pair fragments and hybrized onto the WT array (Affymetrix). The hybridization allowed for binding of the cRNA to specific probe pairs and gene transcript recognition. Excess cRNA was washed off, the array fluorescently stained and the stain amplified to produce different colour arrays. Confocal laser scanning was used to quantify the fluorescent levels, distribution patterns and subsequent transcript levels. Control samples were used at each step. Bioconductor affyQCReport software was used to determine the quality of the chip, Robust Multi-array Average (RMA) algorithms were used for background correction, normalization and summarization of signal values. The scanned array image values were cross validated using model-based expression analysis and statistical analysis by dChip (Zerbini et al., 2006). The samples were checked for reproducibility using correlation and signal-to-noise ratio (SNR) methods for replicate arrays. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to identify Gene Ontology (GO) categories overrepresented in differentially expressed genes. The commercial systems biology package Ingenuity Pathways Analysis (IPA) was used to analyze the interactive networks identified. It calculates the p value for each network and compares the fit of our data to the profiles in the IPA database. The results are displayed as a –log p value score which indicates the likelihood of a gene being found in the network of a pathway by chance. Low p values indicate high confidence that data was not generated by random chance alone. The analysis will provide insight into statistically significant, highly affected (over-represented) GO biological processes/functions and canonical pathways among the predicted microarray target genes and this will help in determining the phenotypic changes observed.

2.4 Connectivity map database analysis

2.4.1 Identification of drugs for *in vitro* analysis

The connectivity map database was used in order to identify DCUN1D1 specific inhibitors of PCa. The database allowed us to employ a genomics approach to drug discovery by using the gene expression signature obtained in the microarray analysis described above to find drugs. The list of up and downregulated genes obtained was uploaded onto the database according to the probe set number of the genes as described in the Affymetrix GeneChip array and based on fold change in gene expression (>2 fold change). The input gene query signature and the spectrum of profiles available in the database were then compared. The output produced consisted of compounds listed according to their enrichment scores and p values, which indicated the correlation between the compound and the input query signature. The enrichment scores range between +1 and -1 indicating a positive or

negative correlation with the query signature profile. A zero or 'null' score was also allocated which indicates no alteration following expression of the query signature. The compounds, in this case the drugs, with a positive correlation were selected for *in vitro* analysis. The top 120 compounds with a positive correlation to our query signature were identified and 30 of these drugs were purchased for analysis. Some of these drugs were under patent restrictions or were not commercially available therefore the drugs finally tested in the study were the highest scoring drugs commercially available.

2.4.2 Drugs

The final concentrations, catalog number and names of the drugs used in the study were: anisomycin (6.25μM) (A9789), monensin (12.5μM) (M5273), lasalocid (7μM) (33339), podophyllotoxin (6.25μM) (P4405), thapsigargin (10nM) (T124), lycorine (12μM) (L5139), econazole (9μM) (E4632), biperiden (11μM) (B5311), eticlopride (11μM) (E101), cyclobenzaprine (13μM) (C4542), guanabenz (14μM) (G110), sulfadimethoxine (12.5μM) (S7007), vinblastine (100nM) (V1377), clorsulon (12.5μM) (33973), trihexyphenidyl (12.5μM) (T1516), E. fumarate (12.5μM) (E100), colistin (3.125μM) (C4461), naringenin (12.5μM) (W530098), acemetacin (12.5μM) (A1674), chenodeoxycholic acid (12.5μM) (C9377), etiocholanolone (12.5μM) (E5126), folic acid (12.5μM) (F7876), guanabenz (12.5μM) (G110), terazosin (12.5μM) (T4680), dapson (12.5μM) (46158), isocarboxazid (12.5μM) (CDS020459), pyrithyldione (25μM) (R279072), nadolol (13μM) (N1892), vigabatrin (31μM) (V8261), gentamicin (12.5μM) (G1272) and josamycin (6.25μM) (59983). All of the drugs were obtained from Sigma-Aldrich, France. The control used was 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, France).

Combination studies were performed with monensin (1μM and 2μM) and podophyllotoxin (15nM and 30nM). 0.1% DMSO (Sigma-Aldrich, France) was used as a vehicle control.

2.4.3 Calculating IC50s using GraphPad Prism version 5.01

The MTT proliferation assay was performed to determine the dose dependent effect of the drugs on PCa cell proliferation. The concentrations of monensin and podophyllotoxin used in the combination study were based on the $IC50_s$ of each drug and the minimum concentration to inhibit proliferation monensin (1μM, 2μM) and podophyllotoxin (15nM, 30nM). Each experiment was performed in triplicate then GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California, USA) was used to construct a sigmoidal dose response curve (variable slope) and non-linear regression was used as a fit for the data.

2.4.4 Isobologram

In order to analyse the combined effect of monensisn and podophyllotoxin treatment on PCa, we performed an isobologram using Calcusyn software (Biosoft). The isobologram is a graphical representation of the interaction between two drugs and is formed by plotting the individual drug

doses required to achieve a single agent effect on their respective x and y axes, drawing a line to connect the two points (known as the line of additivity) and plotting the concentrations of the two drugs used in combination to achieve the same effect on the isobologram. Combination data points that fall on the line represent an additive interaction, whereas points above or below represent antagonism or synergy, respectively.

2.4.5 Statistical analysis: Paired t-test

The proposal investigated in this study was whether DCUN1D1 plays a role in PCa development and progression. Therefore the methodology used, as described in this section, was aimed at establishing whether there was any significant difference in DCUN1D1 expression in human PCa cell lines or human tissue samples relative to normal samples and whether this difference contributed to the growth of prostate tumours. It also aimed to determine if the drugs identified as DCUN1D1 inhibitors using the cmap database significantly inhibited PCa growth in a DCUN1D1-dependent manner. Therefore, our investigations involved comparisons of two data sets including a group representing a reference standard and a group representing the experimental conditions. The averages (means) of the 2 data sets were compared; therefore we used the paired t-test in order to determine if the differences we observed in the experiments were statistically significant. We chose this model because we were comparing two paired/matched data sets by calculating their means and calculating the standard deviation as a measure of variability within each data set. In addition, because we were not only interested in whether there was an association between the two data sets but also the nature of the association, higher or lower, we chose the two tailed distribution.

Therefore with a null hypothesis stating that there is no difference between the matched pairs, based on two-tailed normal distribution and a 95% confidence interval, we used the paired two sample t-test for statistical analysis of our data. A p-value less than 0.05 was considered statistically significant.

Chapter 3: Results

3.1 Differential expression of DCUN1D1 in prostate cancer.

3.1.1 DCUN1D1 is upregulated in human prostate cancer cell lines.

DCUN1D1 is deregulated in several different types of cancers including head and neck and lung carcinomas (Sarkaria et al., 2006). However, its expression level and its role in PCa are unknown. In order to evaluate DCUN1D1 expression in PCa, we screened a variety of PCa cell lines. Using quantitative real time PCR and western blot analysis, four androgen dependent (CW22, CW19, LNCaP and VCaP), four androgen independent PCa cell lines (DU145, DUCaP, PC3 and CL1) and a normal epithelial prostate cell line, PREC were evaluated for DCUN1D1 mRNA and protein expression. This includes CL1, a clonal cell line, which was generated upon conversion of the androgen-sensitive LNCaP cells to an androgen-insensitive state by the removal of androgen in the media. We observed upregulation of DCUN1D1 especially in the androgen independent DU145, DUCaP, PC3 and CL1 PCa cell lines relative to normal cell lines (Figure 7A and B). Quantitative real time PCR analysis showed DCUN1D1 mRNA expression fold induction of 3-4 folds in the androgen independent PCa cell lines with lesser levels in the androgen dependent cell lines. In addition, within the androgen dependent cell lines the cell line representative of more aggressive PCa, VCap, showed higher levels of DCUN1D1 expression. LNCap also showed DCUN1D1 upregulation. Western blot analysis showed similar alterations in the protein levels of DCUN1D1 with DU145, PC3, DUCaP, CL1, VCaP and LNCap showing significant upregulation of DCUN1D1 protein. These findings indicate that DCUN1D1 is upregulated in PCa cells and may play a role in PCa.

3.1.2 DCUN1D1 is upregulated in human prostate cancer tissue.

In order to corroborate the observations made in tissue culture cell lines, we evaluated DCUN1D1 expression levels in human tissue samples. A commercially available panel of 96 human tissue samples (OriGene TissueScan Prostate Cancer Tissue Array I and II) was used which contains cDNA from adenocarcinoma of the prostate, BPH, normal tissue and tissue from other disease controls (bladder carcinoma tissue). Quantitative real time PCR analysis of DCUN1D1 expression found that 42% of the adenocarcinoma tissue samples stages I, II and III showed upregulation of DCUN1D1 relative to normal tissue (Table 1). In addition, immunohistochemistry analysis of DCUN1D1 expression using anti-DCUN1D1 antibody in a separate cohort of 16 human tissue samples was performed with the assistance of Dr Yihong Wang at Brown University, USA. Seven out of the 16 (43.7%) samples showed positive staining for DCUN1D1 (Figure 8). These data provide further evidence for the possible role of DCUN1D1 in PCa.

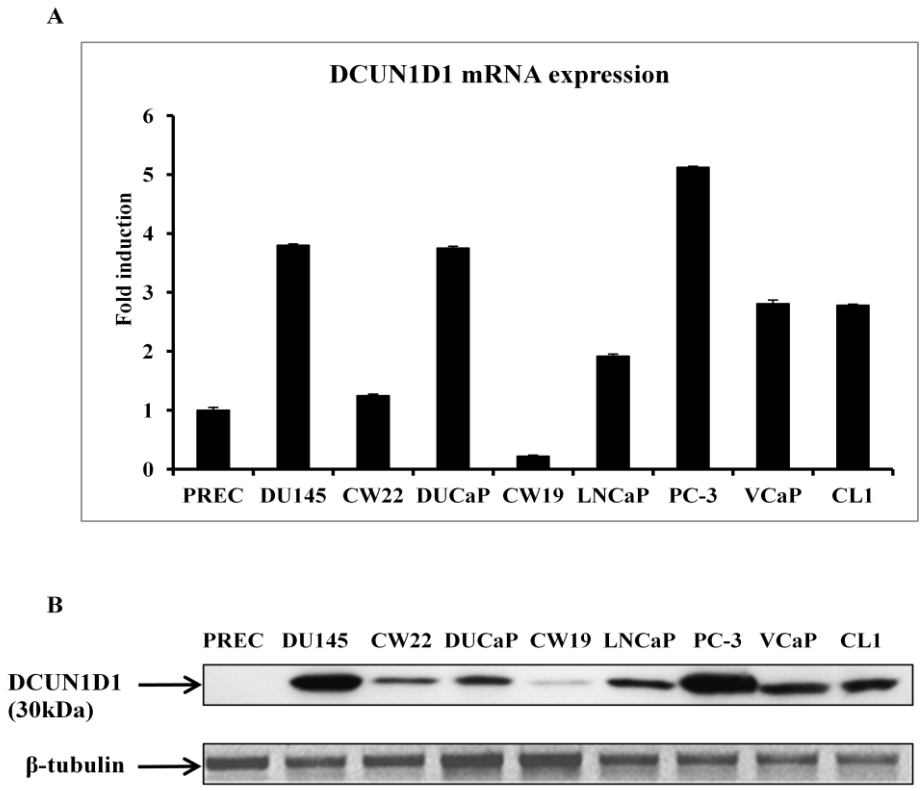

Figure 7. Regulation status of DCUN1D1 in prostate cancer cell lines. A) Quantitative real time-PCR analysis of DCUN1D1 in prostate cancer cell lines. Total RNA was collected from PREC (normal), DU145, DUCaP, PC3, CL1 (androgen independent) and CW22, CW19, LNCaP, VCaP (androgen dependent) cells. Normalization of each sample was carried out by measuring the amount of β-tubulin complementary DNA. B) Western blot analysis of DCUN1D1 expression in the same prostate cancer cell lines. DCUN1D1 expression was probed with anti-DCUN1D1 antibody. Normalization of each sample was carried out by measuring the amount of β-tubulin.

Table 1. Real time PCR analysis of DCUN1D1 gene expression in human prostate cancer tissue.

Tissue type	Number of upregulated samples/number of samples (%)	Fold induction compared to normal prostate tissue
Adenocarcinoma of prostate (stages I, II and III)	29/69 (42%)	2 to 2000
BPH	0/10 (0 %)	0
Carcinoma of bladder, transition tissue	0/2 (0 %)	0

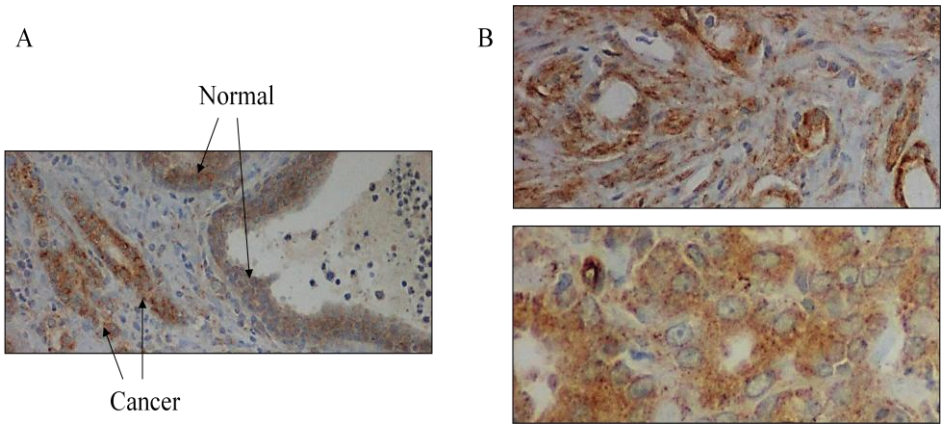

Figure 8. DCUN1D1 is upregulated in prostate cancer tissue. Immunohistochemical staining of prostate cancer biopsies. A) Staining of DCUN1D1 in normal and prostate cancer tissue (magnification 10X). B) DCUN1D1 immunohistochemical staining of prostate cancer tissue (Magnification 20X).

3.2 Blockage of DCUN1D1 inhibits proliferation and migration of prostate cancer cells.

DCUN1D1 overexpression has previously been implicated in proliferation and migration of cancer cells (O-charoenrat et al., 2008; Broderick et al., 2010), therefore we explored these effects in PCa. In order to establish the relevance of DCUN1D1 in PCa proliferation and migration, we generated DU145 and PC3 cells with the DCUN1D1 gene knocked down (DU145 DCUN1D1 -/- or PC3 DCUN1D1-/-) using a lentivirus vector (Mission Lentiviral Transduction Particle, Sigma-Aldrich) specific to DCUN1D1 and green fluorescent protein (GFP) (DU145 or PC3), as the control. Infection of DU145 and PC3 with DCUN1D1 shRNA lentivirus (LV-shRNA) resulted in 95% reduction of DCUN1D1 expression confirmed at the mRNA and protein levels using quantitative real time PCR and western blot analysis respectively, (Figure 9). Inhibition of DCUN1D1 strongly reduced proliferation of DU145 and PC3 PCa cells (Figure 10). Additionally, DCUN1D1 knockdown strongly reduced the migratory capabilities of DU145 and PC3 cells after 24h hour incubation; when compared to DU145 and PC3 cells infected with LV-shRNA GFP. These results demonstrate that DCUN1D1 is an important regulator of PCa cell proliferation and migration (Figure 10).

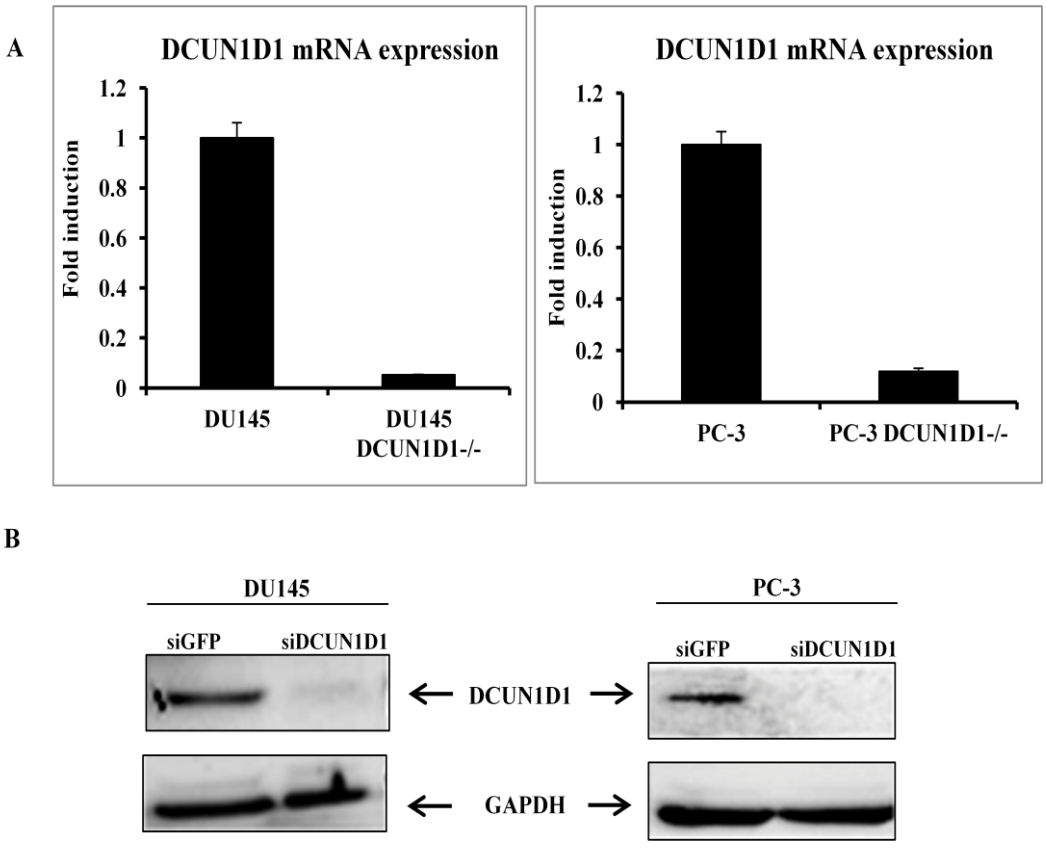

Figure 9. Blockage of DCUN1D1 in DU145 and PC3 prostate cancer cells lines. Prostate cancer cells were infected with lentivirus encoding shRNA against GFP and DCUN1D1. A) Real time-PCR analysis of DCUN1D1 in prostate cancer cell lines. Total RNA was collected from DU145 and PC3 cell lines and normalized by measuring the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) complementary DNA. B) Western blot analysis of DCUN1D1 expression in DU145 and PC3 cell lines. DCUN1D1 expression was probed with anti-DCUN1D1 antibody and normalized by measuring the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

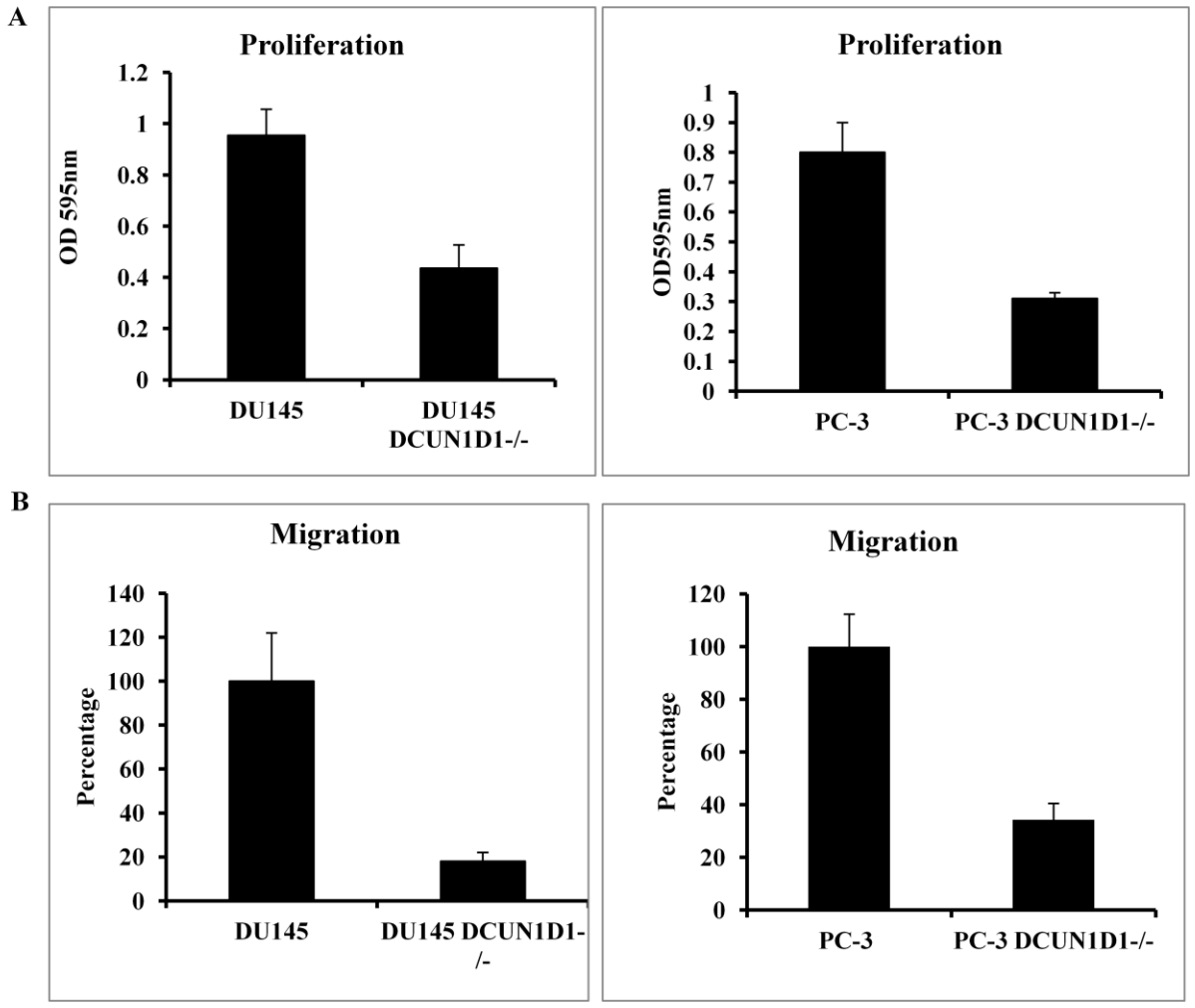

Figure 10. Blockage of DCUN1D1 expression inhibits proliferation and migration in prostate cancer cell lines. DU145 and PC3 cell lines were infected with lentivirus encoding shRNA against GFP and DCUN1D1. A) Proliferation of DU145 and PC3 48h post-infection. B) Migration of DU145 and PC3 cell lines was measured 48 hours post-infection in transwell plates. Data means ±s.d. of triplicate independent experiments.

3.3 Inhibition of DCUN1D1 leads to the induction of apoptosis in prostate cancer cells.

We used the same siRNA approach to determine whether blockage of DCUN1D1 would induce apoptosis in DU145 PCa cells. The Cell Death Elisa Plus kit (Roche, Germany) was used to quantify the induction of apoptosis in DU145 and DU145 DCUN1D1-/- cell lysates. Blockage of DCUN1D1 resulted in the induction of apoptosis when compared to cells infected with control siRNA (Figure 11).

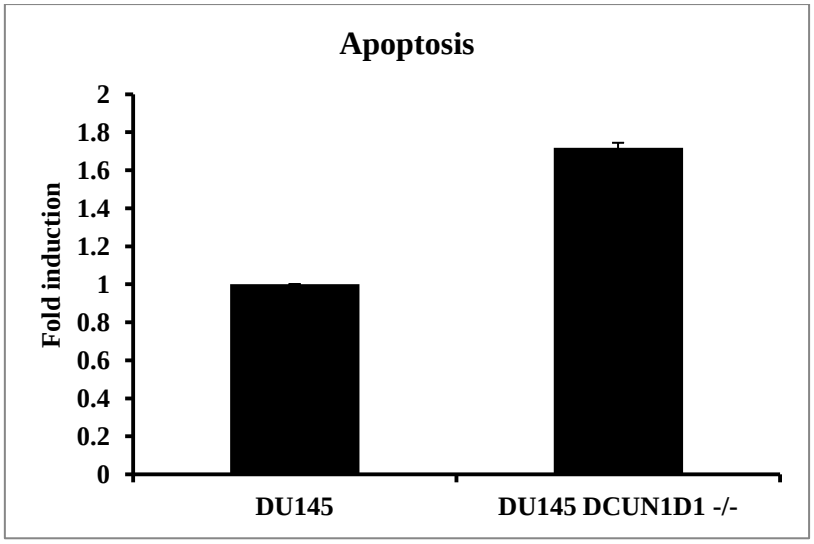

Figure 11. Blockage of DCUN1D1 expression induces apoptosis in prostate cancer cell lines. DU145 cell lines were infected with LV-shRNA DCUN1D1 (DU145 DCUN1D1-/-) and LV-shRNA GFP (DU145) as the control. The Cell Death Elisa Plus kit was used to quantify apoptosis 24 hours post-seeding of the cells. Data means ±s.d. of triplicate independent experiments.

3.4 DCUN1D1 is essential for *in vivo* prostate cancer tumour growth.

Having established the importance of DCUN1D1 for *in vitro* PCa growth, we determined whether inhibition of DCUN1D1 affects tumour formation in MF1 nude mice. Using xenograft mice models, DU145 cells infected with DCUN1D1 shRNA lentivirus (DU145 DCUN1D1-/-) and shGFP (DU145) were subcutaneously implanted into eight week old male MF1 mice and 2 months later, the mice were sacrificed, examined for tumour formation and the tumour weighted. As observed in figure 12, in contrast to the control cells, blockage of DCUN1D1 significantly reduced tumour growth with a 58% reduction in tumour weight observed. This has not been observed previously and it clearly indicates the importance of DCUN1D1 for PCa growth.

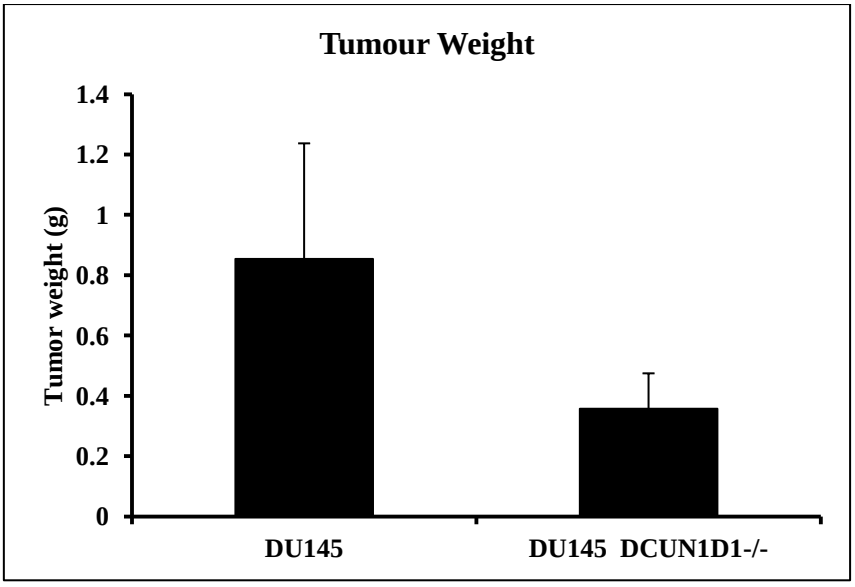

Figure 12. Inhibition of DCUN1D1 reduces tumour formation in MF1 nude mice. DU145 cells (2×10^6) infected with LV-shRNA DCUN1D1 (DU145 DCUN1D1-/-) or LV-shRNA GFP (DU145) were implanted subcutaneously into MF1 nude mice. The tumour weight was measured 2 months after implantation. Values are represented as mean±s.d. of ten individuals.

3.5 Microarray analysis of DCUN1D1 knockdown in prostate cancer cells.

DCUN1D1 has been shown to perform E3 ligase activity in cullin neddylation. However, apart from identifying direct binding of DCUN1D1 and cullin 1-5, no other proteins have been identified as DCUN1D1 substrates and little is understood about its activity (Kim et al., 2008). The main objective in performing the microarray analysis was to understand which biological pathways are being regulated by DCUN1D1 in PCa and which genes are affected by DCUN1D1 expression, in order to obtain a DCUN1D1 -/- gene signature for use in the connectivity map database for the identification of DCUN1D1-dependent inhibitors of PCa. RNA from DU145 cells infected with LV-shRNA DCUN1D1 specific sequences were hybridized to Affymetrix HT U133AAofAv2 GeneChips which contain more than 30 000 probe sets. DCUN1D1 knockdown resulted in the downregulation of 244 genes and the upregulation of 78 genes (≥2 fold change). DCUN1D1 and related analogues have been associated with development and developmental pathways (Kurz et al., 2005; Huang et al., 2011) and similarly, functions associated with development were deregulated upon DCUN1D1 knockdown in DU145 PCa cells. Figure 13 and 14 show the top 10 functions and pathways predicted to be deregulated upon DCUN1D1 knockdown. Gene expression, cellular growth and proliferation, 6 development, cell death and cancer related functions were deregulated. Concurrently, axonal guidance, TR/RXR activation, semaphorin, Wnt/β-catenin, sonic hedgehog signalling pathways were identified which suggests the pathways mediating the developmental functions. PKA, cyclins and cell cycle related pathways were also predicted to be deregulated which suggests the possible mechanisms behind the cellular growth and proliferation dysregulation. Glycolytic and transcriptional pathways

similar to the maturity onset diabetes of young (MODY) pathway were also among the top 10 pathways deregulated. These alterations appear to result in the phenotypic deregulation of the molecular mechanisms of cancer. In addition, the predicted functions and pathways are also reflected in the top 10 up and downregulated genes following DCUN1D1 knockdown (Table 2) (See Appendix 8.1 and 8.2 for top 50 up and downregulated genes). Interesting, among these genes are *UBE2C* and *UBE2J2* which are up and downregulated respectively following DCUN1D1 knockdown. These genes are both E2 ubiquitin conjugating enzymes which also suggests the deregulation of the ubiquitination pathway (Townsley et al., 1997; Oh et al., 2006). Therefore, DCUN1D1 knockdown appears to significantly alter the genetic profile of PCa cancer.

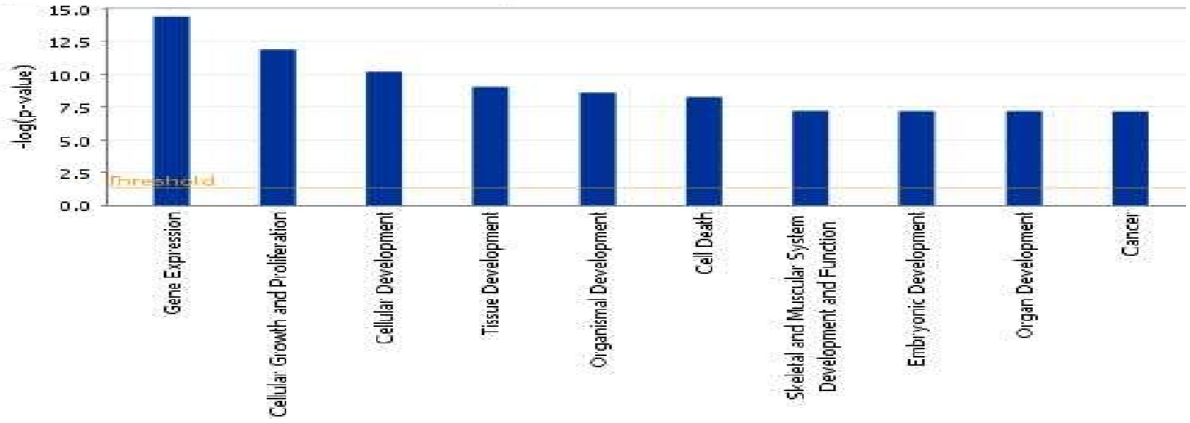

Figure 13. Bioinformatics analysis of the top 10 functions deregulated following DCUN1D1 knockdown in DU145 prostate cancer cells. The list of genes up and down-regulated in DU145 cells expressing the DCUN1D1 shRNA were imported into the Ingenuity Pathway Analysis tool and functions affected were determined. Output represents top 10 deregulated functions expressed as –log (p-value).

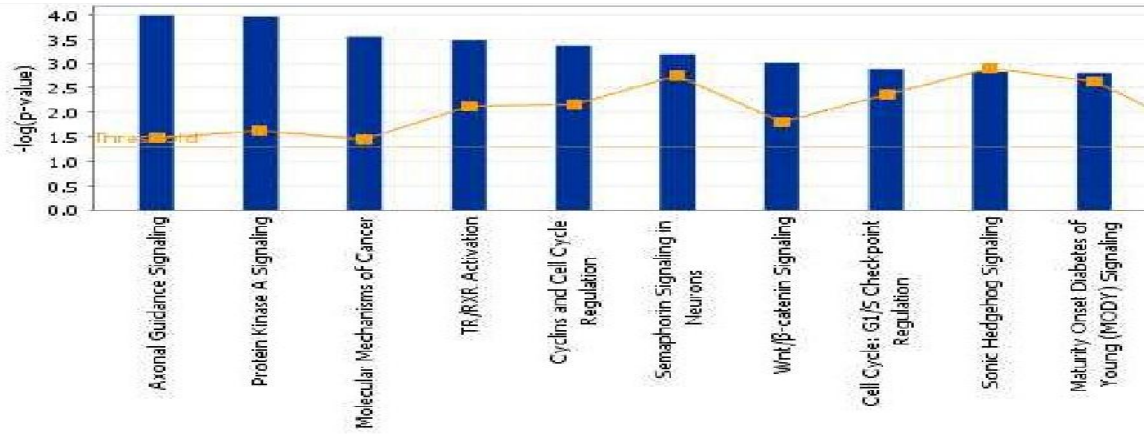

Figure 14. Bioinformatics analysis of the top 10 pathways deregulated following DCUN1D1 knockdown in DU145 prostate cancer cells. The list of genes up- and down-regulated in DU145 cells expressing the DCUN1D1 shRNA were imported into the Ingenuity Pathway Analysis tool and canonical pathways were determined. Output represents top 10 deregulated pathways expressed as –log (p-value).

Table 2: List of top 10 up and downregulated genes after inhibition of DCUN1D1

	Top 10 Upregulated genes				Top 10 Downregulated genes		
	Gene	**Description**	**Fold change**		**Gene**	**Description**	**Fold change**
1	ATP6AP2	ATPase, H^+ transporting, lysosomal accessory protein 2	19.97	**1**	UBE2J2	Ubiquitin-conjugating enzyme E2, J2	-9.5
2	EEF2	Eukaryotic translation elongation factor 2	6.3	**2**	BAT2	HLA-B associated transcript 2	-8.46
3	UBE2C	Ubiquitin-conjugating enzyme 2C	5.9	**3**	ZFPM1	Zinc finger protein, multitype 1	-6.97
4	SPTBN1	Spectrin, beta, non-erythrocytic 1	4.4	**4**	CLDN6	Claudin 6	-6.73
5	BAT2D1	BAT2 domain containing 1	4.04	**5**	TNPO2	Transportin 2	-6.4
6	TNPO1	Transportin 1	3.85	**6**	NMNAT3	Nicotinamide nucleotide adenylyltransferase 3	-5.93
7	SF3B1	Splicing factor 3b, subunit 1, 155kDa	3.68	**7**	EPHB4	EPH receptor B4	-5.38
8	CADM1	Cell adhesion molecule 1	3.59	**8**	ACAA2	Acetyl-Coenzyme A acyltransferase 2	-5.36
9	SH3GLB1	SH3-domain GRB2-like endophilin B1	3.48	**9**	ACAD10	Acyl-Coenzyme A dehydrogenase family, member 10	-5.04
10	HAX1	HCLS1 associated protein X-1	3.24	**10**	FAM109A	Family with sequence similarity 109, member A	-5.01

3.6 Screening of drugs for DCUN1D1 specific anti-prostate cancer activity.

In order to identify compounds that can potentially be used to inhibit PCa growth in a DCUN1D1-dependent manner, we used the connectivity map (cmap) database. As described previously, we performed microarray analysis of DU145 and DU145 DCUN1D1-/- cells and obtained a list of genes up and downregulated upon DCUN1D1 knockdown (>2 fold change). This allowed us to select a gene signature that is more specific to the effect of DCUN1D1 inhibition. Using the probe set number of

these genes as described by Affymetrix Human Transcriptome Array U133AAofAv2 GeneChips we uploaded the gene expression profile into the cmap database and obtained a list of compounds positively or negatively correlated with our query signature. Of the 1309 bioactive small molecules present in the database the pattern matching algorithms identified the compounds based on statistically significant changes represented by p-values and enrichment scores. The enrichment scores ranged between +1 and -1 indicating a positive or negative correlation with the query signature profile. As we are searching for compounds that lead to the same effect as inhibition of DCUN1D1, we focused on compounds with positive enrichment scores (i.e. positive correlation with the profile obtained upon DCUN1D1 knockdown under our experimental conditions). In our first analysis, we selected the top 120 small molecule compounds with a highly significant positive score to our query signature. Nadolol had the highest positive score of 0.911 while vinblastine the compound ranked 30th on our list had a positive score of 0.727. Some of the compounds identified are under patent restriction or were not commercially available therefore; we were able to obtain 30 drugs (listed in Table 3) and performed *in vitro* analysis on them. The compounds used in this study include microtubule inhibitors, protein and DNA synthesis inhibitors, ion channel inhibitors, neurotransmitter agonists/antagonists, anti-inflammatory drugs, antibiotics, an amino acid/ nucleotide synthesis promoter, a liver fluke specific drug and a testosterone metabolite (Table 4). We provide a schematic diagram showing the approach we applied in screening the drugs. Briefly, we first determined whether a compound inhibits PCa cell proliferation and/or induces apoptosis of PCa cells, then we evaluated the effect of the drugs in cells lacking DCUN1D1 expression in order to determine whether the anti-cancer activity was dependent on DCUN1D1 expression.

Table 3: 30 drugs identified through the connectivity map database using p-values and score

Drug	Score	P-value	Drug	Score	P-value
Vinblastine	0.73	0.04	Clorsulon	0.82	0.002
Lycorine	0.67	0.01	Biperiden	0.74	0.003
Anisomycin	0.77	0.005	Gentamicin	0.78	0.004
Lasalocid	0.77	0.005	Chenodeoxycholic acid	0.75	0.007
Thapsigargin	0.79	0.02	Isocarboxazid	0.66	0.012
Podophyllotoxin	0.80	0.003	Colistin sulfate	0.72	0.01
Cyclobenzaprine	0.73	0.01	Sulfadimethoxine	0.67	0.01
Monensin	0.79	0.0002	Dapson	0.68	0.008
Folic acid	0.67	0.028	Etiocholanolone	0.71	0.002
Econazole	0.68	0.02	E. fumarate	0.79	0.02
Guanabenz	0.73	0.003	Trihexypheidyl	0.80	0.02
Acetemacin	0.67	0.027	Josamycin	0.74	0.003
Eticlopride	0.75	0.008	Vigabatrin	0.79	0.018
Terazosin	0.68	0.02	Nadolol	0.91	0.00006
Naringenin	0.66	0.03	Pyrithyldione	0.69	0.018

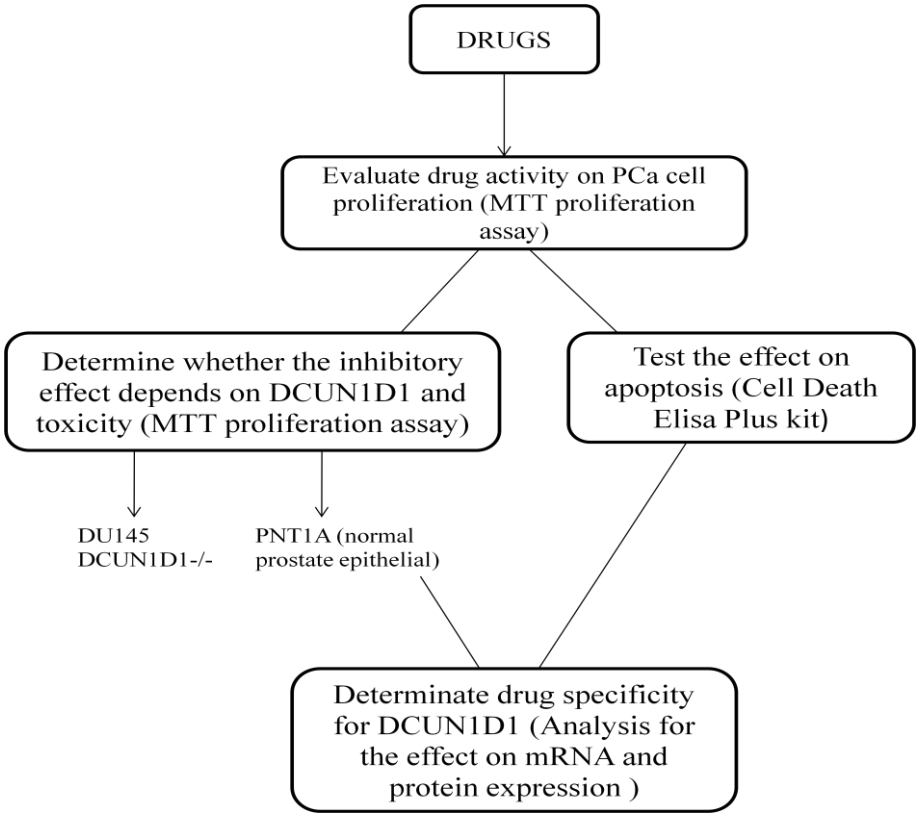

Figure 15. Schematic diagram showing our approach to drug screening.

Table 4: List of drugs identified using cmap database with descriptions of activity and reference articles

Drug	Description	References
Vinblastine	• Alkaloid extracted from *Catharanthus roseus;* inhibits microtubule assembly causing metaphase arrest • Anticancer activity against lympoid, cervical cancer, neuroblastoma and PCa	(Jordan et al., 1991; Jordan et al., 1992; Toso et al., 1993; Vacca et al., 1999; Klement et al., 2000)
Lycorine	• Alkaloid extracted from Amaryllidaceae plants; inhibit protein synthesis, impairs actin cytoskeleton organization; weak inhibitor of acetylcholinesterase activity • Antiviral, antimalarial, antiinflammatory, potent anticancer agent against glioblastoma, NSCLC, oesophageal cancer and melanoma	(Vrijsen et al., 1986; Tanker & Gu, 1998; Sener et al., 2003; Lamoral-Theys et al., 2009; McNulty et al., 2010)
Anisomycin	• Antibiotic isolated from *Streptomyces griseolus;* potent, reversible inhibitor of DNA and protein synthesis*;* pro and antiapoptotic mechanisms • Antiprotozoal; sensitizes PCa cells to apoptosis, anticancer activity against HL60	(Grollman, 1967; Hazzalin et al., 1998; Törocsik & Szeberényi, 2000; Curtin & Cotter, 2002; Stadheim & Kucera, 2002)
Lasalocid	• Antibiotic isolated from *Streptomyces lasaliensis;* carboxylic ionophore; reversible effect on Golgi apparatus • Feed additive to prevent coccidiosis in chickens, horses and cows; toxic to horses and cattle	(Haskell et al., 1965; Somlyo et al., 1975; Jonakait et al., 1979; Hanson & Eisenbeis, 1981; Blanchard et al., 1993)
Thapsigargin	• Sesquiterpene lactone derived from the roots of *Thapsia garganica;* selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), targets calmodulin • Anticancer activity against PCa, NSCLC, glioblastoma cancer but toxicity reported and resistance evident; induces apoptosis even in senescent cells	(Tombal et al., 2000; Kovacs et al., 2005; O'Neill et al., 2006; Linxweiler et al., 2013)
Podophyllotoxin	• Naturally occurring lignan found in Podophyllum plants; inhibits tubulin polymerization to induce cell cycle arrest and apoptosis in cancer cells • Topical gel to treat genital warts, antiviral, anticancer activity	(Jordan et al., 1992; Strand et al., 1995; Imbert, 1998)
Cyclobenzaprine	• Anticholinergic activity • Skeletal muscle relaxant used in the treatment of musculoskeletal pain; structurally similar to tricyclic antidepressants	(Brioschi et al., 2013)
Monensin	• Antibiotic isolated from *Streptomyces cinnamonensis;* carboxylic ionophore that disrupts Golgi apparatus structure and inhibits vesicular transport in eukaryotes; inhibits androgen signalling • Antiprotozoan, antibacterial and antifungal; specific anticancer activity in PCa, lymphoma, lung, colon and renal cancer through cell cycle arrest, oxidative stress and induction of autophagy or apoptosis	(Russell, 1987; Mollenhauer & Morré, 1990; Park et al., 2002; Park et al., 2003; Park et al., 2003; Ketola et al., 2010; Choi et al., 2013)
Folic acid	• Found in fruit and vegetables; required for synthesis of thymidylate, purine nucleotides, serine and methionine • Essential for cell growth and development; contradictory evidence on tumourigenesis	(Huennekens & Duffy, 1987; Butterworth et al., 1992; Bandera et al., 1997; Feigelson et al., 2003)
Econazole	• Imidazole; interacts with mammalian ion channels particularly Ca^{2+} ion channels; affects calcium signalling • Antifungal, antibacterial, antitubercular activity, anticancer activity against PCa, leukemia, breast; evidence of resistant	(Gamberucci et al., 1998; Jan et al., 1999; Hill et al., 2004; Huang et al., 2005; Yu et al., 2008; Slagsvold et al., 2009; Kim et al., 2012)
Guanabenz	• α2-adrenergic agonist; inhibits hepatic cholesterol production, triglyceride synthesis and stimulates fatty acid oxidation • Treatment of hypertension; antiprion activity	(Meacham et al., 1980; Misu & Fujie, 1982; Capuzzi, 1984; Tribouillard-Tanvier et al., 2008)
Acemetacin	• Cyclo-oxygenase inhibitor; prodrug for indomethacin; inhibition of gastric prostaglandin PG synthesis and human leukocyte PG synthesis • Analgesic, non-steroidal anti-inflammatory drug (NSAID); used against rheumatic disease and musculoskeletal disorders	(Chou, 2002; Chávez-Piña et al., 2007; Gil-Flores et al., 2010)
Eticlopride	• Selective dopamine (DA) D2-like receptor inhibitor • Antipsychotic agent but primarily used to study receptor activity in schisophrenia and other brain disorders	(Seeman, 1988; Giuliani & Although, 1995; Giuliani & Ferrari, 1997)

Terazosin	• α1-adrenergic receptor antagonist • Treatment of BPH and hypertension;induces apoptosis in PCa and bladder cancer cells	(Lepor et al., 1992; Itskovitz, 1994; Kyprianou, 2003; Tahmatzopoulos et al., 2005)
Naringenin	• Flavonoid derived from citrus, grapefruits and juices • Antiinflammatory activity; antitumour activity against PCa, breast, stomach and liver cancer	(Kanno et al., 2005; Choi et al., 2007; Knowles et al., 2009; Coelho et al., 2013)
Pyrithyldione	• Psychoactive drug; used as hypnotic or sedative. No longer used	(Becker & Fabing, 1949)
Clorsulon	• Inhibitor of phosphoglycerate kinase and phosphoglyceromutase of *Fasciola* liver flukes • Antiparasitic	(Schulman, 1982; Schulman & Ostlind, 1982; Elitok & Elitok, 2006; Escribano et al., 2012)
Biperiden	• Muscarinic receptor antagonist • Antiparkinsonian; treatment of neuroleptic-induced Tardive dyskinesia, schizophrenia or otherchronic mental illnesses	(Eltze, 1988; Jackisch et al., 1994; Silver & Geraisy, 1995)
Gentamicin	• Antibiotic synthesized by Micromonospora • Inhibits protein synthesis through binding to bacterial ribosome 30S subunit	(Barber & Waterworth, 1966; Weinstein et al., 1967; Buss, 1985)
Chenodeoxycholic acid	• Synthesized mainly from dietary cholesterol; • Treatment of bilary disruptions, gallstones; inhibits inflammation; carcinogenesis	(Bell et al., 1974; Ayaki e et al., 1981; Martin et al., 1981; He et al., 2011; Odunsi-Shiyanbade et al., 2011)
Isocarboxazid	• Monoamine oxidase inhibitor of the brain, heart, and liver; catecholamine transferase inhibitors • Antidepressant; anxiolytic (antianxiety agent)	(Gardner et al., 1960; Joshi, 1961; Barber et al., 1962; Giller et al., 1982)
Colistin sulfate	• Antibiotic isolated from *Bacillus polymyxa* • Active against multidrug resistant organisms; used for cystic fibrosis treatment	(Komura, 1979; Reed et al., 2001; Pitt et al., 2003; Sabuda et al., 2008)
Sulfadimethoxine	• Antibiotic; inhibits bacterial folic acid synthesis • Antibacterial; used in veterinary medicine in the treatment of different species of animals with coccidial (microscopic parasite) infections; antithyroid activity	(Schnitzer et al., 1958; Stowe, 1963; Bridges et al., 1968; Nishikawa, 1983; Mitrovic & Bauernfeind, 2014)
Dapson	• Antibiotic, antiinflammatory • Used primarily for treatment of Dermatitis herpetiformis (leprosy); used in combinations as antimalarial	(Debol & Herron, 1997; Winstanley et al., 1997; Deps et al., 2012)
Etiocholanolone	• Testosterone metabolite; epimer of androsterone a $GABA_A$ receptor modulator and precursor of testosterone in PCa tumours • Anticonvulsant properties, no information on its activity just that it is a metabolite and it's steroidgenesis	(Kaminski et al., 2005)
E. fumarate	• Anticholinesterase; metabolite of physostigmine; • Anticancer activity against mouse neuroblastoma and rat gliomas	(Fürst et al., 1982; Somani et al., 1990)
Trihexyphenidyl	• Muscarinic receptor antagonist • Used in the treatment of neurological movement disorders; antiparkinsonian disease	(Burke & Fahn, 1986; Giachetti et al., 1986; Takahashi et al., 1999)
Josamycin	• Antibiotic produced by *Streptomyces narbonensis.* • Used as antibacterial agent	(Strausbaugh et al., 1976; Privitera & Bonino, 1984; See et al., 2010)
Vigabatrin	• Irreversibly inhibits gamma aminobutyric acid (GABA) transaminase • Antiepileptic drug; used in the treatment of infantile spasms	(Lippert et al., 1977; Tartara et al., 1986; Elterman et al., 2001)
Nadolol	• Non selective β1-adrenergic receptor inhibitor; • Used in the treatment of hypertension and angina (chest pain) and tremor	(Volicer et al., 1979; Wheeldon et al., 1994; Hanania et al., 2009)

3.6.1 Analysis of high scoring drug activity on prostate cancer cell proliferation.

As an initial screening mechanism for drug activity, we performed analysis of proliferation using the MTT assay. This method allowed us to perform high throughput analysis of the inhibitory effect of the drugs at different concentrations and time points. All 30 drugs were used at the concentrations described in the cmap database for a period of 24 and 48 hours and among the selected drugs we identified 12 drugs (anisomycin, monensin, lasalocid, podophyllotoxin, thapsigargin, lycorine, vinblastine, econazole, eticlopride, cyclobenzaprine, guanabenz and biperiden) with significant growth inhibitory properties in PCa cells (Figure 16). In order to determine whether the inhibition of proliferation of PCa cells occurs in a DCUN1D1-dependent manner we evaluated the effect of the drugs on DU145 DCUN1D1 knockdown cell lines (DU145 DCUN1D1 -/-). We also evaluated whether these drugs were toxic to normal prostate cells. As observed in figure 17, anisomycin, thapsigargin, podophyllotoxin, lasalocid, monensin, econazole, biperiden, eticlopride and cyclobenzaprine inhibit proliferation of DU145 PCa cell proliferation but have no effect in DU145 DCUN1D1 -/- cells. In addition, no inhibition of normal PNT1A cell proliferation was observed following treatment with these drugs (data not shown), indicating that these drugs may be inhibiting DU145 proliferation via DCUN1D1. Although vinblastine, lycorine and guanabenz show significant reduction of proliferation in DU145 cells, they were still effective against DU145 DCUN1D1-/- cells and vinblastine was toxic to PNT1A cells. Therefore, anisomycin, thapsigargin, podophyllotoxin, lasalocid, monensin, econazole, biperiden, eticlopride and cyclobenzaprine are the drugs which displayed DCUN1D1-specific anti-prostate cancer inhibitory activity with no effect in normal cells.

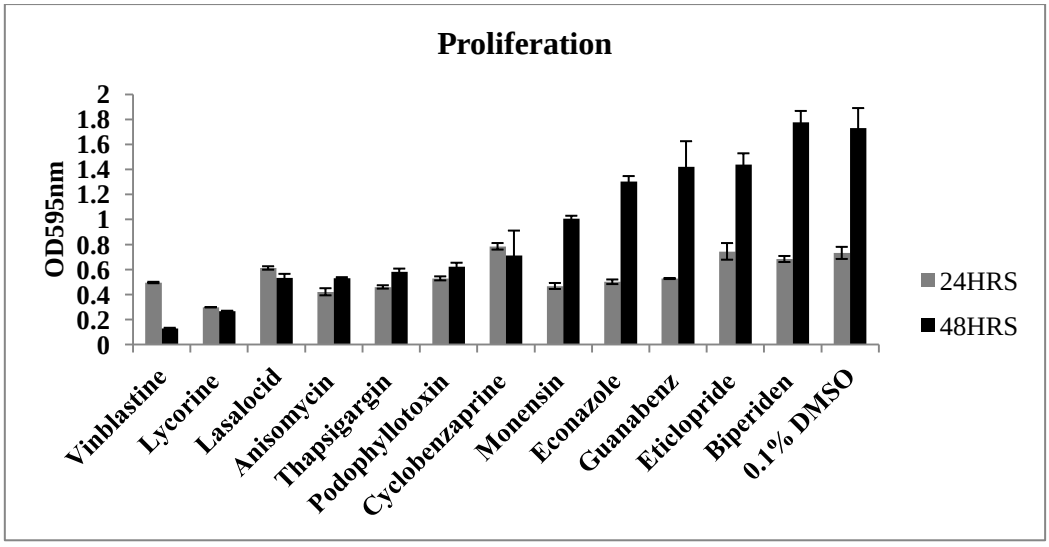

Figure 16. Proliferation assay in DU145 prostate cancer cell lines using drugs at cmap concentrations. Proliferation assay 24 hours post drug treatment. Treatment with anisomycin (6.25μM), monensin (12.5μM), lasalocid (7μM), podophyllotoxin (6.25μM), thapsigargin (10nM), lycorine (12μM), econazole (12.5μM), biperiden (12.5μM), eticlopride (12.5μM), cyclobenzaprine (12.5μM), guanabenz (12.5μM), vinblastine (100nM), 0.1% DMSO as a vehicle control. Data shown are mean±s.d. of triplicate independent experiments. OD, absorbance.

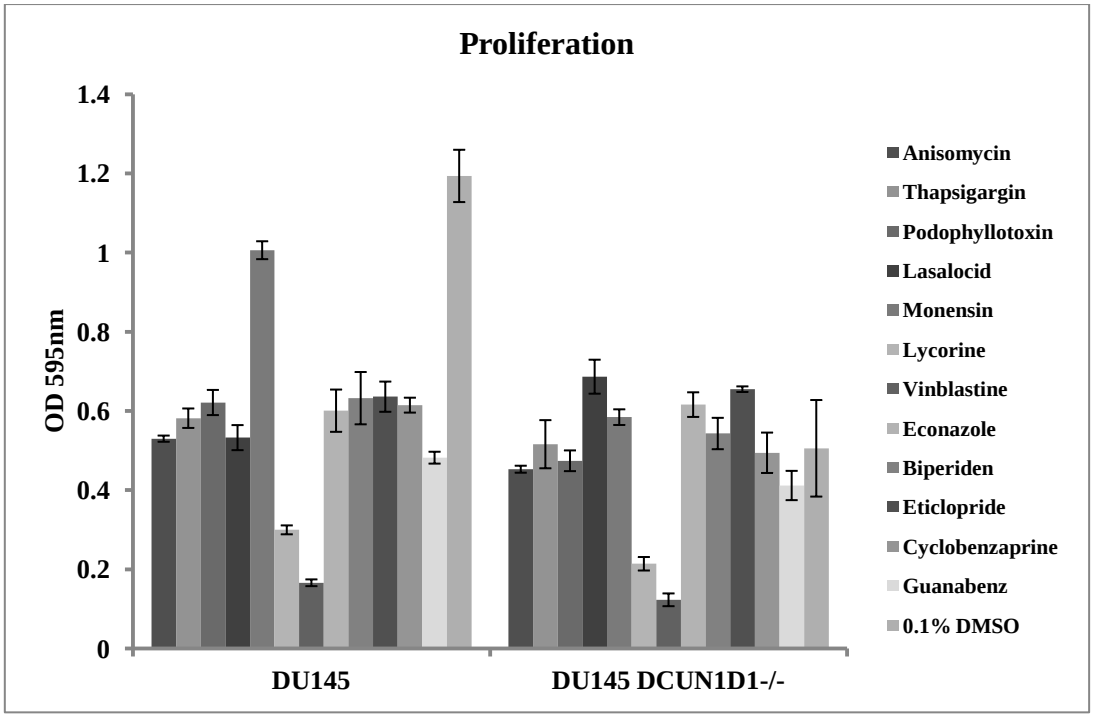

Figure 17. Proliferation assay in DU145 prostate cancer and DU145 DCUN1D1 knockdown. DU145 cell lines were infected with LV-shRNA GFP (DU145) and LV-shRNA DCUN1D1 (DU145 DCUN1D1 -/-) and were evaluated for their drug activity. Treatment with anisomycin (6.25µM), monensin (12.5µM), lasalocid (7µM), podophyllotoxin (6.25µM), thapsigargin (10nM), lycorine (12µM), econazole (12.5µM), biperiden (12.5µM), eticlopride (12.5µM), cyclobenzaprine (12.5µM), guanabenz (12.5µM), vinblastine (100nM) and 0.1% DMSO as a vehicle control. The MTT Proliferation assay was performed 24 hours post treatment. Data shown are mean±s.d. of triplicate independent experiments. OD, absorbance.

3.6.2 Analysis of the induction of apoptosis in response to drug treatment.

We also evaluated the effect of the high scoring drugs from our connectivity map analysis on the induction of apoptosis. A review of the literature revealed that some of the drugs (anisomycin, thapsigargin, monensin) from our analysis have growth inhibitory effects on PCa cells (Tombal et al., 2000; Stadheim & Kucera, 2002; Ketola et al., 2010). As observed in figure 18, treatment with anisomycin, monensin, podophyllotoxin, lycorine, vinblastine, biperiden, eticlopride, cyclobenzaprine, clearly induces apoptosis in PCa cells as measured using the Cell Death Elisa Kit (Roche). However, some of the drugs that demonstrated inhibition of proliferation such as lasalocid and thapsigargin did not induce apoptosis at the same concentrations (Figure 18) which could mean that their inhibitory effect was mediated by an alternative cell death mechanism.

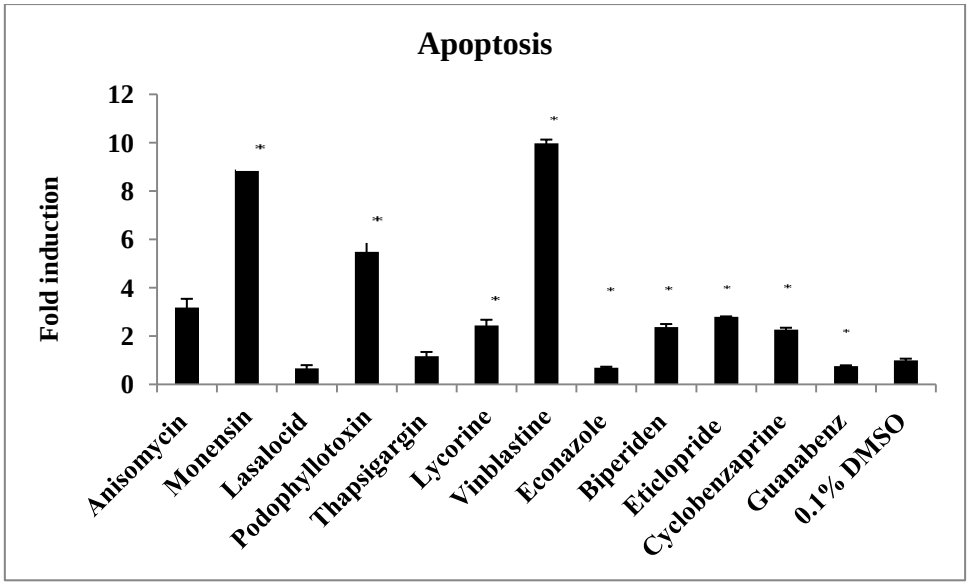

Figure 18. Induction of apoptosis after treatment with high scoring drugs. Treatment with anisomycin (6.25μM), thapsigargin (10nM), podophyllotoxin (6.25μM), lasalocid (7μM), monensin (12.5μM), vinblastine (100nM), lycorine (12μM), econazole (9μM), biperiden (11μM), eticlopride (11μM), cyclobenzaprine (13μM), guanabenz (14μM) and 0.1% DMSO as a vehicle control. The Cell Death Elisa Plus kit was used to quantify apoptosis 24 hours post-seeding of the cells. * represents $p<0.05$

3.6.3 Analysis of high scoring drug activity on DCUN1D1 mRNA and protein expression.

Since the objective of our study is to identify compounds that inhibit PCa growth through direct interference with DCUN1D1, we evaluated the effect of the 12 drugs from our connectivity map analysis on DCUN1D1 expression. Using RT-PCR we determined that monensin, lasalocid, podophyllotoxin, thapsigargin and vinblastine significantly reduced DCUN1D1 mRNA expression 24 hours post treatment, while anisomycin and lycorine significantly increased DCUN1D1 expression (Figure 19 A) compared to the control treatment. The rest of the drugs did not alter DCUN1D1 mRNA expression. Four of the drugs (monensin, lasalocid, podophyllotoxin and thapsigargin) that inhibited DU145 cell proliferation in a DCUN1D1 dependent manner also reduced DCUN1D1 mRNA expression. Messenger RNA expression does not always correlate with protein expression, therefore we utilized the western blot technique to analyse the effects of the drugs on DCUN1D1 protein expression. The same pattern of expression was not observed at the protein level. We observed reduced DCUN1D1 protein expression following treatment with anisomycin, monensin, podophyllotoxin, lycorine, guanabenz and vinblastine (Figure 19 B) and the opposite was true for thapsigargin, lasalocid, econazole, eticlopride, cyclobenzaprine and biperiden. Three of the drugs (anisomycin, monensin, podophyllotoxin) that mediated DCUN1D1-specific inhibition of PCa proliferation reduced DCUN1D1 protein expression. Although lycorine, guanabenz and vinblastine show reduced DCUN1D1 mRNA or protein expression they did not appear as candidates likely to

pass the screening process of our study as they did not mediate DCUN1D1-specific inhibition of DU145 proliferation (Figure 17) and vinblastine showed toxicity towards normal prostate cell. The objective of this study is to identify compounds that inhibit PCa growth through direct interference with DCUN1D1 and we determined this to be the criteria for further analysis: DCUN1D1-specific inhibition of proliferation, minimal toxicity in normal prostate cells; induction of apoptosis and reduction of DCUN1D1 mRNA and protein expression. In this regard, the only drugs that consistently fit our criteria were monensin and podophyllotoxin.

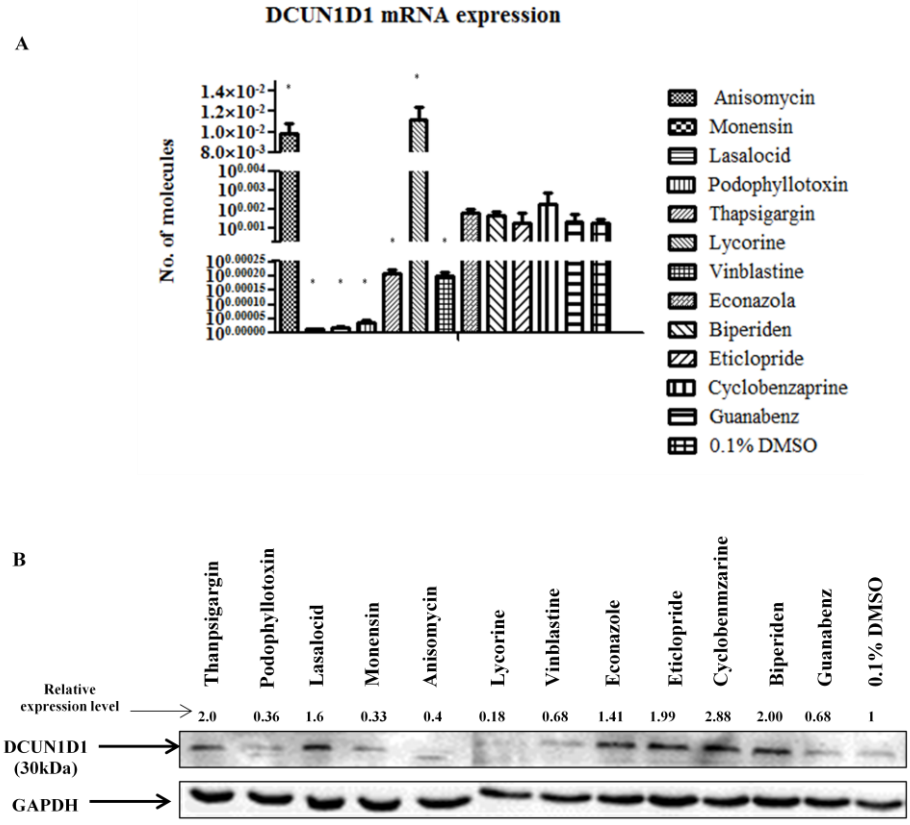

Figure 19. DCUN1D1 expression in DU145 cells 24 hours post treatment with high scoring drugs. A) Real time-PCR analysis of DCUN1D1 in DU145 prostate cancer cell lines following 24 hour treatment with anisomycin (6.25μM), monensin (12.5μM), lasalocid (7μM), podophyllotoxin (6.25μM), thapsigargin (10nM), lycorine (12μM), vinblastine (100nM), econazole (9μM), biperiden (11μM), eticlopride (11μM), cyclobenzaprine (13μM), guanabenz (14μM) with 0.1% DMSO as a vehicle control. Total RNA was collected from DU145 cell lines and normalized by measuring the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) complementary DNA. Data shown are mean±s.d. of triplicate independent experiments. * represents $p<0.05$. B) Western blot analysis of DCUN1D1 expression in the DU145 PCa cells with the same drug set was performed. DCUN1D1 was probed using anti-DCUN1D1 antibody and normalized by measuring the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.6.4 Combinatorial therapy studies

As mentioned previously, using the connectivity map database we employed a genomics approach to drug discovery. After identifying the gene signature of DCUN1D1 knockdown in DU145 PCa cells, the drugs with a positive correlation to the signature were identified. Following initial screening using the MTT proliferation assay, 12 drugs were identified for further analysis and of these drugs, 9 (anisomycin, thapsigargin, podophyllotoxin, lasalocid, monensin, econazole, biperiden, eticlopride and cyclobenzaprine) were determined to mediate DCUN1D1-specific inhibition of proliferation. Of these drugs, monensin, podophyllotoxin and anisomycin induced apoptosis in DU145 cells but most importantly monensin and podophyllotoxin treatment led to significant reduction in both DCUN1D1 mRNA and protein (Table 5). Therefore, we selected monensin and podophyllotoxin for use in combination studies.

Table 5: Summary of drug activity in DU145 cells

	DCUN1D1-specific inhibition of proliferation	Reduction in DCUN1D1 mRNA	Reduction in DCUN1D1 protein	Induction of Apoptosis
Monensin	+	+	+	+
Biperiden	+			+
Podophyllotoxin	+	+	+	+
Guanabenz				
Lasalocid	+	+		
Anisomycin	+		+	+
Eticlopride	+			+
Cyclobenzaprine	+			+
Lycorine			+	+
Thapsigargin	+	+		
Econazole	+			
Vinblastine		+	+	+

In order to establish the IC_{50} of each drug, we performed a dose-dependent proliferation assay and used the GraphPad Prism version 5.01 software to perform the analysis (Figure 20 and 21). The IC_{50s} were determined to be 2μM for monensin and 30nM for podophyllotoxin. These drugs were then used in combination where DU145 PCa cells were treated with monensin 1μM and 2μM and podophyllotoxin 15nM amd 30nM, with 1μM and 15nM representing the lowest concentrations at which inhibitory activity is still observed. Combination of 1μM monensin and 15nM podophyllotoxin demonstrated significant reduction in DU145 proliferation when compared to the monotherapy (Figure 22) ($p<0.001$).

The *in vitro* analysis performed on these two drugs indicates that both drugs are inhibiting PCa growth in a DCUN1D1-dependent manner and combinatorial analysis using the MTT proliferation

assay suggests that when used together, the drugs induce a statistically significant inhibitory effect on PCa proliferation. It is important to determine if the activity of the drugs is leading to a greater inhibitory effect due to combined general inhibitory effects (synergism) or if they are targeting the same pathway and are therefore having an additive effect. Although we have shown that both drugs target DCUN1D1, it could be that DCUN1D1 is affecting a different set of target genes under the mechanism of action of each drug. We used isobologram analysis on Calcusyn3 software (Biosoft) to determine the theoretical combinatorial effect of monensin and podophyllotoxin on DU145 proliferation. The dose equivalence of each drug was analysed and a linear additive isobole was generated to determine the effect of the drugs. The combinations used in the isobole were: monensin 1μM: podophyllotoxin 15nM, monensin 1μM; podophyllotoxin 30nM and monensin 2μM; podophyllotoxin 30nM. As mentioned in section 2.4.4, combination data points that fall on the line represent an additive interaction, whereas points above or below represent antagonism or synergy, respectively. In addition, points that are in close proximity to the line are also considered as having an additive interaction. Significantly, the isobologram analysis predicted the drugs to have an additive effect on the inhibition of PCa proliferation, at the aforementioned concentrations (Figure 23).

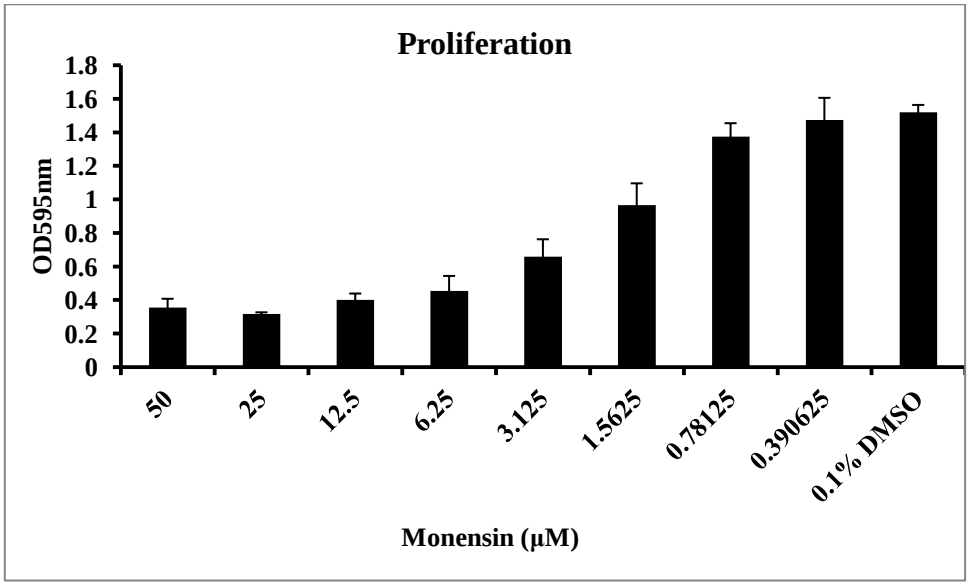

Figure 20. Dose dependent response of DU145 prostate cancer cells to monensin treatment. MTT proliferation assay of monensin and 0.1% DMSO 48 hours post treatment. Data shown are mean±s.d. of triplicate independent experiments. OD, absorbance.

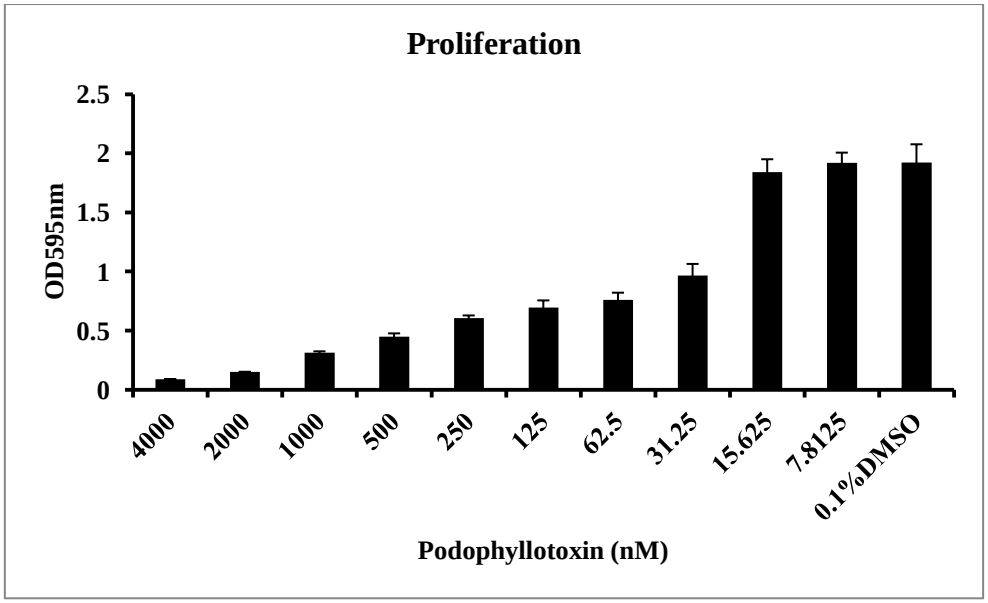

Figure 21. Dose dependent response of DU145 prostate cancer cells to podophyllotoxin treatment. MTT proliferation assay of podophyllotoxin and 0.1% DMSO 48 hours post treatment. Data shown are mean±s.d. of triplicate independent experiments. OD, absorbance.

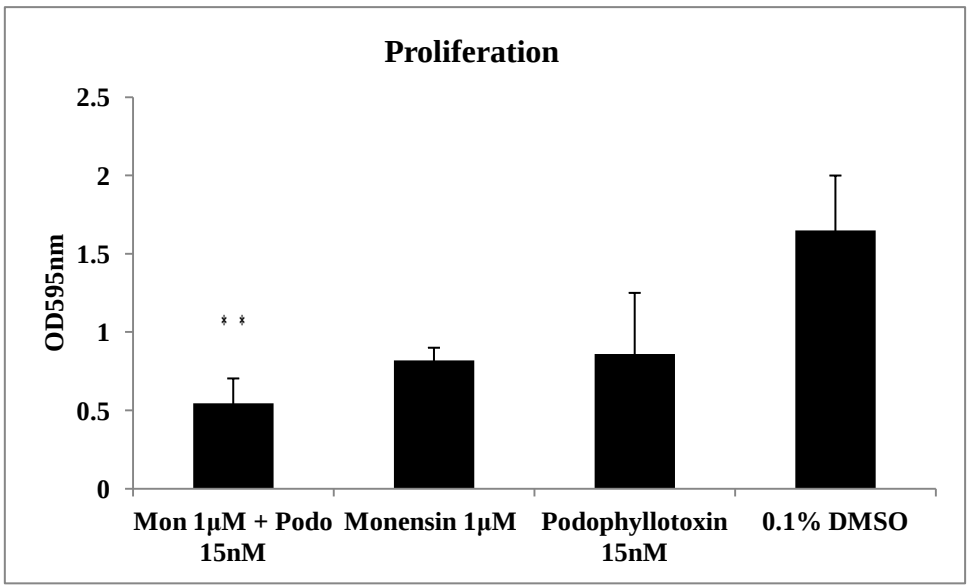

Figure 22. Combinatorial effect of monensin and podophyllotoxin on DU145 prostate cancer cell line proliferation. MTT proliferation assay was performed 48 hours post treatment with mono and combination therapy, 0.1% DMSO control. Data shown are mean±s.d. of triplicate independent experiments. OD, absorbance. $p<0.001$

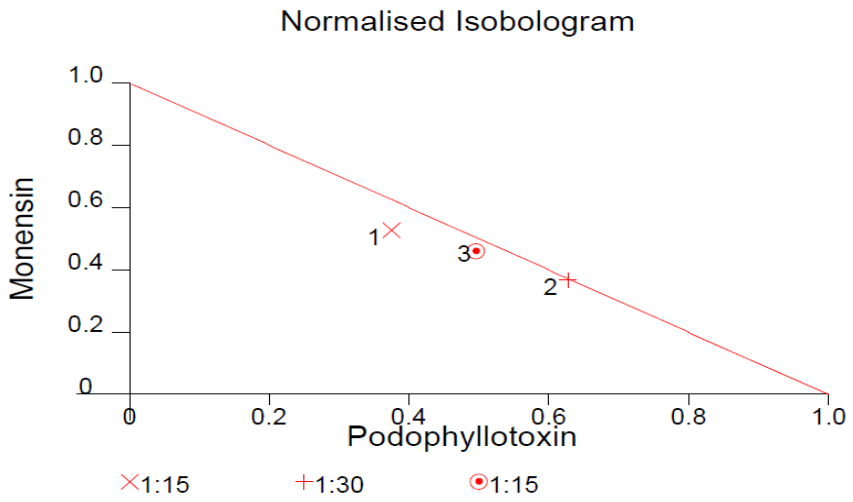

Figure 23. Isobologram analysis of the combinatorial effect of monensin and podophyllotoxin. All three points representing a dose dependent effect on proliferation lie close to the linear isobole (line of additivity) indicating that the drugs have an additive effect on each other's drug activity. Cross (1:15) represents monensin 1μM: podophyllotoxin 15nM, plus sign (1:30) monensin 1μM: podophyllotoxin 30nM and the circle (1:15) represents monensin 2μM: podophyllotoxin 30nM.

Chapter 4: Discussion

Molecular target based treatment of PCa is an evolving approach to PCa therapy. Although initially targeted at androgen deprivation therapy (ADT) due to the dependence of PCa tumours on androgens for growth, the emergence of androgen independent tumours has narrowed the scope of activity of these drugs. In addition, the close correlation between this mode of treatment and depletion in male sexual functioning has made ADT undesirable. Therefore, alternative protein targets have become essential and are currently being tested at clinical trials for anti-PCa activity. Inhibition of protein degradation has been one of the pathways that have emerged as potentially effective in cancer treatment with minimal adverse effects. In the course of this study we have identified DCUN1D1, a neddylation E3 ligase, as a potential new drug target for PCa treatment.

DCUN1D1 has been reported to be upregulated in several squamous cell carcinomas, particularly head and neck, lung, cervical and ovarian cancer (Sarkaria et al., 2004; Sarkaria et al., 2006). It has also been associated with an aggressive phenotype and poor clinical outcomes in these cancers (Sarkaria et al., 2006). In addition, DCUN1D1 expression has been correlated with the T classification in non small cell lung carcinomas where high DCUN1D1 expression was linked to late stages of the disease but interestingly, DCUN1D1was observed to be a marker for brain metastasis (Yoo et al., 2012). Additionally, DCUN1D1 is reported to result in tumour formation and malignant transformation in gliomas *in vivo* (Broderick et al., 2010). In this study, we observed DCUN1D1 mRNA and protein to be upregulated in PCa, which has not been described previously. Additionally, we demonstrate DCUN1D1 to be relevant for human tissue samples where in a panel of adenocarcinoma of the prostate stages I, II and III, BPH, normal tissue and other disease controls, DCUN1D1 was upregulated relative to normal prostate tissue with increased levels observed in 42% of the cancer samples. We also observed this following immunohistochemistry analysis of a separate group of human tissue samples where 43.7% showed high expression of DCUN1D1. We could not extrapolate the exact relationship between DCUN1D1 and adenocarcinoma stages from the tissue array or the immunohistochemistry analysis. However, we have demonstrated that DCUN1D1 is essential for PCa.

In order to determine how DCUN1D1 contributes to PCa tumourigenesis we evaluated its effect on proliferation, migration and apoptosis. DCUN1D1 overexpression has been described to induce malignant transformation and proliferation of cells as well as to induce tumour formation in xenograft mice models (Sarkaria et al., 2006). It has also been implicated in several hallmarks of cancer including increased proliferation, migration, invasion, angiogenesis and inhibition of apoptosis (Sarkaria et al., 2004; Sarkaria et al., 2006; O-charoenrat et al., 2008; Broderick et al., 2010).We have demonstrated that in PCa, blockage of DCUN1D1 inhibits tumour growth in MF1 nude mice with a 58% reduction in tumour weight observed. In addition, our data clearly indicates that upon inhibition

of DCUN1D1, PCa cells lose their ability to proliferate and migrate and they undergo an induction of apoptosis. Although apoptosis was not induced at the levels that we would expect, it is possible that other mechanisms of cell death are responsible for DCUN1D1 activity in prostate cancer such as autophagy. However, DCUN1D1 clearly functions as an oncogene in PCa and a dysregulation in its expression is detrimental to the cancer.

We have demonstrated the individual hallmarks of cancer that DCUN1D1 is implicated in above, however our microarray analysis suggests the broader mechanism behind DCUN1D1 activity in PCa. Blockage of DCUN1D1 may be deregulating key functions and pathways associated with tumourigenesis mainly through developmental pathways. As observed previously in primary lung, head and neck and cervical carcinomas, DCUN1D1 appears to play a role in developmental pathways (Sarkaria et al., 2006). Of the top 10 functions that could be deregulated following DCUN1D1 knockdown in DU145 PCa cells, 6 were associated with development. This may be significant as DCUN1D1 was previously demonstrated to play a role in the embryonic development pathway, hedgehog signalling, in SCC (Sarkariaet al., 2006). Additionally, two E2 ubiquitin conjugating enzymes, *UBE2C* and *UBE2J2*, were also deregulated which suggests the effects of DCUN1D1 knockdown on ubiquitination. Although these two genes are associated with ubiquitination, it was not possible within the scope of this study to conclude the functionality of these E2s in association with DCUN1D1 neddylation. However, *UBE2C* has been implicated in cell cycle regulation through UPP-mediated degradation of cyclin B while *UBE2J2* has been implicated in endoplasmic reticulum associated degradation and could therefore be contributing to the changes in cell cycle and regulation of protein expression observed following DCUN1D1 knockdown (Townsley et al., 1997; Oh et al., 2006). However, the transcription level effects of DCUN1D1 on particular genes involved in ubiquitination, PCa development, gene expression, cellular growth and proliferation pathways and cell death, as suggested by the microarray analysis, needs to be validated by further *in vitro* analysis but was not within the scope of this study. We have instead identified structurally and mechanistically distinct drugs that may affect DCUN1D1 expression using the connectivity map approach.

As mentioned previously, we used the connectivity database (cmap) in order to identify DCUN1D1 specific inhibitors of PCa. This is a novel approach which was developed by the Broad Institute of MIT and Harvard in Cambridge and allowed us to use comparisons of gene expression signatures to link PCa and potential inhibitors of PCa. The approach proved to be successful because of the 30 drugs that we screened, 7 significantly altered DCUN1D1 mRNA (anisomycin, monensin, lasalocid, podophyllotoxin, thapsigargin, lycorine and vinblastine) while 11 altered its protein expression (all except for guanabenz). Although we were interested only in the drugs leading to a significant reduction in DCUN1D1 mRNA and protein expression, the number of drugs altering DCUN1D1 expression means that the theoretical predictions made by the cmap database were relatively accurate and we were able to identify the desired drugs. Significantly, we were able to identify monensin and

podophyllotoxin as compounds mediating PCa inhibition through DCUN1D1 and these were chosen for combinatorial analysis. Additionally, because these drugs are already approved by the US FDA, it means that if evaluations in animal models are successful, these drugs could be approved for use in PCa treatment. We discuss the details of the drugs and their possible mechanisms of activity below.

Of the 30 drugs evaluated in our study, 9 were antibiotics, 4 were plant derivatives and 9 were inhibitors of neurotransmitter receptors. We also identified a SERCAs inhibitor, an amino acid and nucleotide metabolisms synthesis regulator, an ion channel inhibitor, a bile acid, an inhibitor of phosphoglycerate kinase and phosphoglyceromutase and a testosterone metabolite. Following initial screening of the compounds which we identified through the cmap database, 12 drugs (anisomycin, monensin, lasalocid, podophyllotoxin, thapsigargin, lycorine, vinblastine, econazole, eticlopride, cyclobenzaprine, guanabenz and biperiden) were identified as significantly inhibiting PCa proliferation. In depth analysis of the structure and mechanism of activation of the drugs identified in our cmap analysis revealed interesting information. Although all of the drugs appear to have large hydrophobic groups, there does not appear to be any other striking similarity in the structure of these drugs. However, some have similar cellular activity. Monensin, lasalocid and thapsigragin are all involved in ion transport and are linked to Na^+/H^+ exchange and calcium signalling, respectively (Bains, 1980; Russell, 1987; Kovacs et al., 2005). In addition, guanabenz, cyclobenzaprine, eticlopride and biperiden are neurotransmitter inhibitors. Guanabenz is an adrenergic receptor agonist (Misu & Fujie, 1982), cyclobenzaprine, a 5HT2 serotonin receptor antagonist (Brioschi et al., 2013); eticlopride targets the dopamine D2 receptor (Giuliani & Ferrari, 1997), while biperiden is a muscarinic acetylcholine receptor (Eltze, 1988). Anisomycin and lycorine have been described to inhibit protein synthesis while vinblastine and podophyllotxin inhibit microtubule assembly by inhibition of tubulin polymerization (Grollman, 1967; Vrijsen et al., 1986; Toso et al., 1993; Imbert, 1998). As described in chapter 1 of this study, PI3K/AKT, MAPK are pathways that heavily involved in PCa signalling and have been associated with PCa proliferation and survival. Lasalocid, biperiden, cyclobenzaprine, eticlopride, anisomycin, guanabenz, econazole have been described to mediate their activity through inhibition of either both or one of these pathways (Kinsel et al., 1982; Tennant, 1984; Eltze, 1988; Giuliani & Ferrari, 1997; Jan et al., 1999; Stadheim & Kucera, 2002; Brioschi et al., 2013).

However, 9 of these drugs (anisomycin, monensin, lasalocid, podophyllotoxin, thapsigargin, econazole, biperiden, eticlopride and cyclobenzaprine) displayed inhibition of proliferation that was dependent on DCUN1D1 expression. These drugs also showed minimal toxicity in normal prostate cells (PNT1A) which suggests that they could be tolerated in humans. In addition, monensin, podophyllotoxin, biperiden, eticlopride and guanabenz treatment led to a significant induction of apoptosis. However, treatment with lasalocid, monensin and podophyllotoxin decreased DCUN1D1 mRNA, while anisomycin and lycorine increased DCUN1D1 expression. A subsequent decrease in

DCUN1D1 protein expression was observed following treatment with monensin and podophyllotoxin with reductions also observed following treatment with anisomycin, lycorine and vinblastine. Therefore the connectivity map was useful in identifying small molecules that could mediate DCUN1D1-dependent anti-PCa activity. Based on the screening approach set for this study, the drugs that significantly inhibit PCa growth with a subsequent induction of cell death that was mediated specifically through reduction of DCUN1D1 mRNA and protein were monensin and podophyllotoxin. We excluded the drugs that increased DCUN1D1 mRNA or protein as our data indicates that DCUN1D1 is an oncogene in PCa and because its mechanism of action is still unclear. However, it is important to mention that the drugs increasing DCUN1D1 expression could be taking advantage of its role in protein degradation and therefore inhibiting proliferation and inducing apoptosis by increased degradation of crucial cellular proteins. Monensin and podophyllotoxin met the criteria of our screening process and were chosen for further analysis on PCa cell proliferation. Varying concentrations of both drugs were used followed by isobologram analysis demonstrating the drugs to have an additive effect on the inhibition of PCa proliferation.

We identified monensin and podophyllotoxin as DCUN1D1-specific inhibitors of PCa with an additive inhibitory effect and we explore the possible mechanism behind this activity. Monensin is a carboxylic ionophore which mediates Na^+/H^+ transport while podophyllotoxin inhibits tubulin polymerization (Russell, 1987; Imbert, 1998). Previous studies have demonstrated monensin to have a wide spectrum of activity but significantly it is a potent anticancer drug. It has been reported to induce cell cycle arrest, oxidative stress and apoptosis in PCa cells (Ketola et al., 2010). Podophyllotoxin on the other hand is known mainly for its antiviral activity in the treatment of genital warts but its ability to inhibit tubulin polymerisation have also made it a strong anti-cancer drug (Jordan et al., 1992; Strand et al., 1995; Imbert, 1998). In terms of this study, firstly, analysis of the microarray data suggests that axonal guidance and semaphorin signalling, are dysregulated following DCUN1D1 and these pathways have been reported to be dependent on endocytosis. In addition, among the predicted top 10 up and downregulated genes, many genes are associated with endocytosis and lysosomal degradation. Interestingly, monensin has been reported to mediate its activity through prevention of endosomal activity and disruption of Golgi apparatus activity while podophyllotoxin inhibits tubulin polymerization (Berg et al., 1983; Imbert, 1998). Endocytosis and lysosomal degradation have previously been associated with PCa development. The acidic pH of intracellular tumour environment was shown to induce Na^+/H^+ activity which leads to increased proton expulsion and subsequently contributes to the acidic extracellular pH observed in PCa tumour extracellular microenvironments (Steffan et al., 2009). Increased extracellular acidity has been shown to lead to peripheral trafficking of lysosomal vesicles and release of proteases which act under acidic conditions and improve the invasive capacity of tumour cells (Steffan et al., 2010). In addition, the current chemotherapeutic agent used in PCa treatment, docetaxel, has been shown to induce lysosomal permebealization in its

anti-PCa activity (Mediavilla-Varela et al., 2009). Therefore, since these endosomal and lysosomal vesicles are transported along the cytoskeleton and depend on intact tubulin it is likely that monensin and podophyllotoxin are mediating their activity by altering the extracellular microenvironment and disrupting the tubulin network necessary for vesicular transport. They probably target DCUN1D1 to alter these pathways through disruptions in neddylation.

DCUN1D1 is an E3 ligase for the neddylation pathway and alterations in its expression have been reported to alter cullin neddylation. However, as mentioned previously, cullins are not the only substrates of NEDD8. Some of proteins that have been identified as NEDD8 substrates include EGFR, pVHL and BCA3 (Stickle et al., 2004; Gao et al., 2006; Oved et al., 2006). EGFR has been demonstrated to undergo mono and multi-neddylation prior to endocytic degradation, (Oved et al., 2006). Endocytosis is a process which involves internalization of large polar molecules through engulfment (Doherty & McMahon, 2009). The most extensively described endocytosis pathway is the clathrin coat-mediated endocytosis where clathrin coated pits from the cellular membrane detach from the plasma membrane to form clathrin-coated vesicles (Robinson, 1994). These vesicles then mediate early and late endocytosis which leads to lysosomal degradation or recycling of the engulfed molecules. Therefore elevated DCUN1D1 levels could suggest increased NEDD8 conjugation to proteins such EGFR and endosomal-lysosomal degradation of these proteins. Additionally, monensin which has been reported to inhibit particularly intracellular degradation of proteins internalized into the endosome and not inhibition of protein internalization into the endosome could be targeting DCUN1D1 to inhibit protein degradation, however this would need to be validated. In addition, neddylation has been described to mediate fibronectin assembly through pVHL neddylation therefore podophyllotoxin could be targeting inhibition of DCUN1D1-mediated neddylation for cytoskeleton disruption (Stickle et al., 2004). Lastly, other NEDD8 substrates that have been characterized include p53, Mdm2 and BCA3 which recruits SIRT1 to NF-κB (Stickle et al., 2004; Gao et al., 2006). Therefore, inhibition of DCUN1D1 through deregulation of neddylation could be affecting key genes in PCa which could explain its role in PCa and its anti-cancer activity.

In addition, the pathways identified in here as deregulated following DCUN1D1 blockage have been characterized as key pathways of PCa development and progression in other studies. Analysis of three studies done recently (Wang et al., 2011; Li et al., 2013; Wen et al., 2013) employing bioinformatics to identify pathways essential for PCa development revealed interesting similarities to our study. Two of these studies used gene expression data from microarray analysis available in the public database GEO (Gene Expression Omnibus) and compared gene signatures between normal and tumour prostate tissue. The authors then used the gene signatures to identify small molecules that could be used to target PCa treatment. The pathways identified as deregulated were similar to those observed in our study where they observed alterations in cell cycle regulation, Wnt/β-catenin signalling, focal adhesion and actin skeleton regulation. (See Appendices Table A3 and A4). Therefore, not only have

we demonstrated DCUN1D1 to be essential for PCa progression but we have demonstrated that deregulation of DCUN1D1 affects pathways established to be essential in human tissue samples as crucial for PCa development. However, DCUN1D1 is a unique protein due to its link to neddylation and it offers a new and more specific approach to targeting these pathways for PCa inhibition.

As mentioned previously, inhibition of protein degradation pathways is an emerging approach to small molecule based treatment of cancer. Bortezomib and MLN4924 are two drugs that have been identified as chemotherapeutic agents against a wide variety of malignancies and they have proven the efficacy of inhibition of protein degradation as an approach to cancer treatment. Having established the significance of DCUN1D1 in PCa and preliminarily identified small molecule inhibitors of DCUN1D1, our view is that DCUN1D1 is likely a new target for PCa treatment. The signalling pathways and mechanisms of activity of the small molecules used in this study suggest that inhibition of DCUN1D1 is able to reverse PCa gene expression profiles likely due to its role as an E3 ligase in the neddylation pathway, downstream of the NAE currently targeted by MLN4924. Thus targeting DCUN1D1 expression and activity is likely to be a more specific therapy with possibly fewer side effects.

Chapter 5: Conclusion

DCUN1D1 or SCCRO is an under explored protein. Although previous studies have demonstrated it to be upregulated in tumours of squamous origin, we have clearly demonstrated that it plays a key role in solid PCa tumour growth. We have provided *in vitro* and *in vivo* analyses which have demonstrated the pivotal role of DCUN1D1 on PCa tumourigenesis. We also demonstrated that DCUN1D1 could be mediating its activity through deregulation of essential cell growth, proliferation, development, gene expression and cancer-related pathways. In addition, we have demonstrated the potential of DCUN1D1 as a novel drug target for PCa treatment through the identification of monensin and podophyllotoxin as DCUN1D1 specific inhibitors of PCa growth. It is possible that they are targeting DCUN1D1-mediated neddylation for endosomal-lysosomal degradation and microtubule disruptions. Since these drugs are currently approved by the US FDA for use in different disease, following the necessary characterization, they could get approval for use in PCa.

In conclusion, DCUN1D1 is essential for PCa growth and has the potential to be a new therapeutic entry point to fight the disease.

Chapter 6: Areas of further research

In this study we employed a genomics approach to understanding the role of DCUN1D1 in PCa but due to the role of DCUN1D1 in protein modification, it would be essential to determine its role at the protein level. This would require proteomics analysis with an emphasis on identifying direct and indirect protein binding targets of DCUN1D1 but also proteins targeted by DCUN1D1 for neddylation. The role of DCUN1D1 activity in PCa would also need to be evaluated. Although there is significant evidence that alterations in DCUN1D1 expression are detrimental to PCa, *in vitro* and *in vivo* neddylation assays would need to be performed to determine if DCUN1D1 activity is altered. In addition, the exact mechanism of DCUN1D1 activity under physiological and cancer conditions needs to be elucidated. The neddylation pathway plays a pivotal role in mediating ubiquitination of proteins and regulation of numerous biological processes including cancer. In this context our group recently published a review paper where we discussed neddylation substrates which are deregulated in cancer as well as therapeutic approaches to targeting neddylation and our view is that DCUN1D1 could be a key target in cancer therapy (See Appendix 8.6). Its primary role, as described to date, has been through cullin neddylation and ubiquitination as a downstream effector but the mechanisms of action of the drugs found to be specific for DCUN1D1 in this study which include disruption of endocytosis and inhibition of microtubule function suggest that there is a lot to be learnt about DCUN1D1 and the neddylation pathway in general.

Consequently, monensin and podophyllotoxin which were identified in the study as small molecule DCUN1D1 inhibitors of PCa would have to be evaluated further. Determinations need to be made on the best concentrations of these drugs to be used in mono and combination therapy, with these concentrations tested for *in vivo* activity. *In vivo* cytotoxicity of the two drugs used in combination would have to be evaluated. The mechanism of action of these drugs on PCa would also need to be extensively analysed. This study has revealed a dependence of these drugs on DCUN1D1 and the extent of this dependence would have to be established. Significantly, since the current chemotherapeutic agent against PCa, docetaxel, has been described to mediate cell death in PCa cells through lysosomal membrane permeabilization, combination therapy with the drugs identified in this study should be explored. The proposed role of these drugs on DCUN1D1-neddylation mediated endocytosis could prove additive to the activity of docetaxel and thus improve the activity of this drug and concurrently increase the life-expectancy already observed with this drug in humans.

Chapter 7: References

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Chapter 8: Appendix

8.1 Table A1: Top 50 genes upregulated following DCUN1D1 knockdown in DU145 cell lines in descending order.

	Gene	Description	Fold change
1	ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	19.97
2	EEF2	Eukaryotic translation elongation factor 2	6.3
3	UBE2C	Ubiquitin-conjugating enzyme E2C	5.9
4	SPTBN1	spectrin, beta, non-erythrocytic 1	4.4
5	BAT2D1	BAT2 domain containing 1	4.04
6	TNPO1	Transportin 1	3.85
7	SF3B1	Splicing factor 3b, subunit 1, 155kDa	3.68
8	CADM1	Cell adhesion molecule 1	3.59
9	SH3GLB1	SH3-domain GRB2-like endophilin B1	3.48
10	HAX1	HCLS1 associated protein X-1	3.24
11	TXNL1	Thioredoxin-like 1	3.19
12	HUWE1	HECT, UBA and WWE domain containing 1	3.11
13	EIF3A	Eukaryotic translation initiation factor 3, subunit A	3.02
14	TCF4	Transcription factor 4	3
15	C11orf31	Chromosome 11 open reading frame 31	2.98
16	FUNDC2	FUN14 domain containing 2	2.9
17	TNPO1	Transportin 1	2.86
18	AKIRIN2	Akirin 2	2.86
19	CHORDC1	Cysteine and histidine-rich domain (CHORD)-containing 1	2.8
20	PTPN12	Protein tyrosine phosphatase, non-receptor type 12	2.69
21	HBS1L	HBS1-like (S. cerevisiae)	2.69
22	STAG2	Stromal antigen 2	2.64
23	FLOT1	Flotillin 1	2.62
24	HEATR2	HEAT repeat containing 2	2.58
25	MAGED1	Melanoma antigen family D, 1	2.56
26	DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	2.54
27	SON	SON DNA binding protein	2.49
28	TRIP12	Thyroid hormone receptor interactor 12	2.48
29	NUP205	Nucleoporin 205kDa	2.44
30	SHOX2	Short stature homeobox 2	2.44
31	HNRNPL	Heterogeneous nuclear ribonucleoprotein L	2.42
32	GART	Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	2.36
33	SPTBN1	Spectrin, beta, non-erythrocytic 1	2.36
34	RPS3A	Ribosomal protein S3A	2.33
35	AKR1A1	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	2.31
36	PUM1	Pumilio homolog 1 (Drosophila)	2.31
37	APP	Amyloid beta (A4) precursor protein	2.3
38	PTCRA	Pre T-cell antigen receptor alpha	2.3
39	ITCH	Itchy E3 ubiquitin protein ligase homolog (mouse)	2.29
40	ATXN2	Ataxin 2	2.27
41	EXOSC2	Exosome component 2	2.25

42	GPHN	Gephyrin	2.24
43	PRMT5	Protein arginine methyltransferase 5	2.24
44	PHACTR4	Phosphatase and actin regulator 4	2.23
45	KDELR1	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1	2.23
46	CREBBP	CREB binding protein	2.22
47	CWC15	CWC15 spliceosome-associated protein homolog (S. cerevisiae)	2.19
48	RPP30	Ribonuclease P/MRP 30kDa subunit	2.18
49	EZR	Ezrin	2.18
50	NCOA1	Nuclear receptor coactivator 1	2.17

8.2 Table A2: Top 50 genes downregulated following DCUN1D1 knockdown in DU145 cell lines in descending order.

	Gene	Description	Fold change
1	UBE2J2	Ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast)	-9.5
2	BAT2	HLA-B associated transcript 2	-8.46
3	ZFPM1	Zinc finger protein, multitype 1	-6.97
4	CLDN6	Claudin 6	-6.73
5	TNPO2	Transportin 2	-6.4
6	NMNAT3	Nicotinamide nucleotide adenylyltransferase 3	-5.93
7	EPHB4	EPH receptor B4	-5.38
8	ACAA2	Acetyl-Coenzyme A acyltransferase 2	-5.36
9	ACAD10	Acyl-Coenzyme A dehydrogenase family, member 10	-5.04
10	FAM109A	Family with sequence similarity 109, member A	-5.01
11	PI16	Peptidase inhibitor 16	-4.86
12	MRC2	Mannose receptor, C type 2	-4.7
13	SLC6A2	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	-4.67
14	ODF3B	Outer dense fiber of sperm tails 3B	-4.55
15	DOHH	Deoxyhypusine hydroxylase/monooxygenase	-4.31
16	ZNF696	Zinc finger protein 696	-4.11
17	MYL10	Myosin, light chain 10, regulatory	-4.11
18	SARDH	Sarcosine dehydrogenase	-4.09
19	C11orf30	Chromosome 11 open reading frame 30	-4.08
20	SFN	Stratifin	-4.04
21	HKDC1	Hexokinase domain containing 1	-4
22	SMOX	Spermine oxidase	-3.99
23	COL1A1	Collagen, type I, alpha 1	-2.53
24	CASR	Calcium-sensing receptor	-3.94
25	TMEM184A	Transmembrane protein 184A	-3.84
26	TTLL3	Tubulin tyrosine ligase-like family, member 3	-3.82
27	AP2A1	Adaptor-related protein complex 2, alpha 1 subunit	-3.81
28	DLG4	Discs, large homolog 4 (Drosophila)	-3.8
29	FGF18	Fibroblast growth factor 18	-3.78
30	HAB1	B1 for mucin	-3.76
31	TRABD	TraB domain containing	-3.68
32	PSD	Pleckstrin and Sec7 domain containing	-3.63
33	SPTBN4	Spectrin, beta, non-erythrocytic 4	-3.61

34	EPS15L1	Epidermal growth factor receptor pathway substrate 15-like 1	-3.52
35	TEAD2	TEA domain family member 2	-3.5
36	SNX26	Sorting nexin 26	-3.48
37	MEX3A	Mex-3 homolog A (C. elegans)	-3.46
38	BRD4	Bromodomain containing 4	-3.43
39	SIK1	Salt-inducible kinase 1	-3.4
40	CNTROB	Centrobin, centrosomal BRCA2 interacting protein	-3.36
41	FMN2	Formin-2	-3.3
42	JSRP1	Junctional sarcoplasmic reticulum protein 1	-3.27
43	HMGB2	High-mobility group box 2	-3.26
44	MAPRE3	Microtubule-associated protein, RP/EB family, member 3	-3.23
45	TOMM40	Translocase of outer mitochondrial membrane 40 homolog (yeast)	-3.21
46	FAM83G	Family with sequence similarity 83, member G	-3.19
47	MORN1	MORN repeat containing 1	-3.17
48	WTIP	Wilms tumor 1 interacting protein	-3.16
49	HDAC10	Histone deacetylase 10	-3.13
50	TFRC	Transferrin receptor (p90, CD71)	-3.13

8.3 Table A3: Comparison of pathway enrichment between the current study and recent studies

Pathway	Current study	(Wen et al., 2013)	(Li et al., 2013)
1	Axonal guidance signalling	Spliceosome	Focal Adhesion
2	PKA Signalling	DNA replication	TGF-β signalling Pathway
3	Molecular mechanisms of cancer	Cell cycle	MAPK signalling pathway
4	TR/RXR activation	Focal adhesion	Regulation of actin cytoskeleton
5	Cyclins & Cell cycle regulation	ECM receptor	MicroRNAs in cardiomyocyte hypertrophy
6	Semaphorin signalling in neurons	Leucocyte transendothelial migration	Wnt signaling pathway and pluripotency
7	WNT/β-catenin signaling	Pyrimidine metabolism	EGF/EGFR signaling Pathway
8	Cell cycle G1/S checkpoint regulation	Cell adhesion molecules (CAMs)	Apoptosis
9	Sonic hedgehog Signaling	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	Adipogenesis
10	MODY Signaling	Ribosome	Endochondral Ossification

8.4 Table A4: Comparison of functions altered in the current study and following wide spread gene expression analysis

Function	Current study	Wang et al., 2011)
1	Gene expression	Development
2	Cellular growth & proliferation	Signal transduction
3	Cellular development	Apoptosis
4	Tissue development	Survival
5	Organismal development	Cytoskeleton remodelling
6	Cell death	Transcription
7	Skeletal & muscular system development and function	
8	Embryonic development	
9	Organ development	
10	Cancer	

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25 January 2010

REC REF: 009/062

Dr L Zerbini
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Dear Dr Zerbini

PROJECT TITLE: Inhibitions of the proteins AXL and DCUN1D1 in ovarian and prostate cancer cell lines in a mice xenograft model- a pilot study

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Investigators must please contact the office for the latest version of application form (Version 30 March 2009)
A Form for minor amendments (Version 25 April 2005) is also available.
Yearly progress reports submitted to ethics office is a requirement for ongoing approval of studies.
Notification of study closure is also a requirement.
Ethics approval letter and copy of application to be submitted to the Animal Unit when commencing study for release of animals.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF GRAHAM LOUW
CHAIR, HSF ANIMAL ETHICS

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Targeting neddylation in cancer therapy

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The neddylation conjugation pathway has a pivotal role in mediating ubiquitination of proteins and regulation of numerous biological processes. Dysregulation in the ubiquitination and neddylation pathways is associated with many cancers. Ubiquitination involves covalent attachment of ubiquitin to target proteins, leading to protein degradation by the proteasome system. The activity of the E3-ubiquitin ligase family, cullin-RING ligases, is essential for promoting ubiquitin transfer to the appropriate substrates. Neddylation, a process mediated by the protein NEDD8, is required for conformational changes of cullins, a scaffolding protein situated in the core of cullin-RING ligases, and regulation of E3 ligase activity. In this review, we present a comprehensive discussion of the recent findings on the neddylation pathway and its importance during tumorigenesis. The ramifications regarding the potential therapeutic use of ubiquination and neddylation inhibition are also discussed.

The appropriate balance of intracellular protein levels is essential for many cellular processes including the cell cycle and the regulation of gene expression. While protein synthesis is a relatively slow process, protein degradation provides a rapid and irreversible switch-like activity that shuts off key regulatory proteins.

One major pathway for protein degradation is the ATP-dependent three-step process of protein modification with long polyubiquitin chains made up of multiple ubiquitin monomers, with the ultimate aim to target proteins for recognition and processing by the 26S proteasome – a multiprotein complex containing four stacked rings with each ring composed of seven subunits, which is responsible for the ATP-dependent degradation of ubiquitin-tagged proteins [1]. Recently, the ubiquitin-like protein NEDD8 has been identified that similarly targets proteins for degradation via the neddylation pathway as described later in this review. As neddylation and ubiquitination strongly effect one another, the first part of this review will focus on the well-studied ubiquitination pathway.

The other major route for intracellular protein degradation, autophagy, has recently been reported to display active cross-talk with proteasome-mediated degradation [2,3]. While it has been suggested that certain substrates can be targeted by ubiquitylation for degradation via both systems, perturbations in the flux through either pathway has been reported to affect the activity of the other system [3]. The effect of this cross-talk with regard to tumorigenesis is best illustrated by the most common genetic target in human cancers, the tumor suppressor gene p53; inhibition of autophagy was reported to lead to impaired proteasome-mediated p53 degradation, which in turn may predispose cells to apoptosis [3].

The ubiquitination pathway begins with the activation of ubiquitin by E1 (also known as the ubiquitin-activating enzyme) in an ATP-dependent manner [4,5]. The ubiquitin-activating enzyme is the E1 that is thought to primarily perform this function; however, recently a relatively uncharacterized E1 enzyme, Uba6, was found to also activate ubiquitin [6]. The E1 enzyme then transfers the activated ubiquitin molecule to the cysteine residue of the E2-conjugating enzyme [7,8]. Several E2-conjugating enzymes have been identified so far with 35 active E2s already identified in humans. This increase in the number of E2s relative to the known E1s is suggested to account for substrate specificity in that distinct primary sequences within the core domains of E2s can specify cognate E3s, substrate specificity and localization [9]. The E3 ligases then form the final step in the cascade by facilitating the transfer of ubiquitin from E2 to the substrate [10]. Several hundred different E3 ligases have been identified that display substrate specificity and have been grouped into three classes based on the domain found in their core proteins. These include the HECT (homologous to E6-AP carboxy terminus), RING (really interesting new gene) and U-box E3 ligases [11–13]. Of particular

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interest in this review are the cullin-RING E3 ligases that are multicomponent complexes consisting of the RING finger domain-containing protein RBX1/2, a cullin protein that acts as a scaffolding molecule, and other adaptor proteins and substrate-binding sites [14]. The basic function of all the E3 ligases is to recognize specific substrates and to provide optimal conditions for ubiquitin transfer from E2-conjugating enzymes to the substrate.

Targets for the ubiquitin-dependent mechanisms in cancer

Ubiquitination plays a central role in cellular homeostasis and particularly in regulating the cell cycle by targeting and destroying regulatory proteins. Several malignancies and certain oncogenic viral infections are characterized by alterations of the ubiquitin–proteasome pathway, thereby disrupting the ubiquitination of proteins that control cell growth and death, leading to the deregulation of the cell cycle, specifically the G1 phase control and progression to S phase [15]. The development of cancer can be due to the stabilization of oncoproteins or the destabilization of tumor suppressor genes. For example, the tumor suppressor gene p53 has long been known to be degraded by the ubiquitin–proteasome pathway after infection with high-risk oncogenic HPV types [16]. Also, p53 is inactivated in a variety of cancers due to oncogenic activation and/or overexpression of its specific E3-ubiquitin ligase MDM2 [17]. Another well-known tumor suppressor protein associated with susceptibility to breast cancer is BRCA1, which harbors E3-ubiquitin ligase activity. When mutated, intermolecular interactions of BRCA1 with target proteins are affected, thus leading to predisposition to malignancies [18]. In renal cell carcinoma it was found that oncogenic mutations in components of the E3-ubiquitin ligase complex prevents degradation of the HIF1 transcription factor, resulting in its upregulation and predisposition to the formation of renal tumors [19]. High expression of the E3-ubiquitin ligase Smurf2 was found to correlate with poor prognosis in esophageal squamous cell carcinoma as this enzyme interferes with members of and inactivates the TGF-β signaling pathway [20]. Significant adverse impacts on the survival of esophageal squamous cell carcinoma patients was demonstrated by downregulation of the ubiquitin ligase FBXW7, which normally targets positive cell cycle regulators [21]. In colonic carcinoma, the tumor suppressor gene APC is often found as a truncated form that is no longer able to regulate cellular levels of β-catenin. Under normal conditions this protein is tightly controlled by the cellular machinery and targeted for ubiquitination and degradation as it can lead to the transcriptional activation of various oncogenes and cell cycle genes [22].

Therapeutic approaches for inhibition of ubiquitination in cancer

The aforementioned examples clearly demonstrate the central role of the ubiquitin–proteasome pathway for tumorigenesis. Therefore, protein degradation pathways via ubiquitination are potential targets for anticancer therapy. Many inhibitors have been tested and shown to inhibit either the proteasome or substrate-specific E3 ligases [23]. However, only a few drugs could be further developed in clinical trials as many have displayed poor metabolic stability, poor enzyme specificity, poor pharmacological effects and/or irreversible binding (and inhibition) of the proteasomal subunit [23]. The dipeptide boronic acid analogue bortezomib (VELCADE® [Millenium Pharmaceuticals], formerly known as PS-341) has been found to be a metabolically stable, potent, specific and reversible proteasome inhibitor as it selectively inhibits malignant cell growth and induces apoptosis and kills tumor cells, particularly in B-cell malignancies [24,25]. Bortezomib was the first anticancer drug inhibiting the 26S proteasome to be approved for single-agent use in the clinical treatment of patients with newly diagnosed multiple myeloma, relapsed/refractory multiple myeloma and mantle cell lymphoma [26,27]. In clinical trials it was found that bortezomib was relatively well tolerated by the patients, producing efficacious clinical responses with a 35% overall response rate and 10% complete responses [28]. This drug acts by forming a transition state intermediate with the enzymatically active chymotryptic site of the 20S core catalytic component of the proteasome [29]. Although this type of proteasome inhibition could theoretically disrupt many cellular processes, not only the proliferation of cancer cells, bortezomib was found to selectively inhibit malignant cell growth with some toxic effects on normal cells [30]. One explanation is that this drug interferes with the IκB/NF-κB regulatory system, a key characteristic of tumor development and progression [31]. In cancer cells, the transcription factor NF-κB is often constitutively expressed as its inhibitor IκB is degraded via the ubiquitin–proteasome pathway, thereby leading to the uncontrolled activation of NF-κB anti-apoptotic

target genes. Bortezomib is thought to stabilize IκBα through proteasome inhibition, resulting in inhibition of NF-κB activation [29]. G2–M arrest, the upregulation of the pro-apoptotic protein NOXA as well as the stabilization of p53 have also been discussed as possible mechanisms of bortezomib [32,33]. Despite its potent anticancer effects, over the years bortezomib has been shown to have some side effects, toxicities and resistance in individual patients. Several other proteasome inhibitors with improved properties are currently under clinical investigation such as carfilzomib and marizomib, which are still awaiting approval [26].

Rather than the inhibition of the proteasome, which affects a wide range of proteins, the inhibition of substrate-specific ubiquitin E3 ligases would represent potential therapeutic opportunities for targeted anticancer intervention. Currently, various clinical trials are underway testing drugs with such effects, although none of them have been approved for clinical treatment as yet. One of the most promising E3-ubiquitin ligase inhibitors is the small molecule Nutlin-3 that prevents the interaction of MDM2 with its substrate p53, thus stabilizing the tumor suppressor protein [34].

Another therapeutic approach would be the stimulation rather than the inhibition of the ubiquitin–proteasome pathway for the selected degradation of proteins associated with human cancers. One such example is the targeted downregulation of growth factor receptors that are aberrantly activated in a variety of malignancies. The drug trastuzumab (Herceptin®, Genentech), for example, is a monoclonal antibody against the growth factor receptor ErbB2. Binding of the antibody weakly activates ErbB2 resulting in its homodimerization and recognition by the ubiquitin proteasome system, thus leading to its degradation [18]. Trastuzumab has been approved for use in patients whose tumors overexpress ErbB2 and has shown clinical activity alone and in combination with chemotherapy in metastatic breast cancer [35,36].

Neddylation: a pathway of ubiquitin-like protein conjugation

Ubiquitination is not the only process that regulates the degradation of proteins. The ubiquitin-like protein NEDD8 (the mammalian homolog of Rub1 protein in *Saccharomyces cerevisiae*) [37] is involved in neddylation, a modulator of ubiquitin-induced protein degradation. NEDD8 is a small, highly conserved protein with 80% homology to ubiquitin. However, it has a very different conjugation pattern when compared with ubiquitin, and NEDD8-conjugated proteins are highly expressed in the nucleus relative to the cytoplasm [38,39]. The neddylation process involves the E1-activating enzyme complex, also known as the NEDD8-activating enzyme (NAE), composed of two subunits, APP-BP1 and UBA3, and the E2-conjugating enzyme, UBC12 (also known as UBE2M). UBA3 is homologous to the C-terminal end of the E1 enzyme involved in ubiquitination, whereas APP-BP1 is homologous to the N-terminus of the E1 protein [40]. UBC12 tethers selectively to NAE via a unique E1–E2-like interaction, and both the docking peptide and catalytic core domain of UBC12 must bind the NAE simultaneously for optimal transfer of NEDD8 from NAE to UBC12 (**Figure 1**) [41].

The most widely studied class of protein substrates of neddylation are the cullin-RING ligases (CRLs), which are anchored by cullins, a highly conserved family of proteins. The cullins Cul-1, Cul-2, Cul-3, Cul-4A, Cul-4B and Cul-5 are all modified by NEDD8, which is conjugated to them via its C-terminal Gly-76 residue [39,42,43]. The SKP1–Cul-1–F-BOX (SCF) functions as an E3 ligase for CRLs and consists of ROC1 (a small RING protein) bound to the C-terminus of Cul-1 and SKP1 bound to the N-terminus of Cul-1. ROC1 in turn binds to E2 and SKP1 can bind to a variety of F-BOX-containing proteins. CAND1, a 120 kDa HEAT repeat protein, works as a negative regulator of SKP1–Cul binding, by preventing the binding of SKP1 and F-BOX proteins to cullins [44]. The crystal structure of the CAND1–Cul-1–ROC1 complex shows that CAND1 clamps around the elongated Cul-1 [20]. Neddylation of the cullins dissociates CAND1, resulting in the assembly of an active ubiquitin ligase and subsequent substrate ubiquitination [42,44–46]. In this way, NEDD8 regulates the ubiquitination rate of the subset of proteins that are ubiquitinated via CRLs (**Figure 2**).

Therefore, the process of neddylation parallels that of ubiquitination. Each involves their own E1, E2, and E3 proteins in a chronological modification process in which small proteins need to be conjugated, activated and transferred to substrates.

The deneddylation of cullins is carried out by the COP9 signalosome (CSN). COP9 was first discovered as a novel protein complex consisting of eight subunits, which negatively regulates photomorphogenesis in *Arabidopsis thaliana* [47]. It was later rediscovered in various mammalian

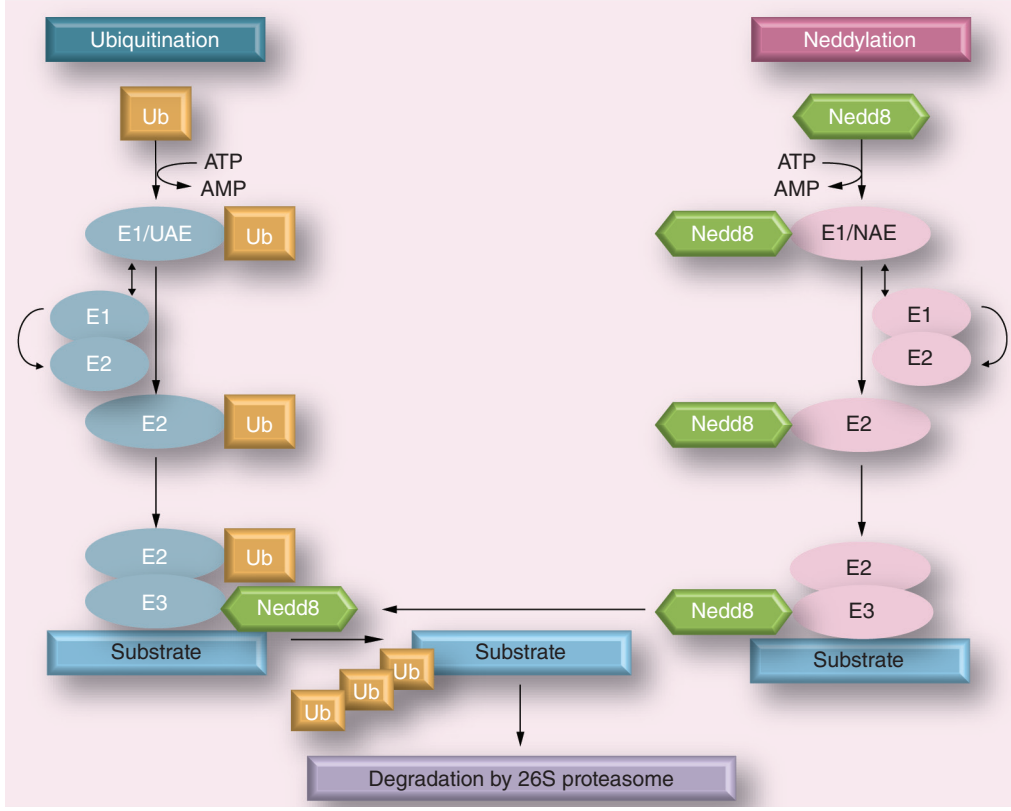

Figure 1. Schematic representation of ubiquitination and neddylation pathways.
In ubiquitination (left-hand panel), E1-activating enzyme activates and transfers ubiquitin to E2 ligase via trans-thiolation reaction. The ubiquitin-charged E2 combines with specific E3 ligases in order to catalyze the formation of the polyubiquitin chain on the substrate protein, which will be recognized and degraded via the proteasome. Neddylation (right-hand panel) is initiated by an E1 enzyme specific to NEDD8 (NAE), which utilizes ATP to generate NEDD8 adenylate. NEDD8 is transferred to a specific cysteine within NAE, generating a NAE–NEDD8 thioester and transferred to specific E2 ligases (such as UBC12 or UBE2F) via a trans-thiolation reaction. Finally, NEDD8 is transferred to a variety of E3 ligases such as the SKP1–Cul-1–F-BOX, which functions as an E3 ligase for cullin-RING ligases. The neddylation process also results in the assembly of an active ubiquitin ligase, assisting E3-catalyzed ubiquitylation and consequently substrate ubiquitination.
NAE: NEDD8-activating enzyme.

tissues [48,49], at which time it was renamed as the CSN complex, consisting of the subunits CSN1–CSN8, named according to their size [50]. The CSN is involved in various protein degradation pathways. However, in deneddylation it serves to remove the NEDD8 protein from the CRL by cleaving NEDD8 from Cul-1 [51–53]. The Jab1/MPN domain metalloenzyme, found in the CSN5 subunit, underpins the NEDD8 isopeptidase activity of the CSN [51]. The CSN binds to the SCF via CUL1 and ROC1, which interact specifically with subunits CSN2, CSN6 and the N-terminus of CSN1 [52–54] (Figure 3). Another protein involved in deneddylation of NEDD8 is DEN1 [55,56], also known as NEDP1. DEN1 is a cysteine protease that demonstrates a 60,000-fold preference for NEDD8 over ubiquitin and acts as a NEDD8 isopeptidase by deconjugating NEDD8 from cullins [57,58]. If DEN1 activity is increased, deneddylation of cullins is strongly affected and it may result in blocking degradation of ubiquitinated proteins with an impact on cell cycle progression. In this context, DEN1 has also been found to remove NEDD8 from hyperneddylated cullins although not from mononeddylated cullins, maintaining the mononeddylated cullins levels necessary for the ubiquitination by SCF ubiquitin ligases [55,56]. However, DEN1 has an antagonistic role as it was shown to cleave off the C-terminal end of NEDD8, to form a mature NEDD8 molecule that is necessary for the neddylation process [59].

Targets for neddylation in cancer

Interestingly, it was recently shown that a variety of cellular stresses lead to a drastic increase in neddylation within the cell, which is mediated via the ubiquitin E1 enzyme Ube1 and

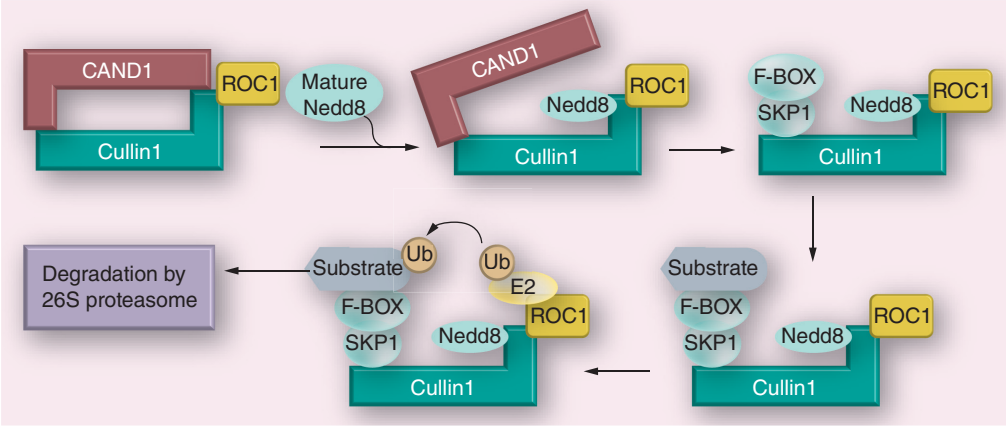

Figure 2. The regulation of cullin–RING ligases via CAND1 and NEDD8. CAND1 binds to the cullin–ROC1 complex blocking the SKP1 binding site. Mature NEDD8 then binds to the cullin–ROC1 complex, displacing CAND1. SKP1 and F-BOX are now free to bind and recruit the substrate. E2 binds to the cullin–RING ligases complex via ROC1 and recruits ubiquitin to the substrate resulting in ubiqitination of the protein leading to proteasome degradation.

not NAE [60]. The most widely studied neddylation targets include the CRLs, the EGF receptor and the E2F-1-transcription factor [61,62]. Very recently there have been a number of papers indicating that parkin and PINK1 are involved in the neddylation cascade. Both proteins are involved in Parkinson's disease [63,64]. Currently, the criteria by which proteins are chosen for the neddylation pathway is still unknown; however, many proteins involved in cancer progression have been identified as neddylation targets. One of these targets is the VHL protein. Mutations in VHL result in some rare familial cancer syndromes as well as the majority of sporadic renal cancers (as a result of bialleic loss of *VHL*) [65,66]. This is just one of many proteins that have been shown to play a role in cancer and are also involved in neddylation. In high-grade neuroendocrine lung tumors, a low level of CAND1, resulting in high levels of neddylated Cul-1, has been associated with disease [67]. Another example is SCCRO (also known as DCUN1D1), which forms part of the E3 ligase complex (Cul-1–ROC1–SCCRO) for neddylation. SCCRO recruits the Ubc12–NEDD8 thioester to the neddylation E3 complex, thereby promoting cullin neddylation. In this way, SCCRO provides an additional level of regulation to the ubiquitination carried out by CRL-containing E3 complexes. Due to the wide range of proteins regulated by CRL-containing E3s, it is not surprising that SCCRO has been implicated in a variety of human cancers [68]. SCCRO, when overexpressed, is associated with an aggressive form of primary squamous cell carcinoma of the lung. Findings thus far indicate SCCRO as an oncogene that induces

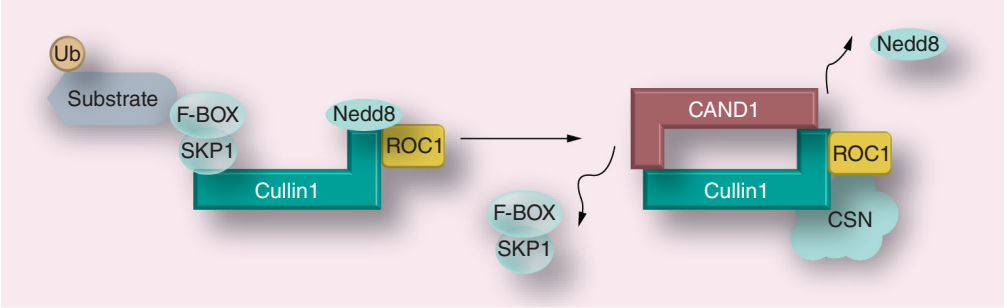

Figure 3. The deneddylation of target proteins via the COP9 signalosome. Once a protein substrate has been ubiquitinated, the SKP1–Cul-1–F-BOX complex is then deneddylated by the CSN. The CSN binds to the SKP1–Cul-1–F-BOX complex via the interaction between Cullin-1–ROC1 and via CSN2, CSN6 and the N-terminus of CSN1, the subunits within the CSN complex. The isopeptidase activity of the CSN results in the cleavage of Nedd8 from the cullin–RING ligases. This in turn allows the binding of CAND1, which displaces SKP1 and F-BOX, returning the cullin–RING ligases to the unneddylated form.
CSN: COP9 signalosome.

Table 1. Relevance of neddylation substrates in different types of cancer.

Type of cancer	Neddylation substrate	Substrate function	Ref.
Renal	VHL	E3-ubiquitin ligase activity, tumor suppressor gene	[65,66]
High-grade neuroendocrine lung tumors	Cul-1	Degradation of proteins that regulate cell cycle progression	[67]
Squamous cell carcinomas	SCCRO, also known as DCUN1D1	Forms part of the E3 ligase complex for neddylation	[68,69]
Liver and colon	Hu antigen R	RNA-binding protein regulating cell de-differentiation, proliferation and survival	[74]
Gastrointestinal	IKKγ	Leads to activation of NF-κB	[75]
Various types of cancer	Mdm2	Promotes neddylation of p53	[73]
Various types of cancer	Cul-1 component of $SCF^{\beta\text{-}TrCP}$	Processes p105, the inactive precursor of a subunit of NF-κB	[76]

the transformation of cells and sustains the malignant cancer phenotype [69]. Mdm2 is well known to be involved in the ubiquitination and degradation of the tumor suppressor gene p53, as well as for its ability to act as an E3 ligase [70–72]. However, Mdm2 in its role as a RING finger E3-ubiquitin ligase is also neddylated and promotes conjugation of NEDD8 to p53. The critical cysteine C462 in the RING finger domain of Mdm2, required for its E3-ubiquitin ligase activity, is also required for Mdm2-dependent neddylation. It is interesting to note that the lysine residues within Mdm2 that are required for Mdm2-mediated ubiquitination and proteasomal degradation of p53 include the lysines that are required for neddylation. This evidence demonstrates a central role for Mdm2 in p53 neddylation and highlighted Mdm2 as an E3 NEDD8 ligase for the first time [73]. Mdm2 also neddylates Hu antigen R (HuR), a central RNA-binding protein regulating cell dedifferentiation, proliferation and survival. In proliferative hepatocarcinoma, colon cancer cells and biopsies, HuR is overexpressed due to Mdm2-mediated neddylation, stabilizing HuR and protecting it from degradation [74]. NF-κB, a critical player in cell survival and proliferation, is controlled indirectly via neddylation. Overexpression of TRIM40 (a RING finger domain-containing protein) results in neddylation of IKKγ and inhibition of NF-κB activity, thereby implicating NEDD8 conjugation of IKKγ as a negative regulator for NF-κB activity [75]. Neddylation is required for efficient $SCF^{\beta\text{-}TrCP}$-mediated ubiquitination and processing of p105 following phosphorylation of the molecule by IKKβ. p105 is a precursor of one of the subunits in NF-κB and thus neddylation adds another level of regulation to the NF-κB pathway, via IKKβ and IKKγ [76]. Table 1 summarizes the substrates of neddylation involved in various types of cancers.

Therapeutic approaches targeting neddylation

Targeting proteins involved in protein degradation appears to be an excellent option for cancer therapy. As mentioned before, bortezomib was the first commercially available drug directly targeting ubiquitination by inhibiting the action of the 26S proteosome. However, given that ubiquitination has a wide range of substrate targets, it is not surprising that bortezomib has some side effects. The answer may rely on targeting neddylation specifically.

MLN4924 is a small molecule inhibitor of NAE, discovered in 2009, that has been shown to be very effective, without the undesired side effects seen in bortezomib [77,78]. NAE catalyzes the formation of a covalent NEDD8-inhibitor adduct with MLN4924. By blocking NAE, the turnover of CRL substrates is completely disrupted and leads to the activation of apoptosis as a consequence of the deregulation of S-phase DNA synthesis. *In vivo* tests demonstrated that MLN4924 suppresses the growth of human tumor xenografts at doses that are well tolerated without any serious toxicity problems. In mouse models, a single dose of MLN4924, at 10, 30 or 60 mg/kg, was shown to reduce NEDD8–cullin levels after 30 min in a similar manner across all three concentrations. *In vitro* studies also demonstrated that MLN4924 treatment inhibited overall protein turnover by approximately 9% ($p = 0.023$) 4-h post-treatment, while bortezomib inhibited protein turnover by approximately 50% ($p < 0.001$) [77,78]. In acute myeloid leukemia mouse models, the generation of reactive oxygen species was shown to be a major contributor to MLN4924-induced apoptosis. In this study, MLN4924 led to a significant regression

of disease and inhibition of neddylated cullins [79]. In preclinical models of activated B-cell-like diffuse large B-cell lymphoma, treatment with MLN4924 resulted in potent NF-κB pathway inhibition. *In vivo* studies in mice bearing human xenograft tumors of activated B-cell-like and germinal-center B-cell-like diffuse large B-cell lymphoma blocked NAE pathway biomarkers and resulted in complete tumor growth inhibition [80].

Apoptosis does not seem to be the only route by which MLN4924 exerts its effects. In various human cancer cell lines, MLN4924 treatment induced irreversible senescence independent of pRB/p16 and p53, but dependent on p21, a mediator of senescence and a known substrate of CRL/SCF E3s. MLN4924 action therefore seems to be two-pronged via induction of apoptosis and irreversible senescence [81].

Recent studies have focused on using MLN4924 as a radiosensitizer for the treatment of pancreatic cancer in mouse models and otherwise resistant leukemia cell lines. By using MLN4924 in conjunction with already established radiation therapy treatments, these studies demonstrated the synergistic effect of MLN4924 with other chemotherapeutics [82,83]. The success of MLN4924 in these preliminary studies is encouraging and highlights the importance of designing other neddylation pathway inhibitors that could prove to be novel chemotherapeutic agents in the future.

Conclusion

Neddylation and ubiquitination have been shown to regulate several molecules involved in crucial steps of tumorigenesis. Our current understanding of the mechanism of action of the neddylation process supports the notion that inhibition of this pathway is a novel and promising cancer therapeutic strategy. A good example is the development of MLN4924, a NAE small molecule inhibitor. MLN4924 actions alter the function of vital tumor suppressors and oncogenes and led to encouraging results in preclinical trials as a monotherapy or in combination with other chemotherapeutic agents.

Future perspective

Inhibition of ubiquitination as a new entry point for cancer therapy has been under investigation for many years. Bortezomib has demonstrated certain cancer cell selectivity but also some cytotoxicity as ubiquitination has a wide range of substrate targets. Inhibition of the neddylation pathway is an underexplored therapeutic approach in cancer and it may be a key new candidate for targeted therapy. Our knowledge of the importance of the neddylation pathway obtained over the past decade indicates that selective disruption of the neddylation conjugation process may be an innovative and unique approach to fight cancer that could be less

Executive summary

Background

- Ubiquitination regulates intracellular protein levels and is essential for many cellular processes including the cell cycle and the regulation of gene expression.

Targets for the ubiquitin-dependent mechanisms in cancer

- Several malignancies and certain oncogenic viral infections are characterized by alterations of the ubiquitin–proteasome pathway.
- The deregulation of many E3 ligases, such as MDM2, BRCA1 and Smurf2, has been implicated in many cancers.

Therapeutic approaches for inhibition of ubiquitination in cancer

- Bortezomib was the first anticancer drug inhibiting the 26S proteasome.
- Other proteasome inhibitors with improved properties are currently under clinical investigation, such as carfilzomib and marizomib.

The role of neddylation in cancer

- Neddylation regulates the protein degradation pathway. The most widely studied class of protein substrates of neddylation are the cullin-RING ligases.
- The deneddylation of cullins is carried out by the COP9 signalosome and DEN1, also known as Nedd8-specific protease.

Targets for neddylation in cancer

- Substrates of neddylation and molecules involved in the process, for example VHL, CAND1 and SCCRO, have been implicated in various cancers.

Therapeutic approaches targeting neddylation

- MLN4924 is a small molecule inhibitor of the neddylation-activating enzyme and exerts effects via induction of apoptosis and irreversible senescence. MLN4924 in conjunction with already established radiation therapy treatments shows a synergistic effect.

Conclusion

- Neddylation may serve as a potential target in novel and promising cancer therapeutic strategies.

associated with toxicity. Future work, resulting in a better understanding of this pathway and how it specifically interferes with development and progression of cancer regulation, will provide additional entry points to selectively impede NEDD8 function and is clearly worth exploring. Furthermore, the development of specific neddylation inhibitors that could be applied alone or in combination with other therapeutic agents in clinical trials might lay down the foundation for a more effective cancer treatment.

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▪▪ of considerable interest

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▪▪ **The role of ubiquitination in several types of cancer is discussed in detail.**

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