Characterising the mechanism of DCUN1D1 activity in prostate cancer and identifying DCUN1D1 inhibitors for prostate cancer treatment.

By

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DECLARATION

I, **Akhona Vava**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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I would also like to thank my family for their unwavering support and Olwethu Cata for the treasure of partnership.

"As a man thinketh in his heart, so is he"

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List of Abbreviations

ADT	Androgen deprivation therapy
AJCC	American joint committee on cancer
ATG12	Autophagy-related protein 12
ATG8	Autophagy-related protein 8
BBS	BES-buffered saline
BING	Best inferred genes
CAND1	Cullin associated and neddylation dissociated 1
CMap	Connectivity map
CRL	Cullin RING E3 ligases
DCUN1D1	Defective cullin neddylation 1 domain containing 1
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
DTT	Dithiothreitol
EI	Electron ionization
ER	Endoplasmic reticulum
ESI	Electron spray ionization
FASP	Filter assisted sample preparation
FAT10	HLA-F adjacent transcript 10
FBS	Fetal bovine serum
FDR	False discovery rate
FUB1	Fau ubiquitin-like
GO	Gene ontology
H&E	Haemotoxylin and eosin
HECT	Homolog to E6-AP c terminus
HIFU	High intensity focus ultrasound
IPMS	Immunoprecipitation-coupled mass spectrometry
ISG15	Interferon-stimulated gene 15
KEGG	Kyoto encyclopedia of genes and genomes
LINCS	Library of integrated network-based cellular signatures
MALDI	Matrix-assisted laser desorption/ionization
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NAE	Neddylation activating enzyme
NCCN	National comprehensive cancer network
NEDD8	Neural precursor developmentally downregulated 8
PAST	Palaeontology statistics
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCa	Prostate cancer
PCA3	Prostate cancer antigen 3
PSA	Prostate specific antigen
PTM	Posttranslational modification
RING	Really interesting new gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCCRO	Squamous cell carcinoma related oncogene
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labelling of amino acids in cell culture

snRNA	Small nuclear ribonucleic acid
SUMO	Small ubiquitin-related modifier
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TIC	Total ion current
TMPRSS2-ERG	Transmembrane protease serine 2-estrogen responsive gene
TNM	Tumour node metastasis
tRNA	Transfer ribonucleic acid
UAE	Ubiquitin activating enzyme
UBC	Ubiquitin conjugation enzyme
UBD	Ubiquitin binding domains
UBL	Ubiquitin-like protein
UBL5	Ubiquitin-like protein-5
UICC	Union for international cancer control
UPP	Ubiquitin proteasome pathway
URM1	Ubiquitin related modifier 1
US FDA	United States of America food and drug administration
VACURG	Veterans administration cooperative urological research group
WHO	World health organisation

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Abstract

DCUN1D1 is an E3 ligase of the neddylation pathway. It mediates the posttranslational modification of majority of the cullin family of proteins with NEDD8. This activity is known to enhance ubiquitination of the cullin RING E3 ligases, however, the extent of the impact of DCUN1D1's activity is underexplored. Studies performed previously in our lab demonstrated the role of DCUN1D1 in prostate cancer *in vitro* and *in vivo.* We also identified potential inhibitors of DCUN1D1 which inhibited the proliferation of prostate cancer cells in a DCUN1D1-specific manner. This study seeks to determine the mechanism of action of DCUN1D1 in prostate cancer and to identify DCUN1D1 inhibitors using a proteomics approach. Immunoprecipitation-coupled mass spectrometry was performed to identify DCUN1D1 binding partners and we identified some known substrates of DCUN1D1 in the form of cullin 3, cullin 4B and cullin 5. We also observed that the DCUN1D1 pulldown products implicated the ubiquitin proteasome pathway, transcription, lipid metabolism and inflammatory pathways. SILAC quantitative proteomics analysis was also performed to determine the proteins that were differentially expressed in DU145 DCUN1D1 knockdown cells relative to DU145 control cells. Interestingly, we did not identify the cullin proteins or classical components of the neddylation pathway but identified the ubiquitin activating enzyme, UBA1. We also found that dysregulation of DCUN1D1 in prostate cancer led to a dysregulation in translation-related and protein processing activities such as dysregulation of eukaryotic protein translation, and protein processing in the endoplasmic reticulum. We also observed the recurrence of the WNT signalling pathway across the proteomics approaches. This culminated in the exploration of the mechanism of action of DCUN1D1 in prostate cancer using changes in protein expression as measured by western blot analysis. Significantly, we determined that DCUN1D1 mediates its mechanism of action in prostate cancer, through the neddylation pathway and preferential neddylation of cullin proteins. We also observed that knockdown of DCUN1D1 in prostate cancer led to the dysregulation of the ubiquitination and WNT/β-catenin pathways. Furthermore, advanced connectivity map analysis was performed to identify potential inhibitors of DCUN1D1 based on a proteomics approach. The drugs found to strongly connect with the DCUN1D1 knockdown signature included kinase inhibitors and anti-inflammatory agents. The above observations could lead to improved understanding of DCUN1D1 and its potential for molecular target based treatment of prostate cancer.

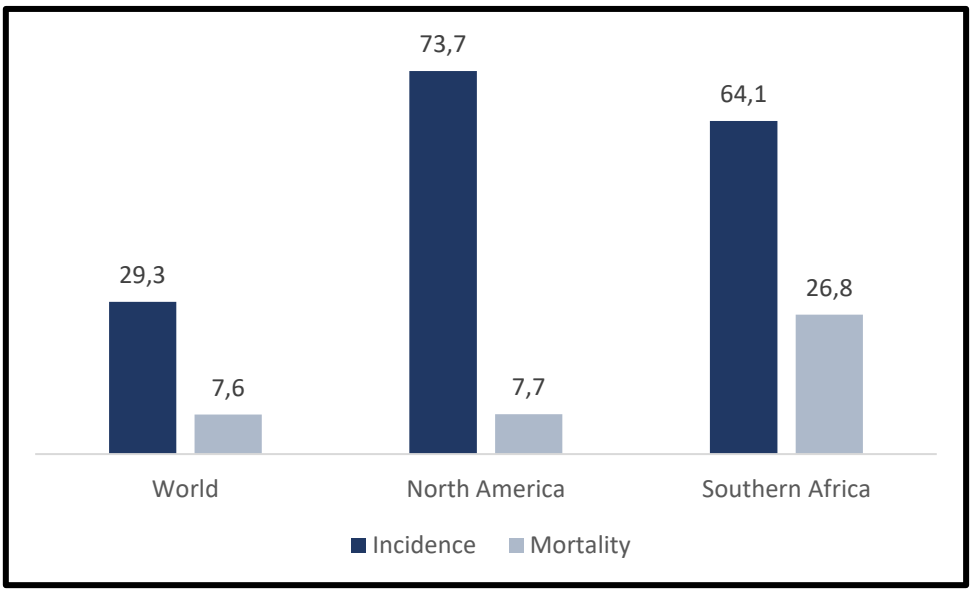
Chapter 1: Introduction

1.1 Prostate Cancer

1.1.1 Background

Prostate cancer (PCa) is the third most common cancer in the world and according to the South African National Cancer Registry 2014, it is the second most common cancer in all South African men (Bray *et al.*, 2018). It develops in the small male reproductive organ situated in the lower body, due to uncontrollable growth of prostate cells. The risk factors associated with it include: age, race, family history of PCa as well as other factors which have not been consistently identified across different studies (Steinberg *et al.*, 1990; B. S. Carter *et al.*, 1992; Hsing, Tsao and Devesa, 2000; Giri and Beebe-dimmer, 2016; Siegel, Naishadham and Jemal, 2017). The diagnosis and treatment options for PCa require exploration of the prostate gland through the rectum or anorectal region, making it difficult to access. This contributes to patient screening, diagnosis and treatment problems with some men being reluctant to be examined with the available approaches. However, PCa has one of the highest cancer-related mortality rates such as in the United States of America (USA), where it was ranked 3rd highest in terms of estimated deaths in 2017 (Ferlay *et al.*, 2015; Siegel, Naishadham and Jemal, 2017). Significantly, according to Globocan 2018, Africa has an increasing PCa burden with increasing incidence and mortality rates (Bray *et al.*, 2018). Figure 1 below illustrates incidence and mortality rates globally, focusing on Southern Africa and North America for comparison.

Figure 1. Estimated age-standardised rates (ASR) per 100 000 of prostate cancer incidence and mortality. Adapted from Globocan 2018.



As is evident in the figure above**,** like a variety of diseases, there are some notable differences in the PCa incidence and mortality rates across the developed and developing regions. While developed regions such as North America show an increased number of new cases due to successful screening programmes, they also have decreased mortality rates. In contrast, a number of African countries such as in Southern Africa, exhibit high incidence and high mortality rates which may be attributed to challenges in treatment options due to the generally lower resource availability (Chu *et al.*, 2011). South Africa is interesting in that, although it's incidence rates are high (46.53 per 100 000; 6807 new cases) and it has better screening programmes than other African countries, it's mortality rates are still relatively high (Ferlay *et al.*, 2015). This is likely due to similar healthcare and finance problems. Therefore, there is a high PCa burden in the world and to understand PCa better, we begin by reflecting on the basic structure and function of the prostate gland.

The prostate gland is a small gland situated in the pelvic region of the body and it is made up of branched glands, with the ducts within the glands composed of secretory epithelial cells, basal cells as well as neuroendocrine cells (Figure 2) (McNeal, 1988). The secretory epithelial cells play a crucial role in normal prostate gland function as they respond to androgen receptor stimuli and produce the important prostate gland antigens e.g. prostate specific antigen (PSA) and prostatic acid phosphatase (Cunha *et al.*, 1987; McNeal, 1988). Significantly, PSA is a widely-used biomarker for PCa that is used together with other predictors of clinical behaviour to help determine if a patient has PCa (Stamey *et al.*, 1987). It is also used combinatorially to determine the extent of disease progression, based largely on observable differences in the prostate gland from its normal to diseased state (Stamey *et al.*, 1987).

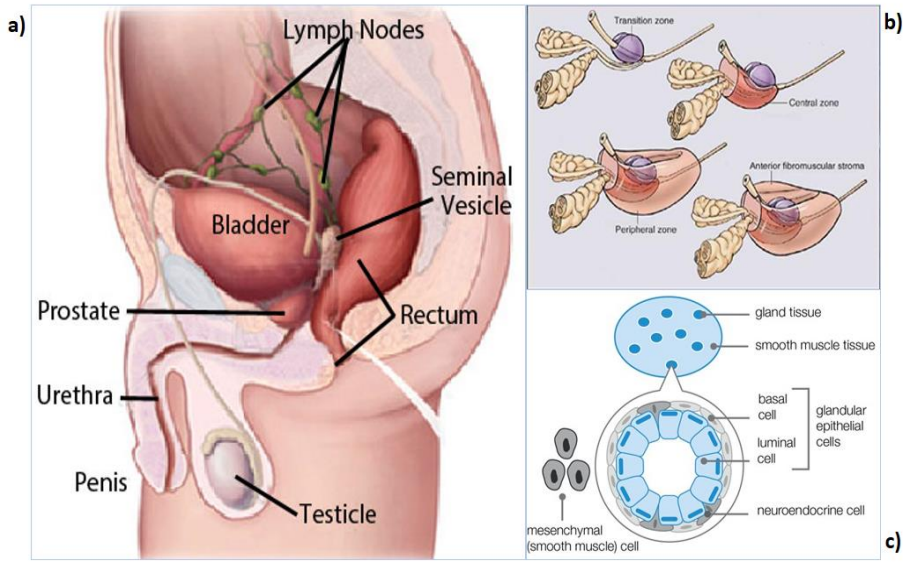


Figure 2: Images of the prostate gland body location, anatomical zones and cellular composition. a) Diagram showing the location and relationship of organs around the prostate from the lymph nodes, seminal vesicle, bladder, rectum, urethra, penis and testicles. The prostate is situated below the bladder and in front of the rectum. b) Anatomical zones of the prostate including the transition, central, peripheral and anterior fibromuscular zones. c) *Top,* cross-section through the prostate gland showing smooth muscle tissue and glandular tissue, *bottom)* cross-section of the prostate duct showing glandular epithelial cells including basal, luminal (secretory), neuroendocrine and mesenchymal cells of the surrounding smooth muscle tissue.
Source: http://www.whathealth.com/prostatecancer/pictures/anatomy, http://prostatecanceruk.org/media/2493051/3836-prostate-tissue-structure-diagrams-v5, http://www.hitachimed.com/self-learning-activity/docs/PelvisImagingModule/images/prostatezones

1.1.2 Prostate cancer screening and diagnosis

Determining whether a patient has PCa normally includes testing for PSA, digital rectal examination (DRE), biopsies and ultrasonography for imaging (Aus *et al.*, 2005; Herbst, 2013). Testing for PSA was initially approved by the United States of America Food and Drug Administration (US FDA) in 1986 for use in monitoring PCa progression in men. In 1994, it was approved for use in conjunction with DRE to test for PCa in asymptotic men during screening and it is currently used worldwide during screening and diagnosis. At a molecular level, PSA is a component of prostatic fluid and is a peptidase that cleaves semenogelin-1 in semen, which liquefies it for sperm motility (Lilja, 1985; Watt *et al.*, 1986; Lilja *et al.*, 1987; Lee *et al.*, 1989). It is also found in the bloodstream but its levels are altered by some drugs, inflammation of the prostate, urinary tract infections, benign prostate hyperplasia and PCa (Crawford *et al.*, 1996; Ulleryd *et al.*, 1999; Hochberg, Armenakas and Fracchia, 2000; Herrala *et al.*, 2001; Hugosson *et al.*, 2004; Andriole *et al.*, 2005; Sävblom *et al.*, 2005; De Marzo *et al.*, 2007; Chang, Harshman and Presti, 2010). Previously, 4.0 ng/mL was considered a threshold for normal PSA with levels above this suggesting irregularities within the gland, however, several studies have demonstrated the inconsistencies in PSA levels and PCa disease stage or progression (Partin *et al.*, 1990; Catalona *et al.*, 1991, 1998, 2000; H. B. Carter *et al.*, 1992; Stenman *et al.*, 1994; Gann, Hennekens and Stampfer, 1995; Thompson *et al.*, 2004; Andriole *et al.*, 2005).

Some patients have been diagnosed as having aggressive PCa while their measured PSA levels are below 4.0 ng/mL (Catalona *et al.*, 2000). Additionally, the use of PSA testing during screening has come under significant scrutiny across the world and as shown in Figure 3 below, different guidelines are suggested for PCa screening in South Africa, Europe and North America. Data obtained from clinical trials demonstrated a lack of specificity in PSA testing for PCa, as such, the United States Preventative Services Task Force recommended in 2012 against the use of PSA during PCa screening (Moyer *et al.*, 2012). Mainly due to concerns that it was leading to the over-diagnosis and over-treatment of patients. Even though PSA testing was detecting potential disease, only 20 - 30% of patients screened progressed to advanced disease but the scare of knowing the potential for disease progression was leading to patients undergoing multiple procedures, which often have complications (Moyer *et al.*, 2012). Additionally, a high number of false positive and false negative detections were occurring when DREs were performed and biopsies were taken following the PSA test and this led to concerns of treating patients unnecessarily or potentially missing patients with high disease risk (Andriole *et al.*, 2005; Schröder *et al.*, 2014). Although some institutions still use PSA testing, they include testing for PSA density, PSA velocity, PSA isoforms and apply age-specific ranges, particularly in the absence of better biomarkers currently (Benson *et al.*, 1992; Oesterling *et al.*, 1993; Smith and Catalona, 1994; Özen and Sözen, 2006).

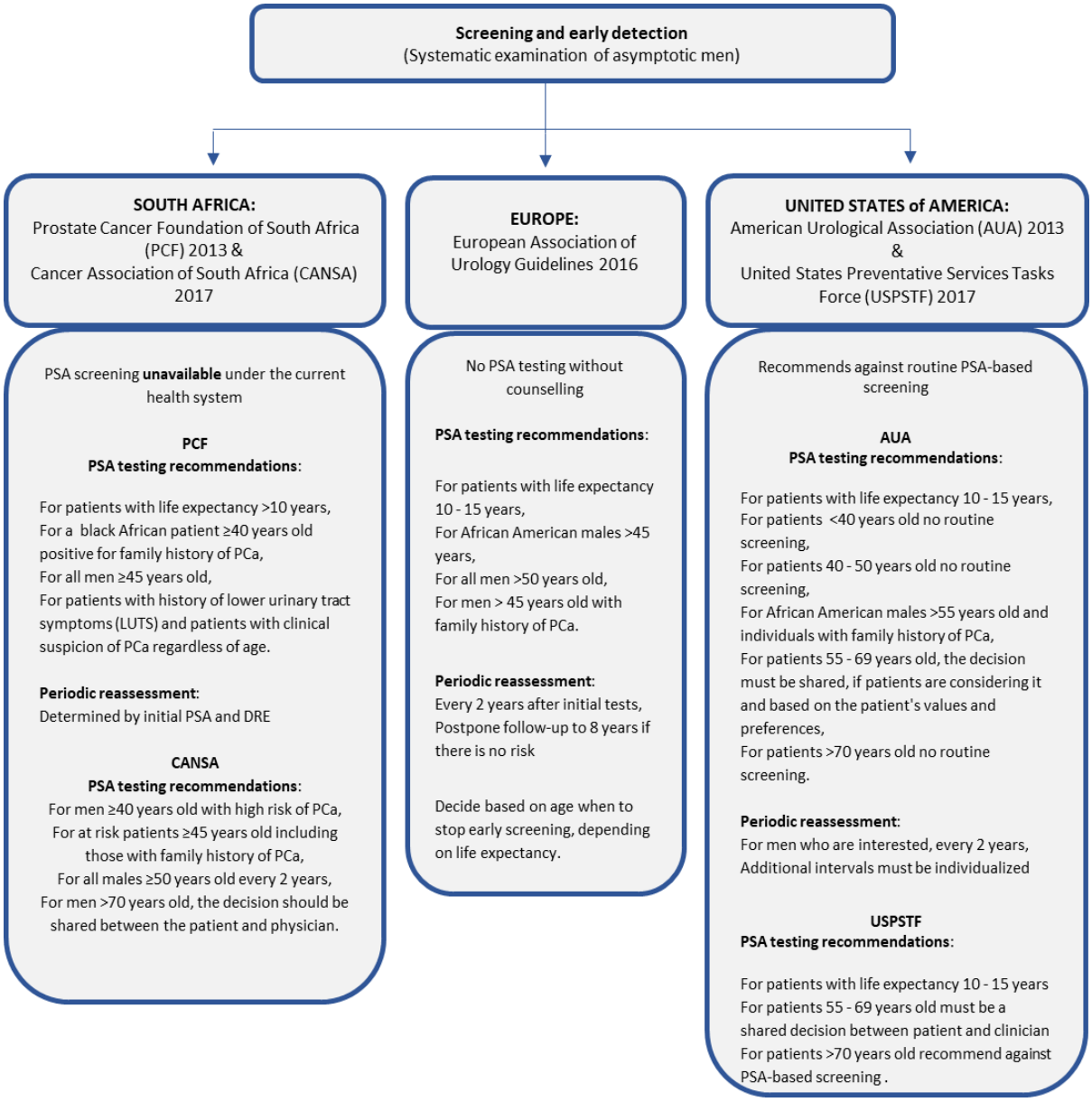


Figure 3. Schematic diagram of prostate cancer screening and early detection guidelines from South Africa, Europe and North America. The information is adapted from the Prostate Cancer Foundation of South Africa 2013 guidelines, the European Association of Urology 2016 guidelines, the American Urological Association (AUA) 2013 guidelines & the United States Preventative Services Tasks Force (USPSTF) 2017 comments on draft recommendation statements from 2012.

Prostate cancer biomarker discovery is an extensive field which aims to identify molecules and tests that can be used for the detection of PCa in patients. It can also be used to assist in stratifying disease risk, in staging, in making decisions on treatment options (where necessary) and for prognosis prediction. Body fluids such as the blood, urine and prostate tissue samples are used to test for molecules that are differentially expressed by quantity, phenotype and/or by body distribution. Less invasive methods such as blood or urine sample collection are normally used during the detection or

screening tests. However, more invasive approaches are required for accuracy of diagnosis. In addition to PSA, other molecules have been studied for use as PCa biomarkers including the prostate cancer antigen 3 (PCA3) and transmembrane protease serine 2-estrogen responsive gene (TMPRSS2-ERG) (Leyten *et al.*, 2014). A detailed description of the molecules and tests currently under investigation is provided in Table 1 and these molecules/tests show variable sensitivity and specificity to PCa relative to PSA. Furthermore, a variety of gene and protein expression assays have been developed to test panels of aberrantly expressed genes e.g. Oncotype Dx, Prolaris score, Decipher, ExoDx, SelectMDx or to test for aberrantly expressed proteins (Prostarix, ProMark) in order to help with decision making (Cuzick *et al.*, 2011, 2012; Cooperberg *et al.*, 2013; Erho *et al.*, 2013; Knezevic *et al.*, 2013; Mcdunn *et al.*, 2013; Shipitsin *et al.*, 2014; Klein *et al.*, 2014; Cullen *et al.*, 2015; McKiernan *et al.*, 2016; Neste *et al.*, 2016). Interesting, the ProMark assay contains 8 proteins including Cullin 2, a known substrate of DCUN1D1 (Kim *et al.*, 2008; Shipitsin *et al.*, 2014). Overall, some of the biomarker tests still require a concurrent urine PSA test and require more costly procedures such as RNA amplification assays, immunofluorescence and professional expertise to perform them, limiting their widespread use. Best practice thus far is still reliant on PSA testing, DRE, biopsies plus other laboratory and clinical information for decision making.

DRE is one of the standard diagnostic procedures and involves a doctor physically exploring the prostate gland to detect any changes in texture, size or shape of the prostate. The prostate gland is composed of four anatomical zones that differ in prevalence of tumour development, they include the peripheral, central, transitional and the anterior fibro-muscular zone (Figure 2) (McNeal J.E., 1981). The zones differ in composition with the peripheral and transitional zones consisting of 70 and 75% glandular tissue, respectively. The central zone contains 20% glandular tissue while the fibro-muscular zone consists of mesenchymal cells (McNeal J.E., 1981). Interestingly, over 90% of PCa tumours are adenocarcinomas and they are found mainly in the peripheral zone followed by the transitional zone (McNeal JE, Redwine EA, Freiha FS, 1988). During DRE, the doctor can feel a significant portion of the prostate, the peripheral zone is easier to access from the rectal region but access to the other zones is limited by the size and location of the prostate relative to surrounding organs. However, the limitations of DRE are improved upon by the examinations being performed by experienced doctors as correlations are observed between the accuracy of diagnosis with experience (Smith and Catalona, 1995). The examination in conjunction with PSA testing and biopsy acquisition assists in making a diagnosis.

Table 1: Summary of current/emerging prostate cancer detection molecules and tests

Test	Description	Sample	Measurement	Predictive value	Approval/ Endorsement	References
4K Score	-Composed of 4 kallikrein proteins -Secondary test either before initial or repeat biopsy -Commercially available	Urine, blood and/or biopsy	Prediction model containing: age, initial biopsy, DRE plus total PSA, free PSA, intact PSA and hexokinase 2	Provides clinician with the patient's risk of biopsy detectable-PCa	National Comprehensive Cancer Network 2016	(Bryant *et al.*, 2015; Parekh *et al.*, 2015)
Prostate health index	-Combines total PSA, free PSA and -2proPSA	Blood	–(p2PSA/fPSA) × PSA½	-Distinguishes between PCa and BPH -Improves detection of high-risk cases -Correlates with adverse tumour features and biopsy reclassification	-FDA approved 2012 -National Comprehensive Cancer Network 2016	(Lazzeri *et al.*, 2013)
PCA3	-Spliced, long, non-coding RNA -Commercially available kit	Urine: Post-digital rectal examination	PCA3 score = PCA3 RNA molecules/ PSA RNA molecules	-Prostate-specific -Good for early detection of PCa -Good for repeat biopsies	-FDA approved 2012 -EAU guidelines	(Crawford *et al.*, 2012; Luo *et al.*, 2014)
TMPRSS2:ERG	-Fusion gene at chromosome 21 -TMPRSS2 is androgen responsive -ERG is oestrogen responsive	Urine	TMPRSS2:ERG score =(TMPRSS2-ERG mRNA/ PSA RNA copies) × 100 000	-Under investigation -Studies differ on prognostic and predictive value	No	(Tomlins *et al.*, 2005; Pettersson *et al.*, 2012; Leyten *et al.*, 2014)
Mi-Prostate Score	-Combines PCA3, TMPRSS2:ERG and PSA -Commercially available	Urine and blood	Logistic regression models containing multivariate Prostate Cancer Prevention Trial risk calculator version 1.0 plus TMPRSS2:ERG and PCA3 score	-More accurate PCa risk assessment -Increased predictive value than PCA3 and TMPRSS2:ERG alone	No	(Tomlins *et al.*, 2016)

Biopsy acquisition is a critical component of diagnosis as it provides visual examination of the tissue, which can provide information on tissue level differentiation. It involves removing small samples of tissue to be observed under the microscope. The most commonly used method is transrectal ultrasound (TRUS) where a thin needle is inserted into the prostate through the rectum under the guidance of an ultrasound for sampling (Hodge *et al.*, 1989; Harvey *et al.*, 2012). The surgeon either locates an area of concern or takes 10 - 12 samples in various areas of the prostate, where the needle retrieves thin, cylindrical sections of tissue that are collected for observation. The samples are stained using haemotoxylin and eosin (H&E) staining which dyes nucleic acids purple and proteins/cytoplasmic components pink/red and allows a pathologist to see the normally transparent tissue components (Fischer *et al.*, 2008). As mentioned previously, the prostate is a glandular organ, which under normal conditions has a typical organized tissue structure. During tumourigenesis the normal cells circumvent several regulatory mechanisms leading to uncontrollable growth and changes in tissue organisation (Hanahan and Weinberg, 2011). The pathologists note the differentiation in prostate tissue and provide a grading based on the Gleason classification system (details provided below).

The decision around the diagnosis of PCa is therefore a step-wise process involving a collection of information including, PSA levels and related variables, feeling for physical/ structural changes in the prostate through DRE and observing histological changes in prostate tissue through biopsy. What follows are mainly risk assessments involving staging of the disease, predictions on potential outcomes and treatment option considerations. Imaging, mainly through ultrasonography is also important as a guidance system to overcome accessibility of the prostate but also during staging and treatment (Sarkar and Das, 2016). Importantly, the steps above require patient considerations, especially in view of the impact that screening and diagnosis procedures have on the quality of life of a patient and the extensive knowledge on the disease burden worldwide.

1.1.3 Prostate cancer risk stratification

As mentioned previously, PCa is a highly prevalent disease with the cumulative risk of men developing PCa in their lifetime being 3.8% (Ferlay *et al.*, 2015). After diagnosis, information is gathered to determine the extent of disease progression and to determine the risk of the patient developing aggressive PCa. One of the key evaluations is grading of PCa tissue. The most widely used system, the Gleason grading systems was initially developed by Dr Donald Gleason together with the Veterans Administration Cooperative Urological Research Group (VACURG) in the 1960s - 1970s (Gleason, 1966; Gleason DF, 1974). The grading score is based on architectural patterns of histological differentiation of prostate tissue. Although established long ago, Gleason grading is still widely used and is considered powerful in predicting patient prognosis and assisting with patient management. Several modifications have been made to this grading system over time with other systems developed that are in use and have been added by the World Health Organisation (WHO) to its recommendations (Epstein *et al.*, 2005, 2015; Humphrey *et al.*, 2016).

The importance surrounding the grading system has been due to its usefulness in making accurate determinations around presence of disease, potential risk for PCa progression and guiding treatment interventions. Although the Gleason grading system has been fairly successful thus far, one of its limitations is the classification of patient risk of PCa following accurate grading. Normally, H&E stained prostate tissue is evaluated by a pathologist and the evaluation involves grading heterogeneous prostate tissue where a grade is given to the most prominent differentiation pattern (primary) and the second most prominent differentiation pattern (secondary) (Gleason, 1992). A score of 1 indicates the best differentiation pattern with a favourable prognosis while a score of 5 indicates the least differentiation pattern and poor prognosis. The scores are combined e.g. 3+4 to give 7 which is the Gleason score assigned. Interestingly, a new grading system has been developed at the John Hopkins Hospital and validated through clinical trials (Pierorazio *et al.*, 2013; Epstein *et al.*, 2016). It is simpler and is considered more accurate in terms of PCa biology (see Figure 4 for schematic diagrams of the systems).

The two systems differ in the reporting of the Gleason score where the latter includes prognostic grade groups which assist in categorizing the pathological findings and is more representative of tumour behaviour (see Table 2 for the different grading systems). It has also been incorporated into the WHO classification of PCa, the National Comprehensive Cancer Network (NCCN) PCa treatment guidelines and the American Joint Committee on Cancer/the Union for International Cancer Control (AJCC/UICC) grading guidelines (Humphrey *et al.*, 2016; Lavery, Kirby and Chowdhury, 2016; Buyyounouski *et al.*, 2017). These developments have occurred alongside significant changes in grading systems that have been adopted at the International Society of Urological Pathology of 2005 and 2014 by consensus from various professionals in the relevant fields (Epstein *et al.*, 2005, 2015). These include the elimination of the grade 1 pattern and noting that the Gleason score 2 was rarely being diagnosed in patients worldwide.

The main conclusions and justifications for adjusting the grading systems is assisting with patient risk stratification and patient management. For example, following the above-mentioned removals from the grading system, the lowest Gleason score associated with a grade group is six. Subsequently, when considered in combination with other clinical information, a favourable outcome is predicted for these patients and as such they can be considered for active surveillance. Previously, a Gleason score of 6

would have placed the same patient at intermediate risk for PCa where more aggressive treatment options would be considered. Therefore, these systems are continuously being modified along with on-going clinical, surgical, cancer research and patient management developments to assist with patient risk stratification. Ideally, to minimize any potential harms and increase the benefits of seeking treatment for PCa.

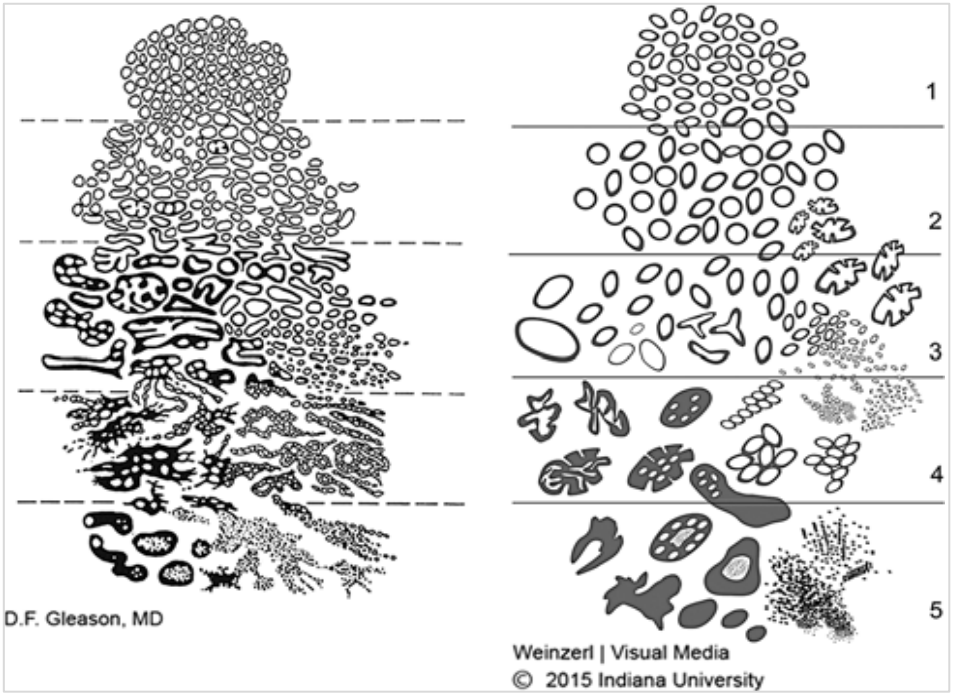


Figure 4: Schematic diagram of the original Gleason grading and modified Gleason grading system. *Left)* the grading system as originally designed by Dr Donald Gleason and VACURG, *right)* the modified grading system as agreed upon at the 2014 International Society of Urological Pathology consensus conference. *Source:* (Epstein *et al.*, 2015)

Table 2: Showing similarities and differences between the original and modified grading systems

Gleason Grading System (original)			2015 Modified ISUP Gleason Grading		
Grade	**Gleason scores**	**Risk stratification**	**Grade**	**Gleason scores**	**Risk stratification: Prognostic Grade Group**
1 **2** **3** **4** **5**	Lowest possible= 2 Highest possible= 10	Low grade PCa (Gleason score 2-5) Intermediate grade PCa (Gleason score 6-7) High grade PCa (Gleason score 8-10)	1 2 3 4 5	Lowest possible= 2 Highest possible= 10	I Gleason score ≤ 6 (most favourable) II Gleason score 3+4 = 7 III Gleason score 4+3 = 7 IV Gleason score 4+4 = 8; 3+5= 8; 5+3= 8 V Gleason score 9-10 (least favourable)

1.1.4 Prostate cancer staging

Staging of PCa involves determining the extent of disease progression using information on the current location and size of the tumour and how far it has spread from the prostate gland. It includes clinical, pathological, post-therapy staging and restaging. Clinical staging involves PSA testing, DRE, biopsy acquisition and imaging, while, pathological staging is for patients that have undergone surgery and is based on surgical determination of spread of the disease plus information from clinical staging (Edge SB *et al.*, 2010; Cheng *et al.*, 2012). Post-therapy staging and restaging involve staging of the disease following therapeutic intervention, at the initial therapeutic intervention or staging after the recurrence of PCa, respectively (Edge SB *et al.*, 2010; Cheng *et al.*, 2012). Critical to PCa staging is the tumour-node-metastasis (TNM) staging system developed and monitored by the AJCC/UICC and is one of the most common cancer staging systems used worldwide (Edge SB *et al.*, 2010).

Prostate cancer staging can broadly be described as localized, locally advanced and metastatic. However, the TNM staging system provides guidelines on PCa classification based on clinical and pathological information (see Figure 5 below for schematic diagrams depicting the different classifications) (Edge SB *et al.*, 2010). It provides detailed descriptions on determining disease severity based on observations made on the prostate tumour, surrounding and distal organs. The tumour extent (T) is classified based on the ability to determine PCa presence through DRE or examination following surgical removal of the prostate gland (Edge SB *et al.*, 2010). It accounts for disease spread within the tumour, spread to the seminal vesicles or spread to surrounding organs other than seminal vesicles. The N classification details the extent of tumour spread in terms of lymph node invasion, detailing whether the tumour has invaded the more proximal lymph nodes in the pelvis or not (Edge SB *et al.*, 2010). The M classification details whether PCa has spread to other sites of the body e.g. non-regional lymph nodes, the bone and other distal organs (Edge SB *et al.*, 2010). The information from the TNM staging system is then used in combination with serum PSA level, histological grading in the form of the Gleason score plus the new grade group classification (described in section 1.1.3 above) to define an AJCC prognostic stage group (Gleason, 1992; Edge SB *et al.*, 2010; Adhyam and Gupta, 2012; Pierorazio *et al.*, 2013; Epstein *et al.*, 2015; Buyyounouski *et al.*, 2017).

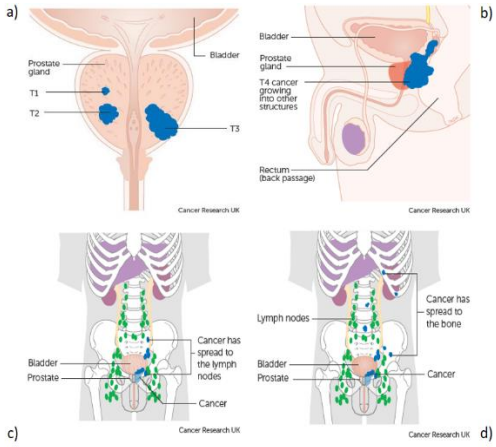


Figure 5: Schematic diagram of the TNM AJCC Staging system. a) and b) showing images associated with T1, T2, T3 and T4 staging. c) Showing the spread of the tumour to the regional lymph nodes (blue dots) and d) showing prostate cancer metastasis including spread to the bone (blue dots). *Source*:http://d4j2i6ubvolvu.cloudfront.net/cdn/farfuture/oFvzd1dSyG7k8MyImjZQ6oGYFpBiJ_grZq11Yy1Qry0/mtime:1473164404/sites/default/files/thumbnails/image/image002.png

Table 3: AJCC Staging system definition. Adapted from AJCC Staging manual 8TH edition (Buyyounouski *et al.*, 2017)

CATEGORY	CRITERIA
Clinical (cT) - T category	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically unapparent tumour that is not palpable
T1a	Tumour incidental histologic finding in 5% or less of tissue resected
T1b	Tumour incidental histologic finding in more than 5% of tissue resected
T1c	Tumour identified by needle biopsy found in one or both sides, but not palpable
T2	Tumour is palpable and confined within prostate
T2a	Tumour involves one-half of one side or less
T2b	Tumour involves one-half of one side or less
T2c	Tumour involves both sides
T3	Extraprostatic tumour that is not fixed or does not invade adjacent structures
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathologic (pT) - T category	
T2	Organ confined
T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
N category	
NX	Regional lymph nodes were not assessed
N0	No positive regional lymph nodes
N1	Metastases in regional lymph node(s)
M category	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

Significantly, the reporting of the grade group was implemented in January 2018 with the new guidelines emphasizing the importance of the histological grade to decision-making (Buyyounouski *et al.*, 2017). However, all the clinical and pathological information contributes to staging of PCa which in turn allows for accurate determination of disease severity, estimation of patient prognosis and guiding recommendations for patient treatment.

1.1.5 Treating prostate cancer

Once a patient has been diagnosed with PCa and the stage of the disease accurately determined, decisions need to be made on the appropriate treatment options. Considerations that inform the decision are the patient's age, health status, the stage of PCa and the patients preferences (Lavery, Kirby and Chowdhury, 2016). Although PCa is more prevalent in elderly men (above 50), younger men are also susceptible due to family history of PCa (Lesko, Rosenberg and Shapiro, 1996; Li *et al.*, 2012; Torre *et al.*, 2015). Additionally, PCa can be "indolent" meaning that, although a tumour is detected, it can either not grow or grow at such a slow pace that it would not require treatment and the patient could die of other co-morbidities (Hall *et al.*, 2005; Droz *et al.*, 2010). Like many other cancers, there are more treatment options for early detected PCa while the more advanced the disease, the more difficult treatment becomes and the chance for recurrence increases. Critical to deciding on a treatment plan is the stage of the disease and several guidelines have been disseminated on how to treat localized, locally advanced and advanced PCa (see Figure 6 below). As mentioned previously, the AJCC/UICC publishes guidelines on staging PCa and based on these guidelines and those standardized based on the individual country's needs, patients are placed into 4 prognostic stage groups (Buyyounouski *et al.*, 2017). These groups then have generalized treatment plans from the relevant institution for localized (stage I and II), locally advanced (III) and advanced (IV) prostate cancer. Additionally, within the advanced PCa group is castration-resistant PCa which is recurrent PCa following hormone therapy and is discussed in more detail below.

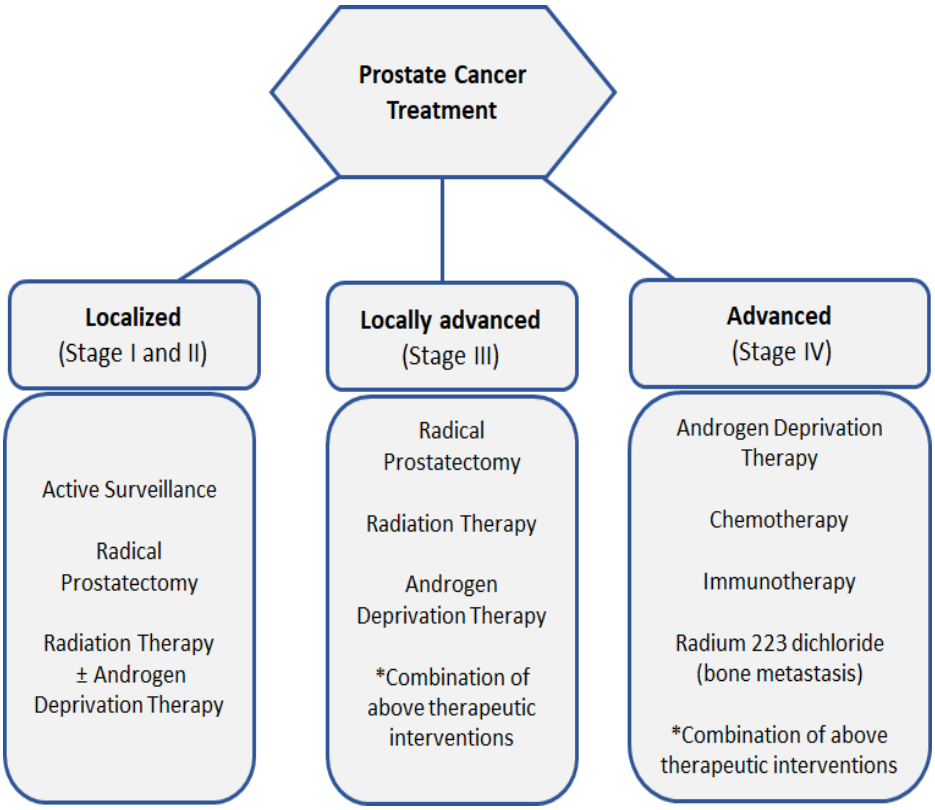


Figure 6: Schematic diagram showing guidelines for standardized prostate cancer treatment based on staging. Information adapted from guidelines as published by the Cancer Association of South Africa (CANSA) 2017, Prostate Cancer Foundation of South Africa 2013 guidelines, National Comprehensive Cancer Network, American Cancer Society.

The treatment options for PCa then include: active surveillance, surgery (radical prostatectomy), radiation therapy, hormone/androgen deprivation therapy, chemotherapy, immunotherapy, cryotherapy and high intensity focus ultrasound (HIFU) (Aus *et al.*, 2005; Aus, 2006; Mohler *et al.*, 2010; Herbst, 2013; Segone *et al.*, 2013; Maia and Hansen, 2017). Active surveillance is the periodic monitoring of PCa progression in patients using PSA testing, DRE and biopsy acquisition (if required) (Matulewicz, Weiner and Schaeffer, 2017). Although it is more of a patient management strategy, due to current challenges in accurately determining patients that will or will not progress to aggressive PCa, it is considered a treatment plan based on the ability to act should it be necessary. This minimizes unnecessary treatment of the patient while allowing for prompt intervention should the disease progress (Matulewicz, Weiner and Schaeffer, 2017).

Upon diagnosis, the standard approaches for treating PCa are radical prostatectomy, radiation and hormone therapy (Aus *et al.*, 2005; Mohler *et al.*, 2010; Herbst, 2013). Radical prostatectomy is the surgical removal of the prostate gland (Lepor, 2005). It is widely recommended for PCa that appears localized and although it can be curative, several complications are possible and current studies differ as to the advantages and disadvantages of open radical prostatectomy vs robotic or laparoscopic surgery in terms of success rate and complications (Tewari *et al.*, 2012; Ilic *et al.*, 2017). Alternatively, PCa can be treated with radiation therapy involving the use of high energy rays to induce DNA damage in cells, leading to cell death or decreased growth of PCa cells, with some impact on normal cells (Dewey, Ling and Meyn, 1995; Mohiuddin, Baker and Chen, 2015). The radiation can be exposed to the areas of interest internally or externally (Bagshaw *et al.*, 1975; Potters *et al.*, 2005). Interestingly, brachytherapy, which involves inserting seeds containing radiation inside the prostate gland, has recently been found to be successful (Blasko *et al.*, 2002; Potters *et al.*, 2005; Shah *et al.*, 2012). Several clinical trials have been performed comparing the efficacy of active surveillance, surgical interventions and radiotherapy in relation to mortality rates, biochemical PCa recurrence (rising PSA levels post-therapeutic intervention) and complications (D'Amico *et al.*, 1998; Hamdy *et al.*, 2016; Ilic *et al.*, 2017). Experts differ on the "best" intervention with some differences occurring depending on whether the study used data from hospitals, specialized treatment/research centres i.e. population-based information versus single institution information (Ilic *et al.*, 2017). Significantly, differences in success rates across the treatment facilities seemed to be attributed to the experience of the treating physician with fewer complications being associated with more experienced physicians (Ilic *et al.*, 2017). Therefore, radical prostatectomy and radiation therapy appear to be equally weighted in terms of curative ability for more localized disease and the other standard of treatment, hormone or androgen deprivation therapy (ADT), is used combinatorially.

Hormone therapy or ADT is a treatment modality around inhibiting the ability of prostate gland cells to grow in response to androgens by decreasing the levels of androgens in the body (Huggins, Stevens and Hodges, 1941; Loblaw *et al.*, 2004; Sharifi, Gulley and Dahut, 2005). Androgens and the androgen receptors which recognize them, play a critical role during PCa development, progression and are extensively targeted for PCa treatment. ADT is used during locally advanced and advanced disease primarily to assist with killing of PCa cells in order to improve the effects of surgery or radiation (Aus *et al.*, 2005; Mohler *et al.*, 2010; Herbst, 2013). They also help to reduce the symptoms of advanced PCa by the same principle (Perlmutter and Lepor, 2007). Reduction of androgens or androgen receptor activity can be achieved by surgical castration or removal of the testes which produce androgens (Huggins, Stevens and Hodges, 1941; Sharifi, Gulley and Dahut, 2005). It can also be achieved by chemical castration, where, synthetic molecules are used to inhibit stimulation of androgen synthesis,

androgen pathways and the inhibition of androgen binding to the androgen receptors (see Figure 7 below for the levels of intervention of ADT) (Huggins, Stevens and Hodges, 1941; Sharifi, Gulley and Dahut, 2005). Although this can be an effective manner of decreasing PCa growth, it has a few side effects that can significantly impact the patient's quality of life. These include changes in sexual activity, hot flashes, changes in reproductive organ size, anaemia, skeletal and bone mass changes (Higano *et al.*, 1996; Smith *et al.*, 2002; Curtis *et al.*, 2008; Galvão *et al.*, 2008). Significantly, following ADT, recurrence of PCa can occur that is characterised as castration resistant PCa (Sharifi *et al.*, 2005; Scher *et al.*, 2008). The cancer cells apply mechanisms that circumvent the inhibitory approaches of ADT such as, blockage of key components of the androgen signalling pathway, to ensure continued expression or function of androgen signalling components and their targets, to retain PCa cell growth (Lonergan and Tindall, 2011).

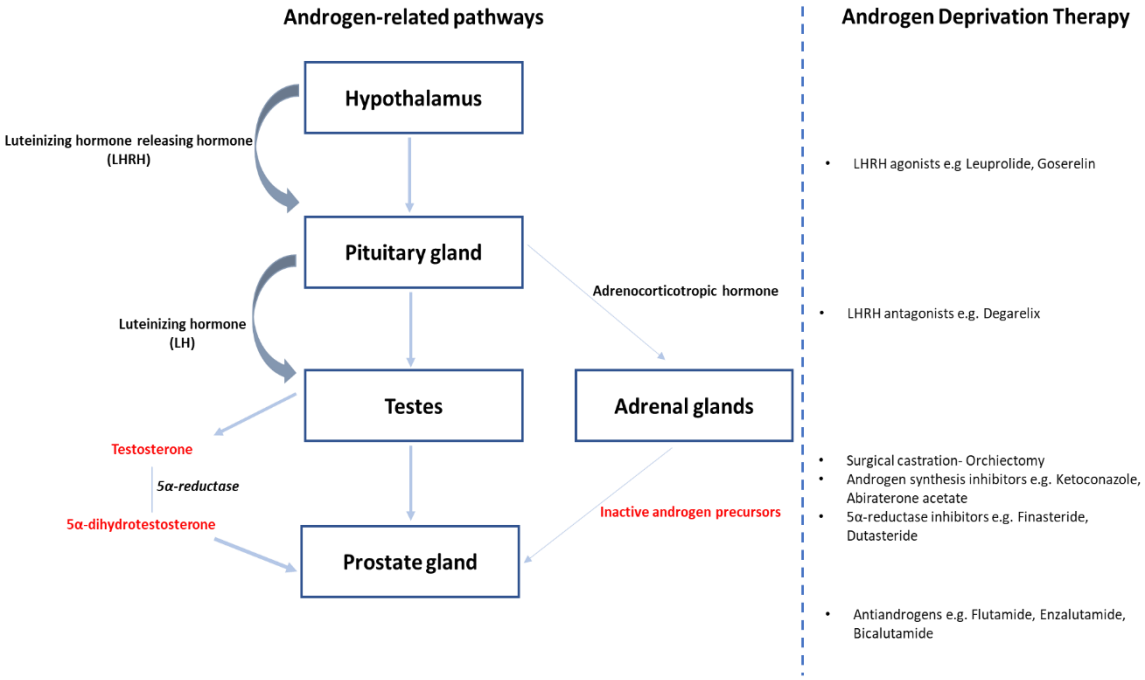


Figure 7. Schematic diagram showing androgen related pathways, organs, synthesis and androgen deprivation therapy approaches. *Left),* a schematic of the androgen-related pathways including the hypothalamus which releases the luteinizing hormone releasing hormone (LHRH) that acts on the pituitary gland to stimulate the release of the luteinizing hormone (LH). LH then acts on the testes to stimulate the release of testosterone and its metabolite 5α-dihydrotestosterone, which is a cleavage product of 5α-reductase cleavage of testosterone. The pituitary gland also releases adrenocorticotropic hormone which acts on the adrenal glands which release inactive androgen precursors, which together with testosterone and 5α-dihydrotestosterone act on the prostate gland to activate androgen-related activity. *Right),* is a depiction of the different points at which androgen deprivation therapy can act in order to inhibit androgen-related synthesis and activities.

Other proposed treatment modalities include chemotherapy and immunotherapy which can be used individually or in combination with ADT (Arlen *et al.*, 2006; Small *et al.*, 2015; Sweeney *et al.*, 2015). Additionally, a variety of chemotherapeutic agents have been studied for PCa treatment covering a wide spectrum of molecular biology approaches. Like other cancers, current chemotherapeutic agents target the high replication rate of PCa cells and these include docetaxel, cabazitaxel, mitoxantrone and estramustine (Petrylak *et al.*, 1999; Tannock *et al.*, 2004; de Bono *et al.*, 2010). There are taxanes such as docetaxel, cabazitaxel and estramustine which bind microtubules, suppressing microtubule assembly/disassembly dynamics and blocking related activities (Ringel and Horwitz, 1991; Dahllöf, Billström and Cabral, 1993; Galsky *et al.*, 2010). Mitoxantrone on the other hand intercalates with DNA and RNA (Alberts *et al.*, 1985). Significantly, treatment of metastatic castration-resistant PCa with docetaxel has shown increased patient survival rates (Tannock *et al.*, 2004). Additionally, in 2012, the androgen receptor antagonists enzalutamide and abiraterone acetate were approved by the US FDA for the treatment of castration-resistant PCa (Yan, 2013). Furthermore, a plethora of other mechanisms have been explored which are at various stages of discovery, have undergone clinical trials and are emerging as key target pathways. The ubiquitin proteasome pathway and neddylation pathway inhibitors have shown great promise in cancer in terms of inhibitory effects and improving patient survival (Adams *et al.*, 1999; Shah *et al.*, 2016). We postulate that molecular targets from pathways such as neddylation can be used as an alternative to or in combination with current chemotherapeutic drugs to improve their activity.

In the interim, immunotherapy is emerging as a treatment intervention which looks to improve the body's natural cancer fighting abilities by boosting the immune system. This approach is also currently used for advanced PCa treatment with Sipuleucel-T (Provenge) approved for the treatment of patients with castration-resistant PCa (Kantoff *et al.*, 2010). It uses a cancer vaccine approach, however, other mechanisms are also under investigation. Alternatively, a variety of newer treatment interventions are available and under exploration such as cryosurgery and HIFU (Cohen *et al.*, 1996; Gelet *et al.*, 1999; Long *et al.*, 2001; Aus, Pileblad and Hugosson, 2002; Blana *et al.*, 2004; Blana, Murat, *et al.*, 2008; Blana, Rogenhofer, *et al.*, 2008; Uchida *et al.*, 2009). However, there is limited long term data on the effectiveness of these treatments as wells as how they fair in comparison to the standard treatment options long term (Aus, 2006).

As demonstrated, PCa has unique challenges due the accessibility of the prostate gland, the potentially slow growing nature of prostate tumours as well as the documented risk of patients developing PCa. Opportunities and controversies have been discovered from screening for PCa, particularly through PSA testing. Though the more developed regions have demonstrated the potential success of PCa screening through patient participation and increased detection of PCa, it also demonstrated the benefits versus the harms for patients. Particularly those detected with lower grade disease. This has contributed to changes being made to the grading system where a new grading system which is more accurate at reporting prognosis has been incorporated into worldwide PCa guidelines. Largely with the hope of improving prognosis and treatment planning, especially for men having early detected, low grade tumours and men with aggressive PCa that require curative treatment. Also for the older men (above 70 years) whose quality of life could be improved by avoiding extensive PCa treatment.

Next, we review topics related to proteomics, which is the method that we will be focusing on in our study, in order to understand current knowledge and practices.

1.2 Proteomics

1.2.1 Introduction to proteomics

Over the last two decades, the field of proteomics has expanded extensively with improvements in molecular biology methodologies, computing power and bioinformatics increasing our understanding of proteins. Following the leaps that have been made in genomics and transcriptomics studies, there has been pressure to improve our understanding of proteins, particularly, the whole cellular protein complement as functional gene products. Proteomics has evolved to meet this demand through improvements in methodology, obtaining detailed information on the physiochemical properties of a protein, accurately identifying proteins and obtaining detailed information on its phenotypic properties. Figure 8 below chronicles the methodological advancements in protein study techniques thus far, highlighting the characteristics of proteins obtained with each method. It includes a timeline of these advancements based on published information plus the concurrent developmental in protein quantification methods. Significantly, it displays the rapid advancements that have occurred in the last two decades and the transformation of protein information from single molecules, to multi-protein complexes and protein networks. This is likely in response to an increasing argument that studying molecules in a manner similar to their natural state in the body can provide more insights into the molecule's activity during normal and diseased states.

Modern-day proteomics has made one of the biggest impacts to systems-biology research by allowing researchers to study proteins in an in-depth manner, obtaining a variety of information on proteins (Figure 9) (Aebersold and Mann, 2003; Sabidó, Selevsek and Aebersold, 2012). Mass-spectrometry (MS) based proteomics has been one of the leading contributors to this understanding, providing large-scale information on simple and complex protein samples, at high dynamic ranges and from diverse sources (Aebersold and Mann, 2003; Sabidó, Selevsek and Aebersold, 2012). This has been through various approaches including shotgun proteomics, targeted proteomics and quantitative proteomics (Wilm, 2009; Yates, Ruse and Nakorchevsky, 2009; Ebhardt *et al.*, 2015). These approaches have allowed for the comprehensive study of the proteome as in shotgun proteomics or analysis of a subset of the proteome as is possible in targeted proteomics (Yates, Ruse and Nakorchevsky, 2009; Ebhardt *et al.*, 2015). Quantitative proteomics includes several metabolic or chemical labelling approaches which allow for quantification of differentially expressed proteins (Wilm, 2009; van der Wal and Demmers, 2015). Shotgun proteomics and label-based quantitative proteomics will be reviewed in detail in Chapter 2 and 3 as they are the approaches used in this study. We begin by providing a review of MS-based proteomics.

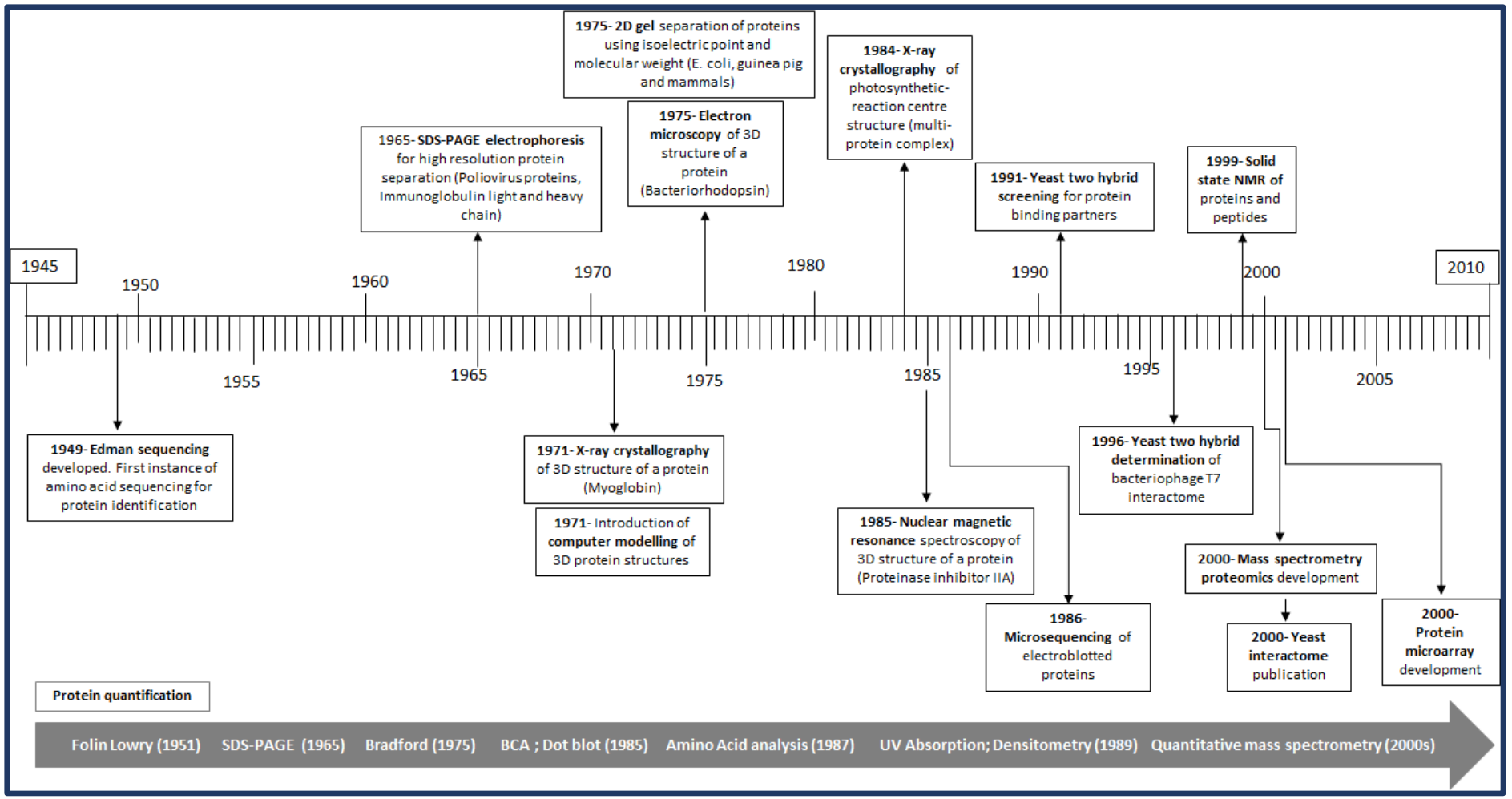


Figure 8. Timeline depicting developments in protein study methodology based on published data including advances in protein quantification methods.

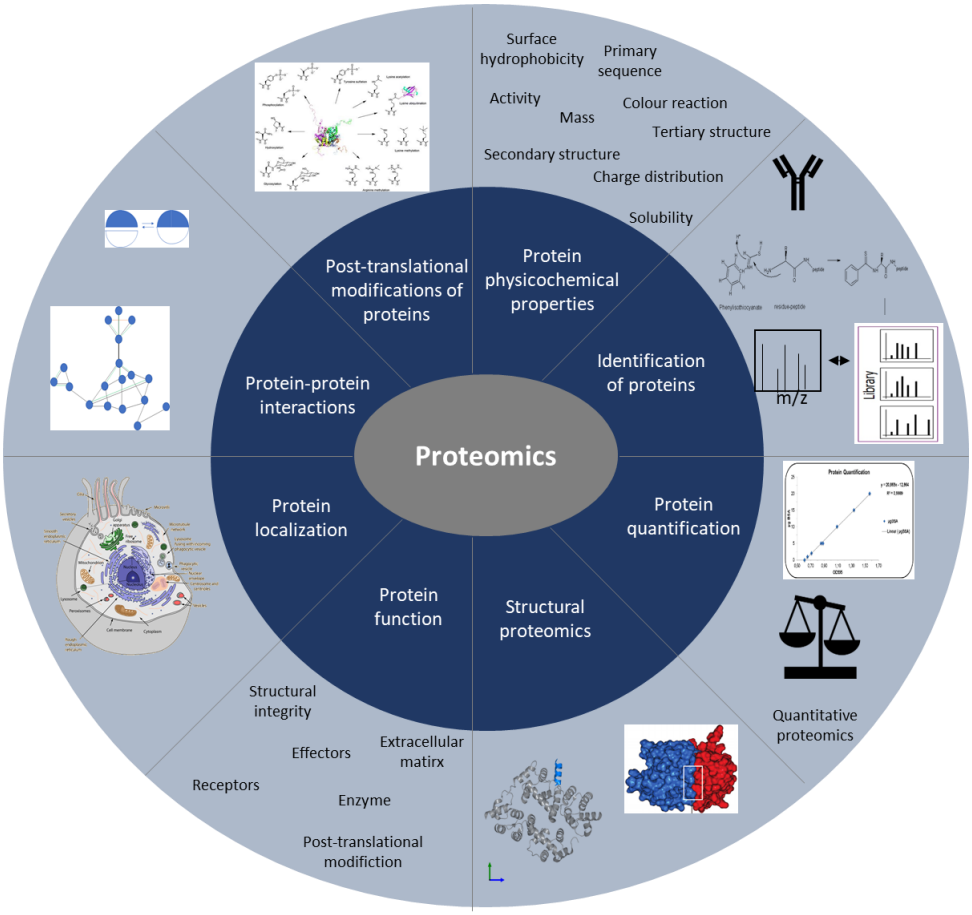


Figure 9. Schematic diagram showing proteomics and the insights it brings to protein studies.

1.2.2 Mass spectrometry

Mass spectrometry (MS) is the detection/identification of molecules on the bases of their mass-to-charge ratio (*m/z*) (Griffiths, 2008). It is used in high-throughput proteomics workflows involving the isolation, purification, identification and quantification of simple and complex protein samples. Currently, proteomics takes mainly a catabolic-approach where complex tertiary protein structures are broken down into peptide components and later peptide fragments availing them for identification (Zhang *et al.*, 2013). This requires conversion of non-volatile, large protein structures into smaller components (peptides or peptide fragments) that can be accurately identified and/or quantified (Zhang *et al.*, 2013). This description follows a "bottom-up" proteomics approach and is more widely used currently, however, analysis of intact proteins is under exploration through "top-down" proteomics (Kelleher, 2004; Catherman, Skinner and Kelleher, 2015). Additionally, "middle-down" proteomics is also emerging as a useful tool for studying proteins through the analysis of large polypeptide chains (3 - 20kDa) by taking advantage of improved ionisation approaches (Cristobal *et al.*, 2017). Generally, proteolytic enzymes such as trypsin are used to digest proteins into peptides and high-resolution machinery is used to separate peptides, converting them into gaseous phase ions that are easier to manipulate and fractionate (Aebersold and Mann, 2003; Olsen, Ong and Mann, 2004). MS has the power to detect these peptides at femtomolar concentrations over a wide dynamic range, analyse them and identify them through inference from peptide fragments (Aebersold and Mann,

2003). We will briefly provide some detail on MS principles, components and the variety of analyses possible.

As mentioned previously, MS measures molecules based on a mass to charge ratio (*m*/*z*) and to do this, non-volatile protein molecules need to be converted to gas-phase ions that are separated for ease of detection and measurement (Figure 10). There are a variety of mass spectrometers that have been developed to do this, that consist of an ion source, mass analyser and a detector (Glish and Vachet, 2003). They compartmentalize different activities for robust, sensitive, high-resolution, precise, mass accuracy measurements (Glish and Vachet, 2003). This is performed in workflows spanning different separation, ionization, detection and measurement approaches (Glish and Vachet, 2003). Mass spectrometers differ in their efficiency and precision to perform these activities with some meeting most but not all of the requirements (Table 4) (Jonscher and Yates, 1997; March, 1998; Schwartz, Senko and Syka, 2002; Douglas, Frank and Mao, 2005; Hu *et al.*, 2005; Scigelova *et al.*, 2011; Boesl, 2017). As a result, modern mass spectrometers consist of a hybrid of components capitalizing on the individual features of each mass spectrometer such as speed, mass resolution and fragmentation strategies for accurate, reproducible analysis of proteins in a high-throughput, automated manner. They can include multiple repetitions of each mass spectrometer such as the triple time-of flight (TOF) or they can be hybrid machines consisting of a quadrupole and Orbitrap like the Q Exactive used in this study (Andrews *et al.*, 2011; Michalski *et al.*, 2011). Overall however, the strength of MS-based workflows lies in the strength of the individual steps and separation is one of the key steps in the ability of mass spectrometers to function.

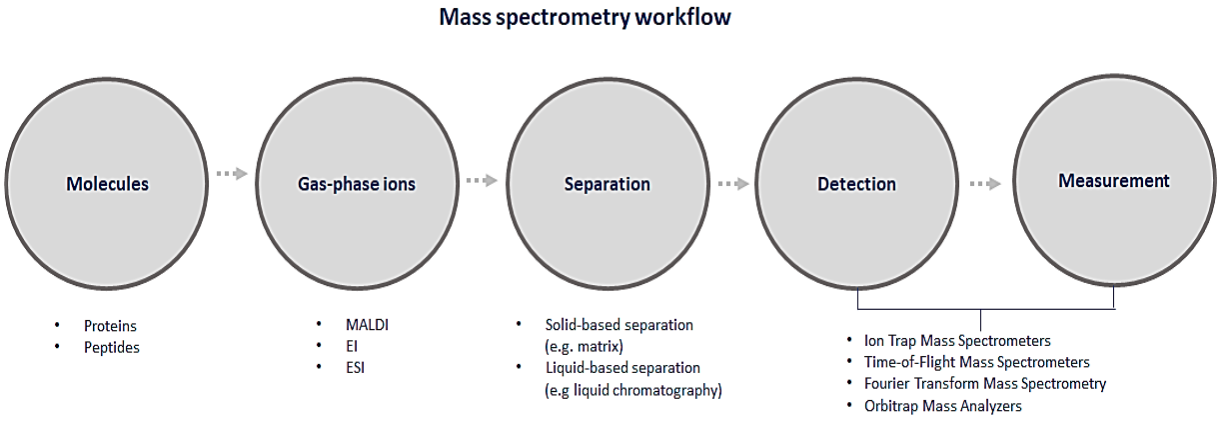


Figure 10. Schematic diagram of mass spectrometry workflow.

Protein separation is a key aspect of proteomics work that is emphasized partly through the importance of sample preparation during MS-based proteomics as well as in the separation principles of each mass spectrometer (Feist and Hummon, 2015). Regardless of the starting material (single cell, human cell lines, body fluids), in order to be able to study proteins they need to be isolated from the other components of the cell such as lipids but also from various reagents and detergents used during sample preparation. As such, part of proteomics workflows are various methods of separation such as ultrafiltration, gel-based chromatography, protein precipitation using organic solvents and filter

assisted sample preparation (FASP) (Chernokalskaya *et al.*, 2004; Jiang, He and Fountoulakis, 2004; Shi *et al.*, 2004; Wiśniewski *et al.*, 2009; Potriquet *et al.*, 2017). The objectives of which are to isolate proteins close to their native state, minimizing sample loss and contamination by other cellular components and reagents prior to enzyme digestion. Following this, the proteins undergo enzymatic digestion to generate peptides, that are introduced to the mass spectrometer of choice for analysis (Olsen, Ong and Mann, 2004; Giansanti *et al.*, 2016). Table 4 below provides examples of mass spectrometers and the principles behind their functions. However, outside of the general properties of the machine and separation, ionization methods are also critical to consider during MS-based proteomics work.

Table 4: Different mass spectrometers and the principles behind their functions

Mass spectrometer	Properties/Physical principles	Separation principle	Performance Analysis	References
Quadrupole Ion Traps	Based on a 3D quadrupole, Ions trapped in the centre of the device, Precursor ions fragmented using collision induced dissociation, Whole mass range transmitted by ion source, Transmission of mass window of chosen precursor ions	Ion transmission	Limited number of ions trapped, Low mass accuracy, Low mass resolution, Robust, Relatively sensitive, Relatively inexpensive	(Jonscher and Yates, 1997; Yates, 2004)
Linear Ion Traps	Based on a 2D quadrupole, Ions trapped at higher volumes than quadrupole ion traps, Stationery ion populations subjected to a Radio Frequency field, Precursor ions fragmented using collision induced dissociation	In time	Limited number of ions trapped, Low mass accuracy, Low mass resolution, Robust, Relatively sensitive, Relatively inexpensive, Improved ion statistics, Faster scanning	(Schwartz, Senko and Syka, 2002; Yates, 2004)
Time-of-Flight (TOF)	Based on the amount of time ions take to fly through a magnetic/electrical field-free tube, Measures intact ions versus fragment ions, Small mass ranges maintain a stable trajectory, Other mass ranges transverse TOF section	In space	Limited ion selection ability, Can't perform true MS/MS due to post-source decay and rapid fragmentation of ions, Improved efficacy for proteomics use, High speed mass analyser	(Yates, 2004; Boesl, 2017)
Fourier Transform Mass Spectrometry	Ions trapped based on high vacuum and magnetic field, m/ƶ determined as a function of the frequency of cyclotronic motion of ions	In space	High resolution, High mass accuracy, Highly sensitive, Wide dynamic range, Low peptide fragmentation frequency, Limited use because of complexity, Expensive	(Yates, 2004; Scigelova *et al.*, 2011)
Orbitrap mass analyzer	Ions trapped based on electrostatic force and axial motion of ions around a central spindle-shaped electrode, Image current of axial motion detected and Fourier Transformed for high mass accuracy mass measurement	In space	High resolution, High mass accuracy, Highly sensitive, Wide dynamic range	(Hu *et al.*, 2005; Han, Aslanian and Yates, 2008)

Ionization has been recognized for the advancements it has made to proteomics, particularly, MS-based proteomics through the 2002 Noble prize in chemistry (Wüthrich, 1986; Tanaka *et al.*, 1988; Fenn *et al.*, 1989). There are multiple ionization methods used to generate gaseous particles which rely on the loss of electrons or gain of protons to charge molecules to ions. The three main approaches are either through, the removal of molecules by laser radiation of a matrix on a planar surface as is done in matrix-assisted laser desorption/ionization (MALDI), by bombarding peptides with electrons in electron ionization (EI) or by subjecting aerosolized peptides to a high voltage as in electron spray ionization (ESI) (Figure 10) (Karas *et al.*, 1987; Fenn *et al.*, 1989; Palma *et al.*, 2011). Currently, MALDI and ESI are the most widely used ionization methods, employing more of a "soft" ionization technique with reduced peptide degradation as opposed to the "hard" ionization technique used in EI (Siuzdak, 2004). Although EI is increasingly being used in molecular biology-based proteomics, MALDI and ESI appear to be preferred, mainly due to their reduced impact on the peptide/protein structure (Siuzdak, 2004). This plays a critical role in peptide/protein identification by retaining the more native form of the molecules, ensuring accurate peptide detection, measurement and identification.

As such, the detection and measurement of peptides/proteins has played a critical role in MS functionality and understanding the proteome. Although the main objectives in these advancements has been to improve mass resolution and mass accuracy, speed, ion trapping capacity and automation have been key contributors. However, Fourier Transform Mass Spectrometry has made the biggest contribution to mass accuracy measurement thus far (Scigelova *et al.*, 2011). The use of frequency to determine mass accuracy has led to significant improvements in mass resolution, which has improved the ability to identify individual peptide fragments at very low parts per million or Daltons, allowing for accurate measurement and identification of peptides (Scigelova *et al.*, 2011; Marshall *et al.*, 2013; Lange *et al.*, 2014). This has also improved our ability to detect small changes in peptide structures such as posttranslational modifications, where, the ability to accurately resolve between peptides using their m/z ratio makes this possible. Additionally, advancements in computing power and bioinformatics have also contributed to peptide/protein identification (Vihinen, 2001; Kumar and Mann, 2009; Verheggen, Barsnes and Martens, 2014).

Database searching and matching of peptide spectra to spectral libraries is required once the peptide fragments have been detected and measured by mass spectrometry (Aebersold and Mann, 2003). Among the challenges to understanding the proteome is the ability to identify peptides/proteins and data repositories of previously identified peptide sequences have played a key role in MS-based proteomics (Ma, 2010; Perez-Riverol *et al.*, 2015). Of the many human proteins known to exist, not all have been identified by MS. This means that even if a researcher maybe able to detect a peptide, they may not know which protein it came from, just by not having something to match it against. Furthermore, the volumes of information, the sizes of the datasets generated also poses a challenge. However, advancements in computing power, particularly, the use of "the cloud" have been irreplaceable and *de novo* sequencing continues to assist in new protein identification (Halligan *et al.*, 2009; Hughes, Bin and Lajoie, 2010; Ma, 2010; Chiva *et al.*, 2018). This includes the use of software packages such as X! Tandem, Mascot that are necessary to condense large mass spectra files and to automate the high-throughput data analysis, including peptide identification, molecular pathway identification and the descriptions of other protein information relevant to the dataset (Eng, McCormack and Yates, 1994; Craig and Beavis, 2004).

Among the goals of proteomics is to catalogue all of the proteins that exist including possible posttranslational modifications and protein isoforms in order to understand protein functions. This will assist us in understanding normal and disease states and improve our ability to treat multiple diseases, including cancer. We use MS-based proteomics in this study to identify the proteins implicated in the activities of Defective in Cullin Neddylation 1 Domain containing 1 (DCUN1D1), in order to understand its mechanism of action in prostate cancer. We aim to use immunoprecipitation-coupled shotgun proteomics plus the quantitative strengths of stable isotopic labelling of amino acids in cell culture (SILAC) to do so and will use western blot analysis for validation.

We will next discuss the approaches used to study posttranslational modifications using proteomics, followed by an in-depth summary of the ubiquitination and neddylation pathways implicated in this study. We will also provide information on current knowledge on DCUN1D1 and its known substrates to understand where our study fits within the knowledge of its activity.

1.3 Posttranslational modifications, cancer and proteomics

Cellular activities are highly regulated to ensure homoeostasis. One critical mechanism used during these regulatory activities is posttranslational modification (PTM) of target proteins. This can involve the covalent modification of proteins by the addition of chemical moieties to amino acids, proteolytic cleavage of peptides or covalent linkage of separate protein domains (Walsh, Garneau-Tsodikova and Gatto, 2005). Mechanistically, PTMs regulate protein activity, protein stability, protein localization and protein-protein interactions of target proteins resulting in structural, chemical, or mechanistic changes (Walsh, Garneau-Tsodikova and Gatto, 2005). These alterations can then lead to the dysregulation of protein expression or protein functions (Walsh, Garneau-Tsodikova and Gatto, 2005). Figure 11 below shows the variety of cellular processes affected by PTMs, from cell growth and division to signal transduction. Additionally, there are multiple PTMs that have been characterised in microbes, plants and mammals including: phosphorylation, acetylation, methylation, glycosylation, ubiquitination, sumoylation, neddylation etc. which contribute in various capacities to cellular activities (Friso and van Wijk, 2015; Grangeasse, Stülke and Mijakovic, 2015; Pagel *et al.*, 2015).

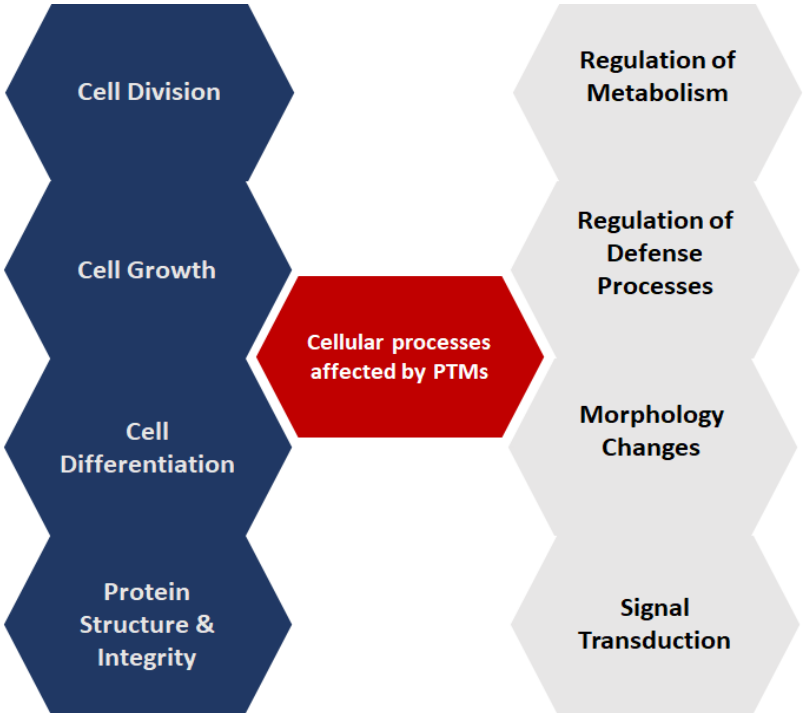


Figure 11. Posttranslational modifications and the cellular processes they regulate.

Reimand et al., 2015 demonstrated using public databases, that of the ~130 000 experimentally determined PTM sites at the time, 72% were phosphorylation sites, 19% ubiquitination, 6% acetylation and 2% were methylation sites (Reimand, Wagih and Bader, 2015). Additionally, Woodsmith et al., 2013 demonstrated that protein complexes tested for either acetylation, ubiquitination, phospho-serine/threonine or phospho-tyrosine modifications, showed complex-specific selective accumulation. The greatest overlap was observed in complexes with ubiquitin and acetyl

modifications with only ~6% of the complexes used in this study showing modification by ≥ 3 of the above modifications (Woodsmith, Kamburov and Stelzl, 2013). It is important to note that although experimentally determined, the data is based on information published at the time and is susceptible to bias due to some PTMs being more extensively studied. Availability of information on the PTMs, ease of detection and limitations due to methodologies available could have also contributed to the PTMs identified. However, the observations are consistent with the understanding that each PTM has specified functional activity and that there is extensive interplay between the PTMs, increasing the complexity of their mechanisms of action (Venne, Kollipara and Zahedi, 2014). Ubiquitination and neddylation, which are explored in this study also show interplay, either through sequential activity on substrates or simultaneous presence within a protein or complex (Petroski and Deshaies, 2005). Overall, the cell allocates a significant portion of its machinery to the regulation of PTMs, with 5 - 10% of the protein coding genome encoding components of PTMs (Woodsmith, Kamburov and Stelzl, 2013). Therefore, PTMs are critical to normal cellular activity and are observed to be highly dysregulated under disease conditions, including cancer (Li *et al.*, 2010; Karve and Cheema, 2011; Martín-Bernabé *et al.*, 2017).

Cancer is the uncontrollable growth of cells leading to tumourigenesis. Although cells and the body, have several mechanisms in place to inhibit tumourigenesis, cancer growth and metastasis, cancer is one of the leading causes of death worldwide (Torre *et al.*, 2015). Hanahan and Weinberg have published two articles in 2000 and 2011 describing the "Hallmarks of Cancer", which summarise the key features/properties or mechanisms that cancer adopts in order to thrive in the body (Hanahan and Weinberg, 2000, 2011). These include, sustaining cell proliferation and growth while inhibiting apoptosis, avoiding and manipulating immune responses and deregulating cellular energetics for sufficient energy supply for the rapidly growing cancer cells (Hanahan and Weinberg, 2000, 2011). Posttranslational modifications are featured extensively throughout the "Hallmarks of Cancer" due to the broad range of cellular activities they influence but also due to the dynamic advantages they offer during tumourigenesis, including speed of signal transduction. Studies performed in our laboratory have demonstrated the role of the receptor tyrosine kinase Axl in PCa and the role of DCUN1D1, an E3 ligase of the neddylation pathway in PCa (Paccez *et al.*, 2012; Vava and Zerbini, 2014). Demonstrating the importance of phosphorylation and neddylation pathways, among other aspects. Figure 12 below illustrates the "Hallmarks of Cancer" as described by Hanahan and Weinberg, 2011, providing an example of the role that different PTMs play within each hallmark.

This role has also been explored in cancer treatment approaches implicating the neddylation and ubiquitination pathways as emerging pathways for cancer treatment. MLN4924 has been characterised for its efficiency in cancer treatment, particularly in myelomas and a wide range of proteasome inhibitors targeting the ubiquitin-proteasome pathway have been tested for cancer treatment (Burger and Seth, 2004; Swords *et al.*, 2010, 2015; Shah *et al.*, 2016). Furthermore, proteomics has played a major role in our understanding of cancer, PTMs and the role they play in cancer.

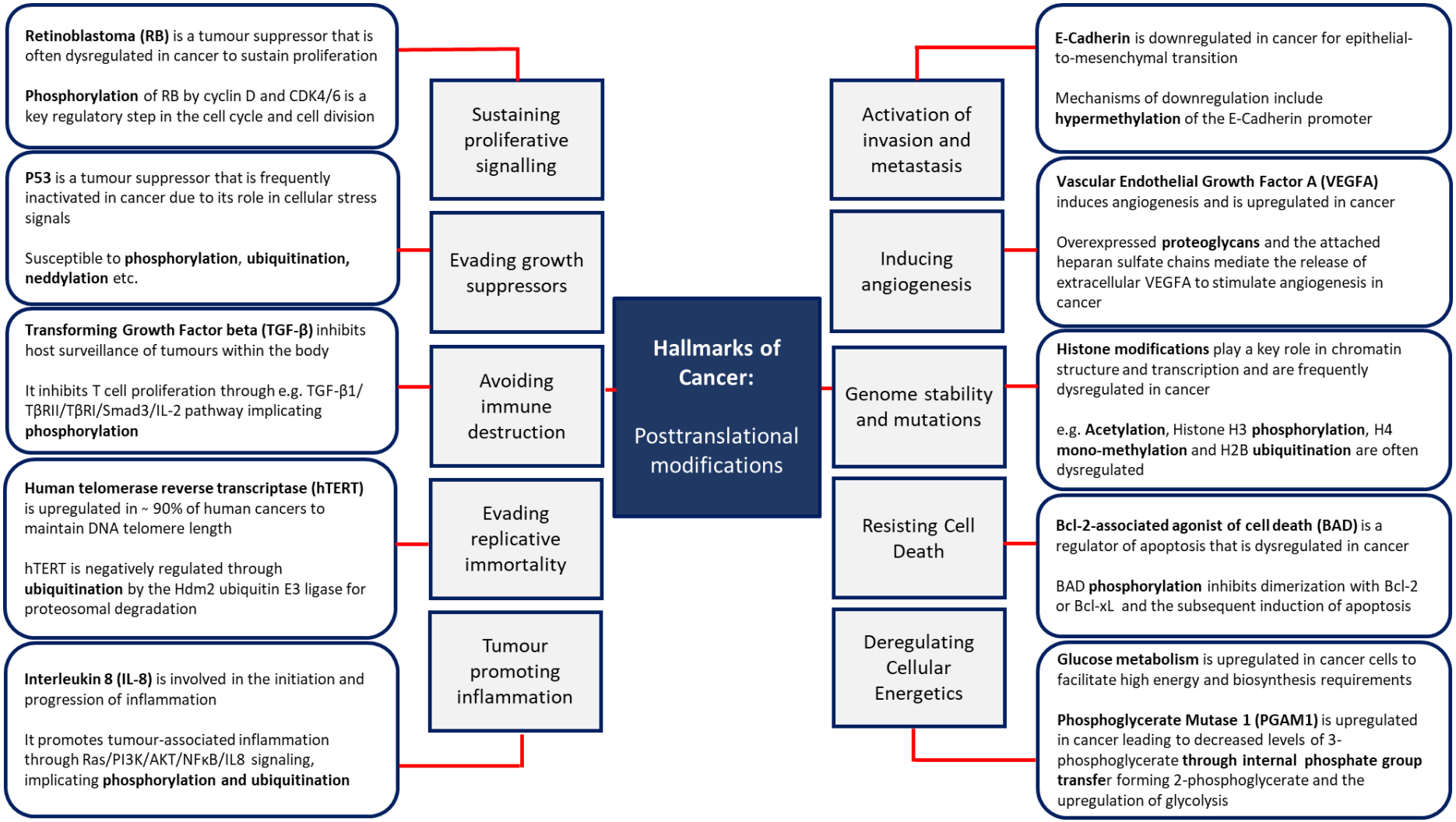


Figure 12. Showing the "Hallmarks of Cancer" as depicted in Hanahan and Weinberg 2011 and examples of the posttranslational modifications that are implicated throughout.

As mentioned previously, ease of detection has played a key role in the number and distribution of PTMs studied to date. This is because although the cell allocates 5 - 10% of its machinery to PTMs, it still leaves 90 - 95% allocated to other components and activities (Woodsmith, Kamburov and Stelzl, 2013). Therefore, PTMs are lowly abundant and MS-based proteomics has demonstrated that they differ in size by detecting PTMs ranging from 42 Daltons to >800 Daltons (Table 5). Furthermore, some PTMs can lead to multi-unit or poly-unit PTM as observed in ubiquitination (Pickart, 2000). They also have a variety of physiochemical properties such as hydrophobicity, stability and being labile, which all play a role in our ability to detect and quantify them (Walsh, Garneau-Tsodikova and Gatto, 2005). Furthermore, protein studies and the understanding of PTMs requires that we not only detect PTMs but determine their sites of modification. Therefore, several methods have been developed to study PTMs such as Edman sequencing, amino acid analysis, SDS-PAGE, immunochemistry, protein arrays, mass spectrometry and isotopic labelling (Edman, 1950; Gerber *et al.*, 2003; Jensen, 2004; Larsen *et al.*, 2006; Mandell, 2008; Jin and Zangar, 2009; Sutandy *et al.*, 2013). MS-based proteomics has made a significant impact to the study of PTMs due to high sensitivity and specificity of PTM detection.

Table 5: Showing the range of sizes of PTMs detected by MS analysis through mass changes

PTM	Mass change (Daltons)
Phosphorylation	80 or 98
Methylation	14
Acetylation	42
Glycosylation	203 to >800
Ubiquitination	114
Neddylation	114

It has been a powerful tool in the study of PTMs due to its ability to fragment proteins to peptides and then into peptide fragments which can be detected and identified (Doll and Burlingame, 2015). It has high mass resolution and mass accuracy capabilities that allow for the detection of PTMs within complex mixtures, allow for PTM site identification, allow for the discovery of new PTM sites and PTM quantification (Doll and Burlingame, 2015). There are also a multitude of approaches that can be employed in combination with MS analysis that can improve PTM detection such as PTM enrichment, PTM chemistry manipulation for sample preparation and PTM quantification, including the use of labelling approaches (Zhao and Jensen, 2009; Chaube, 2014). Additionally, computational tools are available that use algorithms to account for changes in mass values due to PTMs and can also account for MS and MS/MS analysis by-products such as neutral losses and diagnostics ions that can be used for PTM identification (Matthiesen, 2007). They can also be specified for the detection of oxidation of amino acids in order to identity amino acid modifications due to sample processing or due to biological activity such as methionine and cysteine oxidation (Matthiesen, 2007).

Therefore, PTMs are important cellular processes that the cell assigns a significant amount of machinery to and that are implicated in critical cellular processes from cell growth, cell division, cell signalling and to the maintenance of protein turnover at varying speeds (Woodsmith, Kamburov and Stelzl, 2013; Venne, Kollipara and Zahedi, 2014; Reimand, Wagih and Bader, 2015). They play a key role in cancer, spanning the “Hallmarks of Cancer” and cancer treatment approaches (Hanahan and

Weinberg, 2000, 2011; Burger and Seth, 2004; Swords *et al.*, 2010, 2015; Shah *et al.*, 2016). MS-based proteomics through high resolution and high mass accuracy detection as well as through the identification of amino acid sequences has become a critical tool in identifying, mapping and understanding posttranslational modifications (Doll and Burlingame, 2015). At a proteomics level, although "bottom-up" proteomics is extensively used to study PTMs, "top-down" and increasing "middle-down" proteomics is contributing to the aforementioned aspects particularly through the retention of PTM connectivity due to the use of intact and longer polypeptide chains, respectively (Siuti and Kelleher, 2007; Valkevich *et al.*, 2014; Venne, Kollipara and Zahedi, 2014; Shortreed *et al.*, 2015; Sidoli and Garcia, 2017).

In this study, we will use proteomics to understand the role of the NEDD8 PTM-mediating DCUN1D1, in prostate cancer. We begin by reviewing the ubiquitination and neddylation pathways which have been implicated in DCUN1D1 activity below.

1.4 The ubiquitin pathway and related molecules

1.4.1 Ubiquitination

Ubiquitination is the addition of 8.5kDa ubiquitin to specific target proteins leading to a variety of effects in the cell (Clague, Heride and Urbé, 2015). It is expressed in almost all cellular tissue and it is widely considered that throughout the lifetime of any protein, the protein is likely to be affected by ubiquitination. Additionally, more than 1000 proteins are involved in ubiquitin regulation, including the E1, E2 and E3 proteins involved in the cyclic addition of ubiquitin to molecules, the multitude of proteins that act as receptors of ubiquitin through ubiquitin binding domains (UBDs) and through the actions of multiple deubiquitinases (Clague, Heride and Urbé, 2015). As such, ubiquitination is involved in a variety of cellular processes with each type of ubiquitin modification "encoding" a different signal and outcome for the target.

Polyubiquitination of proteins through lysine 48 is a well understood mechanism of ubiquitination that tags substrate proteins for degradation by the ubiquitin proteasome pathway (UPP) (Ciechanover, 1994). It involves the activation of ubiquitin by the E1 ubiquitin activating enzyme (UAE) followed by transfer of ubiquitin to the E2 ubiquitin conjugation enzyme (UBC) and subsequent transfer to the E3 ubiquitin ligase for conjugation to the substrate (Chau *et al.*, 1989; Scheffner, Nuber and Huibregtse, 1995). This can occur for several cycles at the lysine residue of each ubiquitin molecule added to a chain, leading to polyubiquitination of the substrate. This mechanism will be discussed in detail below, however, a variety of ubiquitin-substrate modifications are possible such as monoubiquitination, multi-ubiquitination which involves modification of a substrate by one ubiquitin molecule at multiple lysine residues within the protein and polyubiquitination (Pickart, 2000; H. N. Ramanathan and Ye, 2012). Additionally, ubiquitin is susceptible to modification by other PTMs such as acetylation and phosphorylation, while, polyubiquitination can have branched conformations (Koyano *et al.*, 2014; Meyer and Rape, 2014; Valkevich *et al.*, 2014; Zheng and Hunter, 2014; Ohtake *et al.*, 2015; Wauer *et al.*, 2015). This results in what is considered the "ubiquitin code" referring to the different ubiquitin modifications that proteins can have and the cellular activities for which they encode (Komander and Rape, 2012). Figure 13 below provides a schematic of possible ubiquitin modifications, the different outcomes they can produce, and examples of the cellular pathways/processes implicated, where relevant. The figure demonstrates the impact of ubiquitination on the cell and since ubiquitination is the most extensively described and well understood PTM among ubiquitin-like molecules, this can help our understanding of other pathways, including neddylation. Particularly because ubiquitination and neddylation are linked through the cullin RING E3 ligases (CRLs).

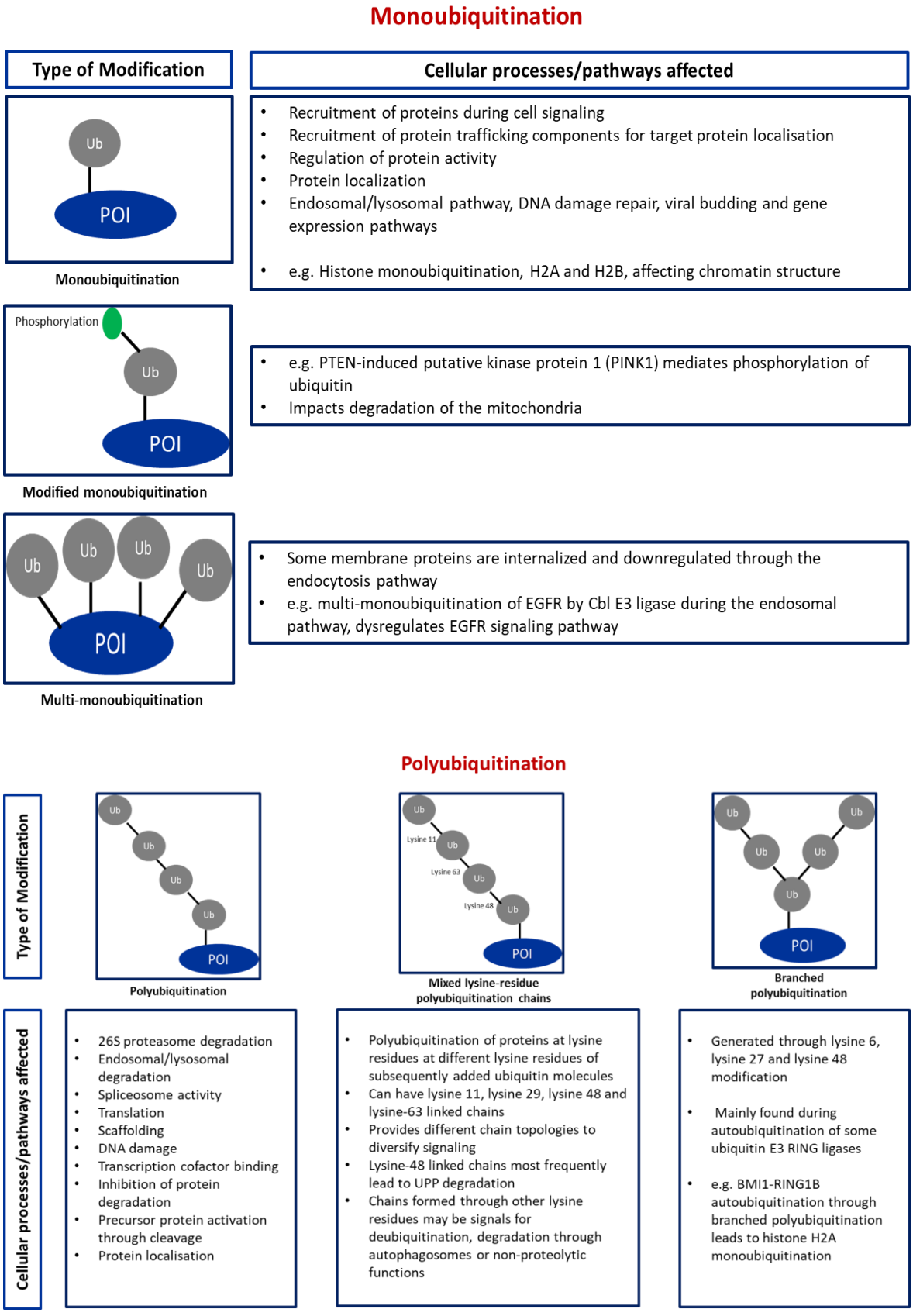


Figure 13. Diagram showing the different types of ubiquitin substrate modifications and the cellular processes/pathways affected.

As mentioned previously, ubiquitin is added to proteins through the cycle involving the E1 UAE, E2 UBC and the E3 ligases. Significantly, a large number of proteins form part of this cycle with approximately 500 E2 UBCs having been identified and thousands of E3 ubiquitin ligases characterised, across multiple species (Sun, 2006; Myung, Kim and Crews, 2008). The E3 ligases make the most significant contribution to substrate identification selectivity, with specificity achieved through three classes of E3 ligases. These include the RING (Really Interesting New Gene), the HECT (Homolog to E6-AP C Terminus) and the U-box E3 ligases (Beaudenon, 1995; Hatakeyama *et al.*, 2001; Hatakeyama and Nakayama, 2003; Petroski and Deshaies, 2005; Deshaies and Joazeiro, 2009; Metzger, Hristova and Weissman, 2012; Berndsen and Wolberger, 2014). The RING E3 ligases are the most widely represented, consisting of the simple RING E3 ligases and multi-protein-complex CRLs (Petroski and Deshaies, 2005). CRLs consist of one member of the cullin family of proteins which acts as a scaffolding molecule, a SKP protein which functions as an adaptor protein, an F box protein which functions as a receptor for the substrate, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase (Petroski and Deshaies, 2005). The different combinations of each protein component allow for the CRLs to recognize multiple proteins with high specificity, leading to protein degradation, among other possibilities. This class of E3 ligases is the focus of this study because the cullin family of proteins undergo neddylation prior to conjugation to the other components of the CRLs (Kamura *et al.*, 1999). In fact, majority of this family of proteins are known substrates of our protein of interest, DCUN1D1, which we will explore in detail below (Kim *et al.*, 2008; Keuss *et al.*, 2016). We will first review the role of ubiquitin pathway components and regulators of ubiquitination in cancer as some have been characterised as tumour suppressors or oncoproteins.

1.4.2 Ubiquitin and cancer

The ubiquitin E3 ligases are the second most prevalent cancer-related family following the kinases (Shi and Grossman, 2010). They can function as tumour suppressors or oncoproteins directly or due to their role in the degradation of some of their substrates, which are known tumour suppressors or oncoproteins. For example, BRCA1, an E3 ligase of ubiquitin, is a well-established tumour suppressor that is dysregulated in several cancers through different mechanisms (Brzovic *et al.*, 2001; Wu *et al.*, 2008). It has been established as part of genetic tests performed for breast cancer risk stratification and breast cancer treatment (Miki *et al.*, 1994; Scully, 2000; Antoniou *et al.*, 2003; Moyer, Services and Force, 2005). Additionally, the regulation of the tumour suppressor p53 is dependent on a few ubiquitin E3 ligases and p53 together with its E3 ligases (MDM2, E6AP and Arf-BP1) are dysregulated in breast, cervical and PCa (Scheffner *et al.*, 1993; Fang, 2000; Chen *et al.*, 2005; Shi and Grossman, 2010). Significantly, the CRLs and their components are implicated in cancer not only through the E3 ligases but also through SKP2 and Fbw7 (Gstaiger *et al.*, 2001; Welcker and Clurman, 2008). These F box proteins function as adaptors/substrate recognition proteins within CRLs whose targets include p27, c-Myc, c-Jun, Notch, mTOR and cyclin E (Gstaiger *et al.*, 2001; Welcker and Clurman, 2008). Disruption in the turnover rates of these proteins leads to dysregulation of the cell cycle, transcription and cell signalling pathways, all of which have been identified among the “Hallmarks of Cancer” (Hanahan and Weinberg, 2000, 2011). Furthermore, deubiquitinases are also implicated in cancer due to their regulation of deubiquitination, including their role in substrate-specific deubiquitination (Wei *et al.*, 2015; Singh and Singh, 2016).

As such, the UPP has been demonstrated as a target for therapeutic interventions for the treatment of cancer, with the US FDA approving the use of the 26S proteasome inhibitor, bortezomib, for cancer treatment (Richardson, Hideshima and Anderson, 2003; Singhal *et al.*, 2003; Papandreou *et al.*, 2004; Richardson *et al.*, 2006). The success of bortezomib has validated the potential of the UPP for therapeutic intervention, surpassing previous concerns related to how widely ubiquitin is expressed and the wide range of protein substrates it targets, which could lead to off-target effects and side effects for patients. Therefore, several drugs are under development targeting other components of the pathway including the E1 UAE and E2 UBC (Figure 14). PYR-41 and TAK-243 have been identified as inhibitors of the UAE through the dysregulation of protein degradation, with TAK-243 identified to form ubiquitin-adducts that impair the cell cycle and DNA repair leading to protoetoxic stress and apoptosis (Y. Yang *et al.*, 2007; Hyer *et al.*, 2018). Additionally, drug library screening through docking and *in vitro* analysis identified several inhibitors of the UBC Rad6 and compound CC0651 was found to inhibit the E2 Cdc34/UBER1 by targeting cell cycle-related proteins (Ceccarelli *et al.*, 2011; Sanders *et al.*, 2013). Ubiquitin E3 ligases have generated the greatest interest as potential drug targets due to the selectivity of their effects and the broader range of mechanisms through which they can be targeted. P53 and MDM2 are examples of this as Nutlins have been developed as antagonists of MDM2 and RITA has been developed to bind p53, all of which inhibit p53-MDM2 interactions in order to disrupt the effects of p53 activity and related pathways in cancer (Figure 14) (Issaeva *et al.*, 2004; Vassilev *et al.*, 2004).

The above-mentioned class of drugs, the inhibitors of deubiquitinases and the inhibitors of 26S proteasome (Figure 14) have introduced the targeting of protein degradation as a mechanism to treat cancer (Richardson *et al.*, 2006; D'Arcy, Wang and Linder, 2015). This in contrast to targeting rapidly dividing cells and the inhibition of cell growth, as was done in earlier chemotherapeutic agents. This has evolved to the identification of MLN4924, an inhibitor of the E1 neddylation activating enzyme (NAE) for cancer treatment with great success observed in myelomas, leading to the increased consideration of targeting other ubiquitin-like proteins for cancer treatment (Swords *et al.*, 2010, 2015; Nawrocki *et al.*, 2012; Shah *et al.*, 2016).

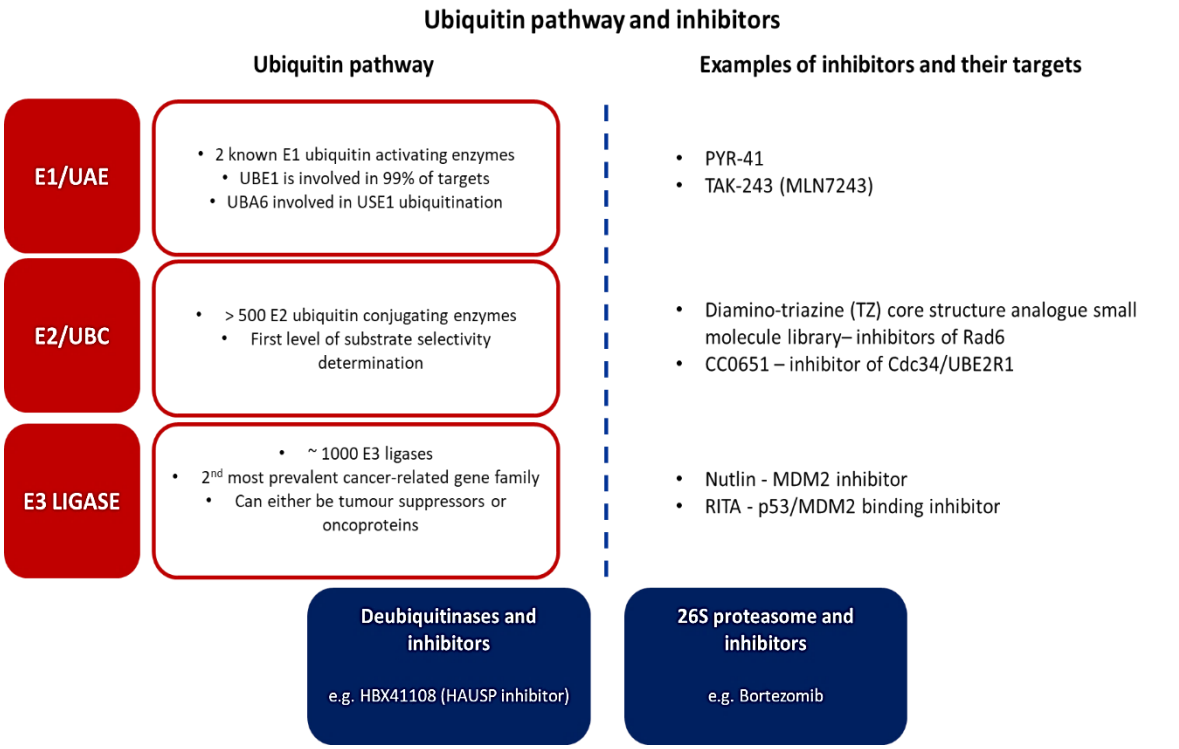


Figure 14. Diagram showing the ubiquitin pathway components and inhibitors that have been identified thus far including their targets.

1.4.3 Ubiquitin-like proteins

Ubiquitin-like proteins (UBLs) are a variety of proteins expressed in the cell that are similar to ubiquitin either through amino acid sequence, protein structure or mechanism of action/regulation. We provide a summary of some of the main UBLs that have been characterised including SUMO, NEDD8, ISG15, FAT10, FUB1, UBL5, URM1, ATG8, ATG12 and the E1, E2 and E3 enzymes implicated in their activity (Table 6) (Wilson and Rangasamy, 2001; Chiu, Sun and Chen, 2007; Geng and Klionsky, 2008; Jones *et al.*, 2008; Aichem *et al.*, 2010; Hatanaka *et al.*, 2011; Petroski, Salvesen and Wolf, 2011; Duncan *et al.*, 2012; Oka *et al.*, 2014). Interestingly, not all UBLs have classical E1, E2 or E3 enzymes but instead have proteins that perform a similar function of mediating transfer of the ubiquitin-like protein. Additionally, although UBL5/HUB1 has low sequence similarity to ubiquitin and has no known E1, E2 or E3, it is still considered a UBL (Lüders, Pyrowolakis and Jentsch, 2003; Yashiroda and Tanaka, 2004). It also cannot conjugate to substrates through the C terminal glycine that is essential for substrate modification as is characteristic of ubiquitin-like proteins (Lüders, Pyrowolakis and Jentsch, 2003; Yashiroda and Tanaka, 2004). Overall however, UBLs have been demonstrated to target specific proteins implicating them in protein degradation, the cell cycle, DNA repair, autophagy and immune responses.

An additional level of importance that UBLs play in the cell is underlined through crosstalk with ubiquitination. SUMO can form mixed chains on polyubiquitinated substrates and neddylation is essential for cullin modification as components of CRLs (Hori *et al.*, 1999; Kawakami *et al.*, 2001; Guzzo *et al.*, 2013). Additionally, FAT10 shares an E1 enzyme (UBA6) with ubiquitin, while the E2 UBC8 is an

E2 for both ISG15 and ubiquitin (Kim *et al.*, 2004; Chiu, Sun and Chen, 2007). Although each modification has unique protein recognition domains, the crosstalk adds to the mechanisms of regulation of cellular processes. As such, UBLs are also under investigation as targets for cancer treatment due to their individual activity and substrate-specific ubiquitin crosstalk (Duncan *et al.*, 2012; Da Silva *et al.*, 2013; Marsh, 2015). Several UBLs have been implicated in cancer through their PTM of tumour suppressors or oncoproteins. Some components of their pathways are dysregulated during tumourigenesis through chromosomal dysregulation, gene mutation and dysregulation of protein expression as is observed in sumoylation and neddylation (Hoeller, Hecker and Dikic, 2006). These two are the better understood PTMs outside of ubiquitination and have been extensively implicated in tumourigenesis. In contrast, FUB1/FAU1 is known as a tumour suppressor based on gene expression studies but its mechanism of action is not well understood (Michiels *et al.*, 1993; Pickard, Mourtada-Maarabouni and Williams, 2011). Therefore, UBLs are implicated in cancer and are among emerging approaches for cancer treatment, which could help reduce the side effects of cancer therapeutics, while retaining drug efficacy.

MLN4924, the inhibitor of the NAE has shown effectiveness in a range of cancers including myelomas and PCa (Swords *et al.*, 2010, 2015; X. Wang *et al.*, 2016). We have also identified DCUN1D1, an E3 ligase of neddylation as playing a role in PCa and have identified potential inhibitors for DCUN1D1 in order to take advantage of the potential of UBLs as therapeutic targets (Vava and Zerbini, 2014). We will provide a detailed review of neddylation below but begin with a review of proteomics and the role it has played in our understanding of ubiquitination and the proteomics-specific methods with which it is studied.

Table 6: Ubiquitin-like proteins, their E1, E2, E3 components and implicated cellular activities

Ubiquitin-like proteins	E1	E2	E3	Cellular activities
Nedd8 (Neural precursor developmentally downregulated 8)	APPBP1/UBA3	UBC12	DCUN1D1 MDM2 PARKIN1	-Regulation of CRLs
SUMO (Small ubiquitin-related modifier)	AOS1/UBA2	UBC9	RanBP2 PIAS Polycomb protein Pc2	-Cell cycle -Nuclear transport -Viral infection response -DNA repair
ISG15 (Interferon-stimulated gene 15)	UBE1L	UBC8	HERC5 ISG15 EFP	-Immune response -Inflammatory response
FAT10 (HLA-F adjacent transcript 10)	UBA6	USE1 (UBE2Z)	-	-Protein degradation -Immune response -Cell cycle regulation
FUB1 (Fau ubiquitin-like)	-	-	-	-Immune response -Binds Bcl-G (pro-apoptotic protein)
UBL5 (Ubiquitin-like protein-5)	-	-	-	-No covalent conjugation to substrates because of absence of C terminal GG motif -Pre-mRNA splicing
URM1 (Ubiquitin-related modifier-1)	MOCS3	-	-	-Oxidative stress -tRNA biosynthesis
ATG8 (Autophagy-related protein 8)	ATG7	ATG3	ATG12-ATG5 perform E3-like activity	-Autophagy
ATG12 (Autophagy-related protein 12)	ATG7	ATG10	No typical E3	- Autophagy

1.4.4 Insights into ubiquitination using proteomics

As mentioned previously, ubiquitin is an 8.5kDa protein. As a precursor, it is composed of 81 amino acids and is cleaved to 76 amino acids, exposing a C terminal GG motif that is essential for substrate modification. During tryptic digestion of a modified substrate, the GG motif remains on the side chain of the modified lysine residue of the substrate resulting in a mass shift (114 Da) that is detectable by MS analysis (Denis *et al.*, 2007). MS-based proteomics has played a key role in detecting, identifying and mapping ubiquitin modifications due to the high sensitivity and specificity of MS technology (Beaudette, Popp and Dittmar, 2016). At a proteomics level, “top-down”, “middle-down” and mainly “bottom-up” proteomics has been used to determine protein ubiquitination and each of these technologies offers different benefits and challenges for detection (Siuti and Kelleher, 2007; Xu and Peng, 2008; Valkevich *et al.*, 2014; Udeshi *et al.*, 2017). “Top-down” and “middle-down” proteomics

analyses intact proteins and longer polypeptide chains respectively. This allows for mass shift detections across longer lengths of peptides and provides information on the connection between ubiquitination of lysine residues at different sites (Xu and Peng, 2008; Valkevich *et al.*, 2014; Lee *et al.*, 2016; Rana, Ge and Strieter, 2017). They have also improved the detection of multi-ubiquitination, polyubiquitination and branched polyubiquitination chain sites (Xu and Peng, 2008; Valkevich *et al.*, 2014; Lee *et al.*, 2016; Rana, Ge and Strieter, 2017). "Bottom-up" proteomics is the most widely used approach thus far and although it lacks the efficiency in connecting ubiquitination sites across polypeptide chains, it provides detail on lysine modification especially when used in combination with other methodologies (Peng, 2008).

Identification of ubiquitin modified proteins, quantification and site mapping currently rely on the detection of the GG motif on modified tryptic digest peptides (Beaudette, Popp and Dittmar, 2016. These can be detected using shotgun, targeted, quantitative proteomics and PTM-enrichment methods (Beaudette, Popp and Dittmar, 2016). Shotgun proteomics allows for the detection of global changes in ubiquitination in samples, while targeted proteomics allows for the detection of known ubiquitin peptide sequences (Beaudette, Popp and Dittmar, 2016; Udeshi *et al.*, 2017). They can detect mono and polyubiquitination, with targeted proteomics having some advantages in terms of the detection of polyubiquitination modified residues (Beaudette, Popp and Dittmar, 2016). Interestingly, neddylation and ISG15 modification also bind substrates using a C terminal GG motif and this can pose a challenge to shotgun proteomics relative to searching for known peptide sequences in targeted proteomics (Jeram *et al.*, 2009). Labelling approaches such as SILAC and chemical labelling can also be used, which offer improved sample preparation techniques and quantification of ubiquitination at different sites, but these can increase MS analysis time and affect the detection of low-level analytes (Xu *et al.*, 2009; Kim *et al.*, 2011; Wagner *et al.*, 2011; Bustos *et al.*, 2012; Stanley and Virdee, 2016). Significantly, PTM-enrichment provides an interesting tool for ubiquitin modification analysis due to its ability to enrich for modified proteins/peptides and can therefore circumvent some challenges experienced in the other approaches, including background (Bustos *et al.*, 2012; Udeshi *et al.*, 2013, 2017).

This can be applied at the protein level using monoclonal antibodies such as the lysine 48 or lysine 63-linkage-specific Fabs, epitope-tagged ubiquitin (His, HA, Flag-tagged ubiquitin) and ubiquitin biotinylation, but also, at a peptide level using ubiquitin site-specific peptide antibodies against lysine 48 and lysine 63 (Xu and Peng, 2006; Newton *et al.*, 2008; Udeshi *et al.*, 2013; Min *et al.*, 2014). Additionally, the natural affinity and substrate specificity of ubiquitin binding domains can also be manipulated for PTM enrichment purposes as well as for the determination of ubiquitin E3 ligase substrates. Fusion proteins such as TUBE (Tandem Ubiquitin Binding Entity) probes composed of fusions between two ubiquitin associated-domains and E3-ligase-ubiquitin fusions called UBAITs (Ubiquitin-Activated-Interaction-Trap), have also been used to improve substrate specificity during PTM enrichment approaches (Hjerpe *et al.*, 2012; O'Connor *et al.*, 2015).

Overall, proteomics and particularly MS-based proteomics is a great tool for identifying, quantifying and mapping PTMs. It has been used in ubiquitination to identify protein substrates, the lysine residues targeted during modification, the connections between ubiquitination at different sites and polyubiquitination chain topologies. It has contributed to the improved understanding of ubiquitination in cellular activities/processes under normal and diseased conditions and, as the most well characterised PTM, ubiquitination provides insights for understanding other PTMs. Neddylation

is similar to ubiquitination and the pathways crosstalk in their activities within the cell. We will review neddylation and current knowledge on it as an emerging pathway in terms of PTMs but also as an emerging target for cancer treatment.

1.5 Neddylation

Neddylation involves the addition of 9kDa NEDD8 to target proteins through isopeptide bond formation (Kumar, Yoshida and Noda, 1993). Precursor NEDD8 is cleaved by DEN1 or UCH L3 to form mature 76 amino acid NEDD8, exposing the conserved C terminal glycine that is essential for substrate binding (Wu *et al.*, 2003; Frickel *et al.*, 2007). NEDD8 is a ubiquitin-like protein and also requires cyclic addition to target proteins, starting with the activation of NEDD8 by the E1 neddylation activating enzyme (NAE), APPBP1/UBA3 (Osaka *et al.*, 1998; Gong and Yeh, 1999). The activated NEDD8 is then transferred to the E2 neddylation conjugation enzyme, UBC12, followed by transfer to the neddylation E3 ligase for substrate modification (Figure 15) (Huang *et al.*, 2004, 2005; Kim *et al.*, 2008). This is done through the C terminal glycine of NEDD8 and the lysine residue of the substrate (Kamitani *et al.*, 1997; Jones *et al.*, 2008). NEDD8 however, is not as ubiquitously expressed as ubiquitin and there is also little evidence of NEDD8 chain modification of proteins (Jones *et al.*, 2008; Ohki *et al.*, 2009; Girdwood, Xirodimas and Gordon, 2011). Nevertheless, it is essential to normal cellular activities and has been implicated in critical cellular functions.

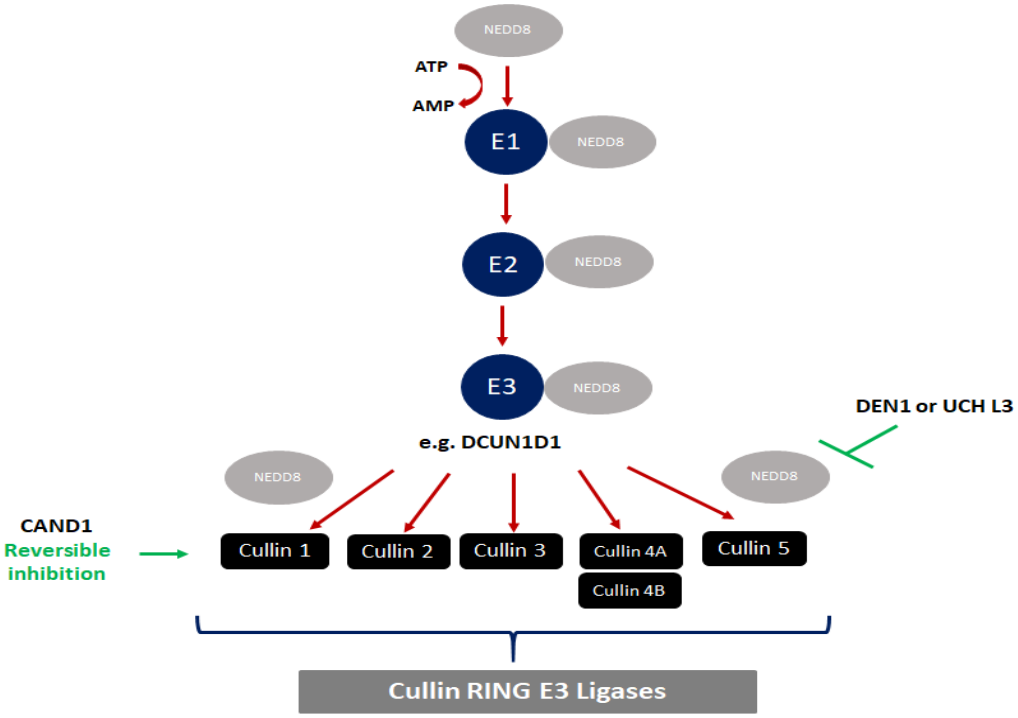


Figure 15. Schematic diagram of the neddylation pathway and DCUN1D1 cullin neddylation. Showing the neddylation pathway and an example of the E3 ligase DCUN1D1 and its known substrates, cullin 1, 2, 3, 4A, 4B and cullin 5. The cullin proteins are scaffolding molecules of the cullin RING E3 ligases (CRLs). It also includes inhibitors of neddylation, namely, CAND1 *(left)* which binds inactive cullin proteins prior to neddylation and DEN1 or UCH L3 *(right)* which are known to process precursor NEDD8 and remove NEDD8 from target proteins.

Model organism experiments have demonstrated that neddylation is an essential pathway because dysregulation or knockout of NEDD8 pathway components leads to lethality in some organisms and developmental defects in others (Tateishi *et al.*, 2001; Kurz *et al.*, 2002; Figueroa *et al.*, 2005; Dorfman *et al.*, 2009; Hosp *et al.*, 2014). Additionally, neddylation has been implicated in transcription, DNA replication, cell growth, cell proliferation, DNA damage repair and apoptosis (Chairatvit and Ngamkitidechakul, 2007; Jones *et al.*, 2008; Soucy *et al.*, 2009; Merbl *et al.*, 2013). As such, it has been implicated in several diseases including neurodegenerative, cardiovascular diseases and cancer (Watson, Irwin and Ohh, 2011; Chen, Neve and Liu, 2012; Duncan *et al.*, 2012; Kandala, Kim and Su, 2014). Neddylation also plays a role in ubiquitin CRL activity, with cullin protein neddylation important for the conformational changes that enhance ubiquitin substrate modification and related activities (Saha A, 2008). The cullin family of proteins: cullin 1, 2, 3, 4A, 4B, 5, 7, except for p53-associated parkin-like cytoplasmic protein (PARC), are among the proteins targeted for neddylation (Skaar *et al.*, 2007; Kim *et al.*, 2008; Sarikas, Hartmann and Pan, 2011).

As mentioned previously, cullin proteins are scaffolding molecules of the ubiquitin CRLs which target a variety of proteins for ubiquitination leading to proteasomal degradation, among other activities (Petroski and Deshaies, 2005). The targets and components of CRLs include regulators of the cell cycle, receptors, tumour suppressors, oncoproteins and knockdown/knockout of cullin proteins can lead to

dysregulation of these proteins or their functions (Sarikas, Hartmann and Pan, 2011). Significantly, cullin 3 knockout in *C. elegans* results in defective embryogenesis and knockout of our protein of interest DCUN1D1, results in developmental abnormalities in mice (Kurz *et al.*, 2002; G. Huang *et al.*, 2011). Additionally, the following proteins have been described as neddylation substrates including p53, MDM2, pVHL, several ribosomal proteins, EGFR and some ubiquitin E3 ligases (Kamura *et al.*, 1999; Stickle *et al.*, 2004; Xirodimas *et al.*, 2004, 2008; Oved *et al.*, 2006; Jones *et al.*, 2008; Watson, 2010). Table 7 below provides a summary of the neddylation substrates that have been identified, including details on p53 and MDM2, which are more well understood. P53 and MDM2 are a NEDD8-substrate and E3 ligase pair, while p53 is also regulated by other NEDD8 substrates such as ribosomal proteins (Xirodimas *et al.*, 2004; Sundqvist *et al.*, 2009; Watson, 2010). This provides insights into the range of mechanisms of action of neddylation and the functions it may perform, including the regulation of protein-protein interactions and regulating protein stability (Figure 16). This is evident also in its role in cancer.

Table 7: Showing neddylation substrates and neddylation-related functions

Neddylation substrate	Function	Neddylation substrate	Function
P53	**MDM2:** Mediates p53 neddylation **Tip60:** Inhibits p53-MDM2-mediated neddylation **NUB1:** Decreases p53 neddylation leading to nuclear export **FBXW011:** Promotes p53 neddylation inhibiting transactivation activity	**HIF1α**	Neddylation results in HIF1α stability
MDM2	**MDM2:** undergoes autoneddylation **P53:** MDM2 neddylation substrate **TAp73β:** MDM2 neddylation substrate **L11:** MDM2 neddylation substrate	**RCAN1**	Neddylation results in RCAN1 stability
RPL11	Neddylation results in RPL11 stability and localization	**TGF β Receptor II**	c-Cbl mediates TβRII neddylation and stabilization
RPL5, RPL6, RPL7, RPL7a, RPL8, RPL9, RPL10a, RPL11, RPL12, RPL13, RPL14, RPL17, RPL18, RPL21, RPL23, RPL24, RPL26, RPL27, RPL29, RPL30, RPL31, RPL35a, RPS2, RPS3, RPS4, RPS6, RPS7, RPS8, RPS11, RPS13, RPS14, RPS15a, RPS16, RPS20, RPS23, RPS26	Targets of neddylation determined from proteomics	**CK1α**	First description of kinase neddylation
PARKIN	Undergoes autoneddylation **PINK1** is its E3 ligase	**SHC**	Undergoes neddylation impacting T cell activity
PINK1	Neddylation results in PINK1 stability	**HUR**	MDM2 mediates HUR neddylation affecting its stability
BRAP2	Brap2 neddylation impacts NF-κB nuclear translocation	**Histone H4**	RNF111 mediates histone H4 neddylation regulating DNA damage
XIAP	Undergoes autoneddylation	**E2F1**	Neddylation controls transcriptional target specificity and induction of apoptosis
AICD	Neddylation results in the inhibition of its transcriptional activity	**ML3**	Undergoes neddylation

Neddylation is dysregulated in multiple cancers including liver, lung, oral squamous cell carcinoma and PCa (Soucy *et al.*, 2009; Lin *et al.*, 2010; Milhollen *et al.*, 2010; Swords *et al.*, 2010; Wei *et al.*, 2012; Shah *et al.*, 2016). Additionally, some of the identified neddylation substrates have been established to play a role in cancer. The tumour suppressors p53 and VHL are dysregulated in many cancers through mutations, PTMs, dysregulation in expression levels and they have been shown to undergo neddylation (Stickle *et al.*, 2004; Xirodimas *et al.*, 2004; Meek and Anderson, 2009; Bieging, Mello and Attardi, 2014; Gossage, Eisen and Maher, 2015). P53 neddylation is regulated by its E3 ligase MDM2, by RPL11, Tip60, NUB1 and FBXW011 (Table 7) affecting its stability, nuclear translocation and transcriptional activity (Xirodimas *et al.*, 2004; Abida *et al.*, 2007; Dohmesen, Koeppel and Dobbelstein, 2008; Sundqvist *et al.*, 2009; Liu and Xirodimas, 2010). However, no direct association has been made between p53 neddylation and cancer thus far. VHL neddylation on the other hand inhibits cullin 2 binding and the role of VHL neddylation in the disruption of fibronectin extracellular matrix assembly is associated with inhibiting tumour development (Stickle *et al.*, 2004; Heir *et al.*, 2013). Several other NEDD8 substrates have been associated with cancer growth and development, independent of neddylation, but affecting different "Hallmarks of Cancer" such as MDM2, HIF1α and BCA3 (Kitching *et al.*, 2003; Vaupel, 2004; Leung and Ngan, 2010; Karni-Schmidt, Lokshin and Prives, 2016; D. Ma *et al.*, 2018). It is however, the development and success of the NAE inhibitor (MLN4924) that highlights the importance of the neddylation pathway in cancer.

The NAE inhibitor MLN4924 has been shown to have potent anti-cancer activity, particularly in myelomas and it is undergoing phase II clinical trials in other cancers (Milhollen *et al.*, 2010; Swords *et al.*, 2010, 2015; Luo *et al.*, 2012; Wei *et al.*, 2012). However, several side effects have been associated with its use in cancer treatment (Milhollen *et al.*, 2010; Swords *et al.*, 2010, 2015; Luo *et al.*, 2012; Wei *et al.*, 2012). In fact, p53 has been tested for the protection of normal cells from MLN4924 treatment side effects, relying on the role of p53 in cell stress regulation (Malhab *et al.*, 2016). MLN4924 has been shown to activate p53 in cancer cells expressing wildtype p53 through deneddylation of some of its regulators, however, cells not expressing p53 or expressing a mutated form of it were still sensitive to MLN4924 (Malhab *et al.*, 2016). Therefore, it has been proposed that, regulation of p53 neddylation may be useful in MLN4924 treatment sensitization when used in combination with p53 inhibitors or it could be used to regulate MLN4924 toxicity in managing side effects (Malhab *et al.*, 2016). This supports our hypothesis that targeting the neddylation pathway may provide cancer treatment efficiencies, with potentially reduced side effects. Studies performed previously in our lab demonstrated the role of DCUN1D1 in PCa using *in vitro* and *in vivo* analyses (Vava and Zerbini, 2014). We also tested drugs to identify DCUN1D1-specific potential inhibitors of PCa and we propose that targeting neddylation, using the downstream DCUN1D1 E3 ligase, may retain potency in cancer treatment and perhaps result in reduced side effects. We therefore want to understand the mechanisms involved in DCUN1D1's activity in PCa inhibition, using proteomics.

Proteomics has already been used to identify some neddylation substrates through affinity purification-based MS analysis and SILAC quantitative proteomics. The studies employed different approaches where affinity purification-based MS was performed following pulldown of proteins bound to NEDD8, after the overexpression of GST-NEDD8 in HEK293 cell lines (Jones *et al.*, 2008). The LTQ mass spectrometer was used and 496 proteins were identified, that were involved in cell cycle regulation, chromatin structure and organization, transcription, DNA replication and DNA damage repair (Jones *et al.*, 2008). The study by Liao et al., 2011 used SILAC-based quantitative proteomics analysis of proteins that were stabilized following MLN4924 treatment of A375 melanoma cells. The

objective was to identify new downstream targets of CRL substrates by inhibiting neddylation, preventing cullin protein neddylation and ubiquitin CRL assembly (Liao *et al.*, 2011). This would lead to the accumulation of proteins normally degraded by the 26S proteasome (Liao *et al.*, 2011). Using the LTQ Orbitrap Velos, 5122 - 6012 proteins were identified over the different time points that they analysed and CRL substrates involved in the cell cycle, DNA damage repair and the ubiquitin pathway were identified (Liao *et al.*, 2011). Additionally, using the overexpression of tandem affinity purification (TAP)-tagged NEDD8 in Hela cells and the Q-Star Pulsar XL mass spectrometer, several ribosomal proteins were identified as targets for neddylation (Table 7) (Xirodimas *et al.*, 2008).

Therefore, neddylation is rapidly emerging as a critical PTM, with many substrates being identified that lead to increasingly complicated cellular activities. Apart from understanding the role that NEDD8 modification plays on protein substrates, one of the biggest questions surrounding neddylation is whether the substrates identified thus far are true/putative neddylation substrates or are artefacts of the overexpression of NEDD8, which leads to NEDD8 transfer being mediated by ubiquitin pathway components. NEDD8 and ubiquitin are 60% identical in terms of amino acid sequence and Leidecker et al., 2012 demonstrated that the UAE UBE1 was able to mediate neddylation, especially of non-cullin molecules under conditions of stress (Kumar, Yoshida and Noda, 1993; Leidecker *et al.*, 2012). However, it is not uncommon for proteins/enzymes to have overlapping activity as demonstrated above with the other UBLs. In fact, some of the neddylation substrates that have been identified are characterized ubiquitin E3 ligases (MDM2 and PARKIN) that have been shown to mediate neddylation of substrates or to undergo autoneddylation (Fang, 2000; Xirodimas *et al.*, 2004; Watson *et al.*, 2006; Choo *et al.*, 2012; Seirafi, Kozlov and Gehring, 2015).

Conversely, it has been proposed that the above-mentioned, maybe a *bona fide* cellular regulatory mechanism demonstrating NEDD8 and ubiquitin crosstalk. Evidence for this can be obtained from how in cases like EGFR, monoubiquitination preceded NEDD8 modification by the same E3 ligase, c-Cbl, suggesting that monoubiquitination may be triggering neddylation (Oved *et al.*, 2006). Additionally, Xirodimas et al., 2008 identified ubiquitin as a NEDD8 substrate using MS, while Leidecke et al., 2012 described mixed chains of ubiquitin and NEDD8 under conditions of stress (Xirodimas *et al.*, 2008; Leidecker *et al.*, 2012). Therefore, neddylation is displaying characteristic molecular level functions and mechanisms that mimic that of ubiquitin, suggesting that it may be as important to cellular function as ubiquitination. Although the neddylation pathway, its substrates and the methods of identification raise interesting questions, there are a variety of pathways that have been implicated in neddylation and neddylation has been demonstrated to be essential, as demonstrated by *in vivo* studies (Kurz *et al.*, 2002; G. Huang *et al.*, 2011).

We have studied one of the neddylation E3 ligases, DCUN1D1, and identified it to play a key role in PCa *in vitro* and *in vivo* (Vava and Zerbini, 2014). We will use proteomics to understand its mechanism of action including attempting to identify any non-cullin proteins that it may be targeting but we begin with a review of what is currently understood about DCUN1D1.

1.6 DCUN1D1

Defective in Cullin Neddylation 1 Domain containing 1 (DCUN1D1) is an E3 ligase of the neddylation pathway currently understood to target some members of cullin family of proteins (Kim *et al.*, 2008; Keuss *et al.*, 2016). It was initially characterised due to its genomic amplification in the 3q locus of a variety of squamous cell carcinomas and is also known as Squamous Cell Carcinoma Related Oncogene (SCCRO) (Sarkaria *et al.*, 2006). DCUN1D1/SCCRO has been demonstrated to play a role in tumourigenesis as a cancer driver gene, it has been linked to cancer progression and has been associated with prognosis in gliomas, lung, cervical cancer, laryngeal squamous cell carcinoma, colorectal and head and neck cancers (Sarkaria *et al.*, 2006; Broderick *et al.*, 2010; Yoo *et al.*, 2012; Wang *et al.*, 2013; Jiang *et al.*, 2016; J. Liu *et al.*, 2017; Xiao *et al.*, 2017). It is increasingly being described as an oncogene in a variety of cancers and studies performed previously in our lab have demonstrated that it plays a role in PCa (Vava and Zerbini, 2014). As observed in other neddylation components, the extent of the function of DCUN1D1 and its mechanism of action are not clearly understood.

Although initially thought to be an enhancer of neddylation, *in vivo* studies demonstrated that DCUN1D1 is essential for neddylation, in part due to its role in the nuclear translocation of cullin 1 (Kim *et al.*, 2008; G. Huang *et al.*, 2011). It has subsequently been demonstrated to mediate cullin 3 neddylation and midbody localization during mitosis (Huang *et al.*, 2017). Furthermore, DCUN1D1 plays a role in cullin 2 neddylation downstream of HIF1α binding to its ubiquitin E3 ligase (VHL) and therefore regulating HIF1α stability, in a manner similar to a feedback mechanism (Heir *et al.*, 2013). It has also been shown that DCUN1D1-mediated cullin 3 neddylation promoted Aurora B ubiquitination during mitosis (Huang *et al.*, 2017). Interestingly, exploring DCUN1D1 activity relative to its family members DCUN1D2, DCUN1D3, DCUN1D4 and DCUN1D5, has provided further insights into its activity. The DCUN1D1 homologues differ in their N-terminal domains but share a conserved C terminal domain through which they bind cullin proteins (Keuss *et al.*, 2016). Additionally, DCUN1D3 has been shown to inhibit DCUN1D1-mediated cullin 1 neddylation, functioning as a tumour suppressor and highlighting the role of DCUN1D1 as an oncoprotein (Huang *et al.*, 2014). Furthermore, a study comparing the cullin-neddylation-specific activity of DCUN1D1 relative to its family members, showed that DCUN1D1 had activity that overlapped with the neddylation activity of some but not all of its family members (Keuss *et al.*, 2016). However, DCUN1D1 also appears to be playing a broader role in cellular activities.

DCUN1D1 has been implicated in embryonic development where DCUN1D1 knockout in mice led to developmental defects or infertility (Broderick *et al.*, 2010; G. Huang *et al.*, 2011). It has also been linked to frontal lobar degeneration, but its mechanism of action is not well understood (Villa *et al.*, 2009). Significantly, dysregulation of DCUN1D1 expression has been shown to inhibit cell proliferation, migration, invasion and epithelial-to-mesenchymal transition (EMT) in several cancers (O-charoenrat *et al.*, 2008; Broderick *et al.*, 2010; Jiang *et al.*, 2016; J. Liu *et al.*, 2017; Xiao *et al.*, 2017; Yu *et al.*, 2017; Zhang *et al.*, 2017). We observed a decrease in proliferation and migration following knockdown of DCUN1D1 in PCa cells and identified two drugs, monensin and podophyllotoxin, as potential inhibitors of DCUN1D1 (Vava and Zerbini, 2014). These drugs were observed to mediate an additive effect when used in combination in PCa (Vava and Zerbini, 2014). Podophyllotoxin is a taxane known to decrease tumourigenesis by inhibiting microtubule polymerisation leading to cell cycle arrest, while, monensin

has been shown to target the WNT signalling pathway, suggesting that these pathways maybe linked to the mechanism of action of DCUN1D1 (Kuo *et al.*, 2004; Tumova *et al.*, 2014). Additionally, DCUN1D1 appears to be highly regulated, with microRNA 195, 218 and 520b directly targeting DCUN1D1 and repressing its expression (Figure 16) (Jiang *et al.*, 2016; Xiao *et al.*, 2017; Yu *et al.*, 2017). Furthermore, DCUN1D1 is inhibited through antagonism by DCUN1D3, p62 has been shown to inhibit DCUN1D1 binding to cullin 2 and VHL regulates the recruitment of DCUN1D1 to initiate cullin 2 neddylation in response to HIF1α binding (Figure 16) (Huang *et al.*, 2014; Chen *et al.*, 2016).

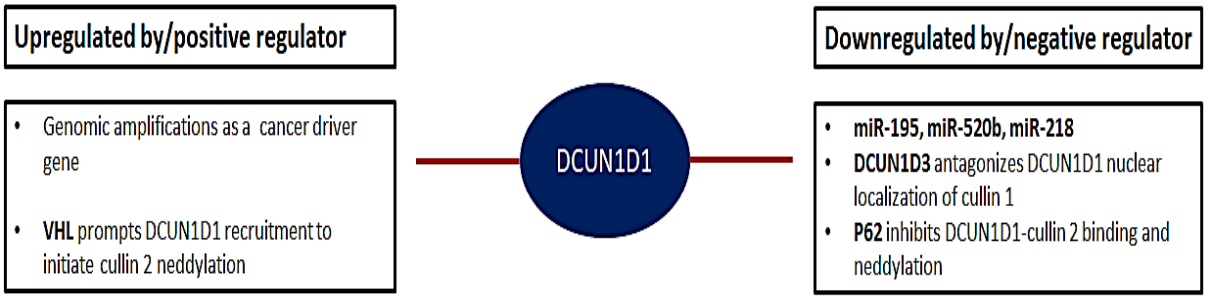


Figure 16. Diagram showing the mechanisms of regulation of DCUN1D1. *Left)* are the mechanisms and proteins currently understood to positively regulate DCUN1D1 activity, VHL regulates DCUN1D1 recruitment during cullin 2 neddylation and DCUN1D1 is selectively upregulated in cancer as a driver gene. *Right)* are the microRNAs that have been found to target DCUN1D1 directly, repressing its expression including miR195, miR520b and miR218, while p62 has been shown to inhibit cullin2 binding also inhibiting its neddylation.

Therefore, as a component of the neddylation pathway, DCUN1D1 may be targeting other neddylation substrates but its upstream and downstream effects have not been extensively characterised. Additionally, by mediating the neddylation of cullin proteins, which are scaffolding molecules of ubiquitin CRLs, DCUN1D1 may be playing a role in the pathways or processes involving neddylation and the wide spectrum of pathways/processes implicated in ubiquitination. Thereby playing a role in protein-protein interactions, protein localization, protein degradation and regulating key cellular pathways. This study seeks to use different proteomics approaches to determine the mechanism of action of DCUN1D1 and to screen for potential inhibitors of DCUN1D1 using a proteomics approach. We would like to determine the broader substrates of DCUN1D1 and identify the mechanism by which it mediates its activities in PCa, which may provide insights into its activity in general. We would also like to identify potential inhibitors of DCUN1D1 which could be used for molecular target-based treatment of PCa. This would enable the understanding of the activity of DCUN1D1 and its contribution to PCa but also allow for targeting of the neddylation pathway downstream of the NAE with potentially fewer side effects.

1.7 Study Aims and Objectives

The objective of this study is to determine the mechanism of action of DCUN1D1 using proteomics and to identify potential inhibitors of DCUN1D1 for PCa treatment based on this approach. This would be critical in providing information on a minimally investigated subject thus far and could provide insights into an emerging approach to PCa treatment.

Aims:

a) Determine the functional consequences of DCUN1D1 activity using immunoprecipitation-coupled MS-based proteomics.

b) SILAC quantitative proteomics analysis to identify differentially expressed proteins in DU145 versus DU145 DCUN1D1 knockdown cell lines.

c) Advanced connectivity map analysis using the DCUN1D1 knockdown cell line protein signature.

d) Validation of DCUN1D1 substrates and proposed mechanism of action using western blot analysis.

Chapter 2: IPMS analysis of DCUN1D1 substrates

2.1 Introduction

DCUN1D1 is an E3 ligase for the neddylation pathway (Kim *et al.*, 2008; Keuss *et al.*, 2016). It plays a role in the transfer of 9kDa NEDD8 to specific protein substrates, following activation by the neddylation activating enzyme (APPBP1/UBA3) and transfer to the neddylation conjugating enzyme (UBC12) (Kim *et al.*, 2008; Keuss *et al.*, 2016). Although the cullin family of proteins (cullin 1, 2, 3, 4A, 4B and cullin 5), which are components of the ubiquitin CRLs, have been demonstrated to be targets of DCUN1D1. The full spectrum of DCUN1D1 target proteins as an E3 ligase for neddylation have not been described previously and the downstream effect of cullin neddylation, by DCUN1D1, has not been fully elucidated (Kim *et al.*, 2008; G. Huang *et al.*, 2011; Keuss *et al.*, 2016). Studies performed previously in our laboratory demonstrated that DCUN1D1 plays a role PCa, where knockdown of DCUN1D1 significantly inhibited PCa *in vitro* and *in vivo* (Vava and Zerbini, 2014). We also identified two potential inhibitors of DCUN1D1 that impede PCa through the reduction of DCUN1D1 mRNA and protein (Vava and Zerbini, 2014). Therefore, understanding its function in the neddylation pathway and its subsequent impact on the ubiquitination pathway can assist in determining the mechanism of action facilitating its response in PCa.

As demonstrated previously, there have been several advancements in the methods of studying proteins (Chapter 1, Figure 8, pg. 18). Importantly, among these have been the developments in methods identifying and characterising protein-protein interactions. From the use of single/multiple tags of one's protein of interest for isolation during immunoprecipitation, to conducting yeast-two-hybrid screening and interactome analysis (Stephens and Banting, 2000; Markham, Bai and Schmitt-Ulms, 2007; Figeys, 2008; Brückner *et al.*, 2009; De Las Rivas and Fontanillo, 2010). Moreover, within MS-based proteomics, there are several approaches that can be used including label-free identification and quantitative MS analysis through chemical and metabolic labelling (Dunham, Mullin and Gingras, 2012). Significantly, immunoprecipitation-coupled mass spectrometry (IPMS) has developed into a highly effective methodology for characterising protein interactions and related network activities (Bauer and Kuster, 2003; Figeys, 2008; Medvedev *et al.*, 2012; Lambert *et al.*, 2013; Morris *et al.*, 2014). It combines the specificity and enrichment properties provided by affinity purification (mainly through antibody recognition) with the sensitivity, accuracy and advancements in MS technology (Bauer and Kuster, 2003; Dunham, Mullin and Gingras, 2012). This has led to the identification of core subunits of protein complexes, weak or transient interactors and has contributed to an improved understanding of their biological functions. However, IPMS can also result in the identification of nonspecific interactors/binding partners due to the affinity of proteins to the components of the immunoprecipitation workflow such as the solid matrices, antibody and the bait (Dunham, Mullin and Gingras, 2012). Therefore, the ability to discriminate between nonspecific binding partners and true interactors is critical and can be performed based on manual elimination using controls during immunoprecipitation as well as statistical probability scoring.

Interestingly, the Crapome database has been developed recently to assist with identifying true interactors from nonspecific binding partners, using data repositories of IPMS studies (Mellacheruvu *et al.*, 2013). It relies on the frequency of detection of certain proteins across multiple different experiments, performed by different laboratories, while accounting for commonly used IPMS reagents and cataloguing the proteins identified routinely (Mellacheruvu *et al.*, 2013). Therefore, we aim to use this overall approach to determine the binding partners of DCUN1D1 and its interaction network.

2.2 Hypothesis

As an E3 ligase of neddylation, DCUN1D1 may be playing a role in the pathways or processes linked to neddylation and the wide spectrum of functions implicated in ubiquitination. Similar to the ubiquitin code (Komander and Rape, 2012), DCUN1D1 may be playing a role in protein-protein interactions, protein localization, protein degradation and other key cellular activities. IPMS enables the identification of proteins based on affinity-based purification, therefore, we postulate that we could identify DCUN1D1 interactors, determine the broader binding partners of DCUN1D1 and the mechanism by which it mediates its activity using this approach. This would enable us to delineate the mechanisms involved in DCUN1D1's activities and provide an improved understanding of the DCUN1D1 mechanism of action. This could contribute to establishing if it is a viable target for PCa treatment.

2.3 Aims and Objectives

The objective of this study is to determine the mechanism of action of DCUN1D1 using an IPMS proteomics approach.

Aim:

Determine the consequences of DCUN1D1 activity using immunoprecipitation-coupled MS-based proteomics.

2.4 Materials and Methods

IPMS was performed in order to identify DCUN1D1 interactors, by isolating DCUN1D1 binding partners from whole cell lysates. The methodological approach is depicted below including sample preparation, co-immunoprecipitation, MS experimentation and data analysis (Figure 17).

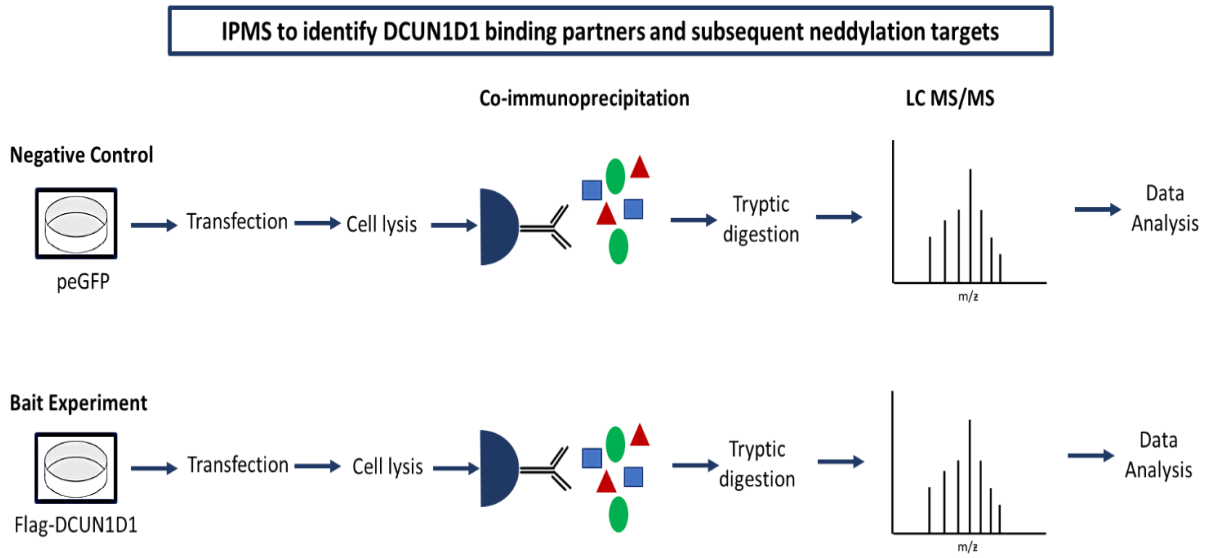


Figure 17. Schematic diagram showing the IPMS workflow. The negative control (peGFP-N3) or bait (pcDNA-Flag-DCUN1D1) plasmids were transiently transfected into HEK293 TT cells, cell lysis was performed followed by enrichment during co-immunoprecipitation for either the control or bait (DCUN1D1) pulldown products. Tryptic digestion was then performed overnight, following which, the peptides underwent LC-MS/MS and data analysis, including database searching and protein identification.

2.4.1 Cell culture and cell maintenance

HEK 293TT cells were used in this study, which were obtained from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy). The cells were grown in complete media containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Germany), 10% Fetal Bovine Serum (FBS) (Gibco, Life Technologies, USA) and 1% penicillin (5000 µg/mL/streptomycin 500 µg/mL). During passaging, the cells were seeded onto 10cm dishes (Greiner, Cellstar Bio-one, Germany) until 80% confluency and then washed using 1× phosphate buffered saline (PBS) pH 7.4 (see Table 8 below). Alternative to the addition of trypsin, the cells were detached by adding 5mL of complete media to the plate and resuspending them in the media using a pipette, due to their loosely adherent nature. They were then plated at the ratio relevant for the experiment, mainly a 1:3 ratio. Two plates each of the control and bait experiment were cultured and allowed to grow until 80% confluency. For the drug treatment studies, two plates each of the GFP-controls and the DCUN1D1 plates were treated either with 0.1% DMSO vehicle control or 1µM MG132. MG132 is a proteasome

inhibitor that has been shown to lead to the accumulation of CRL neddylation substrates and was used in this study to improve DCUN1D1 target identification (Emanuele *et al.*, 2011).

The cell-freezing protocol involved transfer of detached, resuspended cells to a clean, sterile tube and then centrifugation at 4000 rpm for 3 minutes. The cell pellet was then resuspended in a solution containing 50% FBS (Gibco, Life Technologies, USA), 40% DMEM (Sigma-Aldrich, Germany) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, France) and stored either at -80°C or liquid nitrogen.

2.4.2 Calcium Phosphate Transfection

Transient transfection of HEK 293TT cells was performed using the calcium phosphate precipitation method. Calcium and phosphate ions form micro-precipitates in solution, which interact with DNA molecules and form co-precipitates. The co-precipitates then bind strongly to the cell monolayer and allow for the uptake of DNA by the cells (Wigler *et al.*, 1979). Two plates each of the control and bait experiment were cultured and allowed to grow until 80% confluency, then the calcium phosphate solution was prepared. We used clean, sterile 15mL tubes (Corning, Merck, Germany) to which we added 10 - 20µg of plasmid DNA, followed by 2.5M $CaCl_2$. Subsequently, ddH_2O was added to a final volume of 500µL, and then 500µL of 2× BES-buffered saline (BBS) (Table 8) was added, dropwise, to the solution and mixed continuously to obtain a fine precipitate. We then changed the media of the plated cells and incubated them for a few hours at 37°C, under humidified conditions containing 5% CO_2. The transfection solution (1mL) was then slowly added to each plate to improve transfection efficiency. Following this, the plates were incubated for 48 hours until cell lysis.

2.4.3 Cell lysis and co-immunoprecipitation

The cell lysis and co-immunoprecipitation assays were performed in accordance to the laboratories' MS compatible immunoprecipitation protocol from the Proteomics Facility, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy. We initially used the GFP-transfected negative control plates to determine transfection efficiency and following immunofluorescence analysis, we determined the transfection efficiency to be ~90 percent. The cells were then washed using 10mL of cold 1× PBS, aspirated, and 0.5mL of cold cell lysis buffer supplemented with 0.1 mg/mL dextran (Sigma-Aldrich, Germany) (Table 8) was added to each plate. We then scraped the cells using cell scrapers (Greiner, Cellstar Bio-one, Germany) and transferred the lysate to sterile 1.5mL tubes. The lysate was sonicated on ice, for 30 seconds at 80 amps using a 0.5 second cycle, following which, the cell debris and DNA aggregates were pelleted by centrifugation at 13 200 rpm for 10 minutes, at 4°C. The supernatant was then transferred to sterile 1.5mL tubes prior to performing the co-immunoprecipitation assay (Figure 18). The Bradford reagent assay was used to determine protein concentration, where 1mL of premixed Bradford reagent (Sigma-Aldrich, Germany), was added to low volume cuvettes (Sigma-Aldrich, Germany) following which, lysate from the samples was added at 10µL each. An equivalent volume of the cell lysis buffer plus 0.1 mg/mL dextran was used as the blank and after covering all the cuvettes with parafilm, the solutions were mixed and measured using spectrophotometry.

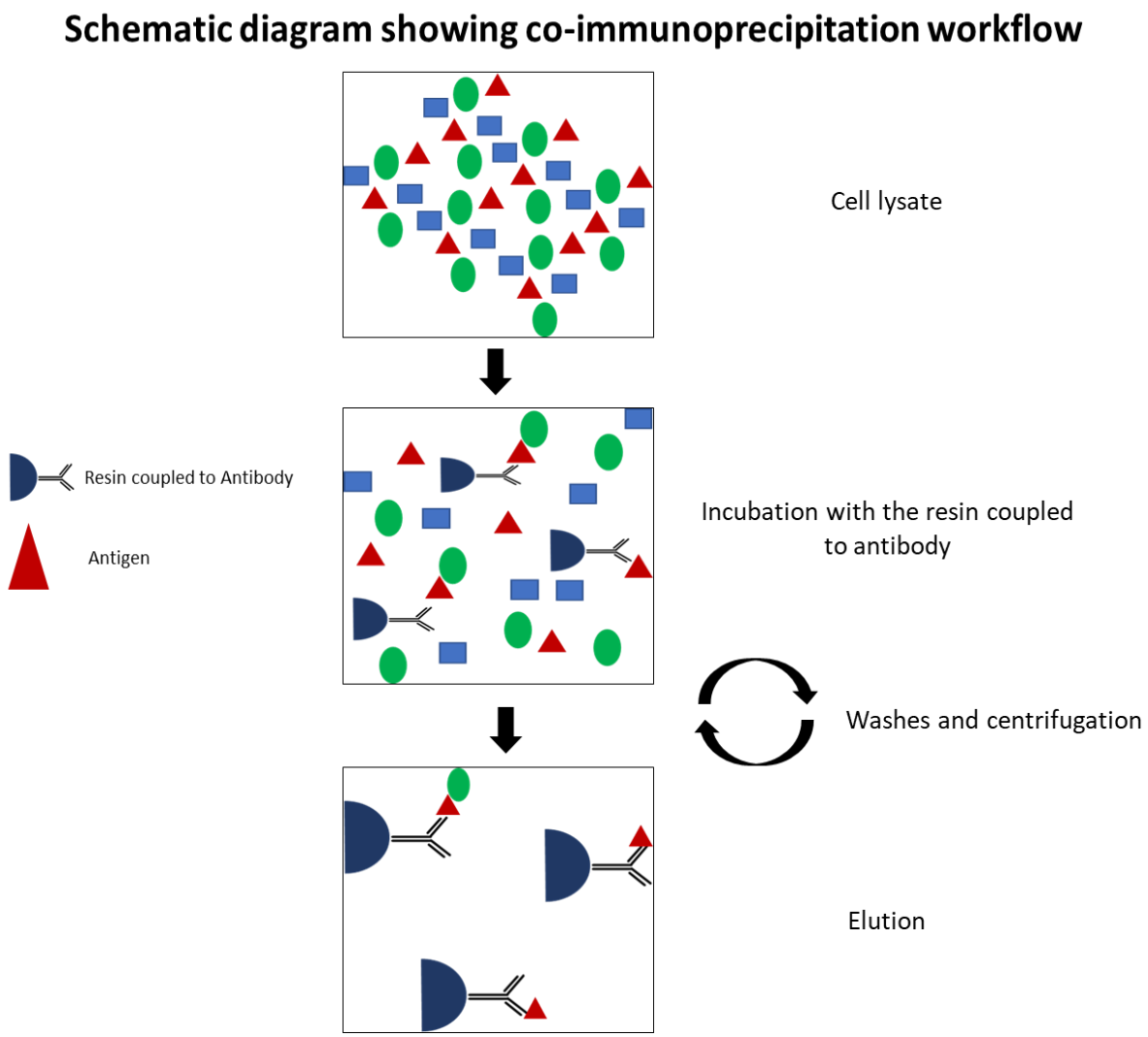


Figure 18. Diagram showing the co-immunoprecipitation workflow. *From top to bottom*, the cell lysate containing the proteins in solution is incubated with the resin containing the antibody coupled to agarose beads. After binding the antigen (protein of interest) and direct/indirect binding partners the pulldown products were washed for several cycles with centrifugation before elution of the bound proteins from the antibodies using an appropriate buffer solvent.

Co-immunoprecipitation was performed using the EZview red anti-Flag M2 affinity gel resin containing beads with anti-Flag antibodies bound to them (Sigma-Aldrich, Germany). First, bottles containing the resin were thoroughly mixed to ensure homogeneity. We then aliquotted 40µL each into sterile 1.5mL tubes using a sterile yellow tip that has been cut at the edge. The beads were then washed 3 times with cell lysis buffer containing 0.1 mg/mL dextran, with centrifugation occurring at 10 000 rpm for 30 seconds in between each wash step. We then discarded the supernatant avoiding bead loss. Lysate from each plate was then pooled into one sample for the control and one sample for the bait experiment then transferred to the tubes containing the washed beads. The samples were then incubated for 1 hour with continuous rotation, at 4°C, followed by centrifugation at 10 000 rpm for 30 seconds. The supernatant was then transferred to sterile 1.5mL tubes and stored at -20°C. The beads with bound proteins were washed 3 times with 1mL of cell lysis buffer only (no dextran) and

centrifuged at 10 000 rpm for 30 seconds. Following this, they were resuspended with 1× PBS and transferred to clean, sterile 1.5mL tubes. They were then washed 2 times with 1mL of 1× PBS prior to further processing.

Gel loading tips (Corning, Costar, USA) were used to remove excess buffers solutions, to prevent disruption of the bead-bound pulldown products and to decrease the contaminants present in the tube, prior to tryptic digestion.

2.4.4 Tryptic digestion and C18 Stage-Tip clean-up

In-solution digestion of bead-bound proteins was performed using sequencing grade modified trypsin (Promega, USA), which was dissolved in 20 mM triethylammonium bicarbonate (TEAB) (Table 8) to a final concentration of 100 ng/µL. The digestion was performed for 16 hours at 42°C, then the supernatant (20µL) was carefully removed using the gel loading tips (Corning, Costar, USA) and transferred to a clean 1.5mL tube. Subsequently, the beads were washed with 20µL TEAB and the supernatant was added to the previously collected peptide solution. The samples were then acidified to a final concentration of 0.1% trifluoroacetic acid (TFA) (Table 8) and the peptides concentrated and purified using C18 Stage-Tip clean-up (Millipore) (Sigma-Aldrich, Germany). The activation solution used was 100% acetonitrile (Sigma-Aldrich, Germany), the equilibration solution was 0.1% TFA and the elution buffer was a 50:50 dilution of 0.2% TFA and 100% acetonitrile (Sigma-Aldrich, Germany). Excess buffer was removed by vacuum drying the samples for 15 - 20 minutes after elution and the samples were stored at room temperature or -20°C prior to MS analysis.

2.4.5 LC-MS/MS

The MS analysis was performed at the Proteomics Facility, at the International Centre for Genetic Engineering and Biotechnology, Trieste, Italy. Peptides were analysed through reverse phase on-line nano-liquid chromatography (LC)-MS/MS on a Bruker EASY-nLC II (Thermo Fisher Scientific, USA), where Bruker Daltonics' Compass software was coupled to the EASY-nLC with the Bruker Daltonics' Amazon Electron-Transfer Dissociation ion trap. The microcapillary column used during LC was discontinuous, made with a pulled fused silica (FS) tip length [75µM internal diameter (i.d.)] plus an Ascentis RPamide (Sigma-Aldrich, Germany), 2.5cm × 2µm. The capillary column was packed with 7.5cm × 3µM C14 Jupiter Proteo Reverse Phase (Phenomenex, USA) and 4cm × 5µM C8 Aguasil (Thermo Fisher Scientific, USA) using a self-assembled high-pressure column loader. The mobile phase was set to ground then -4000 Volts was applied to initiate the electrospray and the duty cycle was 1 survey plus 5 MS/MS scans. The protein-digest products were separated using buffer A, 0.1% formic acid in HPLC-grade water (Sigma-Aldrich, Germany), and buffer B, 100% acetonitrile (Sigma-Aldrich, Germany). The samples were analysed in 60-minute runs with a discontinuous gradient of 0 - 20% acetonitrile for 45 minutes, 20 - 50% acetonitrile for the next 10 minutes, 50 - 80% acetonitrile for 1 minute and any remaining peptides were eluted by adding 80% acetonitrile up to the 60^{th} minute.

2.4.6 Data processing and analysis

The .yep data files generated were processed using the X Tandem software version Alanine (2017.2.1.4), with the Global Proteome Machine (GPM) search engine and then run against the Ensembl human database. Default settings were used in the study, with trypsin selected for enzyme specification and carbamidomethylation of cysteine selected as a fixed modification. The variable modifications selected included single/double oxidation of methionine and tryptophan as well as deamidation of asparagine and glutamine. The dynamic range was set at 100 Daltons, with a mass tolerance of fragment ions set at 0.3 Daltons. The minimum cut-off for peptide mass was set at 150

Daltons, for parent ions 500 Daltons, 50 scans and 1 was set as the maximum permissible number of mixed cleavage sites. The resulting MS/MS spectra were then searched using X Tandem allowing for a 5% False Positive Rate (FPR).

Table 8: Detailed description of the buffers used and reagent composition

Reagent/Buffer	Composition
PBS pH 7.4	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H2O$, 1.4mM KH_2PO_4, ddH2O
BBS pH 7.2	50mM BES (N, N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid, 1.5mM Na2HPO4, 280mM NaCl, ddH2O
Cell lysis buffer	50mM Hepes pH7.4, 150mM NaCl, 50mM NaF, 1mM EDTA, 0.5% NP-40, ddH2O
TEAB pH 8.5	20mM TEAB (Fluka Chemika, Switzerland), HPLC grade water (Sigma-Aldrich, Germany)
0.1% TFA	Diluted down starting from 100% TFA (Fluka Chemika, Switzerland), HPLC grade water (Sigma-Aldrich, Germany)

2.4.7 Software/Tools used in the study

2.4.7.1 Software/Tools used within X Tandem (Alanine 2017.2.1.4)

Gene Ontology

Gene Ontology (GO) is a computational tool compiled and produced by the Gene Ontology Consortium for the unification of biology using genes and gene products (Ashburner *et al.*, 2000; Carbon *et al.*, 2017). It consists of 12 reference genomes including *E. coli*, *A. thaliana*, *S. cerevisiae*, *C. elegans*, *M. musculus* and *H. sapiens*, based on experimental findings from ~140 000 peer-reviewed published articles, which are collated and annotated to represent 600 000 experimentally-supported GO annotations (Carbon *et al.*, 2017). It uses this information together with the extensive information obtained from whole genome sequencing experiments performed on model organisms and species-specific sequencing experiments to infer >6 million functional annotations. These include experiment-supported, phylogenetically-inferred and computationally-inferred GO annotations (Carbon *et al.*, 2017). The analysis is based on the understanding that multiple core biology functions are shared among eukaryotes and that the genes encoding these functions are similar and produce phenotypes that can be extrapolated to other eukaryotes. GO therefore, creates a computational model for biological systems at various cellular levels including cellular components, cellular processes and molecular functions (Ashburner *et al.*, 2000). In addition, following an experiment, GO performs enrichment analysis on input datasets to analyse the underlying molecular changes obtained from measuring the levels of certain molecules (RNA, DNA or protein). It can identify the groups of genes that function together based on over-representation or under-representation of "GO terms" for an annotated gene set, taking into consideration the frequency of the gene within the database (background frequency), the sample frequency and p value determinations (Ashburner *et al.*, 2000; Carbon *et al.*, 2017). In terms of this study, the GO outputs were based on the log (I), log (p) and descriptions based on database and sample frequencies.

KEGG

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a multi-database resource consisting of 18 databases that provide molecular level and high-level information to understand the functions, interactions and uses of biological systems (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017). It includes genomics information in the form of gene-sequencing data obtained from experiments plus inferences from orthologs and protein-sequencing data, based on experimental characterisation of the function of proteins (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017). In addition, it contains chemical information, based on experiments performed using chemical substances and systems information based on "interomics" or network generation and analysis. It also contains health information, using disease and drug-treatment analyses to understand dysregulated biological systems. It then provides a computational representation of these biological systems from more than 4000 genome annotations as well as data generated from the use of viruses and plasmids (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017). In this study, the KEGG PATHWAY database was used within X Tandem to determine the pathways significantly dysregulated following IPMS analysis, based on the log (I), log (p) and descriptions based on database and sample frequencies.

2.4.7.2 Software/Tools used for additional analyses

PAST

PAST (Palaeontology Statistics) is a software that was designed for use in quantitative palaeontology studies that is increasingly being used in other biological studies (Hammer, Ryan and Harper, 2001). It functions on the Windows operating system, based on a user-friendly spread-sheet like platform, to perform "standard numerical analysis and operations", using methods that are specific for palaeontology and biology (Hammer, Ryan and Harper, 2001). These include plotting graphical functions, multivariate analysis, phylogenetic analysis, correlation of geological strata based on geological timelines, curve-fitting, time-series analysis and geometric analysis (Hammer, Ryan and Harper, 2001). In this study, we used PAST version 3 (PAST3) to perform statistical analyses following immunoprecipitation-coupled MS, including univariate and multivariate tests such as the Shapiro-Wilk test for normality, the Welch F test for unequal variance, the Kruskal-Wallis test for equal medians and the principle component analysis (PCA). These were performed according to the default settings, with a significance level of 0.05 and missing values were either supported (using the mean value for the dataset) or deleted depending on the test performed and within the assumptions necessary.

Venny

Venny software is an online program that allows for manual drawing of VENN diagrams to investigate relationships between collections of different datasets, in the form of listed elements (Oliveros, 2007). It uses overlapping circles depicted on a single plane to compare and visualize 4 lists of elements. We used Venny 2.1.0 to compare the relationships within the unfiltered and filtered datasets, using the default settings and the colour style.

STRING

The STRING functional protein association network is a protein-protein interaction database that annotates and provides associations between proteins, based on physical binding and indirect interaction through similar mechanism of action or participation in similar cellular processes (Snel *et al.*, 2000; Mering *et al.*, 2003; Szklarczyk *et al.*, 2015). STRING uses genomics information for annotation and prediction, information based on conserved expression as well as data obtained from high throughput experiments such as "Omics" studies (Snel *et al.*, 2000; Mering *et al.*, 2003; Szklarczyk

et al., 2015). In addition, it uses automated text mining and knowledge obtained from data previously analysed using STRING, to assist with making protein-protein associations (Snel *et al.*, 2000; Mering *et al.*, 2003; Szklarczyk *et al.*, 2015) . The proteins are represented as nodes, while lines (edges) represent the protein-protein associations and any meaningful functional information attributed to that association, is represented as line length, thickness, evidence etc. (https://string-db.org/). These can also be represented in the form of hubs depending on the output (Snel *et al.*, 2000; Mering *et al.*, 2003; Szklarczyk *et al.*, 2015). Currently, the database contains information on 9 643 763 proteins from 2031 organisms including, *E. coli*, *A. thaliana*, *S. cerevisiae*, *C. elegans*, *M. musculus* and *H. sapiens* (https://string-db.org/). Significantly, the number of interactions annotated in STRING, based on the score confidence level are: 1 380 838 440 from low confidence associations (≥ 0.150), 320 182 220 from medium confidence (≥ 0.400), 71 673 028 from high confidence (≥ 0.700) and 25 914 693 from the highest confidence associations (≥ 0.900) (https://string-db.org/).

CRAPOME

Crapome is a central repository for aggregated negative control samples generated from immunoprecipitation-coupled MS experiments for the determination of protein-protein interactions (Mellacheruvu *et al.*, 2013). It consists of negative control samples that are added to the database as raw files including metadata and study protocols, describing the solid matrices (agarose, magnetic beads), antibodies/affinity approach (anti-GFP, streptavidin, calmodulin) and epitope tags (TAP, HA, GFP) used (Mellacheruvu *et al.*, 2013). The deposited raw files are then processed based on a uniform pipeline, where spectral count data is parsed, and protein identifications are mapped according to NCBI gene identifications. They are then organized based on controlled vocabularies, generating standardised information for analysis by the user (Mellacheruvu *et al.*, 2013). This allows researchers to query the polypeptides identified during their study to determine true interactors from non-specific interactors. Based on the understanding that negative control samples are, generally, not dependent on the type of bait used (TAP, HA, GFP) and that comparing studies of different sizes using Crapome, can provide insights from a larger sample of negative control data (Mellacheruvu *et al.*, 2013). It then offers three workflows for use, either by running the user's dataset against the database components or generating custom background protein subsets for data comparison. It contains >360 experiments that were performed by 12 laboratories across different countries, majority of which come from experiments performed using human cell lines (Mellacheruvu *et al.*, 2013). It can be searched against MS data generated using *E. coli*, *S. cerevisiae*, and *H. sapiens* samples. We used Crapome workflow 1.1, selected *H. sapiens* and entered our data using Ensembl protein identification names and where identifications showed "no protein information", we used other annotated protein names (where possible) due to the dynamic nature of data repository-based databases (https://www.crapome.org/).

2.5 Results

2.5.1 Overall sample data analysis

The Bruker Amazon ETD Ion Trap was used for LC-MS/MS analysis, database searches were performed using the GPM interface and X Tandem software version Alanine (2017.2.1.4). The data was searched against the human database using Ensembl, trypsin was chosen for enzyme specifications and a maximum of 1 missed cleavage site was allowed for peptide matching. The overall quality of the data was tested by X Tandem using the rho score, which is a parameter derived from a rho diagram plot of the data, the values of which range from 0 - 100 depicting purely random to non-random assignments. Additionally, the FPR for each sample dataset was determined using a decoy database. The FPR is then defined (according to X Tandem) as the sum of the peptide expectation values measured for the peptides corresponding to the proteins displayed on the page or the dataset (See Table 9 below for sample specific details).

Table 9: Showing quality control information as calculated by X tandem

Sample	Shorthand description	Rho Score	FPR
Experiment 1:			
GFP	1G	60	3.43%
Flag-DCUN1D1	1D	72	2.28%
Experiment 2:			
GFP	2G	64	3.04%
DCUN1D1	2D	66	3.02%
Experiment 3:			
GFP DMSO	3G_dmso	71	2.41%
GFP MG132	3G_mg132	71	2.50%
DCUN1D1 DMSO	3D_dmso	65	2.83%
DCUN1D1 MG132	3D_mg132	67	2.88%

We observed that across the dataset, the range of the rho scores was 60 - 72 indicating that the data was more likely from non-random assignments. Additionally, the FPR was determined to be between 2.28 and 3.43%, which is below the 5% threshold widely used in MS studies. Therefore, in our overall dataset, 3 666 167 513 total peptides were used with 30 938 duplicate peptide identifications, 777 248 total proteins were used, with 121 528 duplicates and of these, 92 were estimated false positive identifications. Furthermore, 3365 total spectra were assigned of which 2304 were unique, therefore the percentage unique spectra assigned was 68.47% (Table 10).

Table 10: Showing overall data analytical parameters/results

Analytical Parameters/Results	Total
Total **peptides** used	3666167513
Duplicate peptide ids	30938
Total **proteins** used	777248
Duplicate proteins	121528
Estimated **false positives**	92
Total **spectra** used	27410
Total spectra assigned	3365
Percentage spectra assigned	12.28%
Total **unique** assigned	2304
Percentage unique spectra assigned	68.47%
Input models	1678
Input spectra	73336
Partial cleavage	15617
Unanticipated cleavage	363
Scoring, maximum missed cleavage sites	1
Scoring, minimum ion count	4
Output, maximum valid expectation value	0.1
Output, maximum valid protein expectation value	0.1
Refine, maximum valid expectation value	0.01

Based on the quality controls performed, we proceeded with further overall data analysis to determine the spread of the data including the main contributors to variance and other descriptive statistics. Broadly, the data was initially processed using Excel and analysed using PAST3 software. The current analysis has been performed on the overall dataset, which is the final protein list and related experimental and bioinformatics information, upon completion of the MS runs. We began by performing a PCA of the dataset using PAST3 (Figure 19). Upon completion of the MS analysis and database searching, Excel files from the *"Model"* tab on the homepage were downloaded for each sample output. We note that we have 8 samples in total, with individual X Tandem outputs per sample for data analysis, where each sample had a unique GPM identification number, which corresponded to a unique X Tandem output and related files. The samples were found to have the following number of protein identifications: 1G (143), 1D (240), 2G (195), 2D (201), 3G_dmso (203), 3G_mg132 (197), 3D_dmso (112) and 3D_mg132 (170). In addition, each Excel file contained tables with multiple column titles used during protein identification and of these variables, we tested the first eight. These were namely: rank, log (e) (log of expectation value), log (I) (log of intensity), % measured (the amino acid coverage of the protein in this assignment), % corrected (the coverage corrected for peptide sequences that are unlikely to be observed using normal proteomics methods), unique (the number of unique peptide sequences associated with this protein assignment), total (the total number of tandem mass spectra that can be assigned to this protein) and molecular mass (M_r) based on the current MS run. The other variables provided qualitative/descriptive data that was not deemed relevant or applicable to the current test.

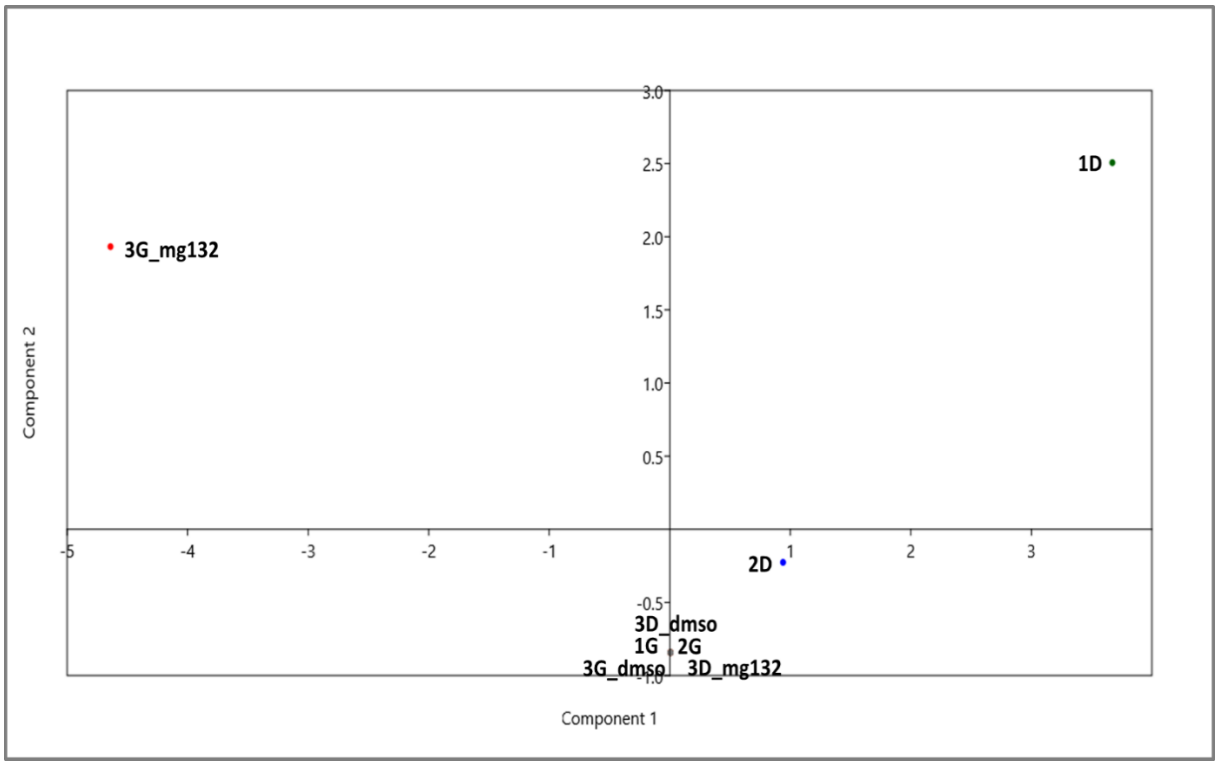


Figure 19. Diagram showing PCA of the unfiltered samples.

From Figure 19 above, we observe distinct clustering of the control samples on the negative Y axis including 1G, 2G and 3G_dmso. Interesting, we observe that samples 3D_dmso and 3D_mg132 also cluster with the control samples while sample 3G_mg132 lies in the top left quadrant and samples 1D and 2D are distinctly variable. We therefore conclude that the experimental samples (1D and 2D) differ from the control samples, meaning, they have distinct protein signatures while samples 3D_dmso and 3D_mg132 have similar protein signatures to the control samples. Additionally, analysis of the protein lists from these samples (3D_dmso and 3D_mg132) found that outside of our protein interest, DCUN1D1, we did not identify any other known substrates of DCUN1D1 or neddylation-related proteins and we therefore did not consider this dataset as suitable for characterising the mechanism of action of DCUN1D1. These samples included the control and DCUN1D1 transfected cells which were treated with DMSO or MG132. MG132 is a proteasome inhibitor and studies have shown that treatment of cells with MG132 results in the accumulation of CRL neddylated proteins (Emanuele *et al.*, 2011). Therefore, we postulated that treatment of our cell lines with MG132 would yield similar results and perhaps improve the identification of DCUN1D1 targets. However, since we observed PCA clusters that were different than would have been predicted, these samples required further optimization such as adjustment of drug concentrations and determinations on whether drug treatments interfered with transfection efficiency. These samples were therefore filtered out of the analysis.

We then performed univariate and multivariate several-sample tests and obtained a normal probability plot with a Shapiro-Wilk test value equals to 0.2668 and a p value of 0, indicating that the data is normally distributed (Figure 20). After performing the Welch F test for unequal variance (p =

4.689E-13) and the Kruskal-Wallis test for equal medians (p = 2.313E-234), we found the samples to be significantly different from each other.

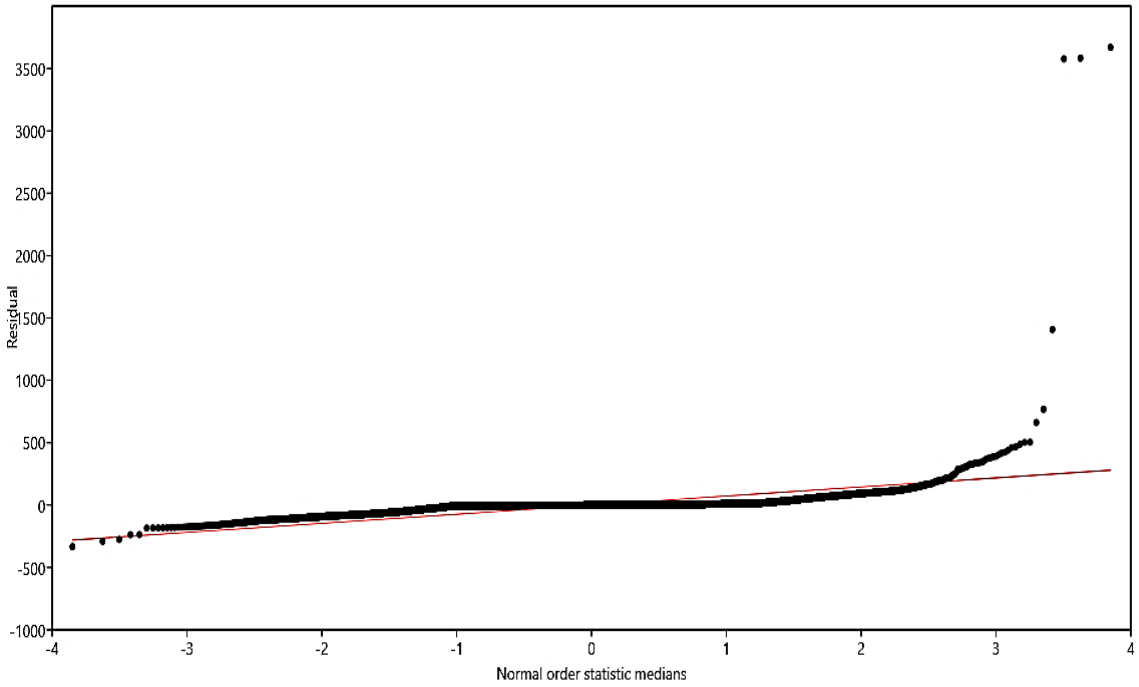


Figure 20. Showing the normal probability plot of the overall dataset.

2.5.1.1 Analysis of enriched molecular biology properties

After having observed the distinct differences between the control (1G, 2G) and experimental samples (1D, 2D), we reported the molecular biology observations made on the experimental samples. We performed the analysis using GO and KEGG outputs, respectively, which were performed within X Tandem based on the protein information generated during MS analysis (Ashburner *et al.*, 2000; Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017; Carbon *et al.*, 2017). From Figure 21 below, we observe the output following GO analysis identifying the biological signatures that were below log (e) -1.2 and report on the top 5 significantly enriched parameters. In terms of sample 1D, the cellular components annotated for the protein list were ubiquitin ligase complexes, the cytoplasm, the nucleus, the membrane and other cellular components. Sample 2D had the following cellular components enriched: the nucleus, ubiquitin ligase complexes, the cytoplasm, the chromosome and other cellular components (Figure 21, bottom). Significantly, we observe that ubiquitin ligase complexes were enriched in both samples, which was consistent with our understanding of the role of DCUN1D1 in ubiquitin cullin RING ligases (Petroski and Deshaies, 2005; Kim *et al.*, 2008; Kurz *et al.*, 2008; G. Huang *et al.*, 2011; Keuss *et al.*, 2016). In addition, we observed cytoplasm and nuclei cellular components to be enriched in both samples. This was consistent with our understanding of the cellular distribution of components of the neddylation and ubiquitination pathways, which are known to shuttle between the cytoplasm and the nucleus during activation, cell stress and under different disease conditions (Kamitani *et al.*, 1997; Boisvert, Hendzel and Bazett-jones, 2000; G. Huang *et al.*,

2011; Chen, Neve and Liu, 2012; Duncan *et al.*, 2012; Leidecker *et al.*, 2012; Kandala, Kim and Su, 2014; Brown and Jackson, 2015; Jayabalan *et al.*, 2016; Wang, Zhu and Xu, 2017).

Additionally, we observed the following biological processes to which our protein signatures contributed in sample 1D: the regulation of transcription, transcription, signal transduction, RNA splicing and other biological processes. Sample 2D on the other hand was enriched in terms of, the regulation of transcription, cell differentiation, signal transduction, multicellular development and other biological processes. Interestingly, the regulation of transcription and signal transduction were found to be enriched in both samples. Therefore, suggesting that although functioning as an E3 ligase for PTM, DCUN1D1 plays a role in the regulation of transcription and signal transduction and maybe contributing to transcription, RNA splicing, cell differentiation and multicellular development. Interestingly, studies performed previously determined that DCUN1D1 plays a role in embryonic and neuronal development-related activities (Sarkaria, Stojadinovic, *et al.*, 2004; Kim *et al.*, 2008; Broderick *et al.*, 2010; Hosp *et al.*, 2014). Lastly, we observed that the molecular functions to which our protein signatures may be contributing were: protein binding, DNA binding, RNA binding, receptor activity and other molecular functions, in sample 1D. On the other hand, sample 2D, was enriched with protein binding, DNA binding, RNA binding, zinc ion binding and other molecular functions. The molecular functions enriched in both samples were protein binding, DNA binding and RNA binding suggesting the biochemical activities implicated in the protein signatures identified in our samples and how they may be fulfilling their biological roles in response to DCUN1D1.

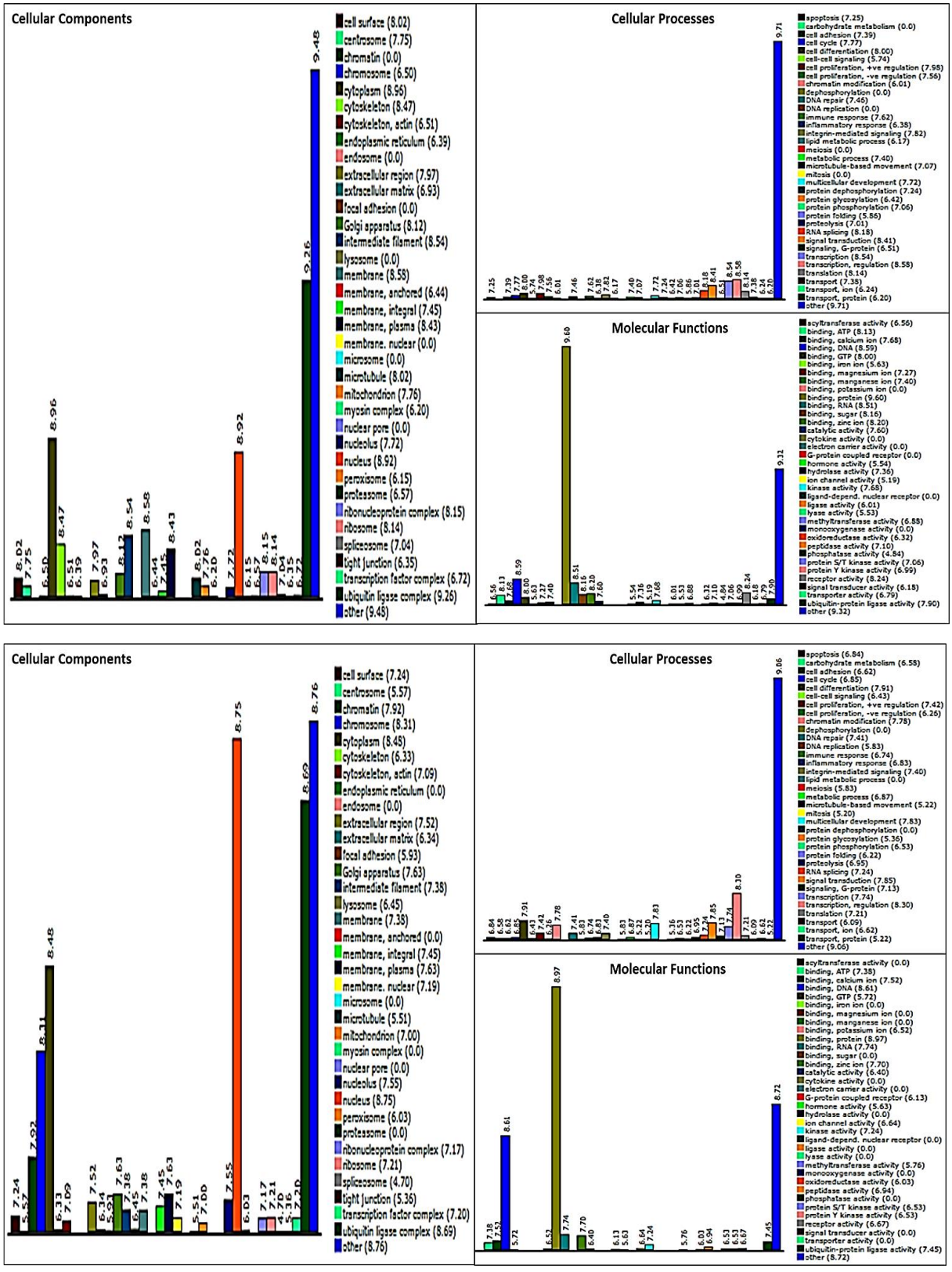


Figure 21. Showing GO outputs for the cellular components, cellular processes and molecular functions enriched in samples 1D (top) and 2D (bottom).

Furthermore, following the analysis of our data using KEGG which was performed within X Tandem based on the protein information generated during MS analysis, we found enrichment in a number of cancer-related pathways across both samples including the WNT/β-Catenin signalling pathway, TGF-β signalling, the cell cycle, apoptosis, VEGF signalling as well as regulators of gene expression such as basal transcription factors, DNA replication and MAPK signalling (Table 11 and 12). Interestingly, we also observed tumour suppressor-related pathways such as the p53 and mTOR signalling pathways and inflammation-related pathways such as B cell receptor, T cell receptor and Toll-like-receptor signalling. Significantly, the top 10 enriched pathways in sample 1D included: ubiquitin-mediated proteolysis, the ribosome, nucleotide excision repair, the gap junction pathway, PPAR signalling, adipocytokine signalling, WNT signalling, regulation of actin cytoskeleton, ErbB signalling and fatty acid metabolism (Table 11). Sample 2D on other hand had ECM-receptor interaction, focal adhesion, the ribosome, nucleotide excision repair, ubiquitin-mediated proteolysis, neurotrophin signalling, Toll-like receptor signalling and leukocyte transendothelial migration enriched (Table 12). Pathways found in common across both samples were: ubiquitin-mediated proteolysis, the ribosome, nucleotide excision repair and PPAR signalling. While pathways such as WNT signalling and the cell cycle were also found in common between the samples, they were enriched at a lower statistical significance.

Based on the evidence-supported annotations provided above, we describe the biological role that DCUN1D1 may be involved in, in terms of its mechanism of action by indicating the cellular components, biological processes, molecular functions and pathways enriched in our samples. This is significant also because they were enriched following an immunoprecipitation-coupled MS experimental approach. Particularly, we observed enrichment in terms of cellular machinery and pathways related to the UPP and the ribosome. Interestingly, Xirodimas et al., 2008 described several ribosomal proteins to be neddylation substrates, however, the neddylation E3 ligase responsible for this function was not identified. We, therefore, begin by performing a more detailed analysis of the protein lists obtained within our dataset to obtain a better understanding.

Table 11. Showing KEGG analysis of pathways significantly enriched in sample 1D

KEGG ID	Pathway	log(I)	log(p)	Protein Description
hsa:04120	Ubiquitin mediated proteolysis	8	-4.1	7/1 proteins of 525
hsa:03010	Ribosome	8	-3.3	6/0 proteins of 261
hsa:03420	Nucleotide excision repair	7.2	-1.2	3/0 proteins of 138
hsa:04540	Gap junction	7.1	-1.2	3/1 proteins of 323
hsa:03320	PPAR signalling pathway	6.4	-1.2	3/0 proteins of 271
hsa:04920	Adipocytokine signalling pathway	6.6	-1.2	3/0 proteins of 275
hsa:04310	Wnt signalling pathway	7	-1	4/2 proteins of 573
hsa:04810	Regulation of actin cytoskeleton	6.2	-0.8	1/3 proteins of 844
hsa:04012	ErbB signalling pathway	6.3	-0.7	2/1 proteins of 373
hsa:00071	Fatty acid metabolism	6.1	-0.7	2/0 proteins of 165
hsa:00512	O-Glycan biosynthesis	6.5	-0.7	2/0 proteins of 106
hsa:04070	Phosphatidylinositol signalling system	6.8	-0.7	2/1 proteins of 357
hsa:03022	Basal transcription factors	8.4	-0.7	2/0 proteins of 124
hsa:04010	MAPK signalling pathway	6.6	-0.7	3/4 proteins of 1129
hsa:04510	Focal adhesion	6.2	-0.6	2/3 proteins of 832
hsa:04144	Endocytosis	7	-0.6	1/2 proteins of 685
hsa:04910	Insulin signalling pathway	7.6	-0.6	2/2 proteins of 553
hsa:04020	Calcium signalling pathway	6.7	-0.6	2/2 proteins of 684
hsa:04062	Chemokine signalling pathway	6.4	-0.6	1/2 proteins of 652
hsa:00120	Primary bile acid biosynthesis	6	-0.4	1/0 proteins of 41
hsa:00380	Tryptophan metabolism	6	-0.4	1/0 proteins of 146
hsa:04650	Natural killer cell mediated cytotoxicity	6.2	-0.4	1/1 proteins of 488
hsa:00600	Sphingolipid metabolism	6.2	-0.4	1/0 proteins of 160
hsa:04730	Long-term depression	6.2	-0.4	1/1 proteins of 299
hsa:00410	Beta-Alanine metabolism	6	-0.4	1/0 proteins of 86
hsa:04115	p53 signalling pathway	5.5	-0.4	1/1 proteins of 309
hsa:04210	Apoptosis	6.4	-0.4	1/1 proteins of 456
hsa:04930	Type II diabetes mellitus	6	-0.4	1/0 proteins of 192
hsa:04370	VEGF signalling pathway	6.2	-0.4	1/1 proteins of 326
hsa:03410	Base excision repair	5.5	-0.4	1/0 proteins of 136
hsa:00640	Propanoate metabolism	6	-0.4	1/0 proteins of 125
hsa:04514	Cell adhesion molecules (CAMs)	5.5	-0.4	1/1 proteins of 505
hsa:03050	Proteasome	6.6	-0.4	1/0 proteins of 132
hsa:00310	Lysine degradation	6	-0.4	1/0 proteins of 208
hsa:04722	Neurotrophin signalling pathway	6	-0.4	1/1 proteins of 519
hsa:04662	B cell receptor signalling pathway	6.4	-0.4	1/1 proteins of 311
hsa:00903	Limonene and pinene degradation	6	-0.4	1/0 proteins of 80
hsa:04270	Vascular smooth muscle contraction	6.2	-0.4	1/1 proteins of 472
hsa:00930	Caprolactam degradation	6	-0.4	1/0 proteins of 14
hsa:00061	Fatty acid biosynthesis	7.2	-0.4	1/0 proteins of 35
hsa:04666	Fc gamma R-mediated phagocytosis	6.2	-0.4	1/1 proteins of 471
hsa:04620	Toll-like receptor signalling pathway	6.4	-0.4	1/1 proteins of 347
hsa:00561	Glycerolipid metabolism	6.8	-0.4	1/0 proteins of 187
hsa:00632	Benzoate degradation via CoA ligation	6	-0.4	1/0 proteins of 64
hsa:00650	Butanoate metabolism	6	-0.4	1/0 proteins of 112
hsa:00280	Valine, leucine and isoleucine degradation	6	-0.4	1/0 proteins of 159
hsa:04720	Long-term potentiation	6.2	-0.4	1/1 proteins of 308
hsa:04110	Cell cycle	6.8	-0.4	1/1 proteins of 390
hsa:04150	mTOR signalling pathway	7.6	-0.4	1/0 proteins of 203
hsa:04664	Fc epsilon RI signalling pathway	6.2	-0.4	1/1 proteins of 318
hsa:00270	Cysteine and methionine metabolism	6.8	-0.4	1/0 proteins of 145
hsa:04916	Melanogenesis	6.2	-0.4	1/1 proteins of 393
hsa:04660	T cell receptor signalling pathway	6.4	-0.4	1/1 proteins of 455
hsa:04350	TGF-beta signalling pathway	6.8	-0.4	1/0 proteins of 237
hsa:04912	GnRH signalling pathway	6.2	-0.4	1/1 proteins of 447

hsa:00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	6.1	-0.4	1/0 proteins of 96
hsa:04670	Leukocyte transendothelial migration	6.2	-0.4	1/1 proteins of 429
hsa:04530	Tight junction	6.2	-0.4	1/1 proteins of 503
hsa:00564	Glycerophospholipid metabolism	6.8	-0.4	1/1 proteins of 338
KEGG:0	other	9.8	-	118 proteins

Table 12. Showing KEGG analysis of pathways significantly enriched in sample 2D

KEGG ID	Pathway	log(I)	log(p)	Protein Description
hsa:04512	ECM-receptor interaction	6.5	-1.8	4/1 proteins of 366
hsa:04510	Focal adhesion	6.6	-1.4	5/2 proteins of 832
hsa:03010	Ribosome	6.6	-1.2	3/0 proteins of 261
hsa:03420	Nucleotide excision repair	7	-1.2	3/0 proteins of 138
hsa:04120	Ubiquitin mediated proteolysis	7.5	-1.2	3/1 proteins of 525
hsa:04722	Neurotrophin signalling pathway	6.9	-0.7	2/1 proteins of 519
hsa:04620	Toll-like receptor signalling pathway	6.9	-0.7	2/1 proteins of 347
hsa:04110	Cell cycle	6.6	-0.7	2/1 proteins of 390
hsa:04660	T cell receptor signalling pathway	6.9	-0.7	2/1 proteins of 455
hsa:04670	Leukocyte transendothelial migration	6.2	-0.7	2/1 proteins of 429
hsa:04062	Chemokine signalling pathway	6.9	-0.7	2/1 proteins of 652
hsa:04530	Tight junction	6.1	-0.7	2/1 proteins of 503
hsa:04010	MAPK signalling pathway	6.9	-0.6	2/3 proteins of 1129
hsa:04144	Endocytosis	6	-0.6	1/2 proteins of 685
hsa:04810	Regulation of actin cytoskeleton	6.1	-0.6	1/2 proteins of 844
hsa:02010	ABC transporters	5.7	-0.4	1/0 proteins of 185
hsa:04740	Olfactory transduction	5.9	-0.4	1/1 proteins of 560
hsa:00830	Retinol metabolism	6	-0.4	1/0 proteins of 305
hsa:00900	Terpenoid backbone biosynthesis	5.2	-0.4	1/0 proteins of 51
hsa:04614	Renin-angiotensin system	6.8	-0.4	1/0 proteins of 39
hsa:04210	Apoptosis	6.8	-0.4	1/1 proteins of 456
hsa:04370	VEGF signalling pathway	5.6	-0.4	1/0 proteins of 326
hsa:03410	Base excision repair	7.2	-0.4	1/0 proteins of 136
hsa:04662	B cell receptor signalling pathway	6.8	-0.4	1/0 proteins of 311
hsa:04070	Phosphatidylinositol signalling system	5.5	-0.4	1/1 proteins of 357
hsa:00061	Fatty acid biosynthesis	5.6	-0.4	1/0 proteins of 35
hsa:00533	Keratan sulfate biosynthesis	5.4	-0.4	1/0 proteins of 58
hsa:04742	Taste transduction	5.7	-0.4	1/0 proteins of 146
hsa:03430	Mismatch repair	5.8	-0.4	1/0 proteins of 116
hsa:00561	Glycerolipid metabolism	5.5	-0.4	1/0 proteins of 187
hsa:04142	Lysosome	6.5	-0.4	1/1 proteins of 402
hsa:00601	Glycosphingolipid biosynthesis - lacto and neolacto series	5.4	-0.4	1/0 proteins of 84
hsa:03440	Homologous recombination	5.8	-0.4	1/0 proteins of 139
hsa:00510	N-Glycan biosynthesis	5.7	-0.4	1/0 proteins of 171
hsa:00650	Butanoate metabolism	5.2	-0.4	1/0 proteins of 112
hsa:00072	Synthesis and degradation of ketone bodies	5.2	-0.4	1/0 proteins of 25
hsa:00280	Valine, leucine and isoleucine degradation	5.2	-0.4	
hsa:04664	Fc epsilon RI signalling pathway	5.6	-0.4	1/0 proteins of 318
hsa:04310	Wnt signalling pathway	6.5	-0.4	1/1 proteins of 573
hsa:04520	Adherens junction	6.1	-0.4	1/1 proteins of 377
hsa:04350	TGF-beta signalling pathway	6.5	-0.4	1/0 proteins of 237
hsa:03320	PPAR signalling pathway	5.2	-0.4	1/0 proteins of 271
hsa:04912	GnRH signalling pathway	5.6	-0.4	1/1 proteins of 447
hsa:03022	Basal transcription factors	7.7	-0.4	1/0 proteins of 124
hsa:04920	Adipocytokine signalling pathway	6.8	-0.4	1/0 proteins of 275
hsa:04612	Antigen processing and presentation	6.5	-0.4	1/0 proteins of 293
hsa:03030	DNA replication	5.8	-0.4	1/0 proteins of 110
hsa:00564	Glycerophospholipid metabolism	5.5	-0.4	1/0 proteins of 338
KEGG:0	other	9.2	-	92 proteins

2.5.1.2 Venn diagram analysis to interrogate the relationships between the protein signatures

We proceeded with the analysis of the protein lists generated for each sample to determine the relationships between and those shared across the samples using Venn diagrams generated using Venny 2.1.0 software. We observed that of the proteins identified, the following percentage were unique to each sample namely, 1G (15.2%), 1D (28.7%), 2G (21.3%) and 2D (21.6%) (Figure 22). The remainder were observed to overlap with one or more of the other samples in the dataset. Interestingly, we identified 18 proteins that overlapped between sample 1D and 2D including DCUN1D1, some known targets of DCUN1D1 such as cullin 3 (CUL3) and cullin 4B (CUL4B) and other proteins such as RBX1 and RPS19 (Table 13).

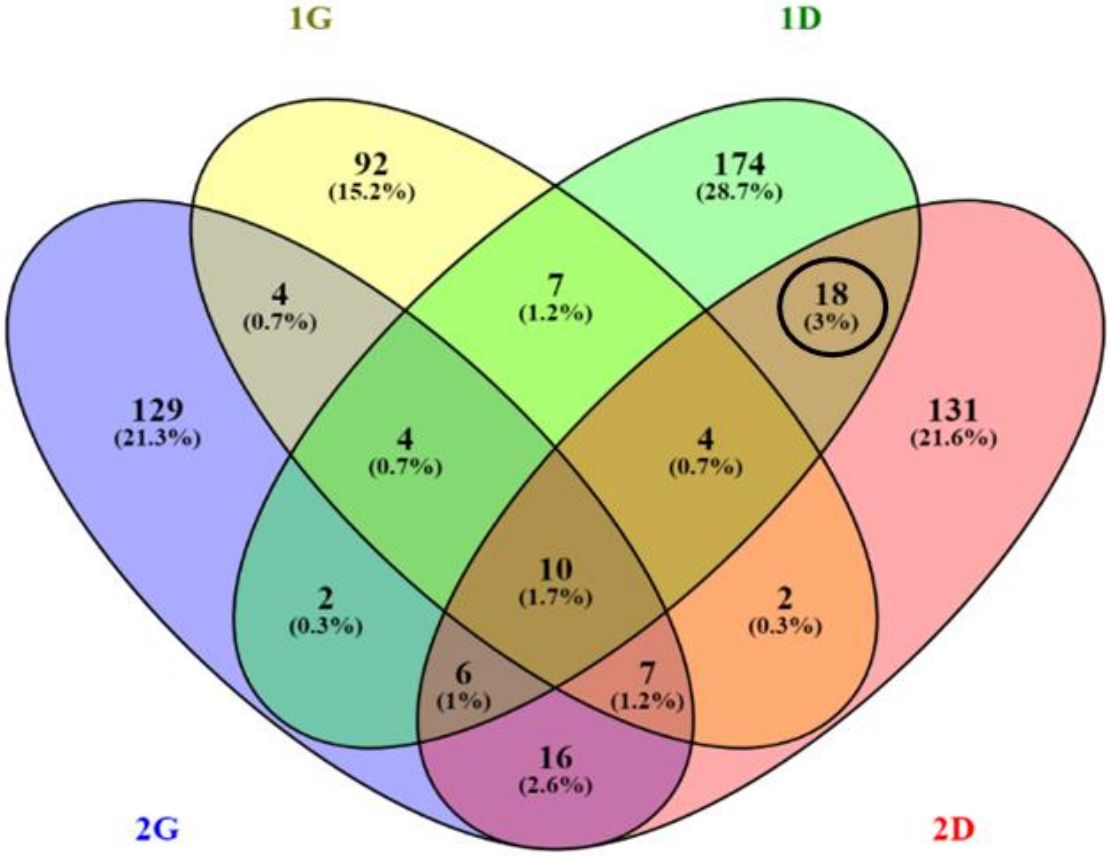


Figure 22. Venn diagram distinguishing between proteins within a sample and those shared between a subset of the samples.

Table 13: Showing details on the 18 proteins found to overlap between samples 1D and 2D

Protein Name	1D Accession number	1D Unique Peptides	2D Accession number	2D Unique Peptides
DCUN1D1	ENSP00000292782	14	ENSP00000292782	11
CAND1	ENSP00000442318	13	ENSP00000442318	25
CUL3	ENSP00000264414	10	ENSP00000264414	9
RPS19	ENSP00000221975	5	ENSP00000221975	2
CUL4B	ENSP00000360373	4	ENSP00000360373	3
RBX1	ENSP00000216225	2	ENSP00000216225	2
BAT2L1	ENSP00000384606	2	ENSP00000384606	1
C1orf77	ENSP00000357683	1	ENSP00000357683	2
MCAT	ENSP00000290429	1	ENSP00000290429	1
C10orf103	ENSP00000394678	1	ENSP00000394678	1
COL19A1	ENSP00000316030	1	ENSP00000316030	1
TMEM14A	ENSP00000211314	1	ENSP00000211314	1
COL1A2	ENSP00000297268	1	ENSP00000297268	2
COL15A1	ENSP00000364140	1	ENSP00000364140	1
G6PD	ENSP00000377192	1	ENSP00000377192	1
ACE2	ENSP00000252519	1	ENSP00000252519	1
COL13A1	ENSP00000381949	1	ENSP00000381949	1
PITRM1	ENSP00000370367	1	ENSP00000224949	1

2.5.2 Filtered sample data findings to identify true interactors of DCUN1D1

After having determined the main trends within the dataset we then performed further analysis applying standards of MS, data repository databases and software to identify those proteins most likely identified as background. We therefore proceeded with the data analysis as outlined in Figure 23 below.

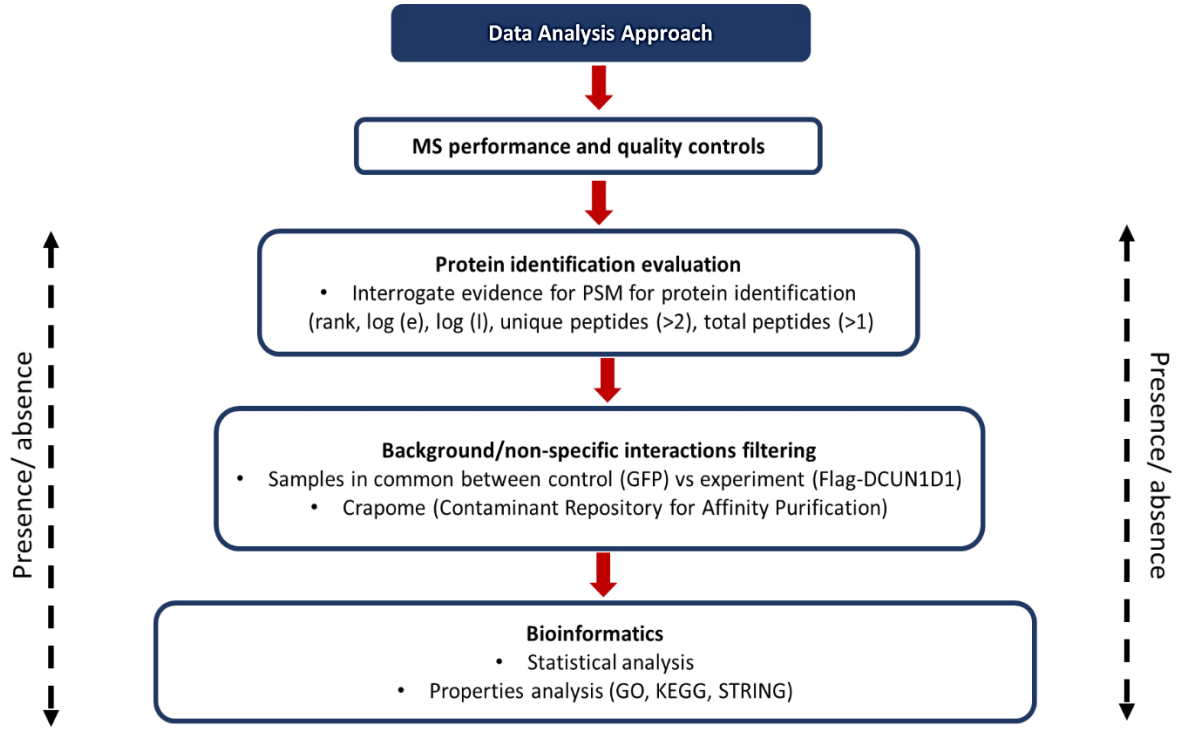


Figure 23. Schematic diagram showing data analysis approach.

2.5.2.1 MS-based and statistical analysis of the filtered dataset

We applied strict bioinformatics criteria where the output for the maximum valid E-value for protein identification was set at 0.1 and further refined to search for and report on protein identifications made from a maximum valid E-value of 0.01. We then selected proteins that were identified from more than 1 total peptide and 2 or more unique peptides and found the following number of proteins in each sample: 1G (17), 1D, (45), 2G (31) and 2D (32). Although we see proteins that are unique to each sample, it is important to determine which proteins are true interactors. As mentioned previously, components of the IPMS workflow such as the solid matrices, antibodies and epitope tags can bind proteins through nonspecific interactions. We used manual filtration by including negative control samples in our experimental approach as well as database repositories to filter out background proteins. In addition, within the GPM interface, there is a list of proteins called the common Repository of Adventitious Proteins (cRAP), which contains the protein sequences for proteins that are commonly found in proteomics experiments. This list includes proteins such as trypsin, FBS etc. as well as other high abundance proteins that can potentially lead to contamination during MS sample preparation. We therefore filtered these proteins out of the list and performed PCA and univariate/multivariate analysis to determine the patterns within the filtered dataset. We provide a scatter plot representing

the individual data points and found that points from across the samples clustered mainly around the origin between -1.5 and 1.5 of the X axes, and thereafter we found data points mainly from sample 1D (dark blue) and 2D (dark purple) (Figure 24). We then determined, using the Shapiro-Wilk test, that the data was not normally distributed with Shapiro-Wilk W value equals to 0.1221 and a p value of 1.955E-58 (Figure 25). However, after performing the Welch F test for unequal variance (p = 4.707E-57) and the Kruskal-Wallis test for equal medians (p = 6.122E-130), we found the filtered samples to be significantly different from each other.

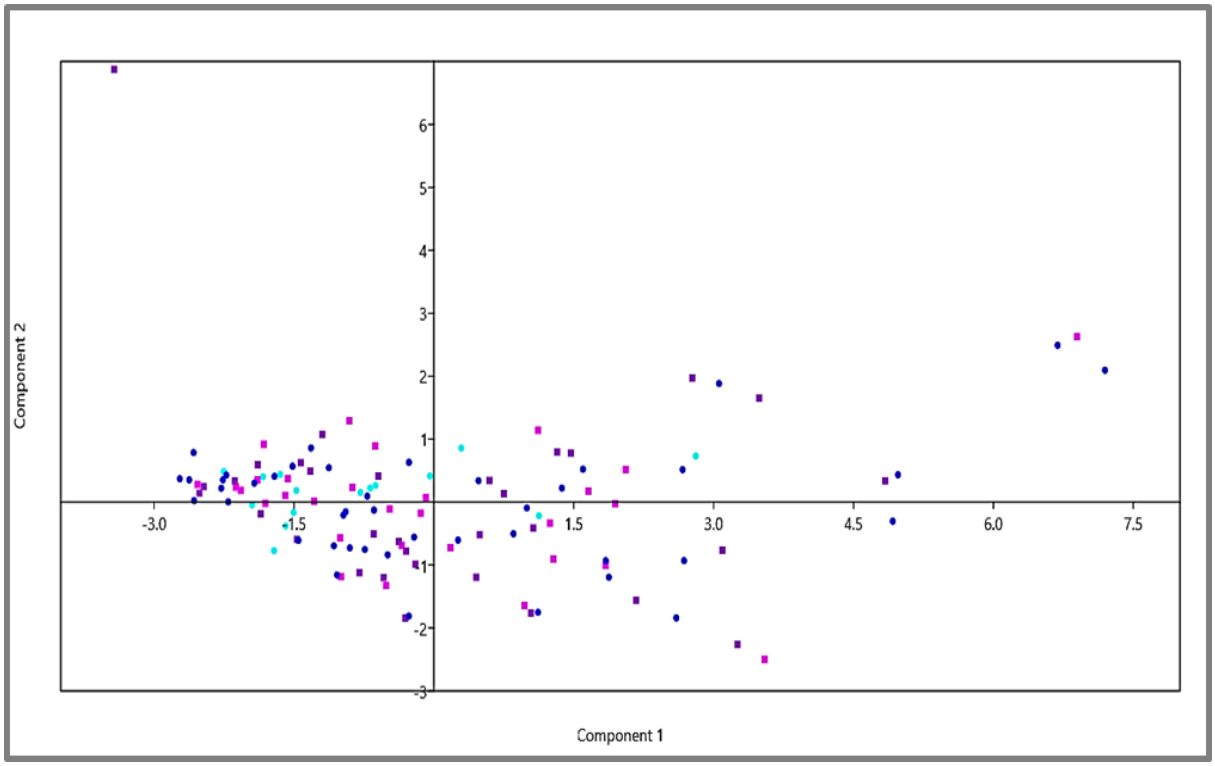


Figure 24. Diagram showing PCA of the filtered samples.

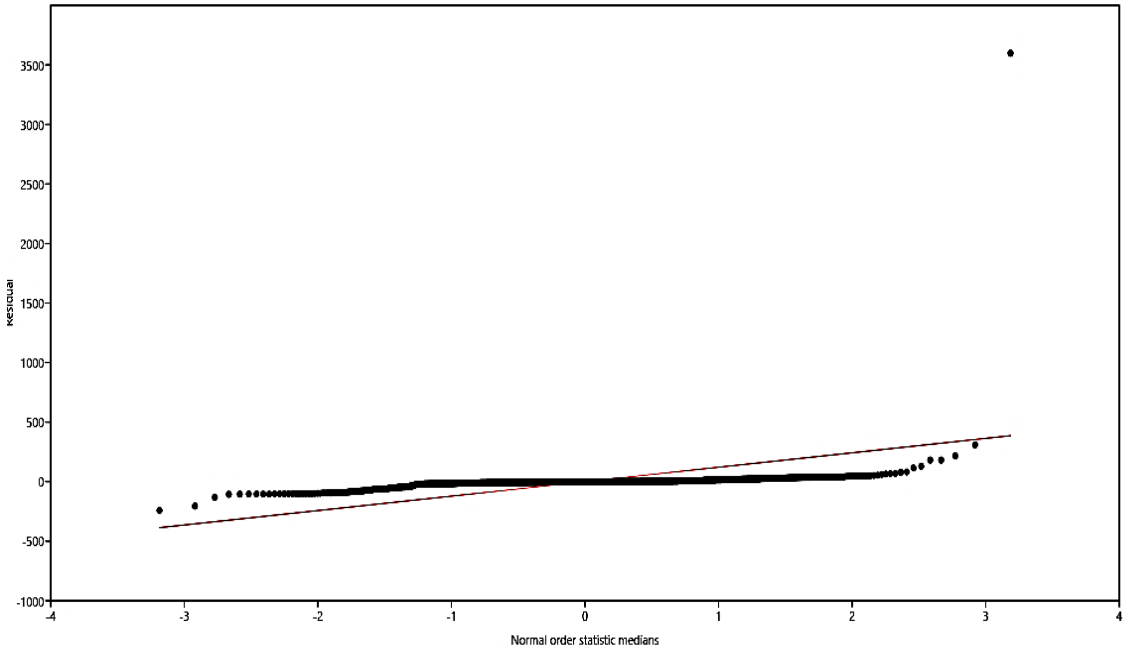


Figure 25. Showing the normal probability plot of the filtered dataset.

2.5.2.2 Determining the relationships between the filtered dataset and the detailed protein list description

We used the Crapome database to test our dataset against a wider collection of background proteins from the aggregated negative control samples. We used Crapome version 1.1, workflow 1, then queried the data from each sample, 1D and 2D, and searched against the *H. sapiens* database. We then filtered the data, based on the proteins that appeared both in our negative controls (1G or 2G), and the Crapome outputs for sample 1D or 2D. Thereafter, we performed Venn diagram analysis of the current list of proteins and found 6 proteins in common between samples 1D and 2D (Figure 26). We provide in Table 14 below a detailed outline of the proteins identified, including the variables that were associated with each protein identification.

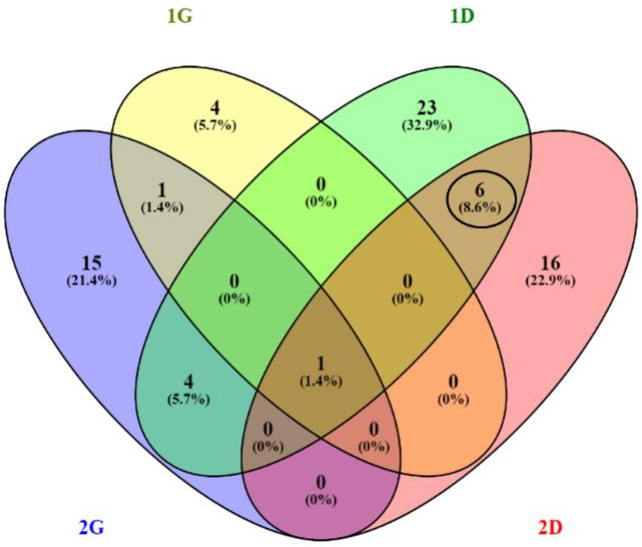


Figure 26. Venn diagram showing relationship between the samples 1G, 1D, 2G and 2D post-filtering.

Table 14: Showing detailed lists of the proteins identified in the filtered samples 1D and 2D

1D_DCUN1D1								2D_DCUN1D1						
Description	Accession	Rank	Log(e)	Log(I)	Unique	Mr		Description	Accession	Rank	Log(e)	Log(I)	Unique	Mr
DCUN1D1	ENSP00000292782	4	-148.4	9.23	14	30.1		CAND1	ENSP00000442318	1	-145.5	7.91	11	136.3
CAND1	ENSP00000442318	5	-144.7	7.99	13	136.3		DCUN1D1	ENSP00000292782	4	-114.9	8.61	11	30.1
CUL3	ENSP00000264414	7	-117.9	7.82	10	88.9		HIST1H1E	ENSP00000307705	5	-104.3	7.41	3	21.9
FUS	ENSP00000254108	8	-85.1	7.57	8	53.4		HIST1H1D	ENSP00000244534	6	-101.3	7.31	4	22.3
HNRNPU	ENSP00000283179	9	-83.2	7.67	7	90.5		CUL3	ENSP00000264414	7	-93.2	7.4	9	88.9
LSM14A	ENSP00000446271	12	-59.9	7.57	7	50.5		YBX1	ENSP00000361621	9	-76.4	7.59	6	34
RPS19	ENSP00000221975	17	-40.9	6.74	5	16.1		HIST1H2BL	ENSP00000366618	13	-34.9	6.64	4	13.9
NME4	ENSP00000395605	18	-36.2	7.23	4	25.4		HIST3H2BB	ENSP00000375736	14	-33.6	6.72	2	13.9
CUL4B	ENSP00000360373	21	-27.4	6.79	4	102.2		H3F3C	ENSP00000339835	18	-27.7	6.76	3	15.2
TRIM21	ENSP00000254436	22	-26.1	6.87	4	54.1		BOD1L	ENSP00000040738	19	-23.9	6.96	4	330.3
BAT2	ENSP00000387477	23	-25.4	6.68	4	228.7		COL4A2	ENSP00000353654	20	-23.5	6.23	4	167.4
BCL11B	ENSP00000349723	25	-24.1	6.23	4	95.5		CUL4B	ENSP00000360373	21	-23.1	6.76	3	102.2
SCRIB	ENSP00000349486	26	-23.7	6.7	3	177.6		HIST2H2AA3	ENSP00000358158	24	-20.6	7.65	3	14.1
RPS4X	ENSP00000362744	28	-21.4	6.96	3	29.6		RPS19	ENSP00000221975	25	-18.7	6.7	2	16.1
THOC4	ENSP00000331817	29	-20.8	7.03	2	26.9		RBX1	ENSP00000216225	28	-15.2	6.52	2	12.3
RPS5	ENSP00000196551	31	-19.6	7.42	2	22.9		ACTB	ENSP00000349960	29	-11.5	6.06	2	41.7
RBX1	ENSP00000216225	32	-18.8	6.76	2	12.3		C1orf77	ENSP00000357683	30	-10.9	6.3	2	26.4
DDB1	ENSP00000301764	34	-17.5	6.52	2	126.9		COL14A1	ENSP00000311809	33	-8	6.32	2	191.8
LRPPRC	ENSP00000260665	35	-17.2	6.76	3	157.8		BIVM	ENSP00000404887	35	-8	7.23	2	181.1
RPS6	ENSP00000369757	36	-17.1	7.1	2	28.7		COL1A2	ENSP00000297268	36	-7.8	5.83	2	129.2
GIGYF2	ENSP00000362664	39	-15	6.52	2	150		COL18A1	ENSP00000383201	37	-7.6	5.63	2	135.7
CIRBP	ENSP00000322887	40	-14.6	6.69	2	18.6		TTN	ENSP00000343764	38	-7.6	6.48	2	3711.3
RPL11	ENSP00000363676	42	-12.9	6.81	2	20.2								
BAT2L1	ENSP00000384606	43	-11.6	5.9	2	165.4								
CUL5	ENSP00000376808	44	-10.8	6.73	2	90.9								
QPCTL	ENSP00000012049	45	-10.8	6.56	2	42.9								
PML	ENSP00000378567	53	-8.6	7.17	2	70								

We also highlight the proteins found in common between the two samples which were DCUN1D1, CAND1, CUL3, RPS19, CUL4B and RBX1. DCUN1D1, our protein of interest, is an E3 ligase of the neddylation pathway, while, CUL3, CUL4B and CUL5 are part of the cullin family of proteins and are known targets of neddylation by DCUN1D1 (Petroski and Deshaies, 2005; Sarkaria *et al.*, 2006; Kim *et al.*, 2008; Kurz *et al.*, 2008; G. Huang *et al.*, 2011; Keuss *et al.*, 2016). We did not identify the other members of the family: cullin 1 (CUL1), cullin 2 (CUL2) and cullin 4A (CUL4A), which could be attributed to the limitation of MS-based proteomics analysis in terms of run-to-run deviations in peptide fragmentation, detection and identification patterns (Tabb *et al.*, 2010). This could also account for the limited overlap between samples 1D and 2D as observed with the lack of identification of histone proteins for example in sample 1D. However, we also identified RBX1, which is a core component of the cullin RING ubiquitin E3 ligases, and is normally bound to some cullin proteins, including those identified in this study (Kamura *et al.*, 1999; Ohta *et al.*, 1999; N. Zheng *et al.*, 2002; Goldenberg *et al.*, 2004; Figueroa *et al.*, 2005; Petroski and Deshaies, 2005; Kim *et al.*, 2008). In addition, we identified CAND1 i.e. cullin associated and neddylation dissociated 1, which is a regulator of the neddylation of cullin/RBX complexes and a regulator of the assembly of the components of the CRLs (Liu *et al.*, 2002; N. Zheng *et al.*, 2002; Min *et al.*, 2003; Goldenberg *et al.*, 2004; Petroski and Deshaies, 2005; Lo and Hannink, 2006; Gray *et al.*, 2008; Schmidt *et al.*, 2009; Wu *et al.*, 2016). It binds un-neddylated cullin/RBX complexes inhibiting neddylation but dissociates upon neddylation. It also functions as an exchange factor in terms of the F box binding proteins recruited for substrate recognition within the relevant CRL complex (Schmidt *et al.*, 2009). Therefore, following IPMS, we have identified 3 members of the ubiquitin CRLs: BCR E3 ligases (BTB-CUL3-RBX), DCX E3 ligases (DDB1-CUL4-XBOX) and the ECS E3 ligases (ELONGIN BC-CUL2/5-SOCS box) (Petroski and Deshaies, 2005). We discuss these in detail below. Interestingly, we also identified RPS19 i.e. ribosomal protein S19 in both samples, and although several ribosomal proteins have been identified as substrates of neddylation, RPS19 was not reported among the ribosomal proteins identified by Xirodimas et al., 2008 as neddylation substrates.

In addition, in respect of sample 1D, we identified other ribosomal proteins namely, RPS4X, RPS5, RPS6 and RPL11. RPS4X, RPS5 and RPS6 are structural components of the small 40S ribosomal subunit, while, RPL11 is a component of the large 60S ribosomal subunit (Thiede, Wittmann-liebold and Otto, 1996; Voronina *et al.*, 2003; Wilson and Cate, 2012). The ribosome is the site of translation within the cell, playing a key role in the regulation of products of transcription as well as protein synthesis. Its main function is the synthesis of proteins from mRNA, however, there are other RNA species within the ribosome that regulate its activity, including 4 ribosomal RNA species (mRNA, tRNA, rRNA and snRNA) and 79 ribosomal proteins (Veldman *et al.*, 1981; Monk, Meyuhas and Robert, 1981; Clark *et al.*, 1984; Tollervey, 1987; Edwards *et al.*, 1989; Moazed and Noller, 1989; Tycowski, Shu and Steitz, 1993; Gutell, Gray and Schnare, 1993; Beven *et al.*, 1996; Naora and Naora, 1999; Frank and Agrawal, 2000; Ben-Shem *et al.*, 2011; Dunkle *et al.*, 2011; Wilson and Cate, 2012). Ribosomal proteins are therefore well established as critical proteins of the cell due to their ribosome-dependent functions, however, there is increasing evidence of ribosome-independent functions (Naora and Naora, 1999; Xiang Zhou *et al.*, 2015).

For example, RPS6 is one of the ribosomal proteins that undergoes PTM through C terminus phosphorylation at 5 serine sites, by various kinases including S6 Kinase 1, S6 Kinase 2, Ribosomal S6 protein Kinase, Protein Kinase A and Casein Kinase 1 (Kabat, 1970; Krieg, Hofsteenge and Thomas, 1988; Wettenhall, Eriksonq and Mallerqll, 1992; Bandi *et al.*, 1993; Pende *et al.*, 2004; Roux *et al.*, 2007; Moore *et al.*, 2009; Hutchinson *et al.*, 2011). It has been shown to regulate cell growth and proliferation in that, knockout of RPS6 in mice resulted in the blockage of proliferation of liver cells (Pende *et al.*, 2004). In addition, RPS6 along with RPS19 have been implicated in p53-mediated cell pigment development and RPS6 has also been implicated in lipogenesis through the

AKT/mTORC1/RPS6 signalling pathway in hepatocellular carcinoma (McGowan *et al.*, 2008; Calvisi *et al.*, 2011). Furthermore, RPL11 has been shown to activate p53, TAp73 and to inactivate c-Myc and PICT1 during tumourigenesis (Dai, Sears and Lu, 2007; Dai and Lu, 2008; Sasaki *et al.*, 2011; Mahata, Sundqvist and Xirodimas, 2012; X Zhou *et al.*, 2015). Significantly, neddylation of RPL11 results in the recruitment of RPL11 to the p53 promoter site, where it binds MDM2 and recruits transcriptional coactivators, activating p53 transcription under conditions of nucleolar stress (Mahata, Sundqvist and Xirodimas, 2012). Moreover, MAPK and the p53 signalling pathways were among the most significantly enriched pathways in sample 1D and 2D (Table 11 and 12 above). MAPK signalling was enriched at a log (p) value of -0.7 and -0.6 in samples 1D and 2D, respectively. P53 signalling on the other hand was enriched at a log (p) value of -0.4 in sample 1D but not significantly enriched in sample 2D. Therefore, following IPMS analysis, DCUN1D1 appears to regulate some ribosomal proteins possibly through the MAPK signalling pathways. However, the activation status of specific MAPK signalling components and subsequent impact on specific ribosomal proteins relative to DCUN1D1 expression/activity would need to be tested.

Furthermore, in respect to sample 1D, we identified TRIM21 and DDB1 which are RING ubiquitin E3 ligases (Shiyanov, Nag and Raychaudhuri, 1999; X. Chen *et al.*, 2001; He *et al.*, 2006; Sabile *et al.*, 2006; Wada and Kamitani, 2006). TRIM21 has been characterised as a member of the SKP-CUL-FBOX (SCF) E3 ligases and has been demonstrated to ubiquitinate SQSTM1/p62 through the KEAP1/NRF2 pathway, in the regulation of cell redox homeostasis (Sabile *et al.*, 2006; Yang *et al.*, 2009; Pan *et al.*, 2016; Lee *et al.*, 2017). KEAP1 is also a scaffolding molecule in the CUL3-RBX1-NRF2-KEAP1 ubiquitin E3 ligase complex and TRIM21 was found to negatively regulate the sequestration of KEAP1 to the complex (Kobayashi *et al.*, 2004; Pan *et al.*, 2016; Lee *et al.*, 2017). Therefore, following IPMS analysis, we describe another link between TRIM21 and the cullin 3-dependent, BCR3 E3 ligases possibly through DCUN1D1-mediated neddylation of cullin 3. This is highly likely because among the cullin family of proteins, cullin 3 has been extensively linked to viral/host interactions and the regulation of the endosomal pathway, while TRIM21 has been characterised as an Fc cytosolic receptor during viral entry and an E3 ligase regulating innate and adaptive immunity (Yang *et al.*, 2009; Huotari *et al.*, 2012; Yoshimi, Ishigatsubo and Ozato, 2012; Versteeg *et al.*, 2013; Mahon *et al.*, 2014; Gschweitl *et al.*, 2016). On the other hand, DDB1 i.e. DNA damage-binding protein 1, is a regulator of the UV-DNA damage response that is a catalytical core component of the DCX E3 ligases (DDB1-CUL4-XBOX) (Feldberg and Grossman, 1976; Dualan *et al.*, 1995; Shiyanov, Nag and Raychaudhuri, 1999; X. Chen *et al.*, 2001). It plays a key role in the regulation of the DNA damage response, through its ubiquitination substrates and through the recruitment of the nucleotide excision repair (NER) pathway components (Feldberg and Grossman, 1976; Shiyanov, Nag and Raychaudhuri, 1999; X. Chen *et al.*, 2001; He *et al.*, 2006; Li *et al.*, 2006; Fei *et al.*, 2011; Li, Bhat and Xiao, 2011).

We also identified multiple RNA binding proteins, regulators of mRNA transcription and processing, translation and nuclear export. These include FUS, HNRNPU, LSM14A, BAT2/PRRC2A, BCL11B (transcription factor), THOC4/ALYREF, GIGYF2, CIRBP, BAT2L1/PRRC2B, YBX1, BOD1L and C1ORF77/CHTOP (Sakura *et al.*, 1988; Didier *et al.*, 1988; Banerji *et al.*, 1990; Kiledjian and Dreyfuss, 1992; Crozat *et al.*, 1993; Nishiyama *et al.*, 1997; Wichmann *et al.*, 1999; Satterwhite *et al.*, 2001; Luo *et al.*, 2001; Giovannone *et al.*, 2003; Albrecht and Lengauer, 2004; Ota *et al.*, 2004; Tanaka *et al.*, 2006; Zullo *et al.*, 2009; Lambert *et al.*, 2012; Morita *et al.*, 2012; Higgs *et al.*, 2015). In addition, within sample 2D, we identified several histone molecules namely, HIST1H1E, HIST1H1D, HIST1H2BL, HIST3H2BB, H3F3C, and HIST2H2AA3 (Albig *et al.*, 1991; Mannironi *et al.*, 1994; Albig and Doenecke, 1997; Marzluff *et al.*, 2002; Hake *et al.*, 2005; Schenk *et al.*, 2011). Histone molecules are part of the nucleosome, consisting of 2 molecules each of H2A, H2B, H3, H4 as well as the linker histone H1 (Kornberg, 1974; Kornberg and Thomas, 1974; Thomas and Kornberg, 1975; Stein, Bina-Stein and

Simpson, 1977; Thoma, Koller and Klug, 1979; Dasso, Dimitrov and Wolffe, 1994; Luger *et al.*, 1997; Lorch, Zhang and Kornberg, 1999). They make up the chromatin within the nucleus and play a key role in a variety of processes including transcription, DNA replication, DNA repair and recombination, chromosome stability, germline DNA packaging, PTM through ADP-ribosylation by PARP1 and functions related to the acrosome during spermiogenesis (Mariño-Ramírez *et al.*, 2005; Ooi and Henikoff, 2007; Messner and Hottiger, 2011; De Vries *et al.*, 2012; Venkatesh and Workman, 2015). Although well known for PTM through acetylation and deacetylation, histones can also undergo PTM by phosphorylation, methylation, ubiquitination, formylation, succinylation and citrullination (Cheung, Allis and Sassone-Corsi, 2000; Nakashima, Hagiwara and Yamada, 2002; Cuthbert *et al.*, 2004; Jiang *et al.*, 2007; Shahbazian and Grunstein, 2007; Wiśniewski, Zougman and Mann, 2008; Wang *et al.*, 2009; Zhang *et al.*, 2011; Cao and Yan, 2012; Xie *et al.*, 2012; Greer and Shi, 2012; Rossetto, Avvakumov and Côté, 2012; Yokoyama, Katsura and Sugawara, 2017). This can disrupt the interactions between the histone proteins and DNA as well as between the core histone components, affecting histone-histone interactions.

Significantly, neddylation has been described to play a role in the regulation of histone molecules, particularly during the DNA damage response (Brown and Jackson, 2015). H2A was found to be neddylated by RNF168 during DNA damage repair and H4 was shown to undergo polyneddylation by RNF111, which antagonized ubiquitination during DNA damage repair (Ma *et al.*, 2013; Li *et al.*, 2014). Neddylation has also been shown to recruit histone deacetylases to repress NFκB-mediated gene expression (Gao *et al.*, 2006). Significantly, cullin 4A has been extensively linked with the regulation of the DNA damage response, however, few studies have explicitly described the role of cullin 4B in this activity (Shiyanov, Nag and Raychaudhuri, 1999; X. Chen *et al.*, 2001; Guerrero-Santoro *et al.*, 2008; Jackson and Xiong, 2009; Brown and Jackson, 2015; Brown *et al.*, 2015; Hannah and Zhou, 2015; Yi *et al.*, 2015). Therefore, we describe the potential role of cullin 4B in the DNA damage response mediated by DCUN1D1. It would be necessary to test this hypothesis by determining the level of expression and functionality of cullin 4B and DCUN1D1 under DNA damage response conditions.

We also identified several components of the extracellular matrix such as COL4A2, COL14A1, COL1A2, COL18A1, ACTB and nuclear matrix components (Ponte *et al.*, 1984; De Wet *et al.*, 1987; Hostikka and Tryggvason, 1988; Just *et al.*, 1991; Bauer *et al.*, 1997; Saarela *et al.*, 1998; Gelse, Pöschl and Aigner, 2003). We identified TTN which plays a role in chromosome condensation and segregation in non-muscle cells and through its ability to connect microfilaments, it is postulated that it may connect the nuclear lamina to chromatin or nuclear actin (Labeit *et al.*, 1992; Labeit and Kolmerer, 1995; Machado, Sunkel and Andrew, 1998; Zastrow *et al.*, 2006). Furthermore, QPCTL, was also identified which mediates the biosynthesis of pyroglutamyl peptides of substrates such as amyloid-β, while SCRIB (sample 1D) is an established regulator of cell polarity, particularly in epithelial cells (Bilder, Li and Perrimon, 2000; Dow *et al.*, 2003; Bilder, 2004; Petit *et al.*, 2005; Cynis *et al.*, 2008; K. Huang *et al.*, 2011). Interestingly, we also identified LSM14A and PML which are key components of RNA-related cellular structures such as the cytoplasmic P-bodies and the PML nuclear bodies, respectively (Ascoli and Maul, 1991; Stuurman *et al.*, 1992; Boisvert, Hendzel and Bazett-jones, 2000; Tanaka *et al.*, 2006; Yang *et al.*, 2006; Brandmann *et al.*, 2018). Both structures play a critical role during the cellular stress response, either through stress granules such as LSM14A and the PML nuclear bodies which assemble and disassemble depending on nuclear stress conditions (Boisvert, Hendzel and Bazett-jones, 2000; Lallemand-Breitenbach and de Thé, 2009; Decker and Parker, 2012; Sahin, De Thé and Lallemand-Breitenbach, 2014; Luo, Na and Slavoff, 2018).

Therefore, DCUN1D1 appears to regulate key proteins of the UPP, the ribosome, transcription and translation machinery as well as regulators of transcription and translation. Including proteins that

form components of the cytoplasmic P-bodies and the PML nuclear body. Significantly, PML undergoes sumoylation during maturation and it would be interesting to explore the role of neddylation in PML nuclear body activities. In addition, a number of inflammation-related proteins were identified in this study suggesting a role for DCUN1D1, likely through DCUN1D1-mediated cullin neddylation. Furthermore, components of the cytoskeleton such as ACTB and cell polarity regulators such as human SCRIB were also identified.

The results described above were obtained following immunoprecipitation-coupled MS and our protein lists are a product of DCUN1D1 protein binding partners following a pulldown assay and MS-based identification. Therefore, we decided to perform STRING analysis to identify the known associations between our final protein lists.

2.5.2.3 Determining the associations between DCUN1D1 and its true interactors using STRING

We combined the lists of proteins generated above and using their Ensembl description/name, we used STRING version 10.5 to determine the associations observed between the final protein lists. Due to the intrinsic properties of MS analysis which mean that not all proteins are detected and/or identified with subsequent MS runs. We postulated that combining the dataset would provide a clearer view of the DCUN1D1 associations and provide a clearer picture of the links between the interactors of DCUN1D1 identified in our IPMS study. We provide STRING output data as a function of the default settings as well as a representation of the data based on "confidence", with the default setting of minimum required interaction score set to 0.400 i.e. medium confidence (Figure 27).

We found 4 main hubs, two of which appear to be better associated with DCUN1D1. Specifically, we observed that DCUN1D1 has direct associations with CUL3, CUL4B, CUL5, RBX1 and CAND1, with edges emanating from the DCUN1D1 node only associated with these nodes (Figure 27). In addition, we observed strong associations between CUL3 and CUL5 with TRIM 21 and subsequent association between TRIM21 and PML. PML is also known as TRIM19 and is among the ~100 TRIM family of proteins that have been described (Reymond *et al.*, 2001; Versteeg *et al.*, 2013). Importantly, STRING outputs are based on associations characterised based on physical binding and indirect interactions as components of a pathway or a mechanism of action. As observed previously, TRIM21 has been linked to CUL3 due to its inhibitory role on the sequestration of the KEAP1 scaffolding molecule targeting SQSTM1/p62 for ubiquitination (Pan *et al.*, 2016; Lee *et al.*, 2017). Therefore, following IPMS analysis and analysis using STRING functional network associations, we hypothesis that TRIM21 may be a component of CUL3 and CUL5 RING E3 ligases. Furthermore, we also observed associations between CUL4B, RBX1 and DDB1 which are 3 components of the RING DCX E3 ligases (DDB1-CUL4-XBOX) (He *et al.*, 2006; Lee and Zhou, 2007). We also found high confidence associations between the aforementioned complex and ACTB suggesting that it could be a substrate of the E3 ligase complex, meditated by DCUN1D1 neddylation.

Furthermore, ACTB appears to be a strong linker node between the "DCUN1D1-hub" and the hub composed of the histone molecules. As described previously, histone molecules are components of the nucleosome complex consisting of 2 molecules each of H2A, H2B, H3 and H4 (Luger *et al.*, 1997; Mariño-Ramírez *et al.*, 2005). ACTB i.e. beta-actin, is found within the cytoplasm, but it plays a critical role in the nucleus as a component of the chromatin-remodelling complex (Olave, Reck-Peterson and Crabtree, 2002; Kapoor and Shen, 2014). It binds all eukaryotic RNA polymerases, DNA Helicase II/RNA Helicase A and regulates gene expression (Zhang *et al.*, 2002; Stüven, Hartmann and Görlich, 2003; Fomproix and Percipalle, 2004; Hofmann *et al.*, 2004; Hu, Wu and Hernandez, 2004). Interestingly, we observe a hub demonstrating associations between C1ORF77/CHTOP, THOC4/ALYREF, FUS, HNRNPU,

LSM14A, and CIRBP. C1ORF77/CHTOP and THOC4/ALYREF are loading components that are normally bound to the TREX (Transcription Export) complex which plays a key role in the coordination and coupling of mRNA-related processes, including mRNA export (Katahira, 2012; Chang *et al.*, 2013).

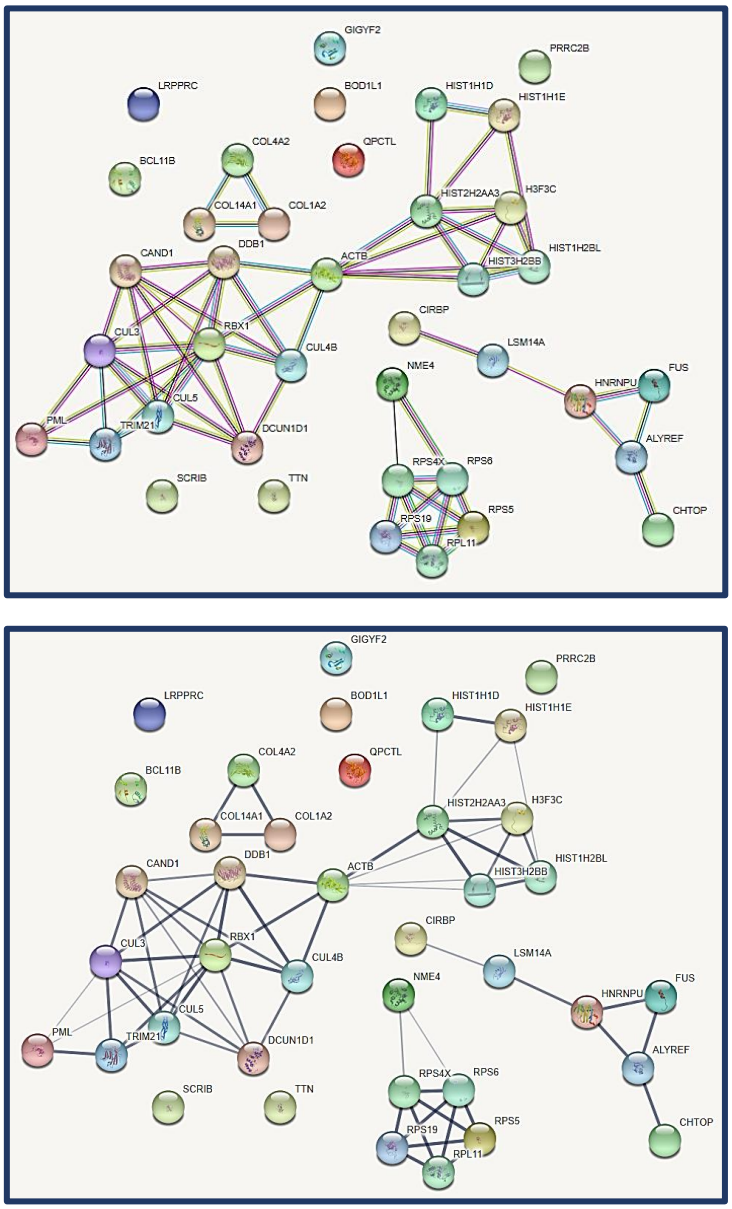


Figure 27. STRING output data represented using all the STRING selections (top) and based on the "confidence" setting (bottom).

2.6 Discussion

Studies performed previously in our lab determined DCUN1D1 to be essential for PCa, where knockdown of DCUN1D1 resulted in a blockage of proliferation and migration (Vava and Zerbini, 2014). We also identified two potential inhibitors that blocked PCa progression in a DCUND1-dependant manner. The aim of this study was to use immunoprecipitation-coupled MS to pulldown DCUN1D1 binding partners following expression of Flag-tagged DCUN1D1 in HEK293TT cells, and to identify the pulldown products using mass spectrometry. In order to determine the broader substrates of DCUN1D1 and the mechanism by which it mediates its activity. In the overall sample dataset, we used 3 666 167 513 total peptides, 777 248 total proteins of which, 92 were estimated false positive identifications. We obtained FPRs between 2.28 and 3.43% across the samples (1G, 1D, 2G, 2D, 3G_dmso, 3G_mg132, 3D_dmso and 3D_mg132) and of the total spectra identified, 2304 (68.47%) were uniquely assigned spectra. We determined the data to be normally distributed and the sample medians to be significantly different from each other. We then performed further analysis with samples 1G, 1D, 2G, 2D and discarded samples 3G_dmso, 3G_mg132, 3D_dmso and 3D_mg132. This was because PCA of the dataset showed that the latter group of samples except for 3G_mg132 clustered with the control samples. Additionally, although we identified DCUN1D1, we did not identify any known interactors within the DCUN1D1 transfected samples. These observations are likely due to experimental errors pertaining to sample preparation and further optimization may be necessary.

Significantly, we analysed the properties enriched within our experimental samples and identified evidence-based annotations relating to the cellular components, cellular processes and molecular functions enriched in samples 1D and 2D (Figure 28). We observed ubiquitin ligase complexes, the cytoplasm and nuclei cellular components to be enriched in both samples, which was consistent with our current understanding of the role of DCUN1D1 in ubiquitin cullin RING ligases (Petroski and Deshaies, 2005; Kim *et al.*, 2008; G. Huang *et al.*, 2011; Keuss *et al.*, 2016). This was also consistent with our understanding of the cellular distribution of components of the neddylation and ubiquitination pathways, which are known to shuttle between the cytoplasm and the nucleus during activation, cell stress and under different disease conditions (Kumar, Tomooka and Noda, 1992; Kumar, Yoshida and Noda, 1993; Hatakeyama, Jensen and Weissman, 1997; Petroski and Deshaies, 2005; Welchman, Gordon and Mayer, 2005; von Mikecz, 2006; Liu and Xirodimas, 2010; G. Huang *et al.*, 2011; H. Ramanathan and Ye, 2012; Karpov *et al.*, 2012; Abidi and Xirodimas, 2015; Brown and Jackson, 2015; Jayabalan *et al.*, 2016; Huang *et al.*, 2017). Significantly, the regulation of transcription and signal transduction were enriched in both samples (Figure 28). This has been observed previously, where DCUN1D1 was demonstrated to regulate the Hedgehog signalling pathway following transfection of DCUN1D1 in NIH-3T3 cells and microarray analysis, resulting in the dysregulation of *Gli1* expression (Sarkaria *et al.*, 2006). Significantly, downstream targets of *Gli1* were also dysregulated including *Gli2, cyclin D1, FGFR, IGF* and *IGFR*. Using a luciferase reporter assay and modified McKay and chromatin immunoprecipitation (ChIP) assays, they demonstrated that DCUN1D1 bound to the promoter region of *Gli1* affecting its transcription (Sarkaria *et al.*, 2006). Therefore, suggesting that although functioning as an E3 ligase for PTM, DCUN1D1 plays a role in the regulation of transcription and signal transduction and may be contributing to transcription, RNA splicing, cell differentiation and multicellular development, as found in this study. Lastly, the molecular functions enriched in both samples were protein binding, DNA binding and RNA binding, suggesting the biochemical activities implicated in the protein signatures identified in our samples and how they may be fulfilling their biological roles in response to DCUN1D1.

Furthermore, pathways found in common across both samples were: ubiquitin-mediated proteolysis, the ribosome, nucleotide excision repair and PPAR signalling (Figure 28). Significantly, within the top

10 enriched pathways identified in each sample, there were pathways found in one sample but not in the other. Therefore, we decided to explore the remaining lists described in Table 11 and 12 above (pg. 61 and pg. 62), to determine whether the pathways were enriched but at lower p values. Specifically, in respect of sample 1D, the following pathways and the log p values were identified outside of the 10 top enriched pathways namely: focal adhesion (log -0.6), Toll-like receptor signalling (log -0.4), neurotrophin signalling (log -0.4), the cell cycle (log -0.4), T cell receptor signalling (log -0.4) and leukocyte transendothelial migration (log -0.4). While sample 2D had the following pathways and the log p values identified outside of the 10 top enriched pathways, namely: PPAR signalling (log -0.4), adipocytokine signalling (log -0.4), WNT signalling (log -0.4), regulation of actin cytoskeleton (log -0.4) and fatty acid metabolism (log -0.4). Although the gap junction pathway was not enriched in sample 2D as observed in sample 1D, the tight junction pathway was enriched at log -0.7. From this analysis, we identified a broader perspective of the potential mechanism of action of DCUN1D1, based on its most likely main mechanism of action (ubiquitin-mediated proteolysis, the ribosome, nucleotide excision repair, PPAR signalling) and the primary effector pathways likely contributing to its mechanism of action.

Therefore, DCUN1D1 appears to mediate its activity primarily through the targeting and regulation of cullin RING E3 ligases, in a manner similar to other proteins that are characterised through the regulation of critical cell complexes/processes such DNA methylases, kinases or phosphatases. We postulate that DCUN1D1 be called “Cullin-Neddylase 1” due to the specificity of targeting the cullin family of proteins and the impact thereof. DCUN1D1 regulates ubiquitin CRLs leading to the regulation of transcription, signal transduction and contributes to transcription, RNA splicing, cell differentiation and multicellular development. This is evidenced by the data obtained from this study following IPMS analysis of Flag-DCUN1D1 pulldown products, statistical analysis, MS-based filtering for accurate protein identifications and identification of true interactors using negative control pulldown products and filtering using the multi-experiment aggregation of negative control samples in the Crapome database. We identified a final list of proteins, using the STRING tool for analysis of functional protein association networks, and found that the edges emanating directly from the DCUN1D1 node were associated with CUL3, CUL4B, CUL5, RBX1 and CAND1. This is also supported by multiple publications which have demonstrated the role of DCUN1D1 in cullin neddylation, through physical binding and mediation of cullin neddylation and also through the translocation from the cytoplasm and to nucleus for neddylation as observed in cullin 1 (Petroski and Deshaies, 2005; Sarkaria *et al.*, 2006; Kim *et al.*, 2008; Kurz *et al.*, 2008; G. Huang *et al.*, 2011; Keuss *et al.*, 2016). Additionally, although there was overlap in the activity of DCUN1D1 and its homologues in terms cullin 1, cullin 2, cullin 3, cullin 4A/4B or cullin 5 neddylation, DCUN1D1 was demonstrated to be among the main contributors to cullin neddylation (Keuss *et al.*, 2016).

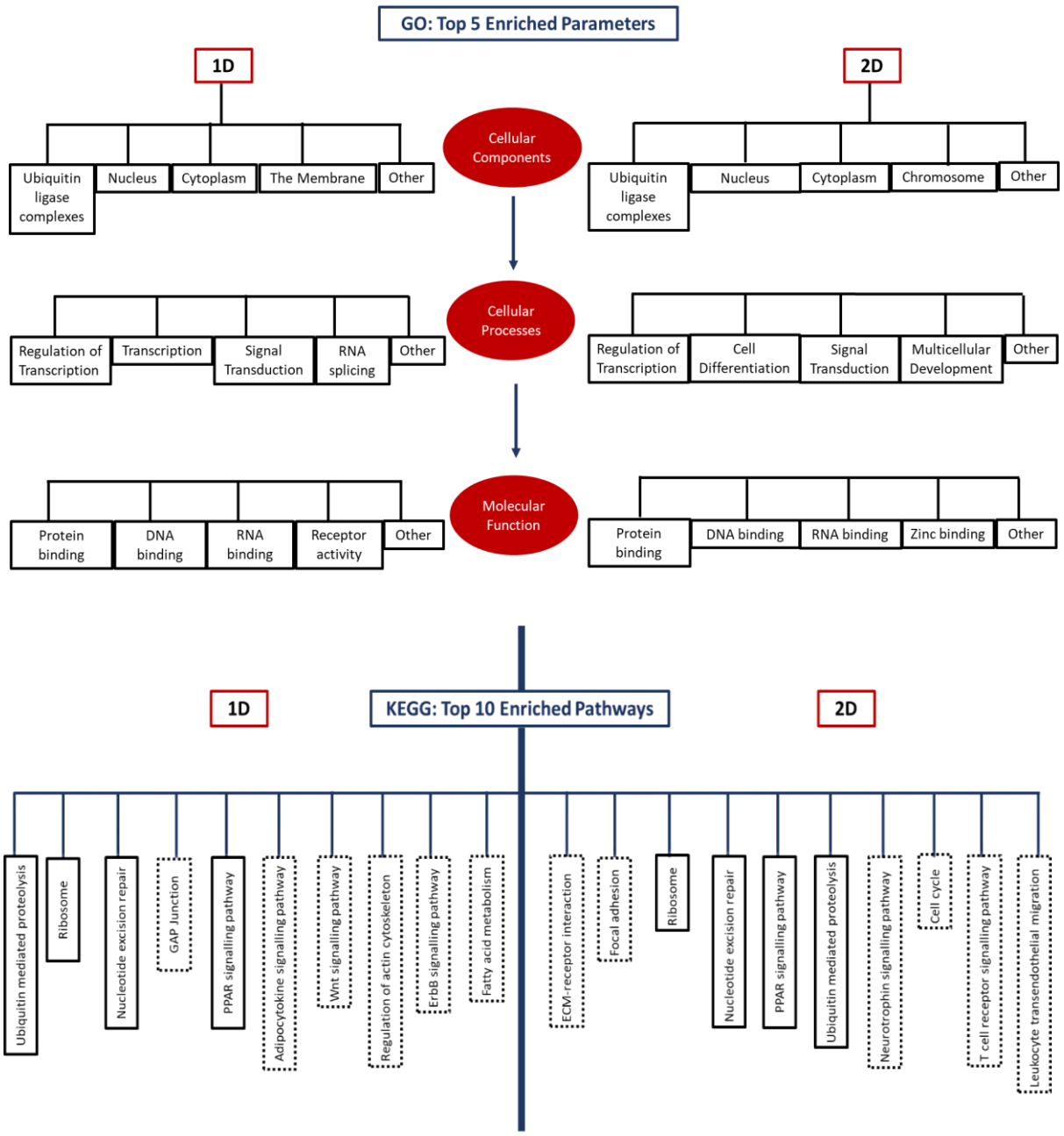


Figure 28. Summarized GO outputs for the top 5 enriched parameters in the samples (top) and the top 10 enriched KEGG pathways with pathways in common represented by solid borders (bottom).

However, there appears to be a distinct ubiquitin CRL, ribosome, transcription, lipid metabolism and inflammation theme across our protein signatures, which could also suggest the mechanism of action of DCUN1D1 in PCa. Cancer is understood to be a disease of gene mutations as observed in "loss of function" and "gain of function" mutations acquired by tumour suppressors and oncogenes, respectively (Hanahan and Weinberg, 2000, 2011; Loeb and Lawrence, 2000; Vogelstein *et al.*, 2013; Pon and Marra, 2015). It is also understood to occur as a result of increased rates of growth and proliferation (Hanahan and Weinberg, 2000, 2011). Significantly, increased rates of ribosomal biogenesis and protein translation have been linked to increased cell growth and proliferation in tumourigenesis (Derenzini, Montanaro and Trerè, 2017; Sulima *et al.*, 2017; Pelletier, Thomas and Volarevi, 2018). In this study, we identified multiple ribosomal proteins including RPS19, RPS4X, RPS5, RPS6 and RPL11 as well as a few ubiquitin E3 ligases which are the 2nd most prevalent cancer-related family, after kinases (Shi and Grossman, 2010). We also identified BCL11B and human SCRIB which have been described as emerging tumour suppressors. Loss of BCL11B alleles resulted in susceptibility of mice to lymphomas and human SCRIB has been demonstrated to function as a tumour suppressor in, PCa, liver and colorectal cancer, through its regulation of cell polarity (Dow *et al.*, 2003; Kamimura *et al.*, 2007; Gutierrez *et al.*, 2011; Pearson *et al.*, 2011; Kominami, 2012; Kapil *et al.*, 2017). Significantly, BCL11B has been demonstrated to regulate the expression of the ubiquitin E3 ligase HDM2 in a p53-dependent (Obata, Kominami and Mishima, 2012). Both of which are substrates of neddylation (Xirodimas *et al.*, 2004; Sundqvist *et al.*, 2009; Mahata, Sundqvist and Xirodimas, 2012; N. Liu *et al.*, 2017). However, increased rates of transcription and translation can also promote cancer development.

We identified multiple RNA binding proteins, regulators of mRNA transcription and processing, translation and nuclear export. These include FUS, HNRNPU, LSM14A, BAT2/PRRC2A, BCL11B (transcription factor), THOC4/ALYREF, GIGYF2, CIRBP, BAT2L1/PRRC2B, YBX1, BOD1L and C1ORF77/CHTOP (Sakura *et al.*, 1988; Didier *et al.*, 1988; Banerji *et al.*, 1990; Kiledjian and Dreyfuss, 1992; Crozat *et al.*, 1993; Nishiyama *et al.*, 1997; Wichmann *et al.*, 1999; Satterwhite *et al.*, 2001; Luo *et al.*, 2001; Giovannone *et al.*, 2003; Albrecht and Lengauer, 2004; Ota *et al.*, 2004; Tanaka *et al.*, 2006; Zullo *et al.*, 2009; Lambert *et al.*, 2012; Morita *et al.*, 2012; Higgs *et al.*, 2015). Interestingly, FUS has been demonstrated to be an E3 ligase for the Erbb3 receptor binding protein, Ebp1, and has been implicated in PCa progression, the regulation of the AR and AR gene transcription as well as PCa hormone resistance (Zhang *et al.*, 2005, 2008; Gannon *et al.*, 2008; Oh *et al.*, 2010). Furthermore, we also identified LSM14A and PML which are key components of RNA-related cellular structures such as the cytoplasmic P-bodies and the PML nuclear bodies, respectively (Ascoli and Maul, 1991; Stuurman *et al.*, 1992; Boisvert, Hendzel and Bazett-jones, 2000; Tanaka *et al.*, 2006; Yang *et al.*, 2006; Brandmann *et al.*, 2018). Both structures play a critical role during the cellular stress response, either through stress granules such as LSM14A and the PML nuclear bodies which assemble and disassemble depending on nuclear stress conditions (Boisvert, Hendzel and Bazett-jones, 2000; Lallemand-Breitenbach and de Thé, 2009; Decker and Parker, 2012; Sahin, De Thé and Lallemand-Breitenbach, 2014; Luo, Na and Slavoff, 2018). However, the PML nuclear body has recently been described to bind nascent RNA on its periphery creating an environment for optimum transcription (Boisvert, Hendzel and Bazett-jones, 2000). Additionally, within sample 2D, we identified several histone molecules namely, HIST1H1E, HIST1H1D, HIST1H2BL, HIST3H2BB, H3F3C, and HIST2H2AA3. Suggesting the implication in chromosome stability as histone molecules are part of the nucleosome, consisting of 2 molecules each of H2A, H2B, H3, H4 as well as the linker histone H1 (Luger *et al.*, 1997; Wolffe, 1997; Mariño-Ramírez *et al.*, 2005). Additionally, histones have also been implicated in DNA replication, DNA repair and recombination (Shahbazian and Grunstein, 2007; Messner and Hottiger, 2011; Cao and Yan, 2012; Venkatesh and Workman, 2015).

Significantly, neddylation has been described to play a role in the regulation of histone molecules, particularly during the DNA damage response (Brown and Jackson, 2015). There is also increasing evidence of the role of neddylation in the DNA damage response through different targets and mechanisms. Furthermore, cullin 4A has been extensively linked with the neddylation-regulated DNA damage response, however, few studies have explicitly described the role of cullin 4B in this activity (Shiyanov, Nag and Raychaudhuri, 1999; X. Chen *et al.*, 2001; Guerrero-Santoro *et al.*, 2008; Jackson and Xiong, 2009; Brown and Jackson, 2015; Brown *et al.*, 2015; Hannah and Zhou, 2015; Yi *et al.*, 2015). Therefore, we describe the association of cullin 4B and DCUN1D1 and their potential role in the DNA damage response. This could also contribute to the mechanism of action of DCUN1D1 in cancer as DNA damage is a key step to tumourigenesis.

Significantly, the metabolism of cancer cells is also markedly altered due to different energy requirements and the requirements of rapidly dividing cells (Hanahan and Weinberg, 2011). This capitalizes on the ability of the cells to generate building blocks of macromolecules such as peptide chains, to the individual amino acids required for protein synthesis and the regulation of fatty acid biosynthesis. Fatty acids are energy sources that are building blocks for lipids and have relationships with carbohydrates, proteins, and nucleic acids (Lindsay, 1975; Randle, 1998; Burdge and Calder, 2015; Araujo *et al.*, 2018). This is important particularly in cancer where, as mentioned previously, cellular energetics are classified as "Hallmarks of Cancer", mainly through the Warburg effect (Warburg, 1956; Hanahan and Weinberg, 2011; Liberti and Locasale, 2016). The preference of cancer cells for glucose as an energy sources impacts the energy-related pathways of cancer cells as well as its building blocks. By exiting at the end of glycolysis, producing quick ATP molecules to cope with the heightened rate of its energy requirements as rapidly dividing cells, cancer cells also produce high levels of lactate and acetyl-coA, relying on fatty acids (Warburg, 1956; Hanahan and Weinberg, 2011; Liberti and Locasale, 2016). Therefore, DCUN1D1 could be regulating the PPAR signalling pathway which has been demonstrated to regulate lipid metabolism including fatty acid degradation, glycerophospholipid metabolism, adipocyte differentiation and gluconeogenesis during tumourigenesis (Youssef and Badr, 2011; Poulsen, Siersbæk and Mandrup, 2012; Ahmadian *et al.*, 2013; Lefterova *et al.*, 2014; X. Ma *et al.*, 2018). Although PPARγ itself was not identified in this study, the PPAR signalling pathway was one of 4 pathways found in common between sample 1D and 2D using the KEGG database. Therefore, DCUN1D1 could be using the pathway to achieve its outcomes in a manner similar to castration resistant PCa, where AR-related pathways are reactivated by tumour cells without targeting the AR itself. Additionally, other pathways that are regulated by the PPAR signalling pathway were significantly enriched individually, such as adipocytokine signalling. The association between DCUN1D1 and PPAR signalling would however require further experimental analysis.

Additionally, across the data obtained in this study we observe a link between DCUN1D1 and inflammatory responses following IPMS analysis. KEGG analysis found enrichment in the Toll-like-receptor, T cell receptor, B cell receptor, neurotrophin signalling and leukocyte transendothelial migration signalling pathways. Neddylation has been implicated in inflammatory responses previously through the regulation of T cell activity via the ERK pathway and neddylation has been demonstrated to regulate NF-κB expression (Gao *et al.*, 2006; Jin *et al.*, 2013). Additionally, the transcription factor NF-κB has been extensively described to increase prostate tumour development by promoting the expression of the cytokines such as, IL-6 and TNFα, while the role of IL-6 in PCa has been well characterised (Sumitomo *et al.*, 1999; Tse, Scott and Russell, 2012; Jin *et al.*, 2014; Nguyen, Li and Tewari, 2014; Culig and Puhr, 2018). Significantly, studies done previously in our laboratory found the receptor tyrosine kinase Axl, to regulate IL-6 in its role in PCa (Paccez *et al.*, 2012, 2014). Furthermore, PCa has been extensively linked to inflammation in that prostatitis, which involves chronic inflammation of the prostate is found mainly in the peripheral zone of the prostate, which is also the

anatomical zone for 70% of PCa (Curtis, 2011; Ho, 2017). Infiltrating lymphocytes have also been found in prostate biopsies with contradicting evidence as to its contribution to PCa progression at various stages of diagnosis (Hu *et al.*, 2015; Strasner and Karin, 2015; Rådestad *et al.*, 2017). However, 20% of PCa-related deaths worldwide have been attributed to persistent inflammation (Mishra and Tewari, 2014). Therefore, DCUN1D1 may be playing a role in inflammatory responses and this may contribute to its role in PCa.

Moreover, the highest ranked proteins identified in our final lists, included proteins that are key regulators of inflammatory responses, mainly as components of the ubiquitin proteasome pathway. As mentioned above, we describe another link between TRIM21 and the cullin 3-dependent, BCR3 E3 ligases possibly through DCUN1D1-mediated neddylation of cullin 3. This is highly likely because among the cullin family of proteins, cullin 3 has been extensively linked to viral/host interactions and the regulation of the endosomal pathway, while TRIM21 has been characterised as an Fc cytosolic receptor during viral entry and an E3 ligase regulating innate and adaptive immunity (Yang *et al.*, 2009; Huotari *et al.*, 2012; Yoshimi, Ishigatsubo and Ozato, 2012; Versteeg *et al.*, 2013; Mahon *et al.*, 2014; Gschweitl *et al.*, 2016). We also identified the transcription factor BCL11B, which regulates the differentiation and survival of T lymphocytes during thymocyte development, regulating the expression of multiple genes including IL-2 (Wakabayashi *et al.*, 2003; Cismasiu *et al.*, 2006, 2009; Albu *et al.*, 2007; Liu, Li and Burke, 2010; Kominami, 2012). Significantly, BCL11B has been demonstrated to undergo sumoylation, and we describe the potential role of DCUN1D1-mediated neddylation on BCL11B (Zhang 2012).

Lastly, the tumour microenvironment and its role in tumourigenesis has been established (Hanahan and Weinberg, 2011; Balkwill, Capasso and Hagemann, 2012; Quail and Joyce, 2013; Belli *et al.*, 2018). We also identified multiple components of the cytoplasmic and nuclei matrix including COL4A2, COL14A1, COL1A2, COL18A1, ACTB, TTN and QPCTL. This activity may go beyond the contribution to the microenvironment as, a lot of the aforementioned proteins have been shown to undergo direct binding with other proteins to mediate certain activities or pathways. For example, ACTB (beta-actin), has been shown to bind to components of the nucleosome as well as RNA polymerases, playing a key role in transcription-related activities (Zhang *et al.*, 2002; Stüven, Hartmann and Görlich, 2003; Fomproix and Percipalle, 2004; Hofmann *et al.*, 2004; Hu, Wu and Hernandez, 2004).

Therefore, based on IPMS analysis we identified proteins that bind to DCUN1D1 which may explain its mechanism of action. We postulate that the primary mechanism of action of DCUN1D1 may be mediated by the interaction of DCUN1D1 with cullin 3, cullin 4B or cullin 5 leading to neddylation. Then, in concert with RBX1 and as part of ubiquitin CRL complexes that target specific substrates, may be implicating pathways involving the ribosome, transcription, lipid metabolism and inflammation. We provide Figure 29 below as a depiction of the proposed mechanism of action of DCUN1D1, based on the proteins identified in this study.

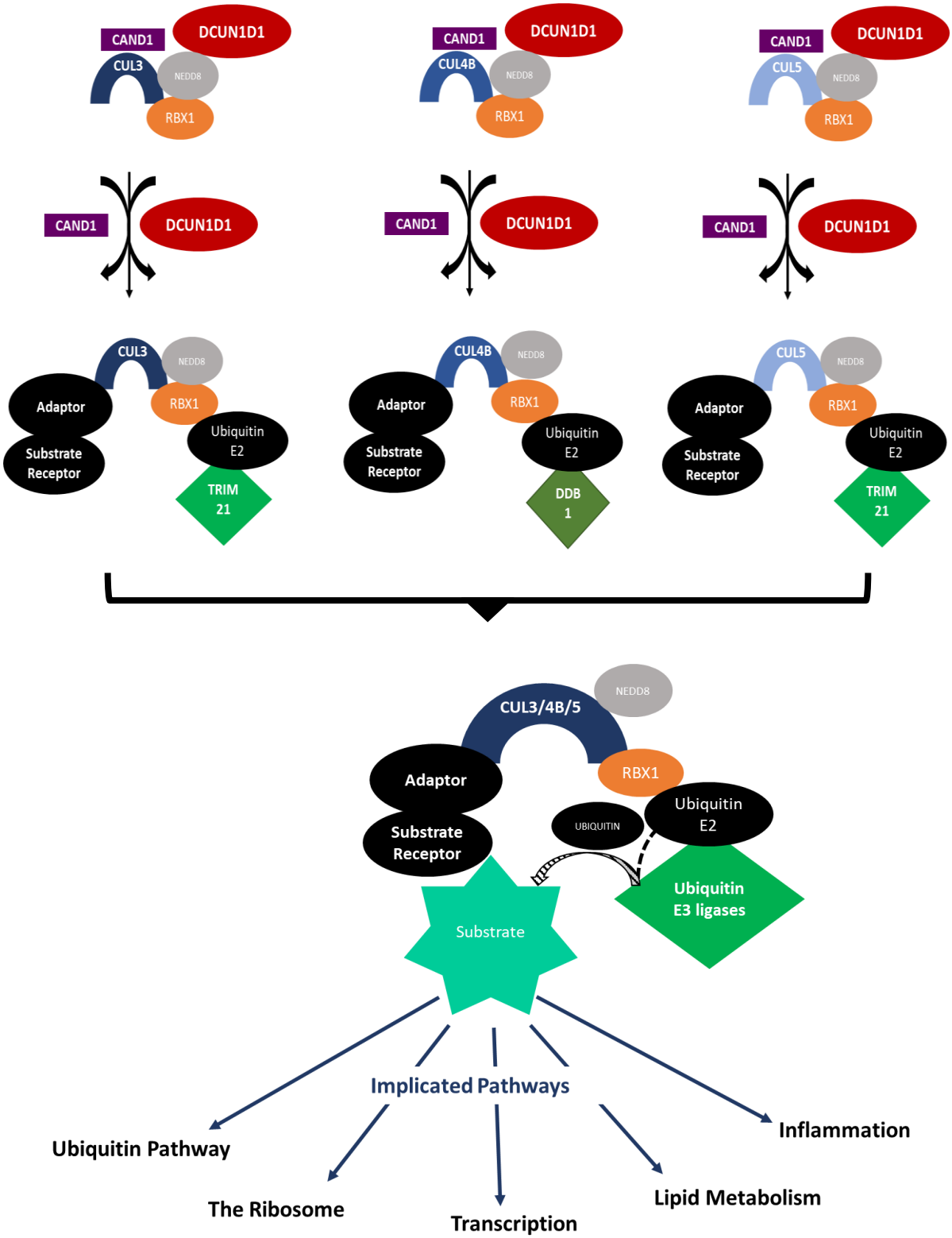


Figure 29. Schematic diagram for the proposed mechanism of action of DCUN1D1.

Chapter 3: SILAC for quantification of differentially expressed proteins

3.1 Introduction

Stable isotope labelling with amino acids in cell culture (SILAC) is a quantitative proteomics method that uses metabolic labelling to quantify protein abundance in samples (Ong *et al.*, 2002; Mann, 2006; Ong and Mann, 2007). Although MS-based proteomics is semi-quantitative it cannot be used as a *bona fide* quantification method because different molecules react differently to mass spectrometry (Tabb *et al.*, 2010), making it difficult to standardise sufficiently enough and consistently enough to make quantitative comparisons. SILAC on the other hand involves metabolic labelling of live cells using non-radioactive isotopes, by labelling proteins through endogenous protein synthesis pathways (Mann, 2006; Park *et al.*, 2012). This creates samples that are chemically identical but differ in molecular mass, which allows for comparative quantification of these samples. Additionally, although there are other labelling techniques such as chemical, hydrolytic and isobaric labelling, SILAC is advantageous in terms of labelling approach, sample preparation, reduced technical errors and improved quantitative accuracy and precision (van der Wal and Demmers, 2015; Ankney, Muneer and Chen, 2018).

SILAC involves labelling of two or more live cell populations with "light" or "heavy" amino acids that are differentiable through MS, based on molecular mass (Ong and Mann, 2007). Amino acid components are substituted with either natural analogues (C or N) or non-radioactive isotopes such as ^{13}C or ^{15}N. This results in a change in mass of the amino acids by a known quantity, depending on the number of elements modified within the amino acid (Ong and Mann, 2007). These amino acids are then incorporated into peptides and then into proteins in the cell where ideally, all the target amino acids are replaced. Although theoretically, any amino acid could be labelled, lysine and arginine are the amino acids most widely used because trypsin cleaves at the C terminus of both, they are more prevalent in protein sequences than other amino acids and are equally prevalent (Ong, Kratchmarova and Mann, 2003). Therefore, following tryptic digestion there is a higher likelihood of every peptide present in the mixture getting labelled, digested and being detectable by mass spectrometry. Therefore, SILAC has experimental strengths that are advantageous for studying proteins and proteomes, but it also provides strong quantitative measures.

One of the key outputs of SILAC-based MS analysis is the fragmentation spectra of peptide pairs from "light" and "heavy"-labelled samples that are chemically identical, that differ in mass and have abundance measurements for each peptide (Ong and Mann, 2007; Cox *et al.*, 2009). Therefore, it is possible to make a quantitative comparison between the peptide pairs by determining the ratio of peak intensities between them and obtaining a measure of abundance of proteins from which the peptides are derived (Ong and Mann, 2007; Cox *et al.*, 2009). This can be done in the form of relative quantification and through absolute quantification. At a quantitative proteomics level, relative and absolute quantification can be performed using stable isotope labelling or label free quantification (Wong and Cagney, 2010; Cox *et al.*, 2014; Chen *et al.*, 2015; Lindemann *et al.*, 2017; Ankney, Muneer and Chen, 2018). The choice between the two depends on the research question. Currently, relative quantification is used more extensively, where one sample is compared to another and a measure of differential expression is reported (Lindemann *et al.*, 2017; Ankney, Muneer and Chen, 2018). Absolute quantification on the other hand requires determining the concentration of the peptides

detected in order to measure protein abundance in the sample (Lindemann *et al.*, 2017; Ankney, Muneer and Chen, 2018). This can be complicated by various factors including the fact that it requires spiking with a peptide/protein of known concentration, that can be detected and identified during MS runs, taking into consideration that, molecules respond differently to MS, variance due to sample preparation and the MS workflow (Lindemann *et al.*, 2017; Ankney, Muneer and Chen, 2018). Furthermore, amongst the challenges to absolute quantification is the limited number and dynamic range of peptides detectable by each mass spectrometer as well as accurate mechanisms of measurement. This is mainly because current methods depend on peptide intensities/spectral counting depending on whether the peptide has been identified and annotated thus far. These challenges can however be mitigated by normalization using peptide, protein or peptide concatemer standards, experimental design and various algorithms (Cox *et al.*, 2014; Ankney, Muneer and Chen, 2018).

These aspects of MS-based proteomics have been particularly beneficial for molecular biology studies, in elucidating and understanding protein functions, the identification of PTMs and protein-protein interactions, among others. Considering the above, we performed relative quantitative proteomics, using SILAC, in order to quantify proteins that are differentially expressed following DCUN1D1 knockdown in PCa cells.

3.2 Hypothesis

DCUN1D1 is an E3 ligase that mediates transfer of NEDD8 to specific protein targets through PTM. Although the cullin family of proteins which are components of cullin RING ubiquitin E3 ligases have been demonstrated to be targets of DCUN1D1 (Kim *et al.*, 2008; Keuss *et al.*, 2016), the full spectrum of DCUN1D1 target proteins as an E3 ligase for neddylation has not been described previously and the downstream effect of cullin protein neddylation by DCUN1D1 has not been fully elucidated.

In Chapter 2 above we postulate that this family of proteins may be its main target and that DCUN1D1 or "Cullin Neddylase 1" plays a role in multiple cellular activities and pathways. We used an IPMS approach to bind DCUN1D1 interactors and to identify the binding partners using MS. Although proteins mediate their activity through covalent and transient binding of specific protein targets, biological signals are also transmitted through changes in the level of expression or changes in the modification patterns of proteins. These can often be indicators of changes in the biological system that are causing or contributing to conditions of stress, disease or are depicting responses to therapeutic interventions. Quantification of these changes can provide information for diagnosis, prognosis and treatment of various diseases.

We postulate that quantification of changes in proteins using SILAC quantitative analysis can be used to obtain a better understanding of the mechanism of action of DCUN1D1 in PCa. We can quantify the proteins whose level of expression is altered following knockdown of DCUN1D1, to understand its mechanism in PCa, its potential broader mechanism of action and identify potential inhibitors. Previously, we identified potential inhibitors of DCUN1D1 using DNA microarray transcriptomics and the Connectivity Map database and currently, we look to screen for drugs using a quantitative proteomics approach.

3.3 Aims and Objectives

The objective of this study is to determine the mechanism of action of DCUN1D1 in PCa using SILAC quantitative proteomics.

Aim:

SILAC quantitative proteomics analysis to identify differentially expressed proteins in DU145 versus DU145 DCUN1D1 knockdown cell lines.

3.4 Materials and Methods

The MS workflow will proceed through sample preparation, MS experimentation and data analysis as depicted in the schematic below.

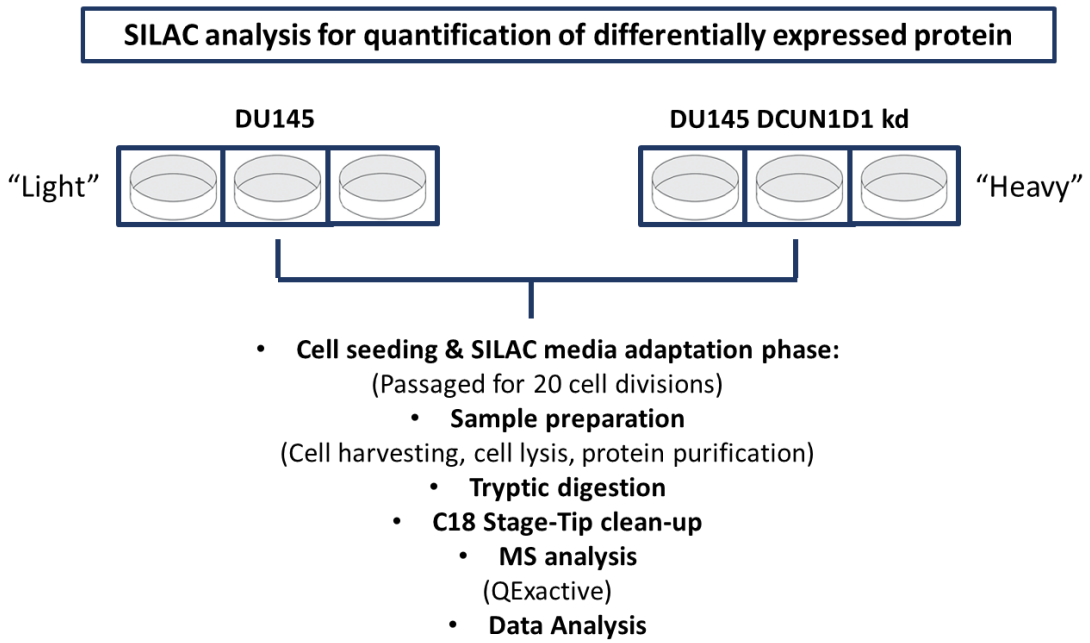


Figure 30. Diagram showing SILAC MS analysis methodological approach.

3.4.1 Cell seeding and SILAC incorporation

DU145 and DU145 DCUN1D1 knockdown cell lines were used to determine the proteins that are differentially expressed following DCUN1D1 knockdown in PCa (Figure 30). DU145 cells were knocked down using the shRNA lentivirus approach and selection was maintained using puromycin (0.1 mg/mL) (Lonza, Walkersville MD, USA). For SILAC analysis, the components used generally included, media without lysine and arginine, dialyzed FBS, "light" or "heavy" amino acid analogues and penicillin/streptomycin (Ong and Mann, 2007). Although the majority of the reagents are components of normal complete media, dialyzed FBS is used because it contains reduced concentrations of small molecules including amino acids. Additionally, because we use media depleted of lysine and arginine it improves the chances of having only the amino acid analogues, "light" or "heavy", incorporated into the cells. This reduces the chances of errors as the introduction of amino acids from other sources such as FBS and trypsin could affect the incorporation of target amino acids, affect the ability to distinguish the cell populations and make it difficult to quantitatively compare the samples.

In this study, we used the SILAC Protein Quantitation Kit DMEM (DMEM-LYS-C) (Cambridge Isotope Laboratories Inc, USA) containing SILAC DMEM media without lysine and arginine, dialyzed FBS, Lysine·2HCl (K^0), Lysine·2HCl (U-$^{13}C_6$, 99%) (K^8), L-Arginine·HCl (R^0) and L-Arginine·HCl (U-$^{13}C_6$, 99%; $^{15}N_4$, 99%) (R^{10}) (Cambridge Isotope Laboratories, Inc, USA). Media for the "light" and "heavy" samples was prepared, and then 1% penicillin (5000 µg/mL/streptomycin 5000 µg/mL) (Lonza, USA) was added prior to filter-sterilization of the media using 0.22µm filters (Techno Plastic Products, Switzerland) (Figure 31). The cells were seeded onto 10cm tissue culture dishes (Wuxi Nest Biotechnology, China) at either 1:40 or 1:25 dilution, from confluent DU145 and DU145 DCUN1D1 knockdown cell lines (Figure 32). They were then maintained at 37°C under humidified conditions, containing 5% CO_2. Following which, they were cultivated taking into account the differences in growth rates between the two cell lines, to allow for adaptation and efficient labelling incorporation.

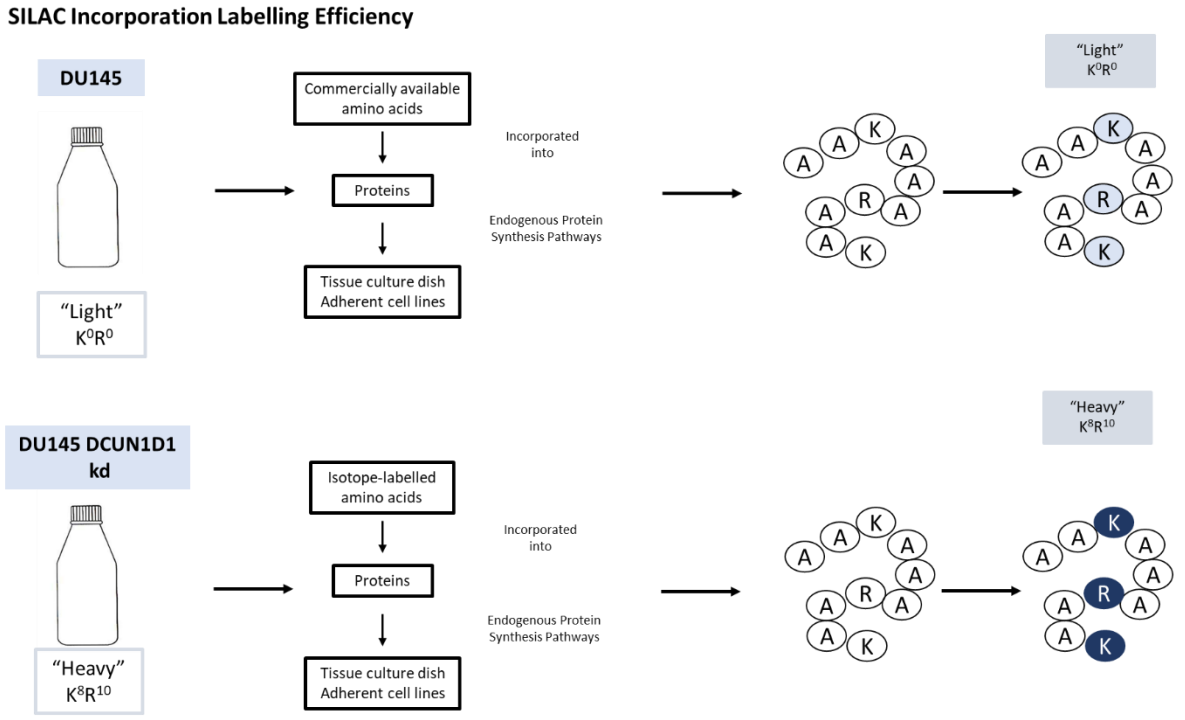


Figure 31. Schematic diagram showing amino acid incorporation labelling efficiency using Stable Isotope Labelling of Amino Acids in Cell culture (SILAC). Representing our experimental layout including the amino acid incorporation of DU145 "Light" (K^0R^0), (*top*) and DU145 DCUN1D1 knockdown (K^8R^{10}) cultures (*bottom*).

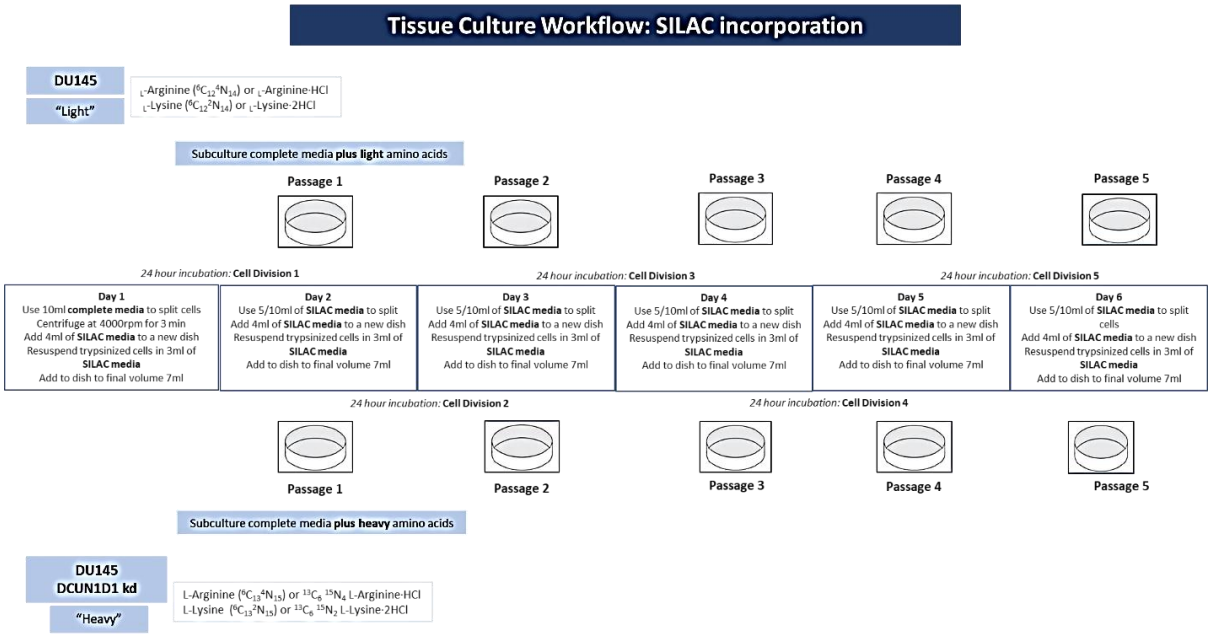


Figure 32. Diagram showing tissue culture workflow for SILAC amino acid incorporation. Showing representation of cell seeding over multiple passages.

3.4.2 Sample preparation

The samples were prepared in triplicate from the DU145 and DU145 DCUN1D1 knockdown cells. SILAC media was aspirated from the DU145 (light) and DU145 DCUN1D1 knockdown (heavy) plates then washed with sterile 1× PBS pH 7.4. Following this, the cells were lysed using 300µL of 1× cell lysis buffer (Cell Signaling Technology, USA) (see Table 15 below for details) and scraped using cell scrapers. The cell lysis buffer was supplemented with protease and phosphatase inhibitors (Roche, Germany) to prevent degradation of the proteins and cell lysis was performed on ice. The samples were then transferred to 1.5mL tubes and cell debris was removed by centrifugation at 13 300 rpm for 2 minutes, retaining the proteins in the supernatant. The concentration of the proteins was quantified prior to further processing. For the SILAC labelling incorporation efficiency test, 100µg each of the lysate was prepared, while, 50µg each of the "light" and "heavy" lysate was prepared. The "light" and "heavy" samples were then mixed to a final mass of 100µg and processed together, decreasing the variability that may occur due to sample processing.

The Pierce BCA Kit assay (Thermo Fisher Scientific, USA) for protein quantification was performed, where a 1:2 dilution series was prepared of the bovine serum albumin (BSA) 2 mg/mL ampule in order to plot a standard curve and measure the protein concentration. The kit working reagent was prepared at sufficient volume for the standards, samples (1:20 dilution) and the blank, where 10µL of each was added to a 96 well flat-bottomed plate. The working reagent was used at 50:1 dilution in terms of the BCA reagent A to BCA reagent B, following which, 200µL was added to each well. The plate was then covered with foil and incubated at 37°C for 30 minutes. The absorbance values were then read at 595nm and the protein concentration determined for further sample processing.

3.4.3 Filter Aided Sample Preparation and Tryptic Digestion

Further sample processing was performed using filter aided sample preparation (FASP) in accordance with the protocol from the IDM Proteomics and Metabolomics Platform, South Africa, standard operating procedure version 2. Tryptic peptides were generated using molecular weight filtration devices, namely, 30 kDa centrifugal filter units (Amicon Ultra, Merck, Germany). First, the water bath was heated to 95°C while the filter units were assembled, following which, 100µg of crude protein lysate was added to 1.5mL clean tubes, and 0.1M dithiothreitol (DTT) (Sigma-Aldrich, Germany) was added to each sample and incubated at 95°C for 3 minutes. Meanwhile, 500µL of urea buffer was added to each assembled filter unit then centrifuged at 14 000 ×g for 10 minutes. The reduced samples were then added to each filter unit, followed by the addition of 200µL urea buffer and centrifugation at 14 000 ×g for 15 minutes. We then repeated the addition of 200µL urea buffer, followed by centrifugation at 14 000 ×g for 15 minutes and the flow-through was discarded when necessary. We then added 0.05M iodoacetamide (Sigma-Aldrich, USA), vortexed the samples at 600 rpm for 1 minute and incubated them in the dark for 20 minutes, without mixing. The samples were then centrifuged at 14 000 ×g for 10 minutes, then washed three times with 100µL urea buffer, followed by centrifugation at 14 000 ×g for 15 minutes during each wash. Finally, the samples were transferred to a clean 1.5mL collection tube then 50mM ammonium bicarbonate (Sigma-Aldrich, USA) was added to the samples at 100µL. We then checked that the pH was 9 and centrifuged the samples at 14 000 ×g for 10 minutes before tryptic digestion.

Tryptic digestion was performed within the filter units using 0.5 µg/µL MS grade trypsin-ultra (New England Biolabs, USA) and 40µL of 2× trypsin-ultra reaction buffer (New England Biolabs, USA), at a final enzyme to protein ratio of 1:100. We then vortexed the samples and placed them in a wet chamber, prior to incubation at 37°C for 18 hours. Following incubation, the samples were centrifuged at 14 000 ×g for 10 minutes with the flow-through containing digested tryptic peptides. We then did an additional centrifugation step at 14 000 ×g for 10 minutes after adding 40µL ammonium bicarbonate to the samples. The flow-through was then transferred to a clean 1.5mL tube where the peptides were acidified by the addition of 0.1% formic acid ensuring that the pH was three.

Table 15: Detailed description of the buffers used and reagent composition

Reagent/Buffer	Composition
1× cell lysis buffer	2mM Tris·HCl pH 7.5, 15mM NaCl, 0.1mM Na_2EDTA, 0.1mM EGTA, 0.1% Triton, 0.25mM sodium pyrophosphate, 0.1mM beta-glycerophosphate, 0.1mM Na_3VO_4, 0.1 µg/mL leupeptin
Urea buffer	24g urea in 50mL of 0.1M Tris in HPLC grade water
Ammonium bicarbonate	0.2g in 50mL of HPLC grade water

3.4.4 C18 Stage-Tip clean-up

We prepared the tryptic peptides for MS analysis by performing C18 Stage-Tip clean-up for offline desalting and removal of other potential contaminants in accordance with the protocol from the IDM Proteomics and Metabolomics Platform, South Africa, standard operating procedure version 1. The C18 solid phase extraction (Sigma-Aldrich, Germany) involved the use of self-assembled microcolumns to purify and concentrate peptides. The apparatus includes a disk that is embedded with silica beads

bound by octadecyl carbon chains (C18), that is placed at the bottom of 20 - 200µL yellow tip (not autoclaved), within a clean 1.5mL tube. The disk composition is optimised to allow for binding of low concentration peptides within comparatively higher aqueous solution volumes, while inhibiting bead dislodgement and peptide loss. We activated the stage-tip resin by adding 100µL of solvent B (80% acetonitrile and 0.1% formic acid) followed by centrifugation at 2000 rpm for 1 minute. We repeated this step three times then performed equilibration by adding 100µL of solvent A (2% acetonitrile and 0.1% formic acid) followed by centrifugation at 2000 rpm for 1 minute, repeated three times. The flow-through was discarded as necessary throughout the centrifugations, then we added 10µg each of the acidified samples, which we centrifuged at 2000 rpm for 1 minute and retained the flow-through. We then washed the samples by adding 100µL of solvent A then centrifuged at 2000 rpm for 1 minute and repeated it twice. At this point, we inserted a clean glass insert into the 1.5mL tube and re-inserted the yellow-tip with the sample-bound disk. We eluted the peptides using 50µL of solvent C (60% acetonitrile and 0.1% formic acid) and centrifuged at 3000 rpm for 1 minute, which we repeated two times. We then dried and concentrated the eluted peptides using the speedyVac concentrator for 1 hour. The samples eluted in glass were either stored at -20°C in a glass autosampler until necessary or resuspended in 50µL solvent A to a final concentration of 200 ng/µL, prior to injection into the mass spectrometer.

3.4.5 LC-MS/MS

We performed MS analysis using the QExactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher, USA) coupled in-line with Dionex Ultimate 3500 RSnano LC systems for proteomics analysis. The SILAC workflow consists of three experimental steps including: the SILAC labelling incorporation efficiency test, the SILAC mixing ratio analysis as well as the final MS/MS run for data analysis (Park *et al.*, 2012; Guan *et al.*, 2014). This is to retain the strength of the quantitative analysis during sample preparation and to safeguard reduced variability. The samples were analysed according to the laboratory standardised default settings, however, the SILAC efficiency test involved running only the “heavy”-labelled samples, namely the DU145 DCUN1D1 knockdown samples. The peptide mixture (5µL) was packed into the 2 cm C18 trap (100 µm ID, packed with Phenomenex Luna 100 Å hollow core beads) and the analytical column used was 30 cm, packed with Phenomenex Aeris peptide C18 (ID 75µm; 3.6µm beads). Peptides were eluted using a 400nL/minute flow rate across the experiments, with a linear gradient of 2% solvent B for 2 minutes, 8 – 23% solvent B for 80 minutes, 23 – 80% solvent B for 8 minutes and 80% solvent B for 10 minutes. Data dependent acquisition was performed using X Calibur in full scan mode with automatic switching between MS and MS/MS at a scan range of 300 – 1750 m/ƶ and an isolation window of 2 m/ƶ. The top 10 most abundant parent ions were selected during initial scans. The mass resolution for MS1 was 70 000, with an AGC target value of 3×10^6 ions, at a maximum injection time of 250 milliseconds. The MS2 mass resolution was 17 500, with an AGC target value of 1×10^6 ions, at a maximum injection time of 80 milliseconds. Peptide fragmentation was performed using high-energy collision dissociation with the normalized collision energy set at 28 and dynamic exclusion of 30 seconds.

3.4.6 Data processing and analysis

The raw data files generated by X Calibur were processed using Maxquant version 1.5.3.12 and searched using the built-in Andromeda search engine. The data was then searched against the human

reference proteome that was downloaded from uniprot.org. The spectra were matched against a reverse decoy database with the false discovery rate (FDR) set at 1%. The protease used was trypsin, allowing for a maximum of 2 missed cleavages, carbamidomethylation of cysteine was selected as the fixed modification and the variable modifications specified were methionine oxidation and N-terminal acetylation. Match between runs was also selected to improve protein identifications. Additionally, all the files were run simultaneously within an experiment, and the replicates were separated through the Maxquant experiment design setting.

3.4.7 Software/Tools used in the study

Perseus

We used Perseus version 1.6.5.0 to perform data filtration and statistical analysis of the Maxquant data outputs (Tyanova *et al.*, 2016). All experiments were initially filtered by removal of proteins identified by site, reverse identifications and those identified as potential MS contaminants. The SILAC incorporation labelling efficiency and mixing ratio experiments were then further analysed using Excel, with the "Intensity" columns specified for calculations. The final MS/MS analysis was performed using the log transformed "Ratio H/L normalized" columns and we selected to remove all proteins without valid values for 100% of the replicates and added uniprot annotations for further enrichment of the dataset. We then performed the two-sample t-test to determine if the means of the replicate samples were significantly different from each other with permutation-based FDR of 5%. We determined the proteins that were significantly differentially expressed following knockdown of DCUN1D1, by calculating the mean and standard deviation for each replicate following the filtration above and selected proteins with 1 standard deviation on either side of the mean, to be significantly differentially expressed.

Gene Ontology/Panther

Gene Ontology (GO), empowered by the Panther classification system version 14.1 was used for functional analysis and to determine the cellular components, molecular functions, biological processes and pathways enriched within our dataset (Ashburner *et al.*, 2000; Mi *et al.*, 2013, 2019; The Gene Ontology Consortium, 2013; Carbon *et al.*, 2017). The GO platform, as described previously, uses gene and gene products for the analysis of data and to understand phenotypes using ontologies and graphical mapping of genes. Additionally, the classification system Panther i.e. Protein Analysis through Evolutionary Relationships, uses 82 genome libraries to enable functional analysis of genes and gene-related attributes, based on evolutionary relationships as mapped using phylogenetic trees, multiple sequence alignments and statistical modelling (Mi *et al.*, 2013). See Chapter 2, section 2.4.7, pg. 51 for detailed GO software background.

STRING

STRING version 11, was used for functional analysis of the associations observed within the networks enriched in our dataset (Snel *et al.*, 2000; Mering *et al.*, 2003; Szklarczyk *et al.*, 2015). See Chapter 2, section 2.4.7, pg. 52 for detailed software background.

3.5 Results

3.5.1 SILAC labelling incorporation efficiency and mixing ratio analysis

The SILAC analysis workflow involved performing the SILAC labelling incorporation efficiency test and mixing ratio evaluation. This was to determine whether during cell culturing, we achieved adequate labelled-isotope incorporation of amino acids into protein sequences and whether mixing of our "light" and "heavy" samples met accurate quantitative requirements (Park *et al.*, 2012; Guan *et al.*, 2014). We provide Figure 33 and 34 below, showing the Total Ion Current (TIC) chromatograms for the labelling incorporation efficiency and mixing ratio tests, respectively. We obtained a bell-shaped mass distribution across the "heavy" samples, i.e. H1, H2 and H3 for the efficiency experiment as well as the mixing ratio samples LH1, LH2 and LH3, respectively. We also obtained a SILAC labelling incorporation efficiency of 97%, 99% and 99% for samples H1, H2 and H3 and the "heavy": "light" SILAC mixing ratios were 0,62, 1,23 and 0,99 in samples LH1, LH2 and LH3, respectively. Comparative quantitative analysis requires that the mixing ratio be as close to 1 as possible, therefore, we performed further statistical and biological analysis with samples LH2 and LH3.

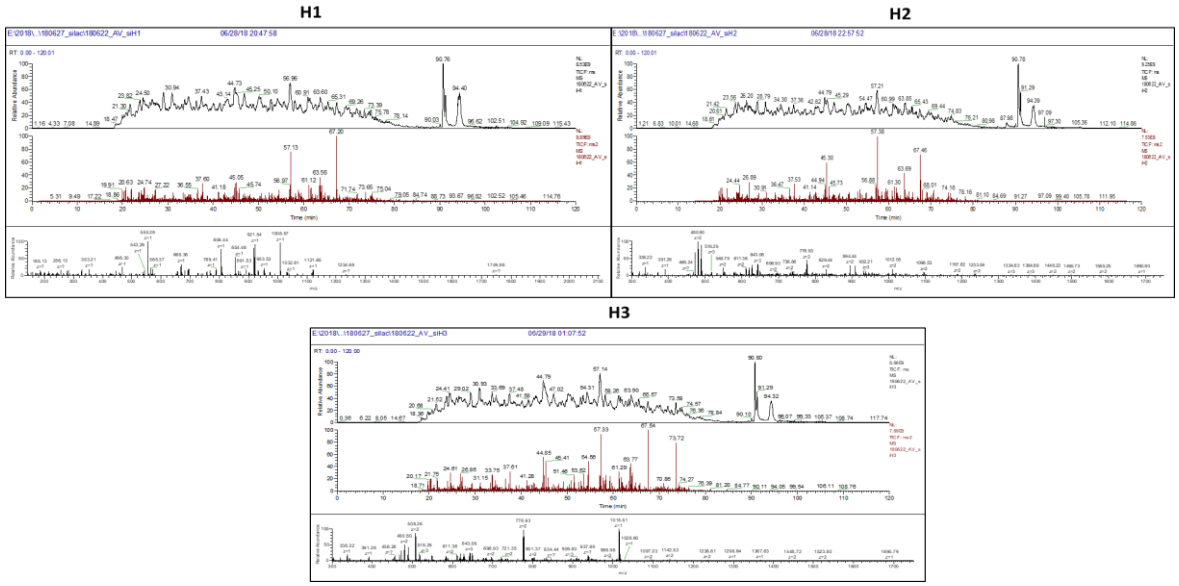


Figure 33. Showing chromatograms for the SILAC labelling incorporation efficiency test. Chromatograms from samples H1, H2 and H3 from the SILAC labelling incorporation efficiency test.

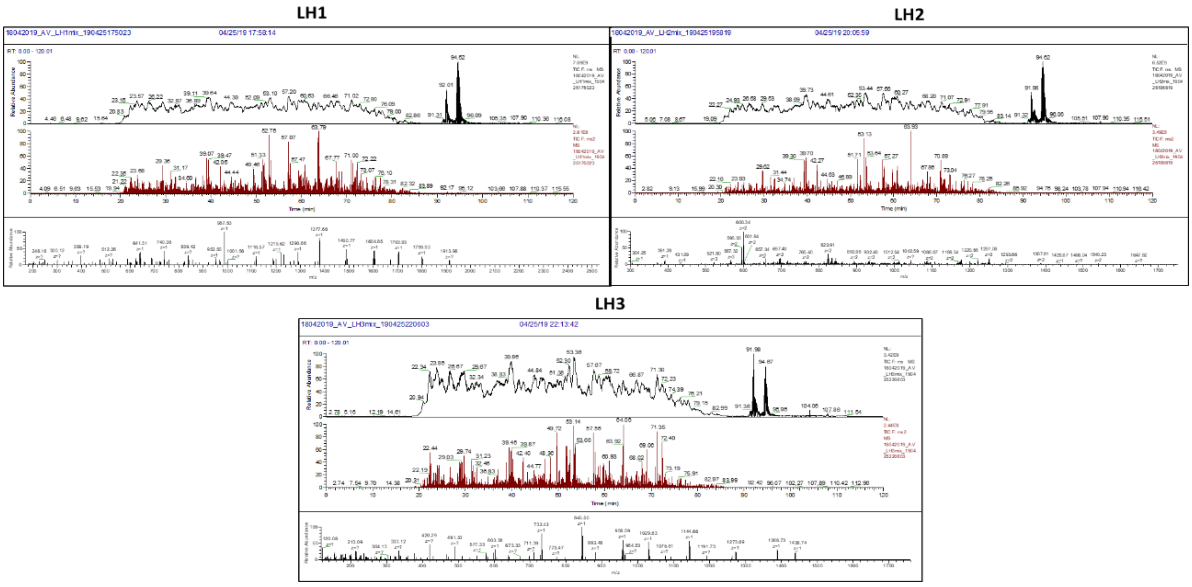


Figure 34. Showing chromatograms for the SILAC mixing ratio test.
Chromatograms from samples LH1, LH2 and LH3 from SILAC mixing ratio evaluation.

3.5.2 SILAC MS/MS data analysis

We performed MS/MS analysis and obtained TIC chromatograms for samples LH2 and LH3 as shown in Figure 35 below. Significantly, we obtained bell-shaped distributions across the samples, and as mentioned previously, the mixing ratios were 1,23 and 0,99 in samples LH2 and LH3, respectively. Analysis of the duplicate samples resulted in the identification of 1443 unique peptide amino acid sequences from 3743 identified spectra. The data was filtered as described above and the number of protein groups observed in our dataset was 456 with the peptide and protein FDR set at 1%. We identified 103 protein groups containing biologically relevant valid values, with the first protein identified in the protein group selected as the best annotated and representative of protein identification.

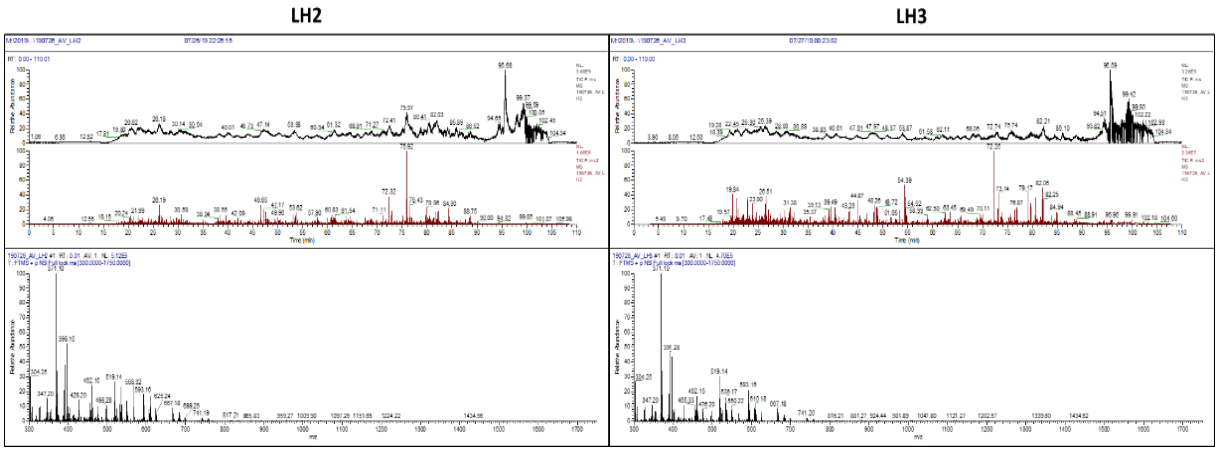


Figure 35. Showing chromatograms for SILAC MS/MS data analysis.
Chromatograms from samples LH2 and LH3 from SILAC MS/MS analysis.

3.5.2.1 Overall sample analysis following SILAC quantitative proteomics

We then performed further analysis beginning by determining the distribution of the replicate samples using histograms and observed that the data were normally distributed with the ratio distributions displaying similar patterns (Figure 36). Additionally, we observed using scatterplots that the ratios had narrow distributions with a moderate Pearson correlation coefficient of 0,572 (Figure 36).

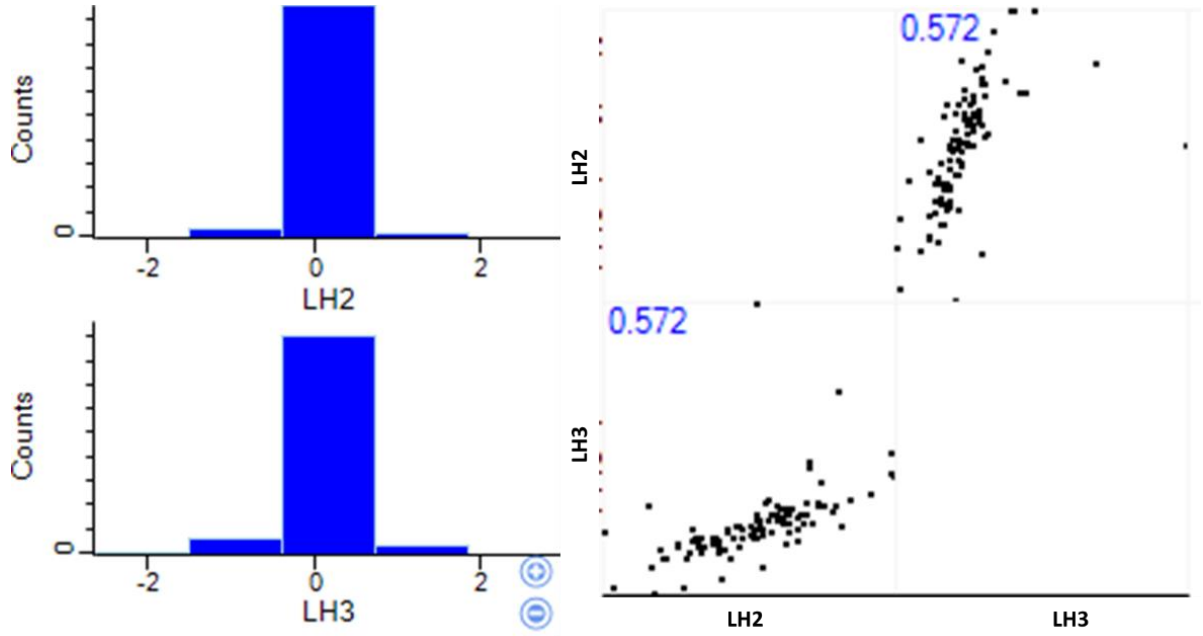


Figure 36. Showing histogram and scatterplot analysis of the samples. *Left)* histograms showing sample ratio distributions and *(right)* showing scatterplot analysis including Pearson correlation coefficient, r.

3.5.2.2 Detailed analysis following SILAC quantitative proteomics

In order to determine the relationships between the proteins identified in each replicate, we performed Venn diagram analysis of our filtered samples within Perseus and found that the 100 proteins observed were represented in both of the samples (Table 16). Additionally, for functional analysis of the overall samples, we first analysed the properties of the samples based on these proteins. We began with functional classification of our data and obtained outputs with respect to cellular components, molecular functions, biological processes and pathways (Figure 37 and 38). We report the data using GO/Panther outputs, based on experimental evidence obtained in published articles and found cellular component classifications which included cellular components of the cell, organelles and protein-containing complexes. We also found molecular functions related to binding, catalytic activity and structural molecule activity. Additionally, we found biological processes related to biological regulation, cellular component organization/biogenesis, cellular processes, localization, metabolic processes and response to stimulus. Interestingly, we also observed biological processes related to developmental processes and immune system processes which have been observed in the previous chapter. Lastly, we observed pathway classifications related to ATP synthesis, apoptosis signalling, cytoskeletal regulation of Rho GTPase signalling, FGF signalling, glycolysis, Huntington's disease, inflammation mediated by chemokine and cytokine signalling, integrin signalling, nicotinic acetylcholine receptor signalling, Parkinson's disease, pyruvate metabolism, serine glycine biosynthesis and the TCA cycle. Significantly, the data includes metabolic and inflammatory pathways and to a lesser extent, the ubiquitin proteasome pathway, WNT signalling and the p53 pathway, which were also observed in Chapter 2 of this study (Figure 38). Overall, the findings provide interesting insights on the molecular biology properties observed based on the proteins identified following MS analysis. It will be interesting to determine which are significantly differentially expressed following knockdown of DCUN1D1. Taking into consideration that the role of DCUN1D1 in the neddylation pathway and UPP is mediated through protein complexes and is implicated in signalling through the regulation of the Hedgehog signalling pathway (Sarkaria *et al.*, 2006; X. Yang *et al.*, 2007; Kim *et al.*, 2008; G. Huang *et al.*, 2011; Heir *et al.*, 2013; Keuss *et al.*, 2016).

Table 16: Showing 100 proteins found across the replicate samples

Protein Name	Protein Name	Protein Name	Protein Name
EIF3C	HSP7C	GRP75	1433E
PHGDH	PABPC1	RS19	RPS14
ACTN4	EF2	RPS9	RS13
HNRPQ	KPYM	RS10	RL7A
LDHA	ENPL	IDHP	RS6
DHE3	DDX5	TCPG	E5RI99
ALDOA	TCPA	EFTU	PPIA
ANXA1	PGAM1	SYAC	RACK1
G3P	FLNA	GDIB	EF1A3
AT1A1	UBA1	TCPQ	TBB4B
ALDH2	ROA2	HNRPF	SLC25A3
ADT2	RS3	SYYC	HNRNPU
EIF2S1	SAHH	XPO2	SRSF2
CDK1	ATPA	TERA	EIF4G1
ATPB	MOES	EIF3B	PRDX1
ENO1	RPL13	MYL6	TIF1B
NPM	VARS	ACTB	IMB1
P4HB	CAD	IF4A1	PLEC
ANXA2	TKT	RS20	PCBP2
TUBB	PDIA3	RS3A	TBA1C
HS90A	PPP2R1A	RPL15	PDC6I
HS90B	GLYM	RL27	NAA15
ODPA	HSPA4	HNRPK	NSF1C
RPSA	MYH9	RS8	RUVBL2
BIP	PRS7	RS16	TLN1

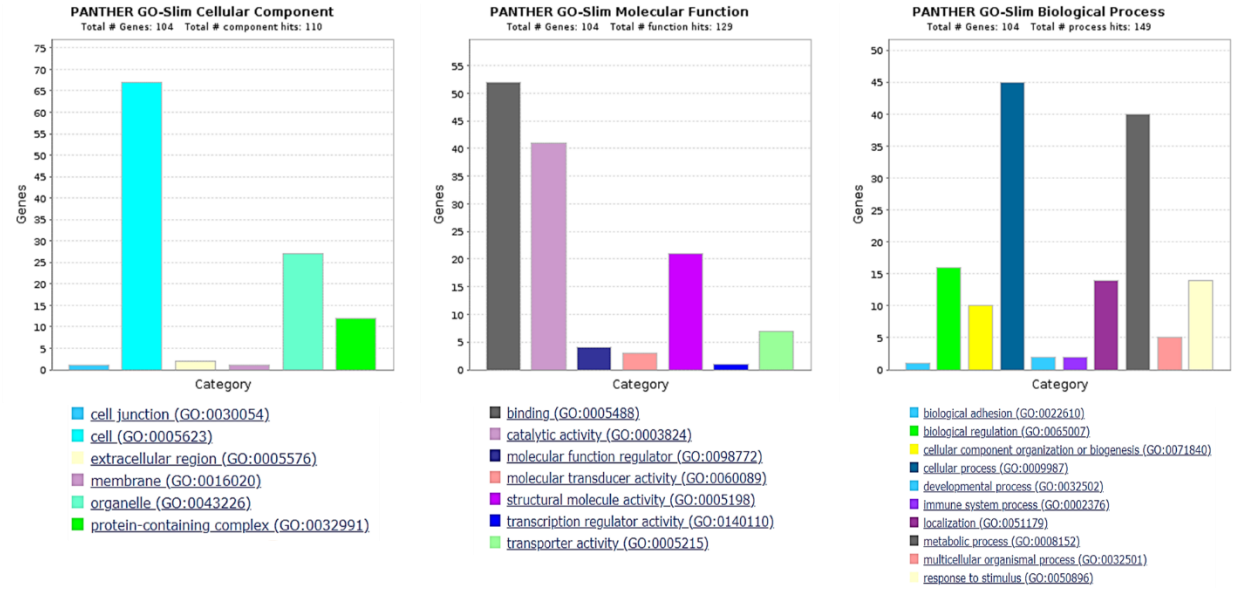


Figure 37. Showing bar charts of Panther GO-Slim outputs. *Left)* experimental data classified according to cellular components, *centre*) experimental data classified according to molecular functions and *(right*) experimental data classified according to biological processes.

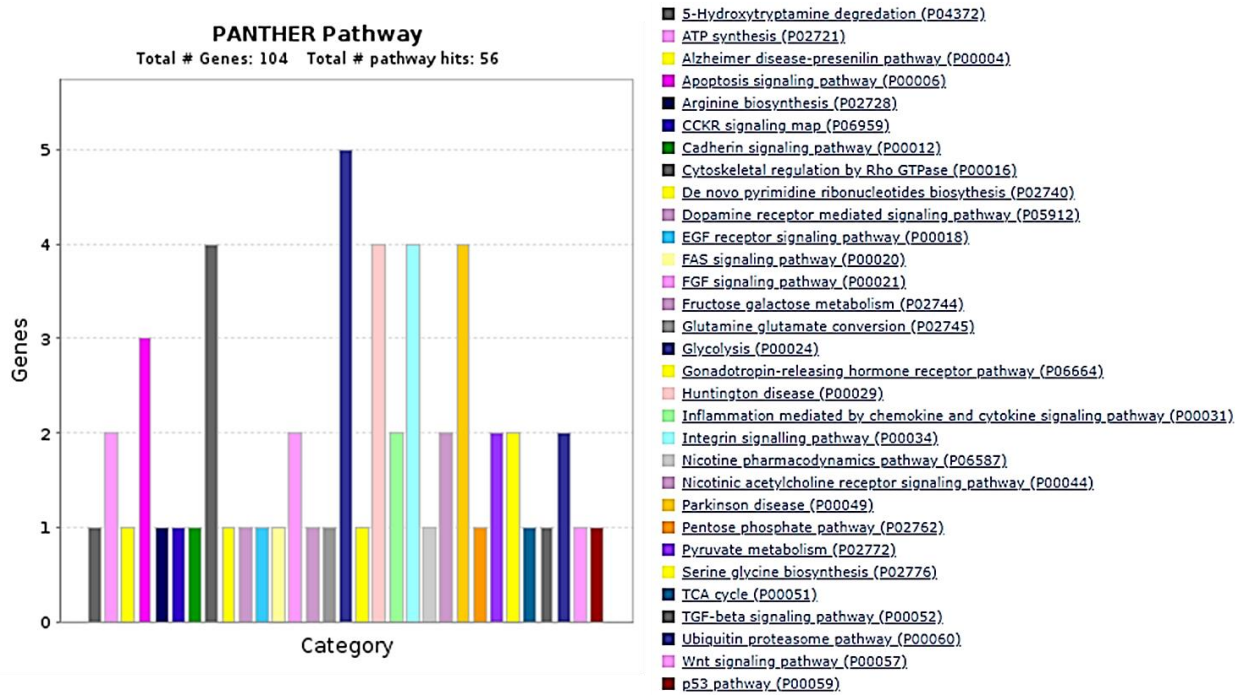


Figure 38. Showing bar chart of Panther Pathway output.

We then performed a paired two-sample Student's t-test analysis on the replicate samples and found a p value equal to 0,442274 meaning that the null hypothesis of equal means was not rejected. We then determined the proteins that were significantly differentially expressed following knockdown of DCUN1D1, by calculating the mean and standard deviation for each replicate following the filtration above and selected proteins that had log transformed H/L ratios of 1 standard deviation on either side of the mean. Following knockdown of DCUN1D1 in DU145 PCa cell lines we found 33 proteins to be significantly differentially expressed in sample LH2 with 14 downregulated and 19 upregulated (Table 17). Additionally, 17 proteins were significantly differentially expressed in sample LH3 with 8 downregulated and 9 upregulated (Table 17). We highlight 11 proteins which were found in common between the replicate samples namely, CCT3, TCP1, EIF2S1, YARS, AARS, SRSF2, NSFL1C, EEF2, HNRNPA2B1, FLNA and PKM.

Majority of the above proteins and those significantly differentially expressed were involved in translation-related activities ranging from involvement in the 43S preinitiation complex, the ribosome, ribonucleoproteins, proteins involved in processing in the endoplasmic reticulum (ER), proteins involved in the spliceosome and those involved in the centrosome. CCT3 and TCP1 are components of the protein-folding complex called chaperonin-containing T-complex, with TCP1 being involved in the folding of 10% of the cell proteome (Leitner *et al.*, 2012). Additionally, both are components of the centrosome, which is the main microtubule organization centre of the cell and plays a role in genome instability in cancer (Kellogg, Moritz and Alberts, 1994; Bobinnec *et al.*, 1998; Krämer, Neben and Ho, 2002; Leitner *et al.*, 2012; Sanchez and Feldman, 2017). EIF2S1 i.e. eukaryotic translation initiation factor 2 subunit 1 is involved in protein synthesis, while, NSFL1C is involved in protein processing in the ER (Chen and London, 1995; Turano *et al.*, 2002; Shih and Hsueh, 2016). HNRNPA2B1 is a ribonucleoprotein that is involved in catalytical activities of the spliceosome, while, SRSF2 is a transcription cofactor that is also a component of the spliceosome (He and Smith, 2009; Mo, Ji and Fu, 2013). We also found YARS and AARS, in common across the samples, which play a role aminoacyl-tRNA biosynthesis (Yao and Fox, 2013). The cell signalling mediators EEF2, FLNA and PKM were also found in common, with PKM playing a role in glycolysis, while, EEF2 is a GTPase that mediates kinase binding activity in cellular differentiation (Nilsson and Nygård, 1995). FLNA on the other hand plays a role in MAPK signalling through actin cytoskeletal activity (Scott *et al.*, 2006).

In terms of sample LH2, ribosomal proteins RPS6, RPS16 and RPL27 were also significantly dysregulated, which are part of the 40S small ribosomal subunit (RPS6 and RPS16) and the 60S ribosomal subunit (RPL27) (Table 17). We also found EIF3C i.e. eukaryotic translation initiation factor 3 subunit C, which along with RPS6 and RPS16 are components of the eukaryotic initiation phase of eukaryotic translation (Jackson, Hellen and Pestova, 2010). Additionally, HSPA5, HSPA8 and PDIA3 were also dysregulated in sample LH2 and they play a role in protein processing in the ER, while, HSPA5 is an ATPase that plays a role in the protein unfolding response (J. Wang *et al.*, 2016). HSPA8 has also been implicated in MAPK signalling and PDIA3 plays a role in antigen processing and presentation in the inflammatory response (Zhang, Wang and Lü, 2014; Zhuang *et al.*, 2015). Some mediators of cellular component organization were also dysregulated including, calcium-dependent ANXA2 which is an RNA binding protein that plays a role in the cytoskeleton (Filipenko and Waisman, 2001; Filipenko *et al.*, 2004; Rescher *et al.*, 2008).

In terms of sample LH3, like LH2, the proteins that were significantly dysregulated played a role in translation-related activities. We found RPS10 which is a component of the 40S ribosomal subunit and the eukaryotic translation initiation factor 3 subunit B (EIF3B). Additionally, DDX5 and SYNCRIP were also dysregulated which are part of the spliceosome and display ATPase and CRD-mediated mRNA stability activity, respectively (Lin *et al.*, 2005; Weidensdorfer *et al.*, 2009). TUBB4B was also

dysregulated which is a component of the cytoskeleton and is implicated in GTPase activity (Isarangkul *et al.*, 2015). Significantly, proteins that were found to be significantly differentially expressed in one sample versus another were all represented in the other sample, with slightly different but correlated H/L ratios.

Table 17: Details on significantly differentially expressed proteins following DCUN1D1 knockdown

LH2						LH3				
Gene names	**Log_2ratio H/L normalized**	**Score**	**Unique peptides**	**Mol. weight [kDa]**		**Gene names**	**Log_2ratio H/L normalized**	**Score**	**Unique peptides**	**Mol. weight [kDa]**
CCT3	-0,58262	72,371	10	60,533		SYNCRIP	-2,58823	36,491	5	69,602
TCP1	-0,574777	67,936	7	60,343		YARS	-1,50332	19,713	3	59,143
EIF2S1	-0,569458	31,17	4	28,865		EIF2S1	-0,750033	31,17	4	28,865
NAA15	-0,466776	17,742	3	101,2		CAD	-0,646632	34,614	3	236,02
PHGDH	-0,373552	50,289	6	45,528		EIF3B	-0,564883	33,541	5	92,48
RPL27	-0,320144	36,012	4	15,798		TCP1	-0,522965	67,936	7	60,343
YARS	-0,308877	19,713	3	59,143		CCT3	-0,477522	72,371	10	60,533
SLC25A5	-0,300413	56,327	7	32,852		AARS	-0,408704	30,771	5	106,81
TRIM28	-0,277132	25,533	4	88,549		EEF2	0,521553	323,31	18	95,337
RPS6	-0,243797	91,042	4	28,68		TUBB4B	0,521754	44,432	2	49,83
AARS	-0,223542	30,771	5	106,81		SRSF2	0,523863	28,006	2	15,156
SHMT2	-0,215781	50,668	4	55,992		RPS10	0,622743	38,65	3	18,898
CCT8	-0,213236	58,234	9	59,62		FLNA	0,631523	125,88	16	278,22
RPS16	-0,185967	56,575	6	16,445		DDX5	0,770618	56,362	6	69,086
HSP90B1	0,357045	108,06	8	92,468		NSFL1C	0,855511	32,882	5	40,572
TUFM	0,36446	13,566	2	49,541		PKM	0,856229	150,14	9	57,936
HSPA5	0,370276	149,12	9	72,332		HNRNPA2B1	0,936817	16,674	2	37,429
ANXA2	0,379843	240,01	11	38,604						
HSPA8	0,40021	323,31	15	70,897						
TLN1	0,40174	51,663	6	269,76						
MYL6	0,410341	34,046	4	16,29						
SRSF2	0,433066	28,006	2	15,156						
NSFL1C	0,444137	32,882	5	40,572						
GOT1	0,462366	25,821	3	46,247						
PDHA1	0,463727	12,989	2	43,295						
P4HB	0,527671	104,88	2	52,502						
EEF2	0,545672	323,31	18	95,337						
ATP1A1	0,551295	105,4	8	112,89						
HNRNPA2B1	0,580531	16,674	2	37,429						
FLNA	0,601792	125,88	16	278,22						
EIF3C	0,615322	42,836	6	105,34						
PKM	0,786178	150,14	9	57,936						
PDIA3	0,838589	51,802	8	56,782						

3.5.2.3 Determining the associations between the significantly differentially expressed proteins

We proceeded with the analysis by combining the list of significantly differentially expressed proteins and searched STRING for associations in the final protein list (Figure 39). We provide STRING output data as a function of the default settings as well as a representation of the data based on "confidence", with the default setting of minimum required interaction score set to 0.400 i.e. medium confidence.

We observed one major hub of interconnected associations with differing strengths of associations based on our analysis. Interestingly, we observed high-confidence associations in the top left-hand corner of the hub consisting of RPS10, RPL27, RPS16, EEF2, EIF2S1, RPS6, EIF3C and EIF3B. We also observed high-confidence associations consisting of SRSF2, DDX5, HNRNPA2B1, HSPA5 intersecting at HSPA8, all of which were components of the spliceosome. These observations highlight the transcription/translation-related properties reflected in the proteins significantly differentially expressed following DCUN1D1 knockdown in PCa. Analysing the associations of the hub from top to bottom reflected the links between the 43S preinitiation complex, the ribosome, ribonucleoproteins, the centrosome and the spliceosome. The proteins involved in processing in the ER, appear to the top right-hand corner of the associations. Taking into consideration the role of DCUN1D1 in protein degradation, the above highlights the dysregulation of translation-related activities following the blockage of DCUN1D1 in DU145 PCa cells.

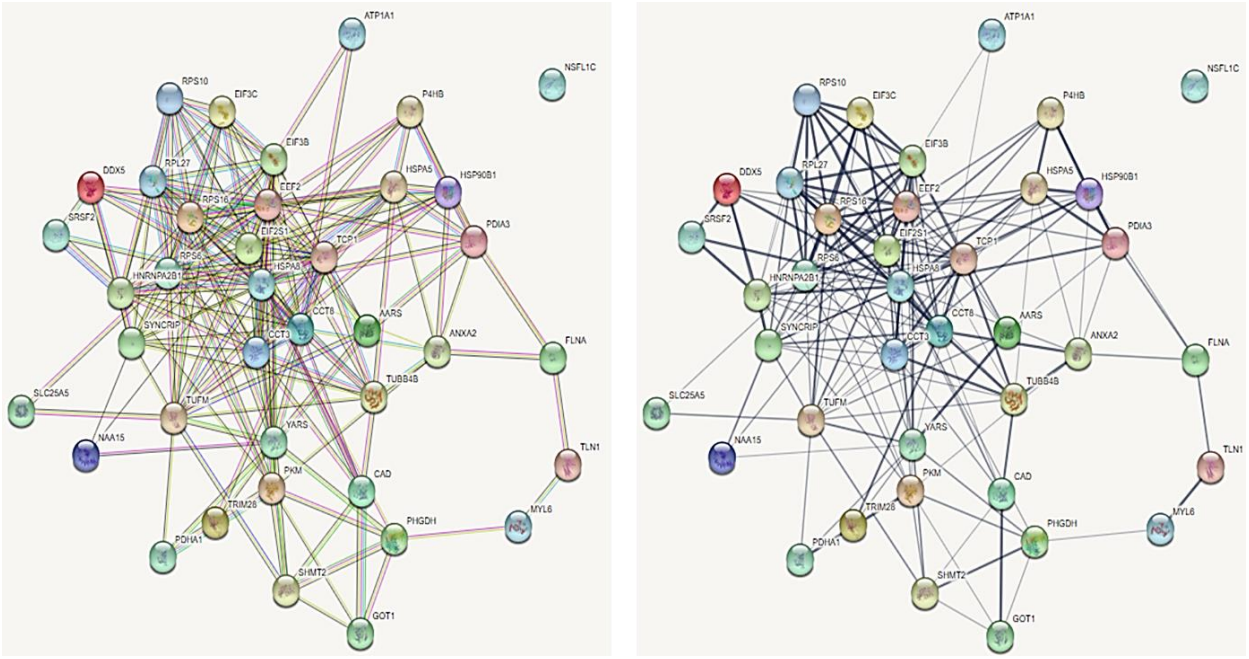


Figure 39. Showing STRING output associations of significantly differentiated proteins using all the STRING selections (top) and based on the "confidence" setting (bottom).

3.6 Discussion

We performed knockdown of DCUN1D1 in DU145 PCa cell lines and found a list of proteins which were differentially expressed as identified by SILAC quantitative proteomics analysis. We observed 103 proteins which were identified in both replicate samples and performed overall analysis on them. The analysis was performed with regards to bioinformatics-classified molecular properties following knockdown of DCUN1D1 and the mechanistic understanding gained. Taking into consideration the dysregulation of these properties in opposition of the known oncogenic role of DCUN1D1 in PCa. The proteins observed classified into cell components, organelles and protein-containing complexes and the molecular functions implicated were binding, catalytic activity and structure molecule activity. This points to the implication of cellular components particularly related to organelles and protein complexes that are involved in binding, catalytical activity and playing a role as structural molecules within the cell. Additionally, the following biological processes were dysregulated: biological regulation, cellular component organization/biogenesis, cellular processes, localization, metabolic processes and response to stimulus. Suggesting that DCUN1D1 knockdown leads to implications on how the cell mediates component organization/biogenesis, biological regulation and processing of the cell, leading to implications on how the cell mediates metabolic processes and responds to stimulus. This is interesting in the context of current knowledge of DCUN1D1, as reviewed in Chapter 1, whose role in the neddylation pathway and UPP is mediated through protein complexes and is implicated in signalling through the regulation of the Hedgehog signalling pathway (Sarkaria *et al.*, 2006; X. Yang *et al.*, 2007; Kim *et al.*, 2008; G. Huang *et al.*, 2011; Heir *et al.*, 2013; Keuss *et al.*, 2016).

Additionally, the pathways implicated following the current analysis were: ATP synthesis, apoptosis signalling, cytoskeletal regulation of Rho GTPase signalling, FGF signalling, glycolysis, Huntington's disease, inflammation mediated by chemokine and cytokine signalling, integrin signalling, nicotinic acetylcholine receptor signalling, Parkinson's disease, pyruvate metabolism, serine glycine biosynthesis, TCA cycle, ubiquitin proteasome pathway, WNT signalling and the p53 pathway. Interestingly, DCUN1D1 has a C terminus UBA domain which is found in proteins involved in the UPP, DNA excision repair, cell signalling via kinases and in proteins with elongation factor binding domains and these encompass the above-mentioned activities (L. Chen *et al.*, 2001; Hartmann-Petersen *et al.*, 2003; Myung, Kim and Crews, 2008; Huang *et al.*, 2015; Wu *et al.*, 2015; Wang *et al.*, 2018). It would be interesting to test the role of the DCUN1D1 C terminus in the pathways identified above, possibly through dominant-negative mutant studies.

Furthermore, we found ATP-synthesis where, ATP has been shown to play a role in multiple of the processes identified and plays a key role in the 26S proteasome and in chromatin remodelling in the modulation of transcriptional activities (Vignali *et al.*, 2000; Peth, Uchiki and Goldberg, 2011). Additionally, similar to the pathways classified here, metabolic and inflammatory pathways were implicated following IPMS analysis of DCUN1D1 pulldown in HEK293TT cells, as described in Chapter 2 above. Chemokine and cytokine signalling were identified in the form of T cell and B cell receptor signalling, however, pathways related to lipid metabolism were identified as opposed to the glycolytic metabolic pathways classified here. We also found HNRNPU and ACTB in both experimental approaches highlighting their association with DCUN1D1 and further implicating the previously linked WNT signalling pathway through ACTB. This suggests the implication of some of the regulatory pathways of the cell, metabolism as well as inflammatory responses. In terms of its role in PCa and potentially in its general mechanism of action.

Interestingly, a number of the "Hallmarks of Cancer", were implicated in the pathways, such as, proliferative signalling e.g. p53 signalling, cell death e.g. apoptosis signalling, inflammatory responses e.g. inflammation mediated chemokine and cytokine signalling and cellular energetics e.g glycolysis, pyruvate metabolism and the TCA cycle. Additionally, we identified disease pathways such as Huntington's disease and Parkinson's disease, implicating DCUN1D1 in these pathways and adding evidence to the link between DCUN1D1 and neurodegenerative diseases, as shown in frontal lobar degeneration (Villa *et al.*, 2009). Furthermore, we observed that proteins identified following knockdown of DCUN1D1 showed minor classification to developmental processes and DCUN1D1 has been shown to play a role in development in model organisms (G. Huang *et al.*, 2011).

Significantly, of the 103 proteins observed in this study, we found several known substrates of neddylation in the form of ribosomal proteins. Seventeen of which were identified in this study, with 5 from the 60S large ribosomal subunit and 12 from the 40S small ribosomal subunit. Among these, we identified RPS3, RPS6, RPS8, RPS13, RPS14, RPS16, RPS20, RPL7a, RPL13, RPL27 and RPL30 which were identified as neddylation substrates in the study by Xirodimas et al., 2008. Some of these were significantly downregulated in sample LH2 including RPS6, RPS16 and RPL27. Additionally, RPS6 was identified following IPMS analysis of DCUN1D1 pulldown in sample 1D. Although this study involves knockdown of DCUN1D1, these proteins may be been linked to DCUN1D1 due to their implication in changes to DCUN1D1 expression. However, further experiments would be required to test the functional relationship of these proteins with DCUN1D1. Interestingly, studies have implicated UPP-mediated protein degradation in the regulation of dysfunctional ribosomal subunits (Hinnebusch, 2009). We understand DCUN1D1 to play a role in neddylation/ubiquitination and here, we identified RACK1, which plays a role in the ribosomal quality control pathway initiated by the ubiquitination of a subset of 40S ribosomal subunits, when ribosomes stall during translation (Adams, Ron and Kiely, 2011; Sundaramoorthy *et al.*, 2017). Furthermore, studies have shown the active role of ubiquitin ligases in the degradation of ribosomal subunits during DNA repair, involving RTT101 (cullin 8) and HRT1 (RING finger protein) of the *S. cerevisiae* (Hinnebusch, 2009).

In terms of the list of proteins significantly dysregulated following knockdown of DCUN1D1 in DU145 PCa cell lines, we found 33 proteins in sample LH2 with 14 downregulated and 19 upregulated. Additionally, 17 proteins were significantly differentially expressed in sample LH3 with 8 downregulated, 9 upregulated and we highlighted 11 significantly dysregulated proteins which were found in common between the replicate samples. We observed limited overlap between the samples, however, this could be attributed to the limitation of MS-based proteomics analysis in terms of run-to-run deviations in peptide fragmentation, detection and identification patterns (Tabb *et al.*, 2010). However, the identified proteins associated with the 43S preinitiation complex, the ribosome, ribonucleoproteins, proteins involved in processing in the ER, the centrosome and the spliceosome. Suggesting the dysregulation of translation-related and protein-processing activities.

Specifically, we found proteins implicated in the centrosome, tRNA biosynthesis and eukaryotic initiation factor 2, which were mostly downregulated in both replicates with contradictory evidence as to the expression status of components of eukaryotic initiation factor 3. During translation, methionine activated tRNA, eukaryotic initiation factor 2 and GTP form a ternary complex as part of the eukaryotic translation initiation phase and bind eukaryotic initiation factor 3 and the 40S small ribosomal subunit (Jackson, Hellen and Pestova, 2010). Taking into consideration the role of DCUN1D1 in protein degradation, the above suggests the dysregulation of translation associated activities following knockdown of DCUN1D1, but it would be necessary to test the status of the whole protein complex following dysregulation of DCUN1D1 to determine the impact on translation. Identification of dysregulation of some of the subunits of the 40S small ribosome and the eukaryotic initiation factors

does not necessarily display a change in catalytical activity and some of the subunits were upregulated as observed with RPS10 in sample LH3. However, several proteins involved in the processing of proteins in the ER were also significantly dysregulated and the ER plays a key role in protein synthesis (Biquan and Amy S., 2013). We also observed upregulation of proteins implicated in the spliceosome and cytoskeletal structure, further suggesting dysregulation in transcriptional/translational machinery.

Interestingly, when performing detailed analysis of the protein lists generated, we did not find any of the cullin family of proteins or classical components of the neddylation pathway. This could be due to the subsequent effect of DCUN1D1 knockdown on these components, where knockdown inhibited their expression. However, we found UBA1, the ubiquitin activating enzyme of the ubiquitination pathway, providing further evidence for the association of UBA1 with the neddylation pathway as demonstrated previously by Leidecker et al., 2012. We also identified PRS7, the 26S proteasome regulatory subunit 7.

The remainder of the significantly differentially expressed proteins included several components of the centrosome and the CCT complex including: CCT3, CCT8 and TCP1. TCP1 has been demonstrated to be involved in the folding of 10% of the cell proteome and has been found to be upregulated in prostate tissue samples using tissue microarray analysis (Leitner *et al.*, 2012). It has also been shown to be inhibited by CT20p, emerging as a promising approach for the treatment of small cell lung cancer (Leitner *et al.*, 2012). Interestingly, the centrosome has been found to play a role in genome instability in cancer (Leitner *et al.*, 2012). Furthermore, the CCT complex has been found to interact with and regulate proteins such as cullin 3, FUS, NEDD1, p53, CDK2 and CDK5 (Aswathy, Pullepu and Kabir, 2016). Significantly, we found associations between DCUN1D1 and cullin 3 and FUS following IPMS analysis of DCUN1D1 pulldown products in Chapter 2 above.

EIF2S1 on the other hand has also been implicated in PCa due to its role in the ER stress response mediated by tannic acid treatment of C4-2, DU145 and PC3 cells, while, phosphorylation of EIF2S1 at serine 51 was implicated in leukotriene receptor antagonist inhibition of PCa through decreased HIF1α translation (Nagesh *et al.*, 2018; Tang *et al.*, 2018). Interestingly, HIF1α has been linked to DCUN1D1 through cullin 2 mediated neddylation (Heir *et al.*, 2013). Furthermore, FLNA is an actin cytoskeleton component that has been implicated in MAPK signalling and has been identified as a PCa biomarker through differential expression in LnCAP, DU145, PC3 cells and in proteomics analysis of clinical samples, with changes observed in its nuclear versus cytoplasmic localisation during metastasis (Ruddat *et al.*, 2005; Bedolla *et al.*, 2010; Narain *et al.*, 2017).

We also identified HNRNPA2B1, SRSF2, DDX5 and SYNCRIP which are components of the spliceosome and were dysregulated following DCUN1D1 knockdown in DU145 PCa cells. RNA splicing is frequently dysregulated during tumour formation and cancer progression through various mechanisms (Anczukow and Krainer, 2016). These alterations can be due to changes in splicing of cancer-related genes or can occur to regulators of splicing such as the components of splicing machinery (Anczukow and Krainer, 2016). Splicing factors have also been shown to play a role in PCa, implicating mainly the AR pathway (Takayama, 2019). Interestingly, changes in SRSF2 have been widely demonstrated through mutations in myeloid and lymphoid lineage tumours, however, somatic mutations in SRSF2 have been identified in 0.4% of solid prostate tumours while alterations in the splicing pathway have been identified in 4% of prostate tumours (Je *et al.*, 2013; Armenia *et al.*, 2018). Moreover, HNRNPA2B1 has been shown to be upregulated in PCa and to regulate the WNT pathway component, β-catenin, a pathway we identified to be dysregulated through ACTB here and in Chapter 2 above (Stockley *et al.*, 2014).

Therefore, we have identified several differentially expressed proteins which have been demonstrated to be aberrantly expressed in PCa and are potential biomarkers for PCa treatment. Some have been shown to interact with proteins identified in the previous chapter such as cullin 3 and FUS, which have themselves been shown to be cancer driver genes. Overall, following SILAC quantitative proteomics analysis of DCUN1D1 knockdown in DU145 PCa cells, we describe dysregulation in cellular components, organelles and protein-complexes (Figure 40). We also describe dysregulation in molecular functions related to binding, catalytic activity and structure molecule activity. Of the significantly differentially expressed proteins, we found ribosomal proteins as well as proteins implicated in protein folding, ER stress, protein-translation and signalling pathways such as Rho GTPase, MAPK signalling and the WNT/β-catenin pathway. We also observed classification of our identified proteins into energy-related metabolic pathways, inflammatory responses as well as minor links to the ubiquitin proteasome pathway, namely through UBA1.

This describes the mechanism of action of DCUN1D1 in PCa following SILAC quantitative proteomics analysis but also provides further insights into the functions associated with DCUN1D1 including protein translation, protein folding and ER stress.

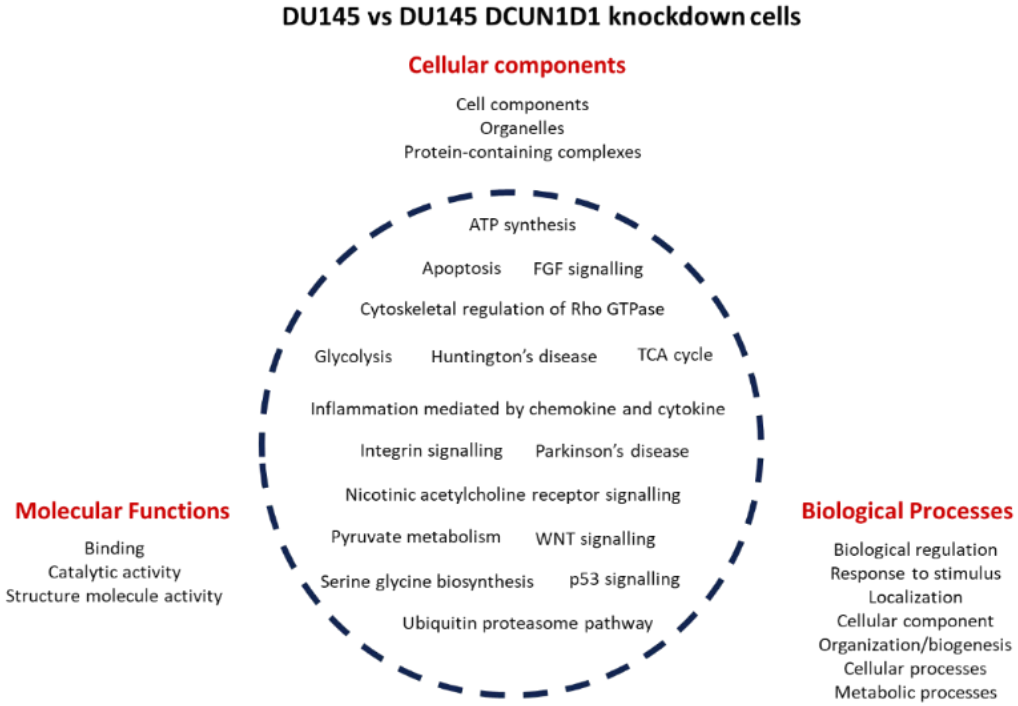


Figure 40. Showing molecular properties identified following SILAC quantitative analysis and the proposed mechanism of action of DCUN1D1 in prostate cancer.

Chapter 4: Advanced Connectivity Map analysis for identifying DCUN1D1 inhibitors using a proteomics approach

4.1 Introduction

The Connectivity Map (CMap) database was developed by the Broad Institute in 2006 (Lamb *et al.*, 2006). It was built around the concept that alterations due to genetic or pharmacological perturbations can have commonality through the gene expression signatures they impact. It functions as an intersection point between genes, drugs and diseases based on the connectivity of gene expression signatures and uses a systems-biology approach to connect them within one platform (Lamb *et al.*, 2006). Initially, it was based on the treatment of a variety of cancer cell lines with different drugs and molecules at different concentrations (including US FDA-approved drugs) and the generation of gene signatures by microarray analysis (Lamb *et al.*, 2006). The gene signatures were identifiable by Affymetrix Genechip identification numbers, generating thousands of profiles (Lamb *et al.*, 2006). Thereby linking the questions of which gene expression profile leads to the development of a disease, which drugs could be used for its treatment and allowing the identification of the mechanisms of action of certain drugs and protein targets (Lamb *et al.*, 2006; Lamb, 2007).

The platform has since developed to the current L1000 assay platform consisting of gene expression signatures generated from 978 transcripts and 80 control transcripts that represent the transcriptional state of the cell (Subramanian *et al.*, 2017). The perturbations have expanded to include genetic alterations due to knockdown or overexpression and to include treatments of cells using small molecule drugs, tool compounds and screening library compounds (Subramanian *et al.*, 2017). Resulting in the database containing over 1 million profiles represented, as part of the NIH Library of Integrated Network-Based Cellular Signatures (LINCS) (Subramanian *et al.*, 2017). This allows for connections to be made to previously unconnected proteins working within the same pathway and to connect small molecules to cellular targets. Additionally, connections can be made between structurally dissimilar small molecules with similar signatures, based on the physiological effect they induce in the cell. Furthermore, it can function as a starting point for drug discovery and drug safety profiling (Subramanian *et al.*, 2017). Significantly, this CMap version permits platform-independent analysis allowing for comparison of gene expression signatures generated from experiments other than microarray analysis. The technology has been used in studies covering a range of diseases including diabetes, skeletal muscle atrophy, inflammatory bowel diseases and cancer (Wang *et al.*, 2008; Saito *et al.*, 2009; Dudley *et al.*, 2011; Stockwell *et al.*, 2012; Dyle *et al.*, 2014; Zhang *et al.*, 2015).

4.2 Hypothesis

Inhibition of protein degradation pathways is an emerging approach to cancer treatment where inhibition of the 26S proteasome and the neddylation activating enzyme (NAE) have been implicated. Proteasome inhibitors are undergoing extensive analysis in clinical trials and have demonstrated potent anti-cancer activity, particularly in multiple myelomas (Yang, Zonder and Dou, 2009; Yong *et al.*, 2018). Additionally, the NAE inhibitor MLN4924 has shown potent activity in a variety of cancers including acute myelogenous leukemia, diffuse large B-cell lymphoma, clear cell renal carcinoma and head and neck squamous carcinoma (Milhollen *et al.*, 2010; Knorr *et al.*, 2015; Tong *et al.*, 2017; Vanderdys *et al.*, 2018). Due to the success of these approaches we postulate that inhibition of DCUN1D1, an E3 ligase of neddylation, may offer targeting of the above pathways in a more specific manner with potentially reduced side effects. This is because DCUN1D1 is activated downstream of the NAE and plays a role in the ubiquitination pathway through the posttranslational modification of the cullin family of proteins through neddylation. Moreover, studies performed previously in our lab involving RNA interference knockdown of DCUN1D1 showed inhibition of PCa *in vitro* and *in vivo* (Vava and Zerbini, 2014). We also identified two potential inhibitors of DCUN1D1 that impede PCa through the reduction of DCUN1D1 mRNA and protein (Vava and Zerbini, 2014). Having identified these inhibitors using a transcriptomics approach followed by CMap database analysis, we postulated that CMap analysis using a proteomics generated signature may expand the scope of drugs identified, include more specific inhibitors of DCUN1D1 or identify drugs that inhibit DCUN1D1 based on an alternative mechanism of action.

4.3 Aims and Objectives

The objective of this study is to perform advanced CMap database analysis for the identification of potential inhibitors of DCUN1D1 from signatures obtained following the analysis of the mechanism of action of DCUN1D1 using SILAC quantitative proteomics.

Aim:

Advanced connectivity map analysis using the DCUN1D1 knockdown cell line protein signature.

4.4 Materials and Methods

We used the CMap database version 1.1.1.42, data version 1.1.1.2 that was available at https://clue.io. We chose the "query app" and queried the proteomics signatures obtained from SILAC quantitative proteomics analysis. The database has over 1 million profiles representing genetic and small molecule perturbations, with signatures generated from an assay platform consisting of 1 058 probes, for 978 landmark transcripts and 80 control transcripts. It uses landmark transcripts which were selected using a data-driven approach, based on their representation in >12 000 Affymetrix gene expression profiles deposited in the Gene Expression Omnibus (GEO) (Subramanian *et al.*, 2017). The platform's gene-profile analysis has been found to be comparable to the gold standard for gene expression analysis, i.e. RNA-seq analysis. Additionally, the landmarks did not display functional enrichment to any protein class or display developmental lineage bias (Subramanian *et al.*, 2017).

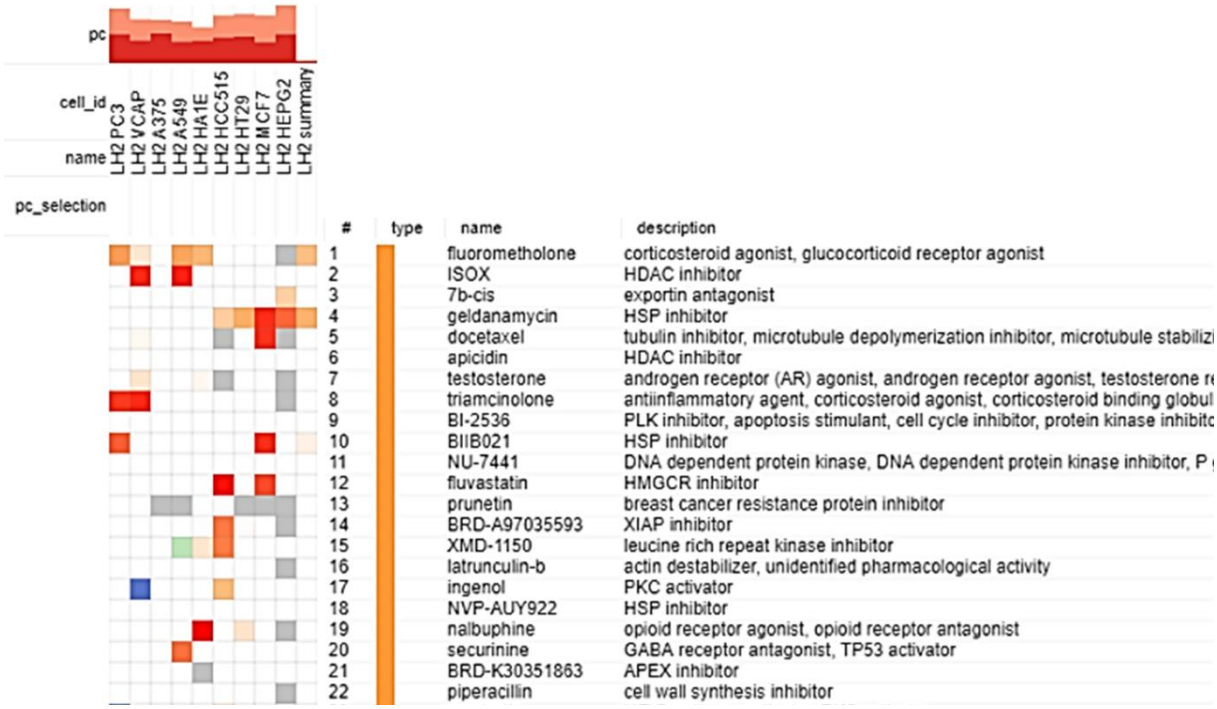
We knocked down DCUN1D1 in DU145 PCa cell lines and queried the proteomics-generated signature against all the signatures in the database. We used the significantly differentially expressed protein lists from our dataset as identified by gene name and inputted them separately into the clue.io CMap platform using default settings. The up and downregulated proteins from samples LH2 and LH3 were queried separately and included 33 and 17 gene names, respectively. The analysis was performed using genes identified as valid on the CMap/Clue app which included those identifiable by valid HUGO symbols or Entrez IDs. They also include the best inferred genes (BING), which include those present in the landmark dataset and genes well-inferred by the CMap database. The data is represented by the connectivity scores, reporting on the similarity and opposition based on closeness to 100 or -100. It was calculated incorporating the p value as determined using Kolmogorov-Smirnov enrichment statistic, multiple hypothesis test adjustment using the FDR and comparison of the observed enrichment score relative to database measurements. We were looking for functional gene signatures that have a strong positive connection with the input signatures because DCUN1D1 has been demonstrated to be an oncogene in some cancers and the interest would lie in drug signatures which could induce similar physiological effect as knockdown of DCUN1D1, possibly by targeting DCUN1D1.

4.5 Results

We performed CMap analysis using the proteins significantly differentially expressed following quantitative SILAC proteomics analysis of DCUN1D1 knockdown in DU145 PCa cell lines. We found 33 proteins to be significantly differentially expressed in sample LH2 with 14 downregulated and 19 upregulated as well as 17 proteins which were significantly differentially expressed in sample LH3, with 8 downregulated and 9 upregulated. We queried the samples in the CMap database using the "query app", according to gene name, with a final number of 14 downregulated and 17 upregulated proteins in sample LH2, which were identified by the database as valid genes for analysis as part of the database BING space. In terms of sample LH3, we had 9 upregulated valid gene name identifications and 8 downregulated valid gene name identifications, which were below the threshold of 10 required for CMap analysis. We therefore included the protein/gene name identification that was nearest to the significant differential expression cut-off (1 standard deviation below the mean), which was P4HB in the upregulated list as well as CDK1, because one of the gene name identifications in the upregulated list was not found within the BING space. Additionally, we included NAA15 and PHGDH in the downregulated list and computed the CMap analysis.

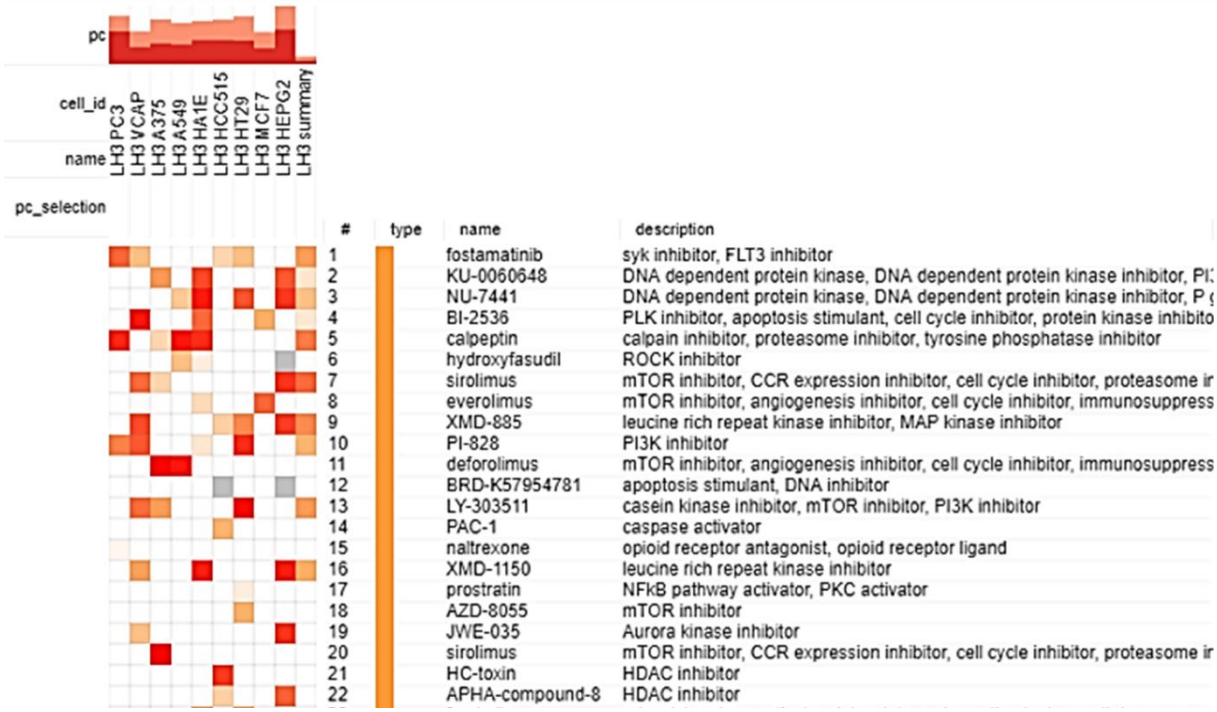
We obtained the query results in the form of a heatmap and detailed tabulated list. We initially performed the analysis by viewing the CMap signatures that persist across the cell lines represented in the database (Figure 41). It is a perturbagen-centric summarization of the data across the 9 cell lines examined in the database, based on the connectivity score of each perturbagen per cell line and summarized across the cell lines. The connectivity score is a measure between -100 and 100, with CMap signatures close to 100 showing strong positive connectivity and those close to -100 showing strong negative connectivity or opposition. We obtained outputs based on the whole reference dataset (Touchstone) which included all the perturbagens (knockdown, overexpression and compounds) and focused our analysis on the outputs obtained based on the compound perturbagen type. Significantly, of the top 22 compounds observed, we found the following compounds in common between the samples: BI-2536 (PLK inhibitor/apoptosis stimulant/cell cycle inhibitor), NU-7441 (DNA dependent protein kinase inhibitor) and XMD-1150 (leucine rich repeat kinase inhibitor). Interestingly, in sample LH2, we found the androgen receptor agonist testosterone and docetaxel, the tubulin inhibitor and current therapeutic agent for PCa treatment. Additionally, we found calpeptin in sample LH3, which is has been described to be a proteasome inhibitor.

LH2



#	type	name	description
1		fluorometholone	corticosteroid agonist, glucocorticoid receptor agonist
2		ISOX	HDAC inhibitor
3		7b-cis	exportin antagonist
4		geldanamycin	HSP inhibitor
5		docetaxel	tubulin inhibitor, microtubule depolymerization inhibitor, microtubule stabiliz
6		apicidin	HDAC inhibitor
7		testosterone	androgen receptor (AR) agonist, androgen receptor agonist, testosterone r
8		triamcinolone	antiinflammatory agent, corticosteroid agonist, corticosteroid binding globul
9		BI-2536	PLK inhibitor, apoptosis stimulant, cell cycle inhibitor, protein kinase inhibitc
10		BIIB021	HSP inhibitor
11		NU-7441	DNA dependent protein kinase, DNA dependent protein kinase inhibitor, P
12		fluvastatin	HMGCR inhibitor
13		prunetin	breast cancer resistance protein inhibitor
14		BRD-A97035593	XIAP inhibitor
15		XMD-1150	leucine rich repeat kinase inhibitor
16		latrunculin-b	actin destabilizer, unidentified pharmacological activity
17		ingenol	PKC activator
18		NVP-AUY922	HSP inhibitor
19		nalbuphine	opioid receptor agonist, opioid receptor antagonist
20		securinine	GABA receptor antagonist, TP53 activator
21		BRD-K30351863	APEX inhibitor
22		piperacillin	cell wall synthesis inhibitor

LH3



#	type	name	description
1		fostamatinib	syk inhibitor, FLT3 inhibitor
2		KU-0060648	DNA dependent protein kinase, DNA dependent protein kinase inhibitor, PI
3		NU-7441	DNA dependent protein kinase, DNA dependent protein kinase inhibitor, P
4		BI-2536	PLK inhibitor, apoptosis stimulant, cell cycle inhibitor, protein kinase inhibito
5		calpeptin	calpain inhibitor, proteasome inhibitor, tyrosine phosphatase inhibitor
6		hydroxyfasudil	ROCK inhibitor
7		sirolimus	mTOR inhibitor, CCR expression inhibitor, cell cycle inhibitor, proteasome ir
8		everolimus	mTOR inhibitor, angiogenesis inhibitor, cell cycle inhibitor, immunosuppress
9		XMD-885	leucine rich repeat kinase inhibitor, MAP kinase inhibitor
10		PI-828	PI3K inhibitor
11		deforolimus	mTOR inhibitor, angiogenesis inhibitor, cell cycle inhibitor, immunosuppress
12		BRD-K57954781	apoptosis stimulant, DNA inhibitor
13		LY-303511	casein kinase inhibitor, mTOR inhibitor, PI3K inhibitor
14		PAC-1	caspase activator
15		naltrexone	opioid receptor antagonist, opioid receptor ligand
16		XMD-1150	leucine rich repeat kinase inhibitor
17		prostratin	NFkB pathway activator, PKC activator
18		AZD-8055	mTOR inhibitor
19		JWE-035	Aurora kinase inhibitor
20		sirolimus	mTOR inhibitor, CCR expression inhibitor, cell cycle inhibitor, proteasome ir
21		HC-toxin	HDAC inhibitor
22		APHA-compound-8	HDAC inhibitor

Figure 41. Showing the top ranked CMap compound signatures based on connectivity score. Heatmaps showing the 9 core cell lines of the reference dataset and the rank-ordered list of compounds for sample LH2 *(top)* and sample LH3 *(bottom)*.

We then performed further analysis of the dataset by selecting for the outputs based on the PCa cell lines within the 9 core cell lines in the CMap database whose cellular signatures were connected to that of the DCUN1D1 differential expression signature (Table 18 and 19). We obtained outputs for both samples and observed that the compounds in common between sample LH2 and LH3 with high connectivity scores (>90) in the PC3 cell line CMap signatures were: talniflumate (cyclooxygenase inhibitor), atorvastatin (HMGCR inhibitor), GR-135531 (melatonin receptor agonist), W-9 (calmodulin antagonist), RX-821002 (adrenergic receptor antagonist) and palmitoylethanolamide (cannabinoid receptor agonist). Additionally, we found ISOX (HDAC inhibitor), ergocryptine (dopamine agonist), XMD-885 (leucine rich repeat kinase inhibitor), mestanolone (androgenic steroid), XMD-892 (MAP kinase inhibitor), carmoxirole (dopamine receptor agonist), and VX-222 (HCV inhibitor) in common in the VCap cell line CMap signatures. Significantly, we found podophyllotoxin and thapsigargin to score highly in the PC3 cell line signature, in sample LH2 and LH3, respectively. These compounds were found previously following CMap analysis of knockdown of DCUN1D1 in PCa and DNA microarray analysis (Vava and Zerbini, 2014). Thapsigargin was found to decrease mRNA expression of DCUN1D1, while, podophyllotoxin was found to decrease mRNA and protein expression of DCUN1D1 and to have an additive inhibitory effect on PCa proliferation in combination with monensin (Vava and Zerbini, 2014).

Lastly, we analysed the data based on perturbagen class (Figure 42). This was defined as a “group of compounds that have the same annotated mechanism of action or genetic perturbagens that are part of the same gene family or are targeted by the same compound”. Of the signatures with the highest connectivity scores we found the following perturbagen classes: leucine rich repeat kinase inhibitor, PKC activator, DNA dependent protein kinase inhibitor, bromodomain inhibitor, IGF-1 inhibitor, bile acid and HMGCR inhibitor in common across the samples.

Table 18. Showing the top ranked CMap compound signatures based on connectivity score (> 90) in PC3 in sample LH2 and LH3.

LH2 - PC3					LH3 - PC3			
Rank	**Score**	**Name**	**Description**		**Rank**	**Score**	**Name**	**Description**
20	99.7	HSP90-inhibitor	HSP inhibitor		16	99.51	RX-821002	Adrenergic receptor antagonist
26	99.42	Talniflumate	Cyclooxygenase inhibitor		26	99.24	Talniflumate	Cyclooxygenase inhibitor
51	98.06	Danusertib	Aurora kinase inhibitor		32	99.09	W-9	Calmodulin antagonist
61	97.25	BIIB021	HSP inhibitor		33	98.94	Phenanthridone	PARP inhibitor
71	96.82	K3644	Kinesin-like spindle protein inhibitor		38	98.83	Cycloserine	Bacterial cell wall synthesis inhibitor
79	96.41	Metoclopramide	Dopamine receptor antagonist		48	98.31	XAV-939	Tankyrase inhibitor
84	96.23	ST-91	Adrenergic receptor agonist		50	98.26	Olaparib	PARP inhibitor
93	95.88	Atorvastatin	HMGCR inhibitor		60	97.99	GR-135531	Melatonin receptor agonist
99	95.5	Fluorometholone	Glucocorticoid receptor agonist		77	97.18	Sulmazole	Adenosine receptor antagonist
107	95.24	Zardaverine	Phosphodiesterase inhibitor		78	97.1	Taurocholic-acid	Bile acid
115	94.69	PCO-400	Potassium channel activator		79	97.08	Spectinomycin	Bacterial 30S ribosomal subunit inhibitor
119	94.43	GR-135531	Melatonin receptor agonist		80	97.04	Fostamatinib	SYK inhibitor
129	93.51	Heraclenol	Vitamin K antagonist		82	96.84	NBI-27914	CRF receptor antagonist
130	93.46	PD-123319	Angiotensin receptor antagonist		86	96.49	QL-X-138	MTOR inhibitor
136	93.13	W-9	Calmodulin antagonist		89	96.23	PI-828	PI3K inhibitor
141	92.66	RX-821002	Adrenergic receptor antagonist		98	95.82	LY-288513	CCK receptor antagonist
145	92.22	Podophyllotoxin	Microtubule inhibitor		121	94.18	WYE-354	MTOR inhibitor
147	92.07	PJ-34	PARP inhibitor		122	94.12	PP-1	SRC inhibitor
156	91.51	Hexylresorcinol	Local anesthetic		123	94.1	Methyllycaconitine	Acetylcholine receptor antagonist
164	90.69	Palmitoylethanolamide	Cannabinoid receptor agonist		131	93.77	PCO-400	Potassium channel activator
168	90.33	Antimycin-a	ATP synthase inhibitor		141	93.05	Oxybenzone	Lipase inhibitor
					144	92.92	AMN-082	Glutamate receptor modulator
					148	92.55	Thapsigargin	ATPase inhibitor
					150	92.42	Colforsin	Adenylyl cyclase activator
					151	92.41	Atorvastatin	HMGCR inhibitor
					153	92.3	Givinostat	HDAC inhibitor
					160	91.57	Hispidin	PKC inhibitor
					161	91.56	SC-19220	Prostanoid receptor antagonist
					164	91.38	Forskolin	Adenylyl cyclase activator
					169	90.92	Skatole	Thrombin inhibitor
					170	90.8	GR-113808	Serotonin receptor antagonist
					175	90.43	Terconazole	Sterol demethylase inhibitor
					176	90.4	Oxprenolol	Adrenergic receptor antagonist
					177	90.37	Palmitoylethanolamide	Cannabinoid receptor agonist
					180	90.3	Phosphodiesterase-V-inhibitor-II	Phosphodiesterase inhibitor

Table 19. Showing the top ranked CMap compound signatures based on connectivity score (> 90) in VCap in sample LH2 and LH3.

LH2 - VCap					LH3 - VCap			
Rank	**Score**	**Name**	**Description**		**Rank**	**Score**	**Name**	**Description**
22	98.8	ISOX	HDAC inhibitor		3	99.82	VX-222	HCV inhibitor
25	98.52	Ergocryptine	Dopamine agonist		12	99.39	ISOX	HDAC inhibitor
34	97.78	XMD-885	Leucine rich repeat kinase inhibitor		14	99.28	BI-2536	PLK inhibitor
35	97.76	Mestanolone	Androgenic steroid		17	99.1	Carmoxirole	Dopamine receptor agonist
36	97.75	Methandriol	Androgenic steroid		19	98.95	XMD-892	MAP kinase inhibitor
43	97.48	Carbamazepine	Carboxamide antiepileptic		32	97.83	XMD-885	Leucine rich repeat kinase inhibitor
49	97.25	Norethisterone	Progesterone receptor agonist		35	97.46	PI-828	PI3K inhibitor
50	97.2	Exemestane	Aromatase inhibitor		36	97.4	Ergocryptine	Dopamine agonist
54	97.05	Formestane	Aromatase inhibitor		44	96.85	LY-303511	Casein kinase inhibitor
62	96.65	XMD-892	MAP kinase inhibitor		51	96.08	Epinephrine	carbonic anhydrase activator
82	94.79	Carmoxirole	Dopamine receptor agonist		59	95.41	Androstenedione	Cytochrome P450 inhibitor
83	94.77	Testosterone	Androgen receptor agonist		60	95.35	XMD-1150	Leucine rich repeat kinase inhibitor
84	94.68	VX-222	HCV inhibitor		61	95.3	Chlorambucil	DNA inhibitor
85	94.68	ZM-39923	JAK inhibitor		62	95.25	Tretinoin	Retinoid receptor agonist
89	94.58	Skatole	Thrombin inhibitor		64	95.13	Chromanol	Potassium channel blocker
94	94.25	Noretynodrel	Progestogen hormone		66	94.98	Irinotecan	Topoisomerase inhibitor
96	94.03	Gestrinone	Progesterone receptor antagonist		74	93.92	Fostamatinib	SYK inhibitor
98	93.79	Vorinostat	HDAC inhibitor		78	93.74	JWE-035	Aurora kinase inhibitor
101	93.59	Dovitinib	EGFR inhibitor		84	92.98	Trimethobenzamide	Histamine receptor antagonist
103	93.39	JLK-6	Gamma secretase inhibitor		90	92.39	QS-11	ARFGAP inhibitor
105	93.14	Flubendazole	Tubulin inhibitor		93	92.29	L-690488	Inositol monophosphatase inhibitor
108	92.99	CD-437	Retinoid receptor agonist		108	91.26	Mestanolone	Androgenic steroid
109	92.97	Enobosarm	Androgen receptor modulator		111	91.2	Pyroxamide	HDAC inhibitor
113	92.74	Linifanib	PDGFR receptor inhibitor		113	90.79	CAY-10577	Casein kinase inhibitor
114	92.73	Aminolevulinic-acid	Oxidizing agent		114	90.75	Midazolam	Benzodiazepine receptor agonist
115	92.61	KU-0060648	DNA dependent protein kinase inhibitor		115	90.75	Alprazolam	Benzodiazepine receptor agonist
122	92.12	MNITMT	Lymphocyte inhibitor		120	90.29	r(-)-propylnorapomorphine	Dopamine receptor agonist
124	91.8	Flurofamide	Urease inhibitor		121	90.17	DR-2313	PARP inhibitor
130	91.49	Fluorometholone	Glucocorticoid receptor agonist		122	90.1	Everolimus	MTOR inhibitor
146	90.4	Docetaxel	Tubulin inhibitor					
149	90.16	Norethindrone	Progesterone receptor agonist					

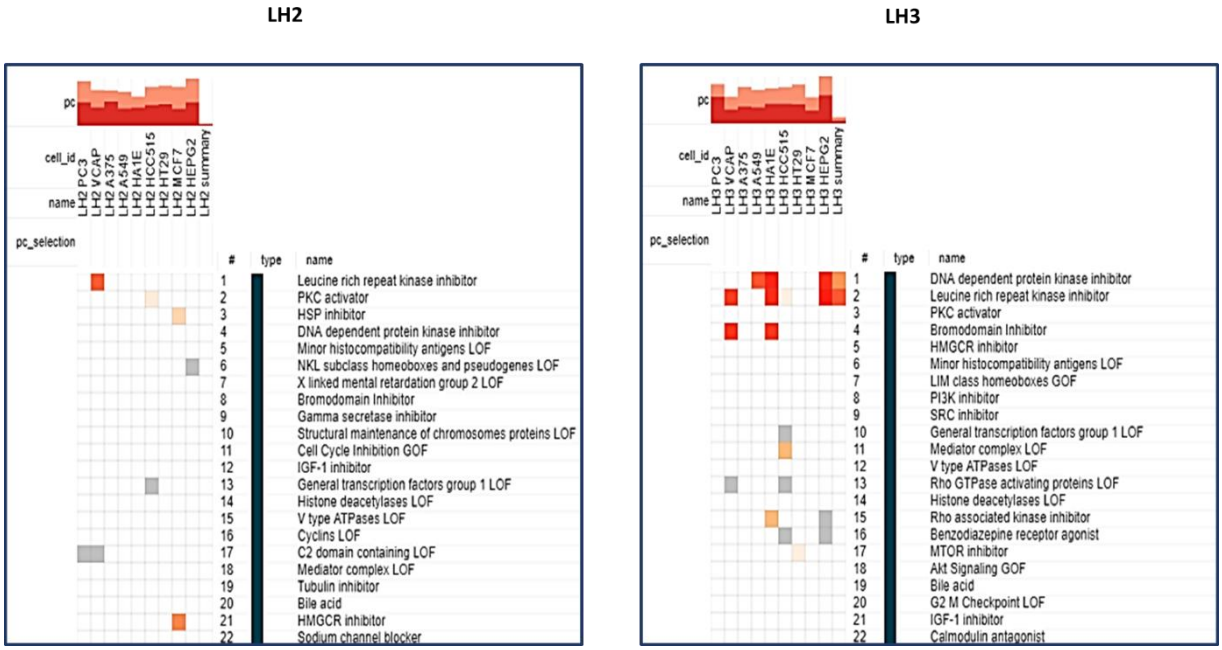


Figure 42. Showing the top ranked CMap signatures based on connectivity score and perturbagen class. Heatmaps showing the 9 core cell lines of the reference dataset and the rank-ordered list of compounds in sample LH2 *(left)* and sample LH3 *(right)*.

4.6 Discussion

The CMap database contains gene expression profiles corresponding to the biological state following treatment of a variety of cell lines with small molecules or genetic perturbagens. We tested the signatures obtained following SILAC quantitative analysis of DCUN1D1 knockdown in DU145 PCa cell lines. When analysing the overall CMap output of the strongly connected signatures that persisted across the 9 core cell lines, we found 3 drugs in common across sample LH2 and LH3, which were BI-2536, NU-7441, and XMD-1150. Although the emphasis of the CMap analysis is the comparisons around similarities in gene expression signatures, we were interested to see the drugs strongly connected to the DCUN1D1 knockdown signature within the PCa cell line data outputs. Analysis of the results in the PCa cell lines found talniflumate, atorvastatin, GR-135531, W-9, RX-821002 and palmitoylethanolamide in common in the PC3 output. We also found ISOX, ergocryptine, XMD-885, mestanolone, XMD-892, carmoxirole and VX-222 in common in the VCap CMap signatures. Moreover, we found the following perturbagen classes in common across the samples and to be strongly connected to the DCUN1D1 knockdown signature: leucine rich repeat kinase inhibitor, PKC activator, DNA dependent protein kinase inhibitor, bromodomain inhibitor, IGF-1 inhibitor, bile acid and HMGCR inhibitor.

Significantly, BI-2536, NU-7441, XMD-1150, XMD-885 and XMD-892 are 5 different kinase inhibitors which were strongly connected to the DCUN1D1 knockdown signature. Interestingly, PhosphoSitePlus® analysis demonstrated two phosphorylation sites (S31 and Y72) in the N terminus of DCUN1D1, however, their contribution to DCUN1D1 functionality and the kinase responsible for this activity has not been determined. In terms of the compounds, BI-2536, is a small molecule inhibitor of polo-like kinase 1 (PLK1) which is a serine/threonine kinase that plays a key regulatory role in mitotic progression and the centrosome cycle (Steegmaier *et al.*, 2007; Cabral *et al.*, 2019). Treatment with BI-2536 was shown to result in mitotic arrest and induce apoptosis, in Hela cells and in NCI-H460 lung tumour mice models (Steegmaier *et al.*, 2007). Additionally, it has been shown to inhibit tumour growth in HCT 116 (colon cancer), BxPC-3 (pancreatic cancer) and A549 (non-small cell lung cancer) xenograft mice models (Steegmaier *et al.*, 2007). Its target PLK1, is upregulated in multiple malignant cells and has been shown to be a prognostic marker in a variety of cancers (Takai *et al.*, 2005; Steegmaier *et al.*, 2007). NU-7441 on the other hand is a potent and selective inhibitor of DNA dependent protein kinase (DNA-PK), which is a nuclear protein kinase that is activated by DNA double strand breaks and plays a role in the detection of DNA damage (Burma and Chen, 2004; Zhao *et al.*, 2006; Ciszewski *et al.*, 2014). NU-7441 has also been shown to improve the chemosensitivity of several breast cancer cell lines and human colon cancer cells in xenograft mice models (Zhao *et al.*, 2006; Ciszewski *et al.*, 2014). Moreover, deficiencies in DNA-PK have been found to increase hypersensitivity to ionising radiation and radio-mimetic drugs (Zhao *et al.*, 2006). Interestingly, the neddylation pathway has been extensively associated with the DNA damage response and this study has found potential linkages between DCUN1D1 and the DNA damage response (Brown and Jackson, 2015).

We also found compounds associated with anti-inflammatory activity and inflammation was one of the pathways dysregulated following IPMS analysis of DCUN1D1 and quantitative SILAC proteomics analysis of DCUN1D1 knockdown. Talniflumate is an anti-inflammatory compound that has been shown to inhibit mucin biosynthesis by targeting core 2 β-1,6 N-acetylglucosaminyltransferase (GCNT3) and to target cyclooxygenase (Vios and Billones, 2015; Rao *et al.*, 2016). It's been shown to increase the survival of cystic fibrosis mice models and is suggested for therapeutic intervention in chronic obstructive pulmonary disease (Donnelly and Rogers, 2003; Walker *et al.*, 2006). Significantly,

treatment with talniflumate has led to decreased cell viability in human pancreatic cancer cells and enhanced the efficacy of the EGFR inhibitor, gefitinib, in pancreatic cancer treatment *in vitro* (Rao *et al.*, 2016). Interestingly, palmitoylethanolamide has also been shown to reduce pain and to have anti-inflammatory activity (Lo Verme *et al.*, 2005). It is an endogenous lipid amide that targets cannabinoid receptors localized to the brain, periphery and within cells of the immune system (Herkenham *et al.*, 1990; Facci *et al.*, 1995; Mackie, 2005). It's also been shown to mediate its anti-inflammatory activity through PPAR-α and following IPMS analysis of DCUN1D1 pulldown, we observed dysregulation of PPAR signalling. Additionally, palmitoylethanolamide in combination with anandamide has demonstrated anti-proliferative activity in breast cancer cells (Di Marzo *et al.*, 2001).

Atorvastatin on the other hand has been shown to reduce the levels of cholesterol in the blood, by lowering the plasma levels of low-density lipoproteins through the inhibition of HMG-CoA reductase (HMGCR) (Baumann *et al.*, 1992; Krause *et al.*, 1993). It's been shown to reduce the risk of cardiovascular diseases and reduce the incidences of strokes (Olsson *et al.*, 2001; de Lemos, Blazing and Wiviott, 2004; Ray *et al.*, 2005; La Rosa and Conti, 2006; Tashkin *et al.*, 2008). Additionally, atorvastatin inhibition of HMGCR has demonstrated anti-cancer activity *in vitro* and *in vivo*, including in metastatic cancer (Ishikawa *et al.*, 2018). Combination studies of atorvastatin and metformin showed strong inhibitory effect on PCa growth *in vitro* by reducing NF-κB activity, decreasing phosphorylation of AKT and led to moderate inhibition of growth *in vivo* (Wang *et al.*, 2017). Clinical trials have explored the potential of atorvastatin in improving patient survival in patients with advanced PCa, however, no significant improvement has been observed with current approaches but the potential for improvement has been postulated with longer exposure to atorvastatin (Murtola *et al.*, 2018).

Interestingly, agonists of endogenous chemical messengers were also identified following CMap analysis. GR-135531, RX-821002 and mestanolone were identified, which are a melatonin receptor agonist, an adrenergic receptor antagonist and an androgenic steroid, respectively. Significantly, the prostate gland and PCa are responsive to hormones with hormone deprivation therapy extensively used in treating advanced PCa (Crawford *et al.*, 2019). It would be interesting to see the manner in which DCUN1D1 is implicated in these hormone pathways. Additionally, we identified ergocryptine and carmoxirole which are dopamine agonists. DCUN1D1 has been implicated in neurodegenerative diseases such as frontal lobar degeneration, while, Huntington's disease, Parkinson's disease were dysregulated following SILAC quantitative proteomics analysis of DCUN1D1 knockdown (Villa *et al.*, 2009).

Therefore, following CMap analysis of SILAC quantitative proteomics signatures of DCUN1D1 knockdown in PCa cells, we observed a variety of compounds with some patterns appearing upon analysis of the compounds found in common across our samples. These include kinase inhibitors, anti-inflammatory drugs, drugs used in the treatment of cardiovascular diseases as well as hormone/neurotransmitter agonists. They also include targets of chromatin-related activities in the form of the HDAC inhibitor ISOX and there is representation of bromodomain inhibitors among the strongly connected perturbagen classes identified in this study. Additionally, known compounds or mechanisms of targeted treatment of PCa were also identified in sample LH2 including docetaxel, testosterone and the oral analogue of the testosterone metabolite dihydrotestosterone (mestanolone) was identified in common in the VCap CMap signatures. Significantly, we found two compounds in sample LH2 and LH3, respectively that we identified and screened as potential DCUN1D1 inhibitors following DNA microarray analysis of DCUND1 knockdown in DU145 PCa cells, namely, thapsigargin and podophyllotoxin. Thapsigargin was found to decrease mRNA expression of DCUN1D1, while, podophyllotoxin was found to decrease mRNA and protein expression of DCUN1D1

and to have an additive inhibitory effect on PCa growth in combination with monensin (Vava and Zerbini, 2014). It would be interesting to explore *in silico* analysis of the binding dynamics of the drugs and DCUN1D1 in order to understand their inhibitory mechanism. Additionally, further *in vitro* and *in vivo* analysis of the drugs and DCUN1D1 in PCa would be promising to investigate.

Chapter 5: Preferential cullin neddylation and dysregulation of the WNT pathway by DCUN1D1 in PCa

5.1 Introduction

DCUN1D1 plays a major role in cullin neddylation (Keuss *et al.*, 2016). It mediates the nuclear localisation of cullin 1 and the assembly of the cullin 1 neddylation E3 ligase complex (G. Huang *et al.*, 2011). It has also been shown to play a role in cullin 2 neddylation, downstream of HIF1α binding to its ubiquitin E3 ligase, VHL (Heir *et al.*, 2013). Additionally, DCUN1D1-mediated cullin 3 neddylation promoted Aurora B ubiquitination during mitosis (Huang *et al.*, 2017). However, the broad spectrum of DCUN1D1 targets has not been fully elucidated.

Therefore, following IPMS analysis of DCUN1D1 pulldown products from HEK293TT cells and SILAC quantitative proteomics analysis of proteins which were differentially expressed following knockdown of DCUN1D1 in PCa, we obtained insights into the mechanism of action of DCUN1D1. We identified, using IPMS, some known targets of DCUN1D1 including cullin 3, cullin 4B, cullin 5, the negative regulator of cullins, CAND1 and the cullin-associated RBX1. We also identified 3 members of the ubiquitin CRLs: BCR E3 ligases (BTB-CUL3-RBX), DCX E3 ligases (DDB1-CUL4-XBOX) and the ECS E3 ligases (ELONGIN BC-CUL2/5-SOCS box) (Petroski and Deshaies, 2005). Although we did not identify the other members of the family such as cullin 1, cullin 2 and cullin 4A, we identified other known neddylation substrates and potential targets of DCUN1D1 such as the ribosomal proteins. Significantly, STRING software analysis performed in this study found that edges emanating directly from the DCUN1D1 node were associated with cullin 3, cullin 4B, cullin 5, RBX1 and CAND1. In addition, studies performed thus far on DCUN1D1 have found only the cullin family of proteins as substrates of DCUN1D1 (Kim *et al.*, 2008; Keuss *et al.*, 2016). Therefore, cullins could be the primary targets of DCUN1D1, perhaps in a manner similar to proteins that are characterised through the regulation of critical cell complexes/processes such as DNA methylases, kinases or phosphatases. The exploration of the downstream targets of DCUN1D1-modified cullins would therefore be critical to understanding its mechanism of action.

Interestingly, following the analysis of the enriched molecular properties using the above-mentioned proteomics approaches, we observed the recurrence of some pathways such as the UPP, the WNT signalling pathway, inflammation-related pathways and the implication of metabolic pathways (See Chapter 2, section 2.6, pg. 82 and Chapter 3, section 3.5.2.2, pg. 101). Furthermore, CMap analysis of signatures from differentially expressed proteins, as quantified using SILAC proteomics, identified compounds with a similar signature to DCUN1D1. This included several different kinase inhibitors, anti-inflammatory compounds, endogenous chemical messenger agonists and a compound that reduces plasma cholesterol levels called atorvastatin (See Chapter 4, section 4.5, pg. 114, 116 and 117). Combination studies of atorvastatin and metformin have shown strong inhibitory activity in PCa *in vitro* by reducing NF-κB activity and decreasing phosphorylation of AKT (Wang *et al.*, 2017). Moreover, studies performed previously in our lab found that the WNT/β-catenin pathway was among the top 10 dysregulated pathways following DNA microarray analysis of DU145 DCUN1D1 knockdown versus DU145 cells (Vava and Zerbini, 2014). We therefore postulate that the WNT/β-catenin pathway maybe key to the mechanism of action of DCUN1D1 in PCa.

The WNT/β-catenin pathway plays a critical role in cell proliferation, cell survival, tissue homeostasis and embryonic development (Masckauchán *et al.*, 2005; Yin *et al.*, 2015; Steinhart and Angers, 2018). It also plays a role in cancer development and progression with multiple components of the pathway implicated, from the activating ligands, to the components of the β-catenin destruction complex and the nuclear cofactors (Zhan, Rindtorff and Boutros, 2017). Furthermore, it has been demonstrated to play an immunosuppressive role in cancer and its activation in the tumour microenvironment may lead to a reduced pro-inflammatory phenotype (Yaguchi *et al.*, 2012). Mechanistically, the canonical WNT signalling pathway is mediated by the β-catenin transcription co-activator that is heavily regulated by the cytoplasmic β-catenin destruction complex (Kimelman and Xu, 2006). The complex consists of Axin, the tumour suppressor gene product *adenomatous polyposis coli* (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) (Kimelman and Xu, 2006). It mediates the phosphorylation of β-catenin which triggers its ubiquitination and proteasomal degradation, preventing its entry into the nucleus and co-transcription of WNT target genes. It is at this point that DCUN1D1 may be associated with this pathway as cullin 1 is a scaffolding molecule in the SKP1/CUL1/β-TrCP ubiquitin ligase that mediates β-catenin ubiquitination and degradation (Winston *et al.*, 1999).

Therefore, taking into consideration the functions and pathways implicated in this study, their overlap with the WNT pathway and its dysregulation following knockdown of DCUN1D1 in PCa, we decided to evaluate the status of the WNT signalling pathway. Moreover, we were intrigued to investigate the mechanism of action DCUN1D1 in PCa including the full spectrum of cullin proteins implicated in its activity.

5.2 Hypothesis

Proteomics enables us to identify and analyse proteins and protein properties using high throughput, automated computational methodologies. This allows us to study simple and complex mixtures while obtaining detailed phenotypic information on gene products and providing functional information on the protein. We used IPMS and SILAC quantitative proteomics analysis to better understand DCUN1D1 and its mechanism of action in PCa. DCUN1D1 mediates posttranslational modification of protein substrates by neddylation. These different but complimentary proteomics approaches enabled us to identify DCUN1D1 binding partners through affinity-based pulldown product analysis followed by MS identification. They also provided us with insights into the broader scheme of proteins affected by DCUN1D1 in PCa through relative quantification of changes in protein levels induced by alterations in DCUN1D1, respectively. Using whole-cell lysates and shotgun proteomics we were able to obtain a global view of the cellular response to DCUN1D1. We then applied statistical and bioinformatics methods to determine the pathways/processes attributed to the observed responses. These included transcription/transcriptional regulation, protein translation related activities, neddylation, the UPP, lipid/energy-based metabolism, cellular signalling and inflammation.

Additionally, advanced CMap analysis of potential inhibitors of DCUN1D1 employed comparative analysis of cellular signatures depicting transcriptional responses from perturbagen treatments, which identified compounds that use different inhibitory approaches. They included kinase inhibition, current PCa therapeutic approaches relying on tubulin inhibition, chemical-messenger agonists, a proteasome inhibitor and anti-inflammatory agents. These were compounds whose signature was connected to the dysregulation signature found upon knockdown of DCUN1D1 in PCa cells and brought new insights, while also spanning the mechanisms described above.

We postulated that using the cellular molecular biology approach of western blot analysis which relies on protein separation, target protein analysis and visualization, we could employ affinity-based antibody recognition and target protein analysis to identify changes in protein levels in complex PCa whole-cell lysates. This would enable investigation of DCUN1D1 substrates and their neddylation status as well as exploration of a signalling pathway consistently identified in our study and whose known activities implicates it in the mechanism of action of DCUN1D1. This would strengthen the evidence we have obtained on DCUN1D1 using different proteomics approaches and demonstrate the robustness of the observations made in earlier chapters that attribute to the mechanism of action of DCUN1D1.

5.3 Aims and Objectives

Our objective is to understand the molecular mechanism of action of DCUN1D1 by identifying potential DCUN1D1 substrates and investigating the dysregulation of signalling pathways implicated. This would be critical in providing information on a subject that has been minimally investigated in the literature thus far and could provide insights into an emerging approach to PCa treatment.

Aim:

Validation of DCUN1D1 substrates and proposed mechanism of action using western blot analysis.

5.4 Materials and methods

5.4.1 Cell lines and culturing

DU145 cells were knocked down using the shRNA lentivirus approach and selection was maintained using puromycin (0.1 mg/mL) (Lonza, Walkersville MD, USA). We then plated DU145 and DU145 DCUN1D1 knockdown cells on 10cm tissue culture dishes and incubated them for 24 hours. Following the 24-hour period, the media in the plates was aspirated and the cells washed using 1× PBS pH7.4. The cells were then lysed using 1× cell lysis buffer containing 2mM Tris·HCl pH 7.5, 15mM NaCl, 0.1mM Na_2EDTA, 0.1mM EGTA, 0.1% Triton, 0.25mM sodium pyrophosphate, 0.1mM beta-glycerophosphate, 0.1mM Na_3VO_4 and 0.1 µg/mL leupeptin (Cell Signaling Technology). This was supplemented with protease and phosphatase inhibitors (Roche, Germany). The cells were then scraped using cell scrapers with rigorous agitation, in order to facilitate lysis and all steps were performed on ice to prevent protein degradation. Cell debris was then separated by centrifugation at 13 300 rpm for 2 minutes and the supernatant containing the proteins was transferred to a clean, sterile 1.5mL tube and stored at -20°C.

5.4.2 Protein quantification and western blot analysis

The proteins were then quantified using the Bradford reagent protein assay, through the generation of a standard curve and calculation of protein concentration from the equation of the line. Ten microliters each of the blank and the protein standards of known concentration (0.5 µg/mL, 0.375 µg/mL, 0.25 µg/mL, 0.1 µg/mL and 0.05 µg/mL) were added to a 96 well flat-bottomed plate. The experimental samples were initially diluted to a ratio of 1:20, then 10µL was used for quantification. The Protein Assay Dye Reagent Concentrate (1×) (Bio-Rad, Germany) was then added at 200µL to all the samples in the plate. Then following quantification, the required lysate volume was added to clean, sterile 1.5mL tubes and boiled at 95°C for 5 minutes for denaturation. Western blot analysis was then performed, involving electrophoresis of the protein lysates using a denaturing SDS polyacrylamide (PAGE) gel and separating gels at 8 and 12% for optimum protein visualization.

The separated proteins were then electroblotted onto 0.2µm nitrocellulose membranes (Bio-Rad, Germany) at 100 volts for 1 hour and 30 minutes. Subsequently, the membranes were blocked with 5% milk and 0.1% imidazole buffered saline with Tween 20 (IBST) at 10mL, to prevent non-specific binding of antibodies. The membranes were then incubated with a primary antibody of interest that was dissolved in 6mL of 5% milk and 0.1% IBST for 16 hours (see Table 20 below for the detailed antibody list). We then washed the membranes three times using 0.1% IBST following which, the membranes were incubated with species-specific secondary antibodies conjugated to horseradish peroxidase (HRP). Specifically, the secondary antibodies used were goat anti-rabbit IgG HRP conjugates (Biorad, USA) and goat anti-mouse IgG HRP conjugates (Biorad, USA) at a dilution of 1:2000 and 1-hour incubation, at room temperature. Excess secondary antibody was then washed from the membranes three times using 0.1% IBST. The protein levels were visualized using chemiluminescent substrate solutions A and B (1:2) (LumiGloReserve, KPL Incorporated, USA) which upon cleavage by HRP emits light.

Table 20: Showing details of the primary antibodies used in the study

Primary antibody	Manufacturer	Catalogue number	Dilution	Species
DCUN1D1	Sigma	HPA035911	1:250	Rabbit
Nedd8	Cell Signaling Technology	CST2745S	1:1000	Rabbit
Ubiquitin	Cell Signaling Technology	CST39365	1:1000	Rabbit
Cullin 1	Cell Signaling Technology	CST4995	1:1000	Rabbit
Cullin 2	Novus Biologicals	NBP1-67535	1:1000	Rabbit
Cullin 3	Cell Signaling Technology	CST2759S	1:1000	Rabbit
Cullin 4A	Cell Signaling Technology	CST2699S	1:1000	Rabbit
Cullin 4B	Bio-Rad	VMA00360	1:1000	Mouse
Cullin 5	Novus Biologicals	NBP1-22970	1:1000	Rabbit
APPBP1	Cell Signaling Technology	CST14321	1:1000	Rabbit
UBA3	Novus Biologicals	NBP2-48628	1:1000	Rabbit
UBC12	Novus Biologicals	NBP1-31459	1:1000	Rabbit
CAND1	Cell Signaling Technology	CST8759S	1:1000	Rabbit
RBX1	Novus Biologicals	NBP2-20113	1:1000	Mouse
β-Catenin	Cell Signaling Technology	CST4394S	1:1000	Rabbit
pβ-CATENIN	Cell Signaling Technology	CST2009S	1:1000	Rabbit
AKT	Cell Signaling Technology	CST92745	1:1000	Rabbit
pAKT	Cell Signaling Technology	CST9271S	1:1000	Mouse

5.5 Results

5.5.1 DCUN1D1 knockdown in PCa cells decreased global neddylation, ubiquitination and decreased expression of neddylation components

The neddylation pathway is a posttranslational modification process that mediates transfer of 9kDa NEDD8 to target proteins (Kumar, Yoshida and Noda, 1993; Soucy *et al.*, 2010). It also plays a role in ubiquitination through NEDD8 modification of the cullin proteins, which are scaffolding molecules in the cullin RING E3 ligases (CRLs) (Hori *et al.*, 1999; Petroski and Deshaies, 2005). Although initially thought to be an enhancer of neddylation, *in vivo* studies demonstrated that DCUN1D1 is essential for neddylation, in part, due to its role in the nuclear translocation of cullin 1 (Kim *et al.*, 2008; G. Huang *et al.*, 2011). We performed proteomics analysis to identify DCUN1D1 substrates and to determine its mechanism of action in PCa. To explore this further, we began by testing the global neddylation and ubiquitination status of PCa following knockdown of DCUN1D1 by performing western blot analysis of DU145 and DU145 DCUN1D1 knockdown cells. We observed significant reduction in DCUN1D1 expression levels in the DU145 DCUN1D1 knockdown cells relative to the control cells (Figure 43A). Additionally, immunoblotting of whole-cell lysates using anti-NEDD8 antibody showed decreased neddylation in the knockdown cells, particularly in the high molecular weight region (Figure 43B). We also observed decreased anti-ubiquitin immunoreactivity in the DU145 DCUN1D1 knockdown cells relative to the control DU145 cells (Figure 43C).

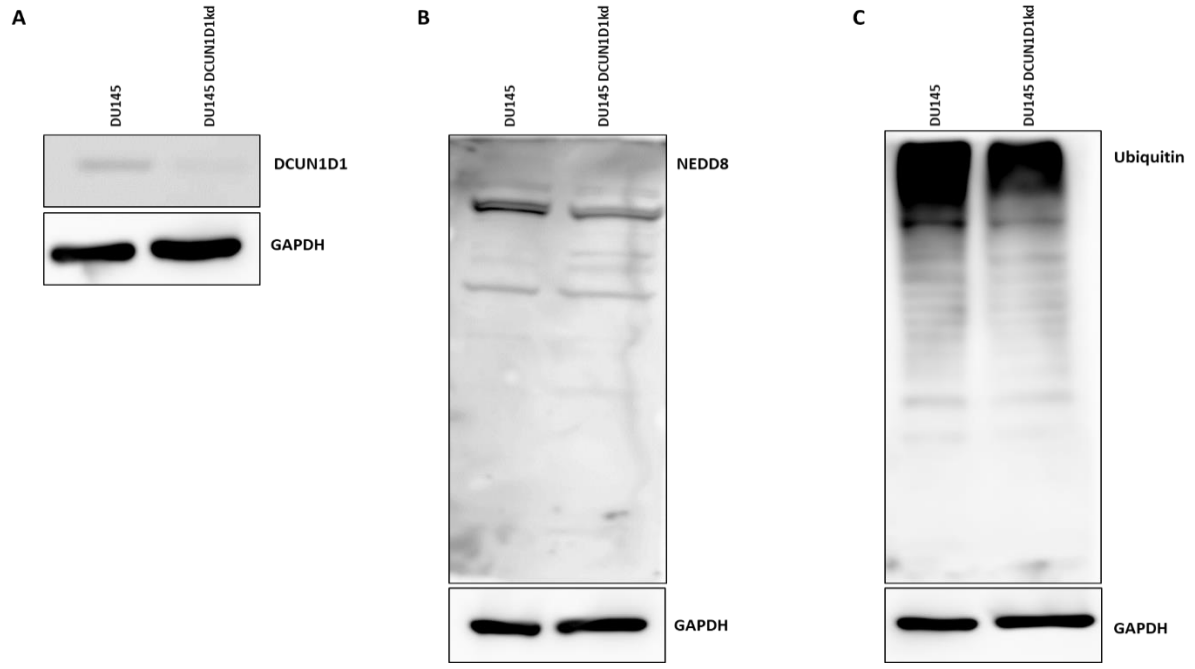


Figure 43. Showing western blot analysis of DCUN1D1, NEDD8 and ubiquitin expression. Protein extracts were obtained from DU145 and DU145 DCUN1D1 knockdown cell lines and tested for *A)* DCUN1D1 expression*, B)* neddylation status using anti-NEDD8 antibody and *C)* the ubiquitination status using the anti-ubiquitin antibody. The GAPDH loading control was probed using the anti-GAPDH antibody.

Having observed the above, we evaluated the expression level of the components of the neddylation pathway. Neddylation is a multi-step process that is mediated by an E1, E2 and an E3 ligase. It begins with the activation of NEDD8 by the E1 neddylation activating enzyme (NAE) heterodimer, APPBP1/UBA3, followed by transfer of the activated NEDD8 to the E2 neddylation conjugation enzyme (UBC12) (Osaka *et al.*, 1998; Gong and Yeh, 1999; Huang *et al.*, 2004, 2005). UBC12 then transfers NEDD8 to the neddylation E3 ligase for substrate modification (Figure 15) (Kim *et al.*, 2008). We wanted to determine whether DCUN1D1 knockdown and the subsequently observed reduction in neddylation was due to a dysregulation in the expression of the other components of the neddylation pathway. This was also of interest because although we identified components of the neddylation pathway following IPMS analysis in HEK293TT, we did not identify any in the SILAC proteomics analysis in DU145 versus DU145 DCUN1D1 knockdown cells. Significantly, knockdown of DCUN1D1 in DU145 PCa cells led to decreased expression of the E1 NAE APPBP1/UBA3 and the neddylation conjugation enzyme, UBC12 (Figure 44A). This data demonstrates that dysregulation of DCUN1D1 led to significant disruption of the neddylation pathway as observed by reduced expression of the components that mediate neddylation. It also indicates that dysregulation of the neddylation pathway was key to the mechanism of action DCUN1D1 in PCa.

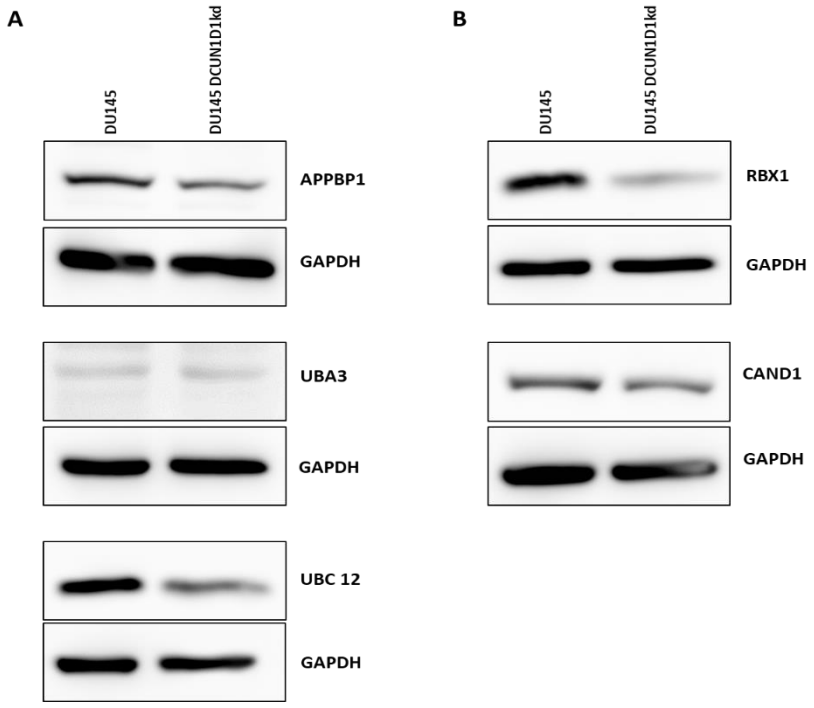


Figure 44. Blockage of DCUN1D1 decreased expression of neddylation pathway components and cullin-associated proteins. Protein extracts containing DU145 and DU145 DCUN1D1 knockdown cell lines were analysed using western blot analysis. *A)* Blockage of DCUN1D1 decreased the expression of the neddylation pathway components including the E1 NAE heterodimer APPB1 *(top panel),* UBA3 *(middle panel)* and the neddylation conjugating enzyme, UBC12 *(bottom panel). B)* Western blot showing decreased expression of the cullin-associated proteins RBX1 *(top panel)* and CAND1 *(bottom panel).* The GAPDH loading control was probed using the anti-GAPDH antibody.

5.5.2 DCUN1D1 shows preferential neddylation activity of cullin proteins in PCa

We explored this further by evaluating the status of proteins that play a role in the neddylation of cullin proteins, which included the cullin-associated RBX1 and the negative regulator, CAND1. RBX1 (RING box protein 1) is a ubiquitin E3 ligase component of the CRLs that interacts with cullins (Kamura *et al.*, 1999; Chen *et al.*, 2000; Petroski and Deshaies, 2005). The cullin-RBX interaction is considered to be the core complex of the CRLs and when unneddylated it is normally bound to CAND1 (J. Zheng *et al.*, 2002; Wu *et al.*, 2016). CAND1 on the other hand binds to cullin-RBX complexes creating sterical hindrance to CRL assembly, acting as a CRL assembly factor (Min *et al.*, 2003; Goldenberg *et al.*, 2004; Wu *et al.*, 2016). It functions as an exchange factor for the F box substrate recognition protein during CRL assembly, to facilitate substrate ubiquitination. It interacts specifically with unneddylated cullin-RBX complexes and is associated with DCUN1D1 (J. Zheng *et al.*, 2002; Kim *et al.*, 2008; Wu *et al.*, 2016). Therefore, to determine the extent of the effect of DCUN1D1 knockdown on the neddylation pathway components involved in cullin neddylation, we evaluated the expression levels of RBX1 and CAND1. We observed decreased expression of RBX1 and CAND1 following knockdown of DCUN1D1 in DU145 PCa cells in comparison to the control (Figure 44B). This demonstrated the dysregulation of cullin-neddylation associated proteins following knockdown of DCUN1D1 in PCa cells. It also provided further evidence for the association of DCUN1D1 with RBX1 and CAND1, as observed previously and as demonstrated following IPMS analysis in this study (Kim *et al.*, 2008).

We then determined whether the dysregulation in the expression of modulators of cullin neddylation had an impact on the status of cullin proteins, upon markedly reduced DCUN1D1 expression. Although cullin 3, cullin 4B and cullin 5 were identified as DCUN1D1 substrates in this study, studies performed previously have demonstrated that RBX1 and DCUN1D1 interact with cullin 1, cullin 2, cullin 3, cullin 4A, cullin 4B and cullin 5 (Ohta *et al.*, 1999; Petroski and Deshaies, 2005; Kim *et al.*, 2008). Therefore, to validate the above as DCUN1D1 substrates and to evaluate the neddylation status of the potential DCUN1D1 cullin substrates in PCa, we performed western blot analysis on cullin 1 - 5. Significantly, we did not observe any changes in the expression levels of these cullins following western blot analysis of DU145 versus DU145 DCUN1D1 knockdown cells, however, we observed selective decreases in NEDD8 modification (Figure 45). We observed decreased NEDD8 modification in cullin 1, cullin 3, cullin 4A, cullin 4B and cullin 5 following DCUN1D1 knockdown. We did not observe this effect in cullin 2 (Figure 45). This data further validates cullin 3, cullin 4B and cullin 5 as DCUN1D1 substrates as observed previously and identified in IPMS performed in this study. It also suggests preferential cullin neddylation of DCUN1D1 in its mechanism of action in PCa. Although DCUN1D1 has been demonstrated to neddylate cullin 1 - 5, this is the first description of DCUN1D1-mediated preferential neddylation of cullin proteins in PCa.

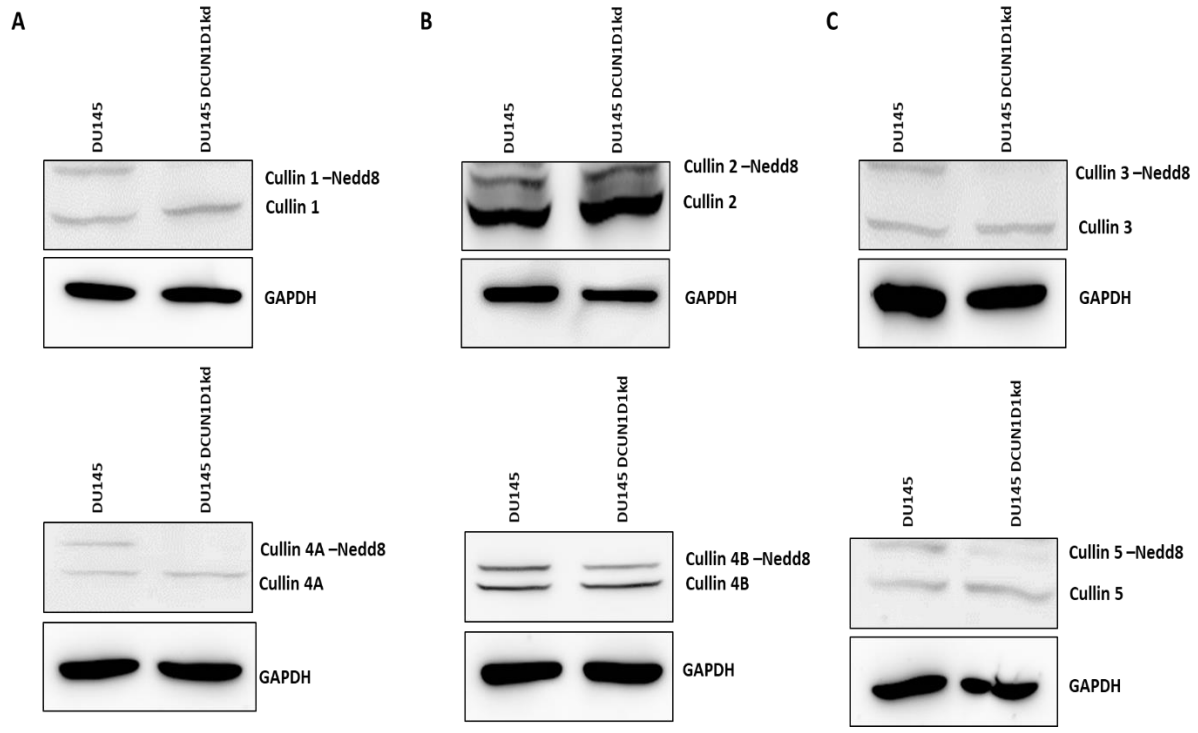


Figure 45. Blockage of DCUN1D1 showed preferential NEDD8-modification of the cullin family of proteins. Immunoblot analysis of DU145 and DU145 DCUN1D1 knockdown cell protein extracts using anti-cullin 1, anti-cullin 2, anti-cullin 3, anti-cullin 4A, anti-cullin 4B and anti-cullin 5. The GAPDH loading control was probed using the anti-GAPDH antibody.

5.5.3 The WNT pathway was inhibited following knockdown of DCUN1D1 in PCa

In order to determine the broader mechanism of action of DCUN1D1 in PCa we explored the WNT pathway which was dysregulated following proteomics analysis in this study and is subject to regulation by CRL ubiquitination (Stamos and Weis, 2013). It was also of interest because studies performed previously in our lab found it to be dysregulated by DNA microarray analysis of DU145 versus DU145 DCUN1D1 knockdown cells (Vava and Zerbini, 2014).

We explored the expression level of β-catenin, which is a widely characterized transcriptional co-activator of the canonical WNT pathway (MacDonald, Tamai and He, 2009). The WNT/β-catenin pathway has also been implicated in a number of pathways/processes that span the mechanism of action of DCUN1D1 as proposed in this study. These include the regulation of transcription, cell differentiation, development and the regulation of inflammation (Masckauchán *et al.*, 2005; Yaguchi *et al.*, 2012; Steinhart and Angers, 2018). β-catenin has also been shown to be associated with PPAR signalling which was dysregulated following IPMS analysis of DCUN1D1 pulldown products (Sharma *et al.*, 2004; Liu *et al.*, 2006). Significantly, we observed increased phosphorylation of β-catenin and a subsequent reduction in the expression level of total β-catenin in the DU145 DCUN1D1 knockdown cells relative to the control DU145 cells (Figure 46). This indicated the deactivation of the WNT signalling pathway following knockdown of DCUN1D1. In the absence of stimulation of the WNT pathway by ligand binding, cytoplasmic β-catenin is phosphorylated then targeted for proteasomal

degradation, preventing its entry into the nucleus and repressing the expression of WNT target genes (MacDonald, Tamai and He, 2009; Stamos and Weis, 2013). Therefore, blockage of DCUN1D1 led to the deactivation of the WNT pathway, validating its role in the mechanism of action of DCUN1D1.

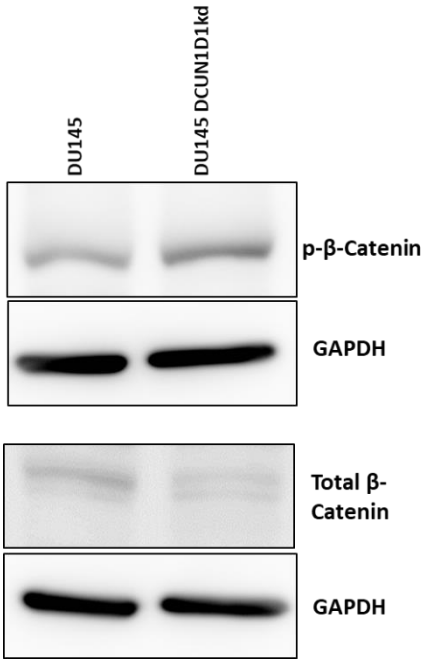


Figure 46. Inhibition of DCUN1D1 deactivated the WNT signalling pathway. Protein extracts containing DU145 and DU145 DCUN1D1 knockdown cell lines were analysed using western blot analysis. Western blot analysis showing increased phosphorylation of β-catenin and decreased expression of total β-catenin. The GAPDH loading control was probed using the anti-GAPDH antibody.

5.5.4 Blockage of DCUN1D1 activated the AKT pathway in PCa

In order to further elucidate the mechanism of action of DCUN1D1, we tested the status of the AKT pathway due to the known association between the WNT and AKT pathway and their key roles in cancer. The dysregulation of the PI3K/AKT/mTOR pathway has been extensively associated with the development and progression of PCa including metastatic PCa (Edlind and Hsieh, 2014). Additionally, we identified PI3K and mTOR inhibitors as the top 8 and top 17 scoring perturbagen classes in sample LH3 following CMap analysis of the DCUN1D1 knockdown signature. Particularly, we identified compounds that target components of the PI3K/AKT/mTOR in sample LH3 including sirolimus (mTOR inhibitor), everolimus (mTOR inhibitor), PI-828 (PI3K inhibitor), deforolimus (mTOR inhibitor) and AZD-8055 (mTOR inhibitor). Therefore, to determine the status of the AKT pathway, we immunoblotted whole-cell lysates using anti-AKT (Ser473) antibody and observed increased phosphorylation of AKT at Ser473, which is the phosphorylation step that completes activation of AKT (Figure 47). This indicates that blockage of DCUN1D1 leads to the activation of the AKT signalling pathway and although the AKT pathway is a pro-survival and proliferation pathway, studies performed in our lab found that knockdown of DCUN1D1 in PCa cells decreased proliferation and migration (Fresno Vara *et al.*, 2004;

Vava and Zerbini, 2014). Therefore, it could be that the activation of the AKT pathway is a response by the cancer cells to the effects of DCUN1D1 knockdown but is not sufficient to impede the inhibition of PCa growth.

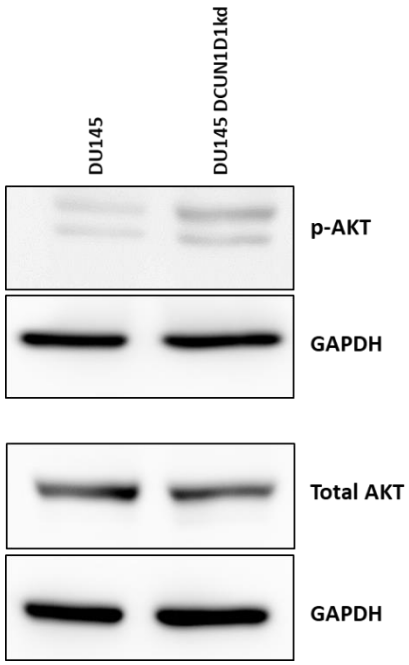


Figure 47. Showing western blot analysis of the AKT pathway. Immunoblot analysis of DU145 and DU145 DCUN1D1 knockdown cell protein extracts using anti-p-AKT, anti-AKT. The GAPDH loading control was probed using the anti-GAPDH antibody.

5.6 Discussion

DCUN1D1 is an underexplored E3 ligase that we have determined to play a role in PCa and to display potential as a molecular target for therapeutic interventions (Vava and Zerbini, 2014). In addition, DCUN1D1 has been associated with PCa progression and prognosis in PCa tissue samples relative to normal tissue (Zhang *et al.*, 2017). Therefore, it is critical to understand the mechanism by which DCUN1D1 mediates its activity in PCa. Using a shRNA approach, we established an *in vitro* knockdown model of DCUN1D1 in DU145 PCa cells and explored the mechanism of action using western blot analysis. We observed decreased global neddylation, particularly within the high molecular weight region as well as a decrease in ubiquitination. Importantly, we determined that knockdown of the DCUN1D1 E3 ligase resulted in decreased expression of the upstream neddylation pathway components. This included a decrease in the expression level of the E1 NAE heterodimer consisting of APPBP1 and UBA3 as well as a decrease in the expression of the E2 neddylation conjugating enzyme, UBC12. This indicates that the neddylation pathway is dysregulated following knockdown of DCUN1D1 and plays a role in the mechanism of action of DCUN1D1. This may also provide evidence for why the neddylation pathway components were not identified following SILAC proteomics analysis of DU145 versus DU145 DCUN1D1 knockdown cells in Chapter 3 above. However, it is not clear whether the observed reductions in expression are due to regulation by DCUN1D1 of these enzymes as they are found upstream of DCUN1D1 or whether the reduction in the E3 ligase expression induced a systematic reduction in the pathway components in the cells. Nevertheless, the blockage of DCUN1D1 led to significant dysregulation of the neddylation pathway. It also indicates that dysregulation of the neddylation pathway was key to the mechanism of action DCUN1D1 in PCa and the critical role of DCUN1D1 in PCa by impacting key cellular pathways such as neddylation and ubiquitination.

Having observed this effect, we then evaluated the status of the neddylation components which are known modulators of cullin neddylation and DCUN1D1 interactors, RBX1 and CAND1. This is due to the identification of RBX1, CAND1, cullin 3, cullin 4B and cullin 5 as DCUN1D1 interactors as reported previously and following IPMS analysis in HEK293TT cells in this study (Kim *et al.*, 2008). RBX1 together with cullins form the core complexes of CRLs and CAND1 is a negative regulator of cullin neddylation. Therefore, analysis of their expression levels would help determine their association with DCUN1D1 in PCa and their role, if any, in the status of cullin 3, cullin 4B and cullin 5 following knockdown of DCUN1D1. Importantly, we observed decreased expression of RBX1 and CAND1 upon blockage of DCUN1D1.

We then explored the neddylation status of the mammalian cullin family of proteins to validate cullin 3, cullin 4B and cullin 5 as DCUN1D1 substrates and to explore the status of the remainder of the family. Significantly, we did not observe any alterations in the expression levels of the proteins but observed selective decreases in NEDD8 modification. We observed decreased NEDD8 modification in cullin 1, cullin 3, cullin 4A, cullin 4B and cullin 5 following DCUN1D1 knockdown in DU145 PCa cells relative to the control. We did not observe this effect in cullin 2. Interestingly, knockout of DCUN1D1 in U2OS bone osteosarcoma cells using CRISPR/Cas9 led to a similar result, with a reduction in neddylation observed in cullin 1, cullin 3, cullin 4A and cullin 5 but not in cullin 2 and cullin 4B (Keuss *et al.*, 2016). Therefore, although DCUN1D1 has been demonstrated to neddylate cullin 1-5, this suggests selective DCUN1D1-mediated cullin neddylation. Significantly, the results above are the first description of DCUN1D1-mediated preferential neddylation of cullin proteins in PCa as depicted by selective reduction in NEDD8 modification of cullins following blockage of DCUN1D1. It would be interesting to test this observation in other PCa cell lines to obtain a comprehensive view of DCUN1D1-mediated preferential neddylation as well as to test it against a normal epithelial prostate cell line.

Furthermore, when determining the broader mechanism of action of DCUN1D1, we found that knockdown of DCUN1D1 led to deactivation of the WNT signalling pathway. Phosphorylation of β-catenin was increased, and we observed a reduction in the expression level of total β-catenin in DU145 DCUN1D1 knockdown cells relative to control DU145. Proteasomal degradation of β-catenin prevents its entry into the nucleus resulting in the repression of the expression of WNT target genes (Kimelman and Xu, 2006). This strengthens the evidence found in our lab around the association between DCUN1D1 and the WNT signalling pathway (Vava and Zerbini, 2014). We identified it following IPMS analysis in HEK293TT cells and SILAC quantitative proteomics analysis of DU145 versus DU145 DCUN1D1 knockdown cells. We also previously identified the WNT pathway as one of the pathways dysregulated following DNA microarray analysis of DU145 versus DU145 DCUN1D1 knockdown cells (Vava and Zerbini, 2014). Therefore, using a transcriptomics and different proteomics approaches the WNT pathway has been implicated in the mechanism of action of DCUN1D1, which was validated using western blot analysis. Moreover, the WNT/β-catenin pathway has been implicated in a number of pathways/processes that span the mechanism of action of DCUN1D1 as proposed in this study. These include the regulation of transcription, cell differentiation, development and the regulation of inflammation (Masckauchán *et al.*, 2005; Yaguchi *et al.*, 2012; Steinhart and Angers, 2018). β-catenin has also been shown to be associated with PPAR signalling which was dysregulated following IPMS analysis of DCUN1D1 pulldown products (Sharma *et al.*, 2004; Liu *et al.*, 2006). Additionally, it has been shown that mitochondrial-WNT signalling was downregulated by reduced ATP synthesis and the induction of ER stress, where, ATP synthesis and the ER stress were dysregulated following SILAC proteomics analysis of DU145 versus DU145 DCUN1D1 knockdown cells (Costa *et al.*, 2019). Therefore, the WNT signalling pathway plays a role in the mechanism of action of DCUN1D1 in PCa.

Interestingly, β-catenin is targeted for degradation through polyubiquitination that is mediated by the β-TRCP E3 ligase in complex with cullin 1, SKP1 and the FBXW11. Having observed decreased neddylation of cullin 1 following knockdown of DCUN1D1, but continued degradation of β-catenin, it would be interesting to determine the mechanism of degradation at play in the downregulation of the WNT pathway. Soucy et al., 2009 found an accumulation of CRLs substrates such as CDT1, p27, and NRF2 following inhibition of their neddylation by treatment with the NAE inhibitor MLN4924 (Soucy *et al.*, 2009). Therefore, it could be hypothesized that decreased cullin 1 neddylation would increase β-catenin levels, however, its degradation is mediated by a multi-protein complex and it would be critical to determine the expression levels and binding dynamics of the whole complex, particularly the β-TRCP E3 ligase. Interestingly, Tripathi et al 2007, demonstrated that cullin 4B negatively regulated β-catenin, therefore, it may be possible that other members of the cullin family of proteins regulate β-catenin in an overlapping manner (Tripathi, Kota and Srinivas, 2007). Meaning that cullin 2, which did not display a decrease in neddylation in this study could be functioning as a scaffolding molecule in a CRL permutation that facilitates the proteasomal degradation of β-catenin. This would need to be explored experimentally.

On the other hand, we evaluated the WNT associated AKT pathway in order to determine its role in the mechanism of action of DCUN1D1. We observed increased phosphorylation of AKT at Ser473, indicating that blockage of DCUN1D1 led to the activation of the AKT signalling pathway. This demonstrates a negative correlation in the expression of DCUN1D1 and AKT activation. Interestingly, we identified PI3K and mTOR as the top 8 and top 17 high-scoring connected perturbagen classes in sample LH3 following CMap analysis of the DCUN1D1 knockdown signature, with several inhibitors identified. The AKT pathway which is a part of the PI3K/AKT/mTOR pathway regulates cell survival and growth and has been associated with resistance to androgen deprivation therapy in the treatment of PCa (Edlind and Hsieh, 2014). Therefore, the activation of the AKT pathway could be a survival response to the inhibitory effects of DCUN1D1 knockdown in PCa. However, studies performed

previously showed that blockage of DCUN1D1 inhibited proliferation and migration in PCa, which suggests that the pro-survival effects of the AKT pathway activation were not sufficient to overcome the inhibitory effects of DCUN1D1 knockdown (Vava and Zerbini, 2014).

Therefore, blockage of DCUN1D1 in PCa led to a dysregulation in the neddylation and ubiquitination pathways through decreased neddylation of its substrates: cullin 1, cullin 3, cullin 4A, cullin 4B and cullin 5. It also resulted in the deactivation of the WNT/β-catenin pathway. This demonstrates that the mechanism of action of DCUN1D1 in PCa involves its neddylation activity and implicates the WNT/β-catenin pathway.

Chapter 6: General Discussion, Conclusion and Future Work

6.1 General Discussion

Molecular target-based therapeutics are emerging approaches to cancer treatment which rely on targeting a specific molecule that can affect the growth, progression or metastases of cancer. This is opposed to current chemotherapeutic agents which rely on the inhibition of rapidly dividing cancer cells, which often have an effect on normal cells. We postulate that DCUN1D1, which is an E3 ligase of the neddylation pathway and plays a role in multiple cancers, including PCa, maybe an optimal target for molecular target-based therapeutics (Estilo *et al.*, 2003; Sarkaria, Pham, *et al.*, 2004; Sarkaria *et al.*, 2006; Kim *et al.*, 2008; Broderick *et al.*, 2010; Zhang *et al.*, 2017). In 2012, of the 23 new cancer medicines approved for treatment by the US FDA, 12 were first-time approved chemical or biological drugs that included 10 molecular target agents (Yan, 2013). Among these were the androgen receptor antagonists, enzalutamide and abiraterone acetate which were proposed for the treatment of castration-resistant PCa (Yan, 2013). They also included the irreversible inhibitor of the 20S proteasome, carfilzomib and omacetaxine mepesuccinate, the protein translation inhibitor (Yan, 2013). Interestingly, proteasome and neddylation pathway inhibition are among emerging cancer treatment strategies with bortezomib and the NAE inhibitor MLN4924, showing promising results (Richardson *et al.*, 2006; Soucy *et al.*, 2009; Chen *et al.*, 2011; Nawrocki *et al.*, 2012). Moreover, there has recently been the development of an inhibitor that blocks the interaction of DCUN1D1 with the neddylation conjugating enzyme, UBC12, resulting in selective inhibition of cullin 3 neddylation (Zhou *et al.*, 2017). Therefore, targeting of DCUN1D1 could enable targeting of the neddylation pathway downstream of the NAE, leading to the inhibition of PCa, while minimizing off-target effects and therefore side effects.

This is why understanding the mechanism of action of DCUN1D1 is important. This study has postulated the mechanism of action of DCUN1D1 in PCa using high throughput proteomics and molecular biology analysis of protein expression using western blot analysis. It also used advanced CMap database analysis to screen for drugs that potentially inhibit DCUN1D1, based on the similarities in signatures observed. Having previously demonstrated the role of DCUN1D1 in PCa and identified potential inhibitors using a transcriptomics approach, we wanted to employ a functional and systems-based analysis using proteomics (Vava and Zerbini, 2014). MS-based proteomics enabled studying of the cellular protein complement providing information on proteins and protein properties as functional gene products. We used IPMS and label-based relative quantitative proteomics in the form of SILAC to identify proteins implicated in the mechanism of action of DCUN1D1 based on protein identification, protein-protein interactions and protein quantification.

Using the IPMS approach, we determined from the cellular components, cellular processes, molecular functions and pathways classified that the overall mechanism of action of DCUN1D1 implicated the ubiquitin proteasome pathway, the ribosome, transcription, lipid metabolism and inflammation. We used HEK293TT cells in order to obtain insights into DCUN1D1 interactors since it is more amenable to transfection. We observed a mechanism mediated primarily by interaction with cullin proteins and cullin-associated neddylation components that had an impact on the ubiquitin proteasome pathway. STRING analysis of functional protein association networks found that the edges emanating directly from the DCUN1D1 node were associated with cullin 3, cullin 4B, cullin 5, RBX1 and CAND1. We also identified ubiquitin E3 ligases, TRIM21 and DDB1. Therefore, we identified 3 members of the ubiquitin CRLs: BCR E3 ligases (BTB-CUL3-RBX), DCX E3 ligases (DDB1-CUL4-XBOX) and the ECS E3 ligases

(ELONGIN BC-CUL2/5-SOCS box) (Petroski and Deshaies, 2005). Based on this work and evidence published previously on DCUN1D1 substrates, we suggest that DCUN1D1 may be "Cullin Neddylase 1" (Kim *et al.*, 2008; K. Huang *et al.*, 2011; Heir *et al.*, 2013; Keuss *et al.*, 2016; Huang *et al.*, 2017). It appears to specifically target the cullin proteins but to play a role in critical cellular activities. This could be in a manner similar to kinases and phosphatases where the posttranslational modification signals or triggers critical cellular activities. DCUN1D1 may be targeting cullin proteins then based on the functions associated with that cullin and related ubiquitination substrates, impacting cellular functions. This could be attributing to the mechanism of action of DCUN1D1 in PCa based on the proteins targeted downstream of the neddylated cullin RING E3 ligases or through other mechanisms that require in-depth exploration.

Furthermore, following SILAC quantitative proteomics analysis we identified proteins differentially expressed in DU145 DCUN1D1 knockdown cells relative to DU145 PCa cells. The changes in the proteome we quantified classified into cellular components, organelles, structure molecule activity with the biological processes dysregulated including biological regulation, cellular component organization/biogenesis, cellular processes, localization, metabolic processes and response to stimulus. We also observed associations as depicted by STRING analysis, between the components of the 43S preinitiation complex (whose assembly triggers the eukaryotic translation initiation), the ribosome, ribonucleoproteins, the centrosome, the spliceosome and processing in the endoplasmic reticulum. Interestingly, as observed in the dysregulation of the centrosome here, DCUN1D1 has previously been implicated in mitosis, where, DCUN1D1-mediated cullin 3 neddylation promoted Aurora B ubiquitination during mitosis (Huang *et al.*, 2017). Additionally, we found dysregulation in TCP1 which has been demonstrated to be involved in the folding of 10% of the cell proteome and has been found to be upregulated in prostate tissue samples using tissue microarray analysis (Leitner *et al.*, 2012). We also found RPS10 which is a component of the 40S ribosomal subunit and the eukaryotic translation initiation factor 3 subunit B (EIF3B) which both play a role in protein synthesis through protein translation (Pestova *et al.*, 2001). This suggested that the knockdown of the protein-degradation linked E3 ligase of neddylation (DCUN1D1) in PCa, led to alterations in translation-related and protein-processing activities.

Similar to the IPMS analysis, we observed the implication of metabolic processes and inflammatory responses. However, the metabolic pathways pertained to glycolysis and pyruvate metabolism as opposed to lipid metabolism and changes in cellular energetics is a known "Hallmark of Cancer". Inflammatory responses were also identified through dysregulation in the inflammation mediated by chemokine and cytokine biological processes. There was also dysregulation in cellular signalling as observed with the dysregulation of cytoskeletal regulation of Rho GTPase signalling, FGF signalling and integrin signalling. Significantly, we found the dysregulation of the WNT pathway specifically through ACTB and potentially, HNRNPA2B1 which has been shown to be upregulated in PCa and to regulate the WNT pathway component, β-catenin (Stockley *et al.*, 2014). Moreover, although the substrates of DCUN1D1 identified in the pulldown assay such as the cullins and the cullin-neddylation components were not identified, we found the ubiquitin activating enzyme UBA1, providing further evidence for the association of UBA1 with the neddylation pathway as demonstrated previously by Leidecker et al., 2012. We also observed the dysregulation of potential neddylation substrates in the form of ribosomal proteins including, RPS6, RPS16, RPL27, where, RPS6 was also identified following IPMS analysis of DCUN1D1 pulldown products.

Interestingly, DCUN1D1 has a C terminus UBA domain which is normally found in proteins involved in the UPP, DNA excision repair, cell signalling via kinases and in proteins with elongation factor binding domains and these encompass the above mentioned activities (L. Chen *et al.*, 2001; Hartmann-

Petersen *et al.*, 2003; Myung, Kim and Crews, 2008; Huang *et al.*, 2015; Wu *et al.*, 2015; Wang *et al.*, 2018). The UBA domain-associated activity could also allude to the insights obtained from the targets and the mechanisms of the compounds strongly connected to the DCUN1D1 knockdown signature following CMap analysis.

We found compounds related to perturbagen classes that were strongly connected to the DCUN1D1 knockdown signature which included: leucine rich repeat kinase inhibitors, PKC activators, DNA dependent protein kinase inhibitors, bromodomain inhibitors, IGF-1 inhibitors, bile acid and HMGCR inhibitors. We also found anti-inflammatory compounds, such as talniflumate and palmitoylethanolamide as well as chemical messenger agonists such as the androgenic steroid mestanolone and dopamine agonists, ergocryptine and carmoxirole. Interestingly, blockage of DCUN1D1 in PCa cells produced a signature that strongly connected to kinase inhibitors. PhosphoSitePlus® analysis has demonstrated the presence of two phosphorylation sites (S31 and Y72) in the N terminus of DCUN1D1. Furthermore, transfection of DCUN1D1 in LnCap cells has been shown to inhibit invasion and migration involving the focal adhesion kinase (FAK) and DCUN1D1 has been found to be an endogenous activator of FAK in non-small-cell lung cancer (Zhang *et al.*, 2017; Li *et al.*, 2019). However, we did not identify FAK in our study and there has not been extensive description of the role of kinases in DCUN1D1-mediated activity nor the identification of a kinase or kinases that phosphorylate DCUN1D1. Therefore, the strong connection observed with the DCUN1D1 knockdown signature and kinase inhibitors suggests the potential role of the kinases such as DNA dependent kinases in DCUN1D1-mediated activities and the potential of treatment of DCUN1D1-regulated kinase pathways in PCa.

Interestingly, although we may have expected the identification of the NAE inhibitor among the strongly connected compounds due to linkages to the neddylation pathway, our search did not identify a strong connection with the NAE inhibitor MLN4924. We did however find strong connections to the proteasome inhibitor, calpeptin in one of the samples. It's important to note that we performed CMap database searches using the minimum acceptable number of gene names, but the range of values accepted by the database is 10 – 150. Therefore, it is possible that a greater sample size may strengthen connections with some lower scoring compounds and provide further insights. Significantly, although not found in common across our samples, we found two compounds that we identified and screened as potential DCUN1D1 inhibitors following DNA microarray analysis of DCUND1 knockdown in DU145 PCa cells, namely, thapsigargin and podophyllotoxin. We observed podophyllotoxin to decrease mRNA and protein expression of DCUN1D1 and to have an additive inhibitory effect on PCa growth in combination with monensin (Vava and Zerbini, 2014). Identifying them using a proteomics approach and transcriptomics approach strengthens the evidence for their potential as DCUN1D1 inhibitors.

The collective information we obtained following the above-mentioned high throughput data analyses led us to a proposed mechanism of action. Western blot analysis of changes in specific protein expression levels following knockdown of DCUN1D1 determined that blockage of DCUN1D1 led to dysregulation of the neddylation pathway, ubiquitination and deactivation of the WNT/β-catenin pathway. Additionally, although DCUN1D1 has been demonstrated to neddylate cullin 1 - 5, our data suggests selective DCUN1D1-mediated cullin neddylation in PCa (Kim *et al.*, 2008; Keuss *et al.*, 2016). This would be the first description of DCUN1D1-mediated preferential neddylation of cullin proteins in PCa. Moreover, the observed deactivation in the WNT/β-catenin pathway following blockage of DCUN1D1 strengthens the evidence previously found in our lab around the association between DCUN1D1 and the WNT signalling pathway using transcriptomics and currently using different proteomics approaches (Vava and Zerbini, 2014). The WNT/β-catenin pathway has been shown to play

a critical role in cell proliferation, embryonic development and the pro-inflammatory phenotype in the tumour environment (Masckauchán *et al.*, 2005; Yaguchi *et al.*, 2012; Steinhart and Angers, 2018). These activities overlap with the observations that we have made of DCUN1D1 and implicate it in the mechanism of action of DCUN1D1.

This study has provided further insights into the cellular activities into which DCUN1D1 may be playing a role. Analysis of DCUN1D1 binding partners identified cullin proteins as target proteins of DCUN1D1 which along with studies published previously on DCUN1D1, we postulate may be the primary targets of DCUN1D1. We also identified neddylation substrates such as ribosomal proteins of which DCUN1D1 is associated, however, it was not clear whether this could be as an E3 ligase or as part of a broader mechanism such as through the implication in eukaryotic translation or dysregulation of ribosome biogenesis in PCa. In addition, key cellular pathways were implicated including proteasome degradation, transcription, translation, metabolism and inflammation. Analysis of the drugs strongly connected with the DCUN1D1 knockdown signature in PCa showed aspects of the implicated pathways as well as strong connections with kinase inhibitors, raising interesting questions around the role of DCUN1D1-mediated kinase signalling pathways in PCa. We validated the role of cullin 3, cullin 4B and cullin 5 neddylation in the mechanism of DCUN1D1 in PCa, demonstrating preferential decreases in cullin neddylation of cullin 1, 3, 4A, 4B and cullin 5. Following the strength of evidence obtained in our lab at a transcriptomics and proteomics level, the WNT/β-catenin pathway was shown using western blot as playing a role in the mechanism of action of DCUN1D1 in PCa.

6.2 Conclusion

DCUN1D1 mediates its mechanism of action in PCa, through the dysregulation of the neddylation and ubiquitination pathways. This mechanism was mediated through the preferential neddylation of cullin proteins. Additionally, the WNT/β-catenin pathway was deactivated following knockdown of DCUN1D1 in PCa, implicating it in the mechanism of action of DCUN1D1. We also identified several kinase inhibitors, bromodomain inhibitors, chemical messenger agonists and anti-inflammatory agents amongst the high scoring compounds in terms of connectivity to the DCUN1D1 differential expression signature.

6.3 Future Work

Western blot analysis was used to validate and explore the mechanism of action of DCUN1D1 in PCa. The specificity of this mechanism could be strengthened by determining whether re-introduction of DCUN1D1 by transfection into DU145 DCUN1D1 knockdown cells reverses the observed effects in neddylation, ubiquitination and the WNT/β-catenin pathway. However, immunoprecipitation analysis of the changes in binding dynamics upon dysregulation of DCUN1D1 expression would also be important to determining the extent of functional impact on the observed pathways. Additionally, the functional neddylation assay could be performed to test the ability of thioester linked UBC12 enzyme to mediate NEDD8 transfer in DU145 relative to DU145 DCUN1D1 knockdown PCa cells.

Furthermore, investigation of the ubiquitin E3 complex composition responsible for the degradation of β-catenin would be critical to understanding in what way the disruption in DCUN1D1 has dysregulated the WNT/β-catenin pathway. We observed decreased neddylation of cullin 1 which is a known scaffolding molecule in the SKP1/CUL1/β-TrCP ubiquitin complex that has been demonstrated to mediate β-catenin degradation in the absence of stimulation of the WNT pathway. However, Tripathi et al 2007, demonstrated that cullin 4B negatively regulated β-catenin, therefore, it may be possible that other members of the cullin family of proteins regulate β-catenin in an overlapping manner (Tripathi, Kota and Srinivas, 2007). Meaning that cullin 2, which did not display a decrease in neddylation in this study could be functioning as a scaffolding molecule in a CRL permutation that facilitates the proteasomal degradation of β-catenin. Therefore, it would be interesting to test whether this mechanism was at play in the dysregulation of the WNT/β-catenin pathway following knockdown of DCUN1D1 in PCa. Additionally, it is established that degradation of β-catenin prevents its entry into the nucleus and the co-transcription of WNT target genes. We observed that transcription and regulation of transcription were dysregulated following the analysis of DCUN1D1 pulldown products. Therefore, it would be interesting to determine the impact of the DCUN1D1-associated dysregulation of the WNT/β-catenin pathway by probing the protein-DNA interactions altered using the chromatin-immunoprecipitation assay. It would be interesting to determine if this mechanism is at play in the WNT/β-catenin pathway as DCUN1D1 has been shown to regulate the Hedgehog signalling pathway by binding to the promoter of *Gli1* (Sarkaria *et al.*, 2006). Furthermore, the chromatin-immunoprecipitation assay could provide some insights into the nature of the relationship between DCUN1D1 and the histone molecules also identified following IPMS.

Additional exploration of the potential inhibitors of DCUN1D1 would also be interestingly, particularly, in understanding how the inhibition of DCUN1D1 by these compounds leads to blockage of PCa growth. Initially, through screening of the new list of DCUN1D1 signature connected drugs for specificity for DCUN1D1 through exploration of their impact in the presence and absence of DCUN1D1. Particularly, in terms of podophyllotoxin and thapsigargin which have been found using a transcriptomics and proteomics approach. Further analysis could include *in silico* docking experiments and investigation of their effect on DCUN1D1-mediated neddylation using the functional neddylation assay against the panel of cullin proteins. It would also be interesting to explore drugs such as palmitoylethanolamide which is an anti-inflammatory agent that targets the PPAR signalling pathway and regulates lipid metabolism. The exploration of the phosphorylation sights of DCUN1D1 that play a role in its activity and perhaps identification of the kinase responsible for this activity would also be interesting. This would advance the understanding of DCUN1D1 activity and potentially identify another approach to targeting DCUN1D1 in PCa treatment.

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