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**Investigating the effects of drug combinations
and loss of the PRH/Hhex oncoprotein in CCA
cells.**

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Declaration

I declare that this thesis is my own work that was generated during the registered period at the University of Nottingham. No part of this thesis has been submitted for any other academic degree at any other institution.

Grace Martin, September 2024

Abstract:

Cholangiocarcinoma (CCA) is a group of malignancies that arises from the biliary epithelial cells that line the bile duct. Despite the emergence of new treatment modalities, patient prognosis is very poor and novel therapeutics are urgently needed. This project investigates Niclosamide, an anti-helminth drug, that was identified from a drug repurposing screen. In this thesis, I show that Niclosamide treatment over 72 hours can reduce CCA cell viability significantly more than normal Biliary Epithelial Cells (BECs). This reduction in cell viability is due in large part to Niclosamide-induced mitochondrial uncoupling that causes cellular stress and an imbalance in protein homeostasis. Niclosamide treatment is also shown to promote apoptosis after 24 hours of treatment and to have an anti-proliferative effect over 72 hours. The Proline Rich Homeodomain (PRH) protein, also known as the haematopoietically expressed homeobox (HHEX) protein, has recently been shown to act as an oncoprotein in CCA cells increasing cell proliferation and cell migration. Interestingly, Palbociclib was shown to abrogate cell proliferation in PRH-driven CCA cells through the inhibition of CDK4/6 activity. Here, Palbociclib is shown to selectively reduce the viability of CCA cell lines compared to BECs and to synergise with Niclosamide. At a phenotypic and protein level, the Niclosamide-Palbociclib combination is shown to effectively promote cell cycle arrest through multiple mechanisms. Thus, the combination of Niclosamide and Palbociclib is shown to provide a potent anti-CCA effect in 2D and 3D *in vitro* cancer models. PRH is substantially decreased by Niclosamide and Palbociclib dual therapy, which occurs through two independent mechanisms, whereby Niclosamide treatment induces PRH degradation by the proteasome, and PRH protein level also decreases upon Palbociclib-induced cell cycle arrest. Also, the drugs as single agents, and as a combination, are more effective in cells with high levels of PRH expression compared to cells with low levels of PRH expression. To understand the

role of PRH in CCA further, the effects of PRH knockdown on CCA cells was studied through siRNA and shRNA protein knockdown and for the first time CRISPR-Cas mediated PRH knockout. These models shed light on the functions of the PRH protein in cell cycle and highlight previously undocumented roles of PRH in CCA cells in cell survival, cell metabolism and apoptosis. In conclusion, this thesis provides the first preclinical evaluation of Niclosamide-Palbociclib dual therapy for CCA and provides more information about the role of PRH in CCA biology that may lead to improved methods for the targeting of PRH in PRH-driven CCA.

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List of Abbreviations:

ACD	Accidental Cell Death
AID	Activation-induced Cytidine Deaminase
AKT/ PKB	Protein Kinase B
AML	Acute myeloid leukemia
AMMF	Alan Morement Memorial Fund
APC	Adenomatous Polyposis Coli
APS	Ammonium persulfate
ATG	Autophagy Related
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 related
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2 associated X-protein
BECs	Biliary Epithelial Cells
BCL-2	B-cell Lymphoma 2
BCL-XL	B-cell Lymphoma extra large
BENC1	Beclin-1
BF	Bright Field
BIM	Bcl-2 like protein 11
BMP	Bone Morphogenic Protein
BRCA	Breast Cancer gene
BSA	Bovine Serum Albumin
CBA	Conjugated Bile Acids
CBP	CREB Binding Protein
CCA	Cholangiocarcinoma
CCND2	Cyclin D2
CDK1	E-cadherin
CDK	Cyclin Dependent Kinase
CDK1	Cyclin Dependent Kinase 1
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin Dependent Kinase 4
CDK6	Cyclin Dependent Kinase 6
CDKI	Cyclin Dependent Kinase Inhibitor
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CI	Combination Index
CK2	Casein Kinase 2
Cmax	Maximum Serum concentration
COX	Cyclooxygenase
CpG	Cytosine phosphate Guanine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycles Threshold
DDR	DNA Damage Response
DEGs	Differentially Expressed Genes

DISC	Death Inducing Signalling Complex
DLL	Delta Like Ligand
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EC50	Half maximal Effective Concentration
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eGFP	Enhanced Green Fluorescent Protein
eIF4E	Eukaryotic initiation factor 4E
E _{max}	Efficacy
E _{miss.}	Emission Wavelength
EMT	Epithelial to Mesenchymal Transition
ENG	Endoglin Transforming Growth Factor B Co-receptor
ERK	Extracellular signal-regulated kinase
ES	Enrichment Score
ESM-1	Endothelial cell specific molecule 1
Excit.	Excitation Wavelength
F _a	Fraction Affected
FACS	Fluorescent Activated Cells Sorting
FADD	Fas-associated death domain
FBS	Foetal Bovine Serum
FC	Fold Change
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDA	Food and Drug Administration
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
FPKM	Fragment per Kilobase Million
FOX	Forkhead Box Protein
F _u	Fraction Unaffected
FWD	Forward
FXR	Farnesoid X Receptor
FZD	Frizzled
G1	Gap Phase 1
G2	Gap Phase 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCS	Goosecoid Homeobox
GPCR	G Protein-coupled Receptor
GPC3	Glypican 3
gRNA	Guide RNA
GSEA	Gene Set Enrichment Analysis
GSK3	glycogen synthase kinase 3
GTF	General Transcription Factors
HAT	Histone Acetyltransferase
HCC	Hepatocellular Carcinoma
HD	Homeodomain

HDAC	Histone Deacetylase
HDM	Histone Demethylases
HER2	Human Epidermal growth factor receptor 2
HES1	Hairy and Enhancer of Split homolog-1
HHEX	Haematopoietically Expressed Homeobox
HMT	Histone Methyltransferase
HNF	Hepatocyte Nuclear Factor
HOX	Homeobox
HP1	Heterochromatin Protein 1
HR	Hormone Receptor
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis
IDH	Isocitrate dehydrogenase
IGF	Insulin-like Growth Factor
IL-6	Interleukin 6
INK4	Inhibitor of CDK4
KD	Knockdown
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KO	Knockout
JAG	Jagged
JAK	Janus kinase
LB	Lysogeny broth
LEF/TCF	T-cell factor/lymphoid enhancer factor family
lncRNA	Long Non-Coding RNA
LRP5/6	Low-density lipoprotein-related receptors 5 and 6
miRNA	Micro RNA
mTOR	Mammalian target of rapamycin
M	Mitosis Phase
MAPK	Mitogen-Activated Protein Kinase
MBP	Methyl Binding Proteins
MCL-1	Myeloid Cell Leukemia-1
MEK	Mitogen-activated protein kinase kinase
MET	MNNG HOS transforming gene
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MSigDB	Molecular Signature Database
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	Normalised Enrichment Score
NFK-B	Nuclear Factor Kappa Beta
NICD	Notch Intracellular Cellular Domain
NICE	National Institute for Health and Care Excellence
NPAT	Nuclear Protein, Coactivator of Histone Transcription
NTC	No Template Control
NTRK	Neurotrophic receptor tyrosine kinases
OD	Optical Density
OE	Overexpression
PAM	Protospacer Adjacent Motif
PBC	Primary Biliary Cholangitis
PBS	Phosphate-buffered saline
PcG	Polycomb Group
PCR	Polymerase Chain Reaction

PCR2	Polycomb Repressive Complex 2
PDAC	Pancreatic Ductal Adenocarcinoma
PDGF	Platelet Derived Growth Factor
PD-1	Programmed Cell Death 1
PD-L1	Programmed Cell Death Ligand 1
PEI	Polyethylenimine
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PIC	Preinitiation Complex
PML	Promyelocytic Leukaemic protein
PMSF	Phenylmethanesulfonyl fluoride
PRH	Proline Rich Homeodomain
PROX1	Prospero homeobox protein 1
PS	Phosphatidylserine
PSC	Primary Sclerosing Cholangitis
PTEN	Phosphatase and tensin homolog
PTM	Post Translational Modification
qRT-PCR	Quantitative Reverse Transcription PCR
RB	Retinoblastoma Protein
RCD	Regulated Cell Death
REV	Reverse
RIN	RNA Integrity Number
RIPA	Radioimmunoprecipitation assay buffer
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
S	Synthesis Phase
SASP	Senescence-associated Secretory Phenotype
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering Ribonucleic Acid
shRNA	Short Hairpin Ribonucleic Acid
SKI	Sloan-Kettering Institute proteins
SMAC/DIABLO	Second Mitochondria-derived Activator of Caspases/DIABLO
SMAD	Suppressor of Mothers against Decapentaplegic
sncRNA	Short Non-Coding RNA
SOCS3	Suppressor of cytokine signalling 3
SOX	SRY-box Transcription Factor
STAT	Signal transducer and activator of transcription
STR	Short tandem repeat
SUZ17	Suppressor of Zeste 17
SVC	Scrambled Vector Control
SWI/SNF	SWItch/Sucrose Non-Fermentable
S1R	Sphingosine 1-phosphate receptor
TAE	Tris-acetate-EDTA Buffer
TBP	TATA Binding Protein
TBS	Tris-Buffered Saline
TEMED	Tetramethylethylenediamine

TERT	Telomerase Reverse Transcriptase
TGF-B	Transforming Growth Factor Beta
TF	Transcription Factor
TLE	Transducin-like enhancer
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
TrxG	Trithorax Group
TP53	Tumour Protein 53
UV	Ultraviolet
VC	Vehicle Control
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor Receptor
WNT	Wingless-related integration site
WT	Wildtype
2-HG	2-Hydroxyglutarate

CHAPTER 1:

Introduction

1.1 Cancer and Hallmarks of Cancer

1.1.1 Cancer

Cancer is a group of diseases defined by an accumulation of abnormal cells, called a tumour. The abnormal cells occur as a consequence of alteration in the cell's genome, called mutations, which lead these transformed cells to possess unnatural characteristics including uncontrolled growth (Cooper, 2000). The tumour increases in size over time and may acquire other features as the tumour evolves in this neoplastic environment. Often this may include other mutations (Gerstung et al., 2020). Over time, tumours can become complex, accumulating multiple cell types, and distinct regions (Mayer et al., 2023). It is the physical burden of the tumour mass that manifests the pathology of cancer, and thus, it is dependent on the tissue of origin as to which bodily systems are affected and the symptoms that may present. The ability of the cancer cells to spread from the primary site and form distant tumours, or metastasis, is usually the point at which the disease becomes lethal and multiple processes in the body are dysfunctional (Boire et al., 2024). Cancer cells can spread to neighbouring tissues, or via the bloodstream to other organs, bone or lymph nodes, to cause detrimental effects (Riihimäki et al., 2018).

The evolution of a normal cell to a cancer cell is a multi-factorial process and although the disease can originate in multiple locations in the human, the characteristics of this process are similar for all cancers. Hanahan and Weinberg classified these commonly observed characteristics in proposing the 'Hallmarks of Cancer' (Hanahan & Weinberg, 2000). These Hallmarks allow the sustained growth of a cancer cell population over normal cells, which is governed by several homeostatic controls. The Hallmarks include sustained

proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, resisting cell death and inducing angiogenesis (**Figure 1.1**). However, there have been new concepts added since the original six hallmarks hypothesised, and the hallmarks expand as more knowledge is acquired about the disease (Hanahan & Weinberg, 2011).

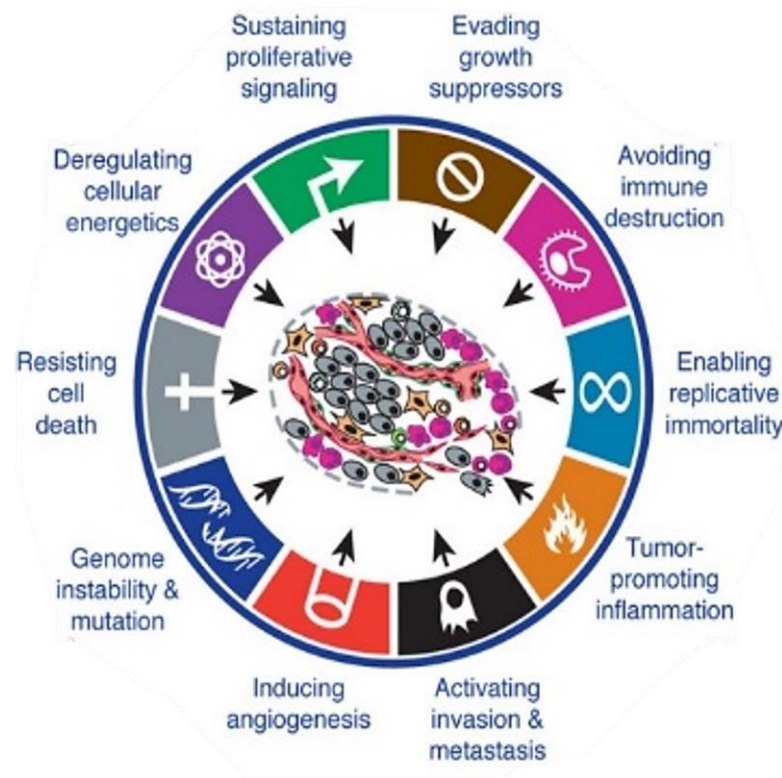


Figure 1.1: The Hallmarks of Cancer.
From Hanahan and Weinberg, 2011.

1.1.2 Sustaining Proliferative signals

One of the most fundamental hallmarks of cancer is sustained cell proliferation. The proliferation of cells is controlled by pro-growth and anti-growth signals to maintain the structural and functional capability of the tissue. Cancer cells deregulate these signals to over-ride any homeostatic control of proliferation and result in the growth of a population of cells. Sustained proliferation can be brought about by a range of factors including cell cycle related proteins or various upstream signalling pathways that regulate cell cycle.

1.1.2.1 Cell cycle signalling

The cycle of the cell contains four phases: G1, S, G2 and M. The majority of cells in the adult body have a non-proliferative status. However, upon stimulation, cells can enter the cell cycle at G1 phase. In G1 phase, the cell grows and prepares to divide, however cells can also enter G0 to become quiescent. The cell will stay in G1 until the cell has surpassed a threshold of signalling to overcome the S phase entry commitment point, or restriction point (R), during the G1 decision window. In synthesis or S phase, the cell replicates its DNA content. There is another decision window called the mitotic entry commitment point in the post-replicative G2 phase. Once the chromosomes are condensed and aligned correctly, this promotes the cell to enter mitosis or M phase. During mitosis, the genetic material and cellular contents are divided into two daughter cells and the cell cycle returns to the dominant interphase state (reviewed: Matthews et al., 2022).

The pivotal mediators of cell cycle transitions are cyclin dependent kinases (CDKs) and their activating proteins, cyclins. The expression of different cyclins follows a sequence through cell cycle that promotes the activity of different CDKs and thus the strict control of cell cycle transitions. There is an expansive and tightly regulated network of signals that control cyclin expression and degradation. Different CDK-cyclin complexes are associated with the different transitions. The CDK4/6-cyclin D complexes are thought to be essential for G1 entry. To promote G1 to S phase, cyclin E associates with CDK2 and CDK2-cyclin A complexes are also required during S phase. In mitotic commitment, cyclin A binds to CDK1 and CDK1-Cyclin B complexes are associated with mitosis (reviewed: Ding et al, 2020)

CDKs promote transitions by phosphorylating substrates such as retinoblastoma protein (RB). Upon phosphorylation, RB dissociates from partner proteins HDAC and E2F, E2F is then able to promote the transcription of cell cycle progression genes, including cyclin A and E. RB is hyperphosphorylated for the rest of the cell cycle making p-RB a marker of dividing cells. Cyclin E-CDK2 complexes phosphorylates NPAT (nuclear protein mapped to the Ataxia-Telangiectasia Mutated locus) in S phase entry, and also phosphorylate histone H1 to help condense chromatin in S phase. Cyclin A-CDK2 phosphorylate DNA polymerase alpha primase for DNA replication. Finally, CDK1 activity promotes the phosphorylation of cytoskeletal proteins required for mitosis (nuclear lamins, microtubules, vimentin) as well as cell cycle regulator proteins like WEE1 and CDC25 (reviewed: Vermeulen et al., 2003) (Figure 1.2).

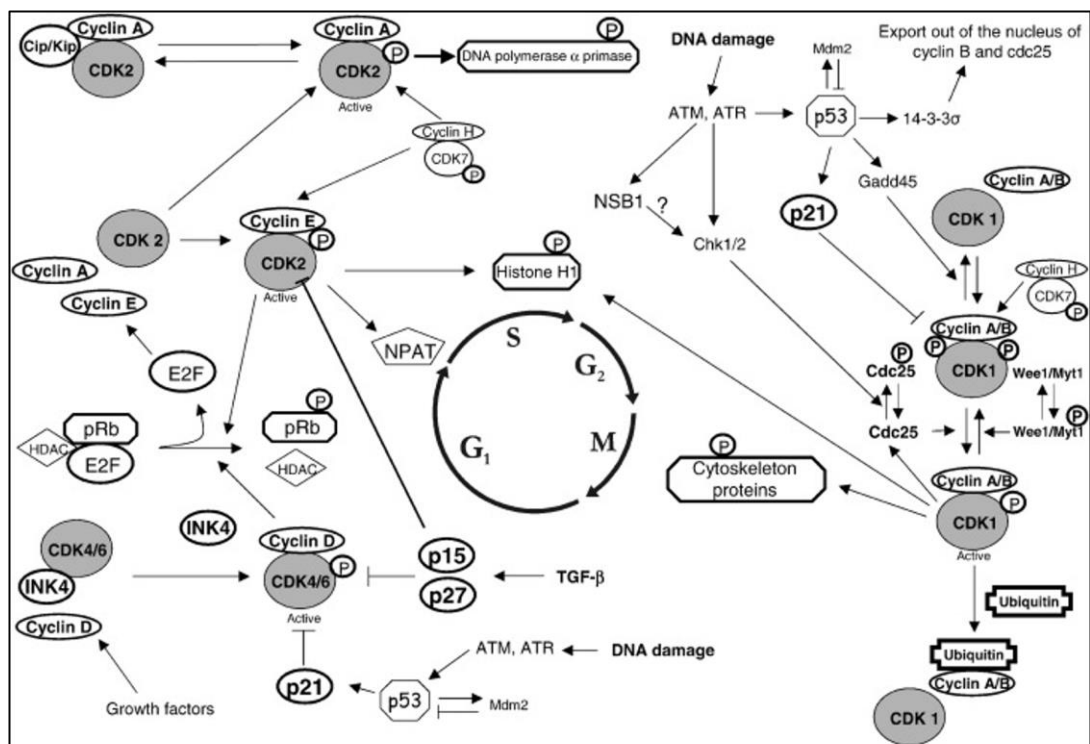


Figure 1.2: The Regulation of the Cell Cycle through Cyclin-CDK complexes.
From Vermeulen et al., 2003.

1.1.2.2 The Manipulation of Proliferative Signalling in Cancer:

Cyclin-CDK activity is extensively regulated by many systems, including the synthesis and degradation of cyclins, the phosphorylation of CDKs, and the binding of CDK inhibitors, such as p21 and p27 (Zheng et al., 2016). Aberrant regulation of Cyclin-CDK activity can cause cancer through manipulating these mechanisms. One common mechanism of dysregulation is the overexpression or amplification of Cyclins or CDKs, for example, Cyclin D1 or CDK4/6, which can initiate the cell cycle, leading to uncontrolled cell proliferation (Montalto et al., 2020). In addition, mutations or deletions to genes that are responsible for the cell cycle or spindle assembly checkpoints, such as Tumour Protein p53 or *TP53*, Ataxia-Telangiectasia Mutated protein or *ATM*, or Ataxia-Telangiectasia and Rad-3 related or *ATR*, can cause the cell to bypass signalling for DNA damage response and promote cell survival. Cancer cells can lose tumour suppressor genes function, such as *RB1* or members of the INK4 family (reviewed: Sami, 2023).

Usually, cells require upstream stimulation to enter the cell cycle however cancers can sustain proliferation by stimulating their own growth signals and become independent of extrinsic factors for growth. This manipulation by cancer cells can occur at any point in the pathway. Cancers may aberrantly express pro-growth factors to stimulate pathways, alternatively cancers may dysregulate the expression or activity of cell surface receptor and/or downstream mediators to render a pathway overactive. Similarly, loss of proteins that are responsible for the negative feedback of growth signalling can result in sustained cell proliferation. There are common growth transduction pathways that are deregulated in cancer such as the mitogen MAPK signalling pathway, the Akt/PI3K axis and mTOR pathway (Duronio & Xiong, 2013).

1.1.3 Evading growth suppression

Cells have multiple feedback loops and networks which are responsible for suppressing growth to maintain healthy tissue. Mostly these mechanisms involve tumour suppressor genes (Cooper, 2000). Frequently mutated tumour suppressor genes in cancer are *TP53* and *RB1*. The p53 protein is a molecular sensor that, upon cellular stress signalling, induces transcription programmes (Laptenko & Prives, 2006) to arrest cell cycle and depending on the damage to the cells, may promote apoptosis, a form of programmed cell death (Rozan & El-deiry, 2007). RB protein (so called as it is commonly deleted or mutated in retinoblastoma tumours) and its inactivation by phosphorylation, are determinants of cell cycle initiation. Thus, loss of *RB1* means the cell has less suppression to signals promoting cell proliferation (Velez-Cruz & Johnson, 2017). Other common tumour suppressor genes include members of the INK4 cell cycle inhibitors and Phosphatase and Tensin homolog or *PTEN*. Adenomatous Polyposis Coli or *APC*, and Mothers Against Decapentaplegic homolog 2 or *MADR2*, are tumour suppressor genes commonly associated with colon cancer (Zhang & Shay, 2017) (Eppert et al., 1996), and mutation of the Breast Cancer genes, *BRCA1/2*, are associated with breast and ovarian cancer (Rebbeck et al., 2015). At a tissue level cells, in response to high cell density, can suppress proliferation through contact inhibition, which ensures regular tissue homeostasis (Eagle & Levine, 1967). This inhibition is signalled through strengthening cell-cell contacts and stabilising epithelial cell polarity and structure. Cancer cells lose or reduce this feature (McClatchey & Yap, 2012).

1.1.4 Inducing Angiogenesis

The formation of blood vessels or angiogenesis is required for the maintenance of healthy tissues as it supplies oxygen and nutrients to the cells. The formation

of vasculature mainly occurs in embryogenesis; in the adult, it is usually only transiently activated, for example for wound healing. Cancers can activate an angiogenic switch to increase the supply of oxygen and nutrients to meet the demands of the growing tumour (Hanahan & Folkman, 1996). To promote angiogenesis, cells can increase Vascular Endothelial Growth Factor or *VEGF* signalling, through receptor (*VEGFR1-3*) overexpression or the release of ligand from the extracellular matrix with matrix degrading proteins (Kieran et al., 2012). Other signalling pathways in oncogenic angiogenesis are other growth factor signalling, angiopoietin and Tie signalling, signalling via integrins and Notch signalling (Lui et al., 2023). The growth of new vascular structures in tumours is usually malformed, haemorrhagic and permeable; this can promote cell invasion and metastasis (Bielenberg & Zetter, 2015).

1.1.5 Invasion and Metastasis

As mentioned previously, cancer can metastasise from the primary origin to a secondary site; this increase in tumour burden correlates with cancer fatality. Metastasis is a multi-step process (Fares et al., 2020). The cell must invade and disseminate, and these cells must adapt to a more migratory phenotype, for example through Epithelial-Mesenchymal-Transition or EMT. During EMT, the cell loses their epithelial features, such as E-cadherin expression, cell junctions and polarity, to become mesenchymal, showing increased motility and in many cases increased Vimentin expression (Kalluri & Weinberg, 2010). Once the cells have invaded the bloodstream, and survived the circulatory system, cancer cells extravasate to their secondary site where they can undergo mesenchymal to epithelial transition or MET (Fares et al., 2020).

1.1.6 Replicative Immortality

Repetitive DNA-protein complexes located at the end of chromosomes called telomeres are important in genome stability and by protecting the chromosome ends from damage. The telomeres are maintained through telomerase enzyme, a large ribonucleoprotein complex responsible for synthesis of telomere repeats (Jafri et al., 2016). In normal cells that have undergone many growth-and-divide cycles, telomere shortening occurs and if the telomeres become dysfunctional, it promotes the DNA damage response (DDR) and cells either become senescent or apoptotic. Cancers can overcome limited proliferation by maintaining telomeric DNA and thus avoiding the DDR signalling and downstream events. This can occur by the over activity or overexpression of the telomerase catalytic subunit, *TERT* (Jafri et al., 2016). As this replicative immortality causes the cell to exceed the cell's pre-programmed growth-and-divide cycle limit, *TERT* mutations can cause further aberrations within the cancer especially in mutant *TP53* cancer where general stress signalling is lost.

1.1.7 Evading Cell Death

Cancer cells frequently harbour adaptations that affect the homeostatic balance of the cell. Thus, the cancer cell also requires mechanisms of evading apoptotic signalling that is a result of the cellular stresses created by these mutations. Attenuated cell death signalling can also generate tumours that show chemotherapy resistance.

1.1.7.1 Cell Death Pathways:

Cell death can be classified into regulated cell death (RCD) or accidental cell death (ACD). ACD is promoted by an injury stimulus, but RCD occurs as a result of a cell death programme controlled by the cell. There are several types of

RCD: autophagy, apoptosis, necrosis, necroptosis, pyroptosis, lysosome-dependent cell death and others (Peng et al., 2022). Apoptosis and autophagy are commonly manipulated in cancers to overcome RCD.

There are two mechanisms for inducing apoptosis: intrinsic apoptosis is mediated through the mitochondria and extrinsic apoptosis is mediated through death receptors (Elmore, 2007). Upon intracellular stress signalling, the intrinsic apoptosis pathway increases the B-cell lymphoma 2 or Bcl2 family proteins. The accumulation of the pro-apoptosis Bcl2 protein at the mitochondria leads to the release of cytochrome C and other apoptosis mediators, such as the second mitochondria-derived activator of caspase, SMAC/DIABLO. Cytochrome C forms the apoptosome when complexed with Apaf-1 and caspase 9. The apoptosome initiates caspase 9 activation, via cleavage and this activates effector caspases, like cleaved caspase 3. Caspases are endoproteases and caspase 3 cleaves key proteins to induce the disassembling of the cell (Saelens et al., 2004). Whereas SMAC/DIABLO promotes apoptosis by neutralising a group of endogenous caspase inhibitors called the Inhibitor of Apoptosis (IAP) proteins (Du et al., 2000). During extrinsic apoptosis, external death ligands, such as Fas Ligand, tumour necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL), bind to their respective death receptor, Fas, TNF Receptor 1 (TNFR1) and death receptor 4/5. This triggers the binding of the mediator protein, Fas-associated death domain (FADD), to the receptor at the cell surface, along with caspase 8, to initiate the Death-Inducing Signalling Complex or DISC. DISC activates caspase 8, which in turn activates caspase 3 causing cell death (Peter & Krammer, 2003).

Autophagy is the breakdown of abnormal, excessive or damaged material or organelles via an autophagosomic-lysosomal system (Hollenstein & Kraft, 2020). Autophagy related (Atg) proteins accumulate to form a membraned

vesicle called the autophagosome (Suzuki et al., 2001). The formation of the autophagosome is a multistep process with many protein complexes. The mature vesicle then fuses with lysosomes and the release of the lysosomal hydrolases catalyse degradation of the encapsulated cargo. Autophagy cargo receptors target specific material for autophagy (Zaffagnini, et al., 2016), such as ubiquitylated proteins that are recognised by p62, but autophagy can also recruit cargo without selectivity, for example in starvation where autophagy functions to recycle biological matter (Hurley & Young, 2017). Cell death pathways are illustrated in **Figure 1.3**.

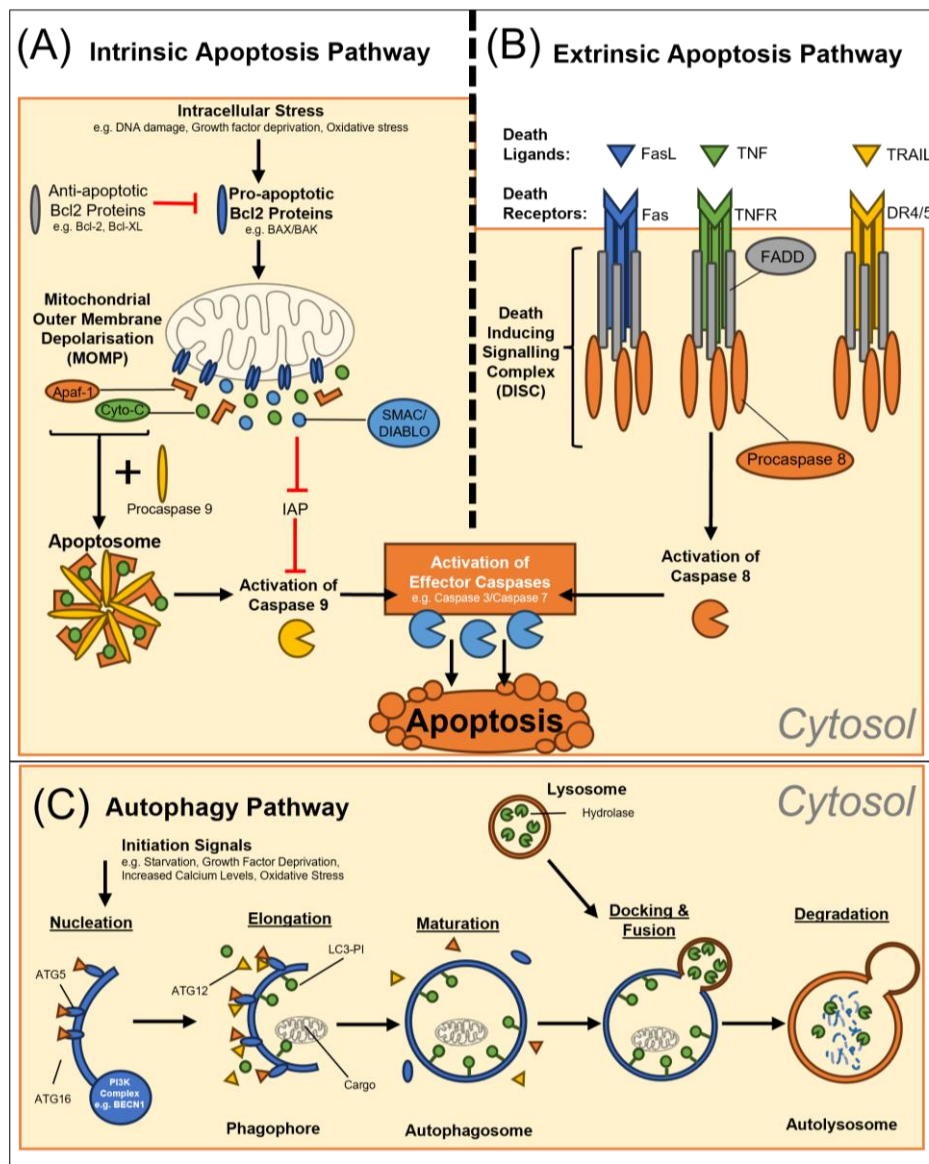


Figure 1.3: The Apoptosis and Autophagy Signalling Pathways.

The signalling pathways for both intrinsic (A) and extrinsic (B) apoptosis and autophagy (C).

1.1.7.2 Cell Death Pathways Manipulated in Cancer:

Cancers can lose the function of apoptotic sensors that would normally promote cell death signalling. As mentioned previously *TP53* is mutated in a high percentage of cancers. P53 protein is a potent apoptosis inducer and promotes the expression of Bax and Puma, BH3-only proteins (Ozaki and Nakagawara, 2011). Cancers can promote the abnormal expression of decoy death receptors to sequestering apoptosis signalling (Pitti et al., 1998), or decoy DISC mediator proteins that prevent caspase 8 activation to evade cell death (Micheau et al., 2003). Tumour cells can favour the expression of the anti-apoptotic Bcl-2 proteins, namely Bcl-2 and Bcl-XL, over the expression of pro-apoptotic Bcl-2 proteins Bax, Bim and Puma (Adams et al., 2007). The Inhibitor of Apoptosis (IAP) proteins, which are canonically expressed upon the upregulation of survival signalling, can be utilised by tumour cells to promote resistance to apoptosis (Michie et al., 2020).

Interestingly, autophagy has been shown to have roles in tumour suppression but also has been manipulated by cancers to maintain their survival (Debnath et al., 2023). The mutation of autophagy-related genes has been found in cancer, such as *BECN1* in breast cancer and the *ATG* genes in GI-tract and liver cancers (Liang et al., 1999) (Kang et al., 2009). Similarly, the deletion of these genes results in tumours in mouse models (Qu et al., 2003). However, autophagy has pro-tumour roles in clearance of abnormal organelles (Miller & Thorburn, 2021), escaping immune detection (Yamamoto et al., 2020) and has some roles in promoting metastasis (Sharifi et al., 2016).

1.1.7.3 Senescence and the role of p53/p21 in Cancer

Reports by Hayflick and Moorhead showed that primary human cells present a limited lifespan and coined the term cellular senescence (Hayflick and

Moorhead, 1961). Cellular senescence is characterised by an irreversible cell cycle arrest, which can occur in response to variety of damaging stimuli such as telomere shortening, specifically called repetitive senescence. In the normal tissue, senescence is used by the cell to halt cellular processes and assess damaged cells. However, senescence can also occur through the activation of oncogenes (Palikowski et al., 2013). The senescent cell has a senescence-associated secretory phenotype (SASP) where the cells express and secrete cytokines, extracellular matrix enzymes and other factors (Coppe et al., 2010). The SASP is partly dependent on DNA damage response (DDR) activation.

The protein p53 plays a critical role in initiating senescence and is triggered downstream of the DDR, through activation by ATM (Mijit et al., 2020). P53 is responsible for many cellular processes as discussed previously, however it is thought that stable stimulation of p53 triggers senescence, whereas short stimulation results in a transient block of cell cycle and potent stimulation induces apoptosis (Mijit et al., 2020). Senescence-associated functions of p53 are mediated through its downstream target, p21, which can cause cell cycle arrest through CDK inhibition, and can also block apoptosis by binding and sequestering caspase enzymes (Yosef et al., 2017).

The p53/p21 axis is dysregulated in multiple types of cancer (Kneis et al., 2019). P21 is downregulated in loss of p53 function (i.e. p53 mutation) and this can result in poor prognosis as cell cycle progression cannot be controlled. Conversely, p21 overexpression is also seen in a variety of cancers where its anti-apoptotic function is up regulated, and tumours are associated with chemotherapy resistance. The dual roles of p21 as pro-oncogenic or anti-oncogenic are dependent on its cellular localisation, and pro-oncogenic p21 is usually associated with p21 translocation to the cytoplasm from the nucleus. Thus, alterations in p21 can be anti or pro-oncogenic (reviewed in Abbas &

Dutta, 2009). Similarly, induction of senescence can be anti-oncogenic, by inhibiting the cell cycle, and also pro-oncogenic by supporting tumour expansion, dysregulation of the immune response and promoting chemotherapy resistance (Gonzalez-Meljem et al. 2018).

1.1.8 Additional Cancer characteristics

Other emerging hallmarks of cancer include deregulating cellular energetics, avoiding immune destruction, and the tumour microenvironment (Hanahan & Weinberg, 2011). Cancers can switch their glucose metabolism to become oxygen independent (the Warburg Effect) and it is thought that this can benefit the cancer by providing rapid energy supplies, promoting biosynthesis flux and promoting tumour microenvironment changes, such as inducing hypoxia or acidosis, which can both promote cancer invasion (Liberti & Locasale, 2015). To overcome immune detection and attack, cancer cells can recruit immune-suppressive cells such as regulatory T cells (Tregs), harbour defective antigens to evade detection, and promote Th1-Th2 polarisation (Vinay et al., 2015). The tumour microenvironment (TME) promotes cancer survival and progression and permits cells to become invasive and spread through metastatic cascade (Wang et al., 2023). The TME has many pro-oncogenic features including M2 macrophages that promote growth and induce immunosuppression, cancer exosomes that promote cell migration and cancer-associated fibroblast that promote cancer progression (Wang et al., 2023).

1.2 Transcription and Transcriptional Regulation

1.2.1 Introduction to the Central Dogma: Gene to RNA to protein:

A genome is a complete set of DNA that is required to build and maintain an organism and is contained in almost every cell in the organism. The human genome is approximately three billion base pairs in length and is estimated to encompass 20,000-25,000 genes (Amaral, 2023). In multicellular organisms, cells differentiate by having varying gene expression profiles (Levine & Tjian, 2003). This means a certain cell type will express a specific program of genes, produce the corresponding RNA transcripts, and a particular set of proteins, to determine cell structure and function. This concept or the 'central dogma of molecular biology' was proposed by Francis Crick in 1958, and the theory describes the flow of the transfer of information from DNA to RNA and eventually a functional protein product (Crick, 1958). From the 1960, the process of converting DNA to RNA, or transcription, and the process of converting RNA to protein, or translation, have become both well understood and accepted processes (Alberts et al., 2002). There has been extensive research to unravel the mechanisms involved in the regulation of gene expression and how it can be manipulated, for example in cancers. The following section will describe transcription and transcriptional regulation by offering a brief summary of chromatin, the transcription initiation complex, epigenetics, transcription factors and gene expression with a focus on cancer.

1.2.2 Chromatin Structure:

In 1953, Crick and Watson colleagues proposed the structure of DNA, a double strand helix of two phosphate sugar chains held together by pairs of purine and pyrimidine bases (Crick & Watson, 1953). In the human genome the DNA is present as 23 pairs of chromosomes (Tjio & Levan, 1956). The condensed chromosome seen at mitosis is the highest order of DNA compactness. Chromosomes are composed of supercoiled chromatin, and chromatin is made from repeating structural units called nucleosomes along with other proteins. In electron microscopy, chromatin is comparable to beads on a string (Olins & Olins, 1974). The beads consist of around 146bp of DNA wrapped around an octamer of histones proteins, two histone H3/H4 dimers and two histone H2A/H2B dimers, with the complex referred to as nucleosomes. Histones are positively charged proteins so bind tightly to the negatively charged DNA. The addition of histone H1 linkers between the nucleosome forms a chromatosome. The 'beads on a string' are compacted into a 30nm fibre, which forms loops in a supercoiled 250nm-wide fibre (**Figure 1.4**). For transcription to occur, chromatin is re-structured by chromatin remodelling- and modifying-complexes. It is largely due to the work of Kornberg and colleagues, who characterised the nucleosome (Kornberg, 1974), that we understand chromatin structure and its effect on transcription (Kornberg & Lorch, 1999).

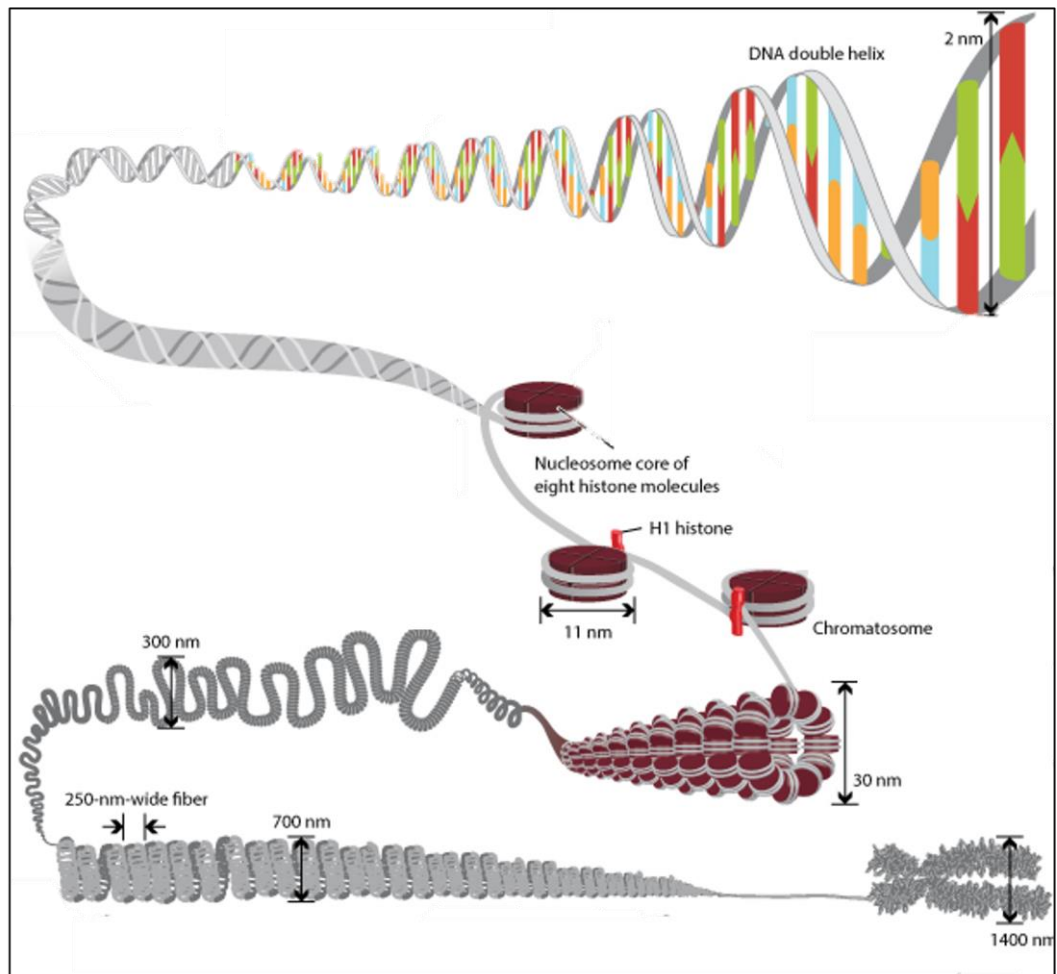


Figure 1.4: The Compaction of Chromatin.

The DNA double stranded helix is wrapped around histones proteins to form nucleosomes. The addition of histone H1 linkers between the nucleosome forms a chromatosome. The 'beads on a string' are compacted into a 30nm fibre, which forms loops in a supercoiled 250nm-wide fibre. This supercoiled chromatin is forms chromosomes, the highest order of DNA compaction. From Annunziato, 2008.

1.2.3 Chromatin Remodelling:

For gene expression, the DNA must be made accessible to the various proteins involved in transcription by remodelling its chromatin structure. Eukaryotic genomes exist in a constitutively repressed 'ground state' as highly condensed chromatin, which make the genes highly inaccessible. This closed chromatin is often referred to as heterochromatin. The unwrapping of chromatin from its tightly wound state to an open structure, or euchromatin, is required for gene

expression and is called chromatin remodelling. The remodelling is associated through histone modification (reviewed: Morrison, 2021).

Histones are rich in residues, including lysine, serine, threonine and arginine, which allow for a range of post-translational modifications (PTMs) such as: acetylation, methylation, ubiquitylation and phosphorylation. Specific patterns of histone modifications, or histone 'marks', result in different structure and functions, and tell us whether a region of chromatin is active or repressed. Whilst early studies demonstrated that hyper-acetylated histones could be associated with activated genomic regions and methylated regions with gene repression (Allfrey et al., 1964), methylation is more complex. Generally, the methylation of Histone 3 Lysine 4 (H2K4me3) and acetylation of Histone 3 Lysine 27 (H3K27ac) is associated with activation (Santos-Rosa, 2002) (Creyghton et al., 2010). In contrast, the methylation of Histone 3 Lysine 27 (H3K27me3) and ubiquitylation of histone H2A at Lysine 119 (H2AK119ub1) result in gene silencing (Rea et al., 2000) (de Napoles et al., 2004).

These modifications are a result of the activity of a spectrum of enzymes that precipitate in the recruitment of transcriptional activators or repressors that are often part of a multi-functional complex. These enzymes can be grouped into 'writer', 'erasers' and 'readers' (Gillette & Hill, 2015). The writer enzymes are histone methyltransferases (HMTs) and histone acetyltransferases (HATs) that respectively add methyl and acetyl groups to specific residues of the histones. The eraser enzymes are histone demethylases (HDMs) and histone deacetylases (HDACs) that remove histone PTMs. The reader proteins identify these signals through domains that have specific affinity to histone PTMs to mediate either gene expression or repression. Domains that have affinity to methylated histones include the PHD, chromodomain, WD40, Tudor and MBT, and the domains that have affinity to acetylated histones are the PHD domain

and bromodomains (Yun et al., 2011). Polycomb group (PcG) and Trithorax group (TrxG) are transcriptional co-repressors and co-activators, respectively, and are well-documented due to their roles in development and control of *HOX* genes (Ingham, 1983). Canonical PcG complexes contain a chromodomain and are associated with binding methylated chromatin and repression; classically the opposing TrxG complexes contain a bromodomain and are associated with binding to acetylated chromatin and activation (Shuettengruber et al., 2017). The TrxG complex also contains SWI/SNF proteins that can increase chromatin access by nucleosome 'sliding' (Clapier et al., 2017). PcG complexes methylate H2K27 and ubiquitylate H2AK119 which interferes with transcription initiation and causes gene repression. Also, PcG complexes undergo oligomerisation to allow long-range interactions, resulting in chromatin looping and tighter chromatin packaging into heterochromatin (Entrevan et al., 2016).

1.2.4 The Transcription Complex and Transcription

Initiation

RNA synthesis begins with the binding of a DNA-dependent RNA polymerase to the promoter region of a gene. There are several RNA polymerase enzymes in human cells, however it is RNA polymerase II (RNA pol II) that can read DNA template strands to produce messenger RNA transcripts. When studies highlighted purified RNA pol II could not initiate transcription alone (Roeder, 1976) (Weil et al., 1979), it was discovered that cofactors are required for the recruitment of RNA pol II to the gene promoter (Matsui et al., 1980). These are termed the General Transcription Factors (GTFs). There are six involved in the basic transcription machinery or preinitiation complex (PIC), along with the polymerase: TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH. The highly conserved assembly of the PIC complex has been thoroughly reviewed (review:

Orphanides et al., 1996), with all the GTFs assigned a role in a cascade of sequential functions in transcription initiation.

The recognition of the gene promoter occurs through the TFIID complex. Promoters are specialised regions of DNA found directly upstream of a transcriptional unit. They contain binding sites for various protein factors, and result in the nucleation of transcriptional machinery. A common core promoter element is the TATA box. This is a stretch of A and T bases situated 25-30 upstream of the transcription start site (Goldberg & Hogness, 1978). Beyond the core promoter, there are proximal and distal promoter elements that contain regulatory sequences such as enhancers, silencers and insulators that have roles in regulating transcription. TFIID interacts with the TATA box through a TATA box-binding protein (TBP) (review: Hernandez, 1993). TBP is a “saddle-shaped” protein that binds the minor groove of the TATA box through a large DNA-binding domain and induces DNA distortion (Kim et al., 1993). The conformational change in the DNA leaves it partially unwound. In addition, TBP-associated factor (TAF) subunits interact with TFIID and are essential for some kinds of transcription. TAFs have roles in recognising downstream promoter elements, act as coactivators, and interact with nucleosomes (Joo et al., 2017).

TFIIB binds the TFIID-TATA complex (Yamashita et al., 1993) and the TFIIF-RNA pol II complex (Ha et al., 1993), tethering them together, and binds DNA through the TFIIB recognition element (BRE). This strengthens the integrity of the TFIIB-TFIID-TATA complex and aids in positioning the PIC correctly on the promoter (Deng and Roberts, 2005). TFIIA, TFIIIE and TFIIH are then recruited to the PIC complex, which assembles at the +1 nucleosome. TFIIIE is believed to have roles in promoter melting and allows TFIIH to enter the complex (Holstege et al., 1995). TFIIH is a large complex with structural subunits and two helicases, XPB and XPD, that have roles in DNA repair and ATP-dependent melting of

promoter DNA. The XPB translocase in TFHII drives the rotation of +1 nucleosome resulting in PIC-nucleosome complexes and unravelling of the nucleosomal DNA (Wang et al., 2023). Mediator is a co-activator which is recruited by transcription factors and stimulates the phosphorylation of RNA pol II C-terminal (CTD) domain by the TFIIH subunit CDK7 (Abdella et al., 2021). When the polymerase loses affinity to the Mediator, it breaks contact with the PIC in a process called promoter escape (reviewed: Rimel et al., 2018) (**Figure 1.5**).

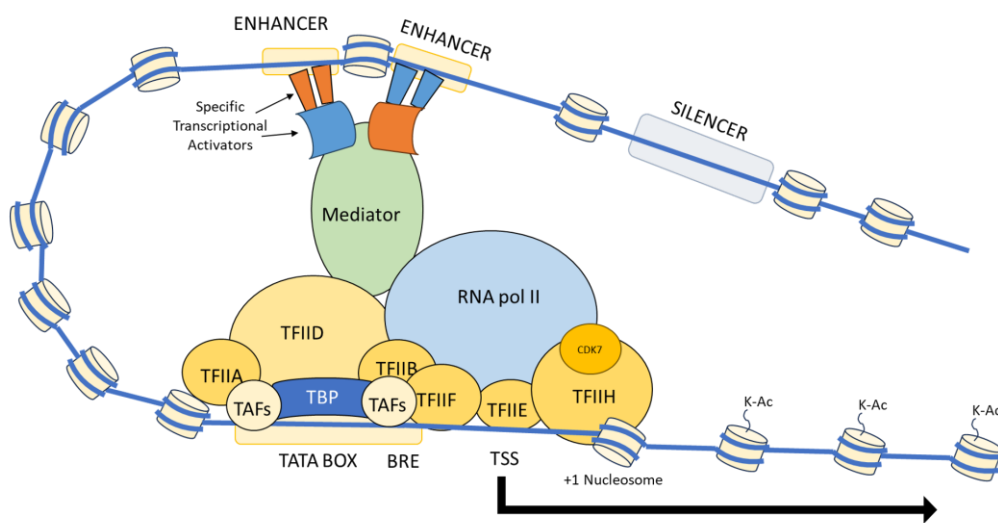


Figure 1.5: The Transcription Initiation Complex.

There are six general transcription factors (GTFs) involved in the basic transcription machinery along with the polymerase: TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. Each GTF has a role in the initiation cascade. Mediator co-ordinates the formation of the initiation complex by binding upstream element like enhancers via specific transcriptional activators to promote gene expression.

1.2.5 Transcription Factors

So far, the 'basal' transcription machinery has been described. This apparatus cannot express a gene unless an 'activator' protein activates it. The activator, or transcription factor (TF), usually has multiple functional domains including motifs for site-specific DNA-binding, to the promoter or enhancer, and domains for binding the transcription initiation complex or motifs for binding other proteins, such as other co-activators or co-repressors (Cooper et al., 2000). These TF proteins occur in a range of forms and do not only activate

transcription. The TFs in fact use a variety of different mechanisms to promote, or repress, various steps of RNA synthesis including removing basal transcription complex associated repressor proteins, covalent modifications, remodelling chromatin conformation, GTF or RNA pol II recruitment as well as promoter clearance and elongation (reviewed: Ma, 2011). Pioneer transcription factors are a class of factors that can directly interact with nucleosomal DNA to orchestrate gene expression or repression (Yu et al., 2020). Overall, it is the balance of activating and repressing transcription factors that determine gene expression.

1.2.6 Epigenetic Regulation of Transcription:

Epigenetic regulation of transcription is heritable gene activity that occurs through mechanisms that do not involve in changes in DNA sequence. As previously mentioned, enzymes that effect chromatin remodelling are often classed as epigenetic regulators of gene expression. Other processes are DNA methylation and RNA based mechanisms (Gibney & Nolan, 2010). Methylation of the 5'-position of cytosine in DNA, to form 5-methyl-cytosine, can affect recognition of DNA by DNA binding proteins to causes gene repression (Newell-Price et al., 2000). DNA is methylated by DNA methyltransferases (DNMTs) and the methylated DNA is condensed by methyl binding protein (MBPs) and demethylation occurs through the loss of their activity and the activity of TET dioxygenases. CpG rich regions of the chromatin called CpG islands (Gardiner-Garden & Frommer, 1987), avoid methylation by encoding demethylation signals and regulate gene expression (Elango & Yi, 2011). Small non-coding RNA (sncRNA) and long non-coding RNA (lncRNA) (Gibney & Nolan, 2010) also control transcription. Small RNA species such as microRNA and siRNA can inhibit translation or promote

mRNA degradation through RISC complex. Small interfering RNA can also cause gene silencing at a transcription level (RITS) (Carthew et al., 2009). Long cnRNAs can change gene expression by targeting proteins like chromatin remodelling proteins to genes to affect transcription, modulating the activity of protein binding partners, modulating the processing of other RNAs, and maintaining structural function in subcellular bodies (reviewed: Wiluz et al., 2009).

1.2.7 Dysregulation of Gene expression in Cancer

The regulation of gene expression therefore occurs at multiple levels, from the effect of epigenetic modifiers to the formation of the transcription initiation complex. However, in cancer dysregulation of gene transcription occurs through multiple mechanisms.

Global hypomethylation of DNA and hypermethylation of CpG were observed in cancer (Feinberg & Vogelstein, 1983). It is thought that the global hypomethylation of DNA can promote gene activation and lead to the overexpression of oncogenes, like Cyclin D2 or *CCND2* (Oshimo et al., 2003). Similarly, the hypermethylation can promote silencing of tumour suppressor genes, such as *RB1* (Sakai et al., 1991). Enzymes and proteins involved in histone modifications are also altered in cancer. For example, *E2F1* activity is increased upon gene acetylation through p300/CBP activators to promote cell cycle progression (Martinez-Balbas, et al., 2000). Similarly, the loss of heterochromatin protein 1 (HP1) and inhibitor of growth (ING) proteins, which both have roles in maintaining silenced chromatin through chromatin remodelling, result in cancer progression (Norwood et al., 2006) (Ythier et al., 2008). The enhancer elements upstream of the promoter can become 'super enhancers' that have a constitutively active gene regulatory state and drive

uncontrolled proliferation in cancer (Sur & Taipale, 2016). The CCCTC-binding factor or CTCF plays a key role in organizing chromatin by insulating cohesion-mediated loops of chromatin. Genetic alterations in *CTCF* have been discovered in cancers (Debaugny & Skok, 2020). Dysregulation of miRNA occurs in cancer and many miRNA are biomarkers of cancers; this can arise from miRNA deletion or amplification, aberrant miRNA-chromatin remodelling protein interactions and defects to miRNA processing (Peng et al., 2016). Finally, in cancer, genetic mutations, and genetic rearrangements and epigenetic alterations aberrations may can often lead to the activations of oncogenes or to the inactivation of tumour suppressor genes and promote aberrant gene expression programs that ultimately promote pro-tumourgenic oncogenic signalling cascades (Bradner et al., 2017).

1.3 The Biliary System and

Cholangiocarcinoma:

1.3.1 Introduction:

The biliary tree is a system of tubules that exist within, (intrahepatic), and outside of, (extrahepatic), the liver. The main function of the biliary system is to drain waste products from the liver, and to collect bile from bile-producing cells and control its release to either the gall bladder for storage, or to the small intestine to aid in absorption of fats and fat-soluble vitamins.

1.3.2 The Structure of the Biliary System:

The most upstream structures of the system are tiny vessels called canaliculi, which are situated within the liver parenchyma. The canaliculi are channels

formed between two cords of bile-secreting hepatocyte cells, which only have a width of 0.5-2µm in diameter, and they merge to form the canals of Hering (Saxen et al., 2004). The canals of Hering are part of the liver lobule; these structures are important in supplying the liver with circulatory blood from the hepatic artery and filtering the nutrient-enriched blood from the portal vein. The surrounding hepatocytes excrete bile salts into the canaliculi, and due to diffusional pressures, the salts flow into the small bile ducts. These small bile ducts are lined with biliary epithelial cells or cholangiocytes. Cholangiocytes are characterised by their cuboidal shape and by the cilia on the apical surface membrane. The salts cause water influx and allow the flow of bile along the ducts via diffusion (Ezzat et al., 2020). The bile duct runs in parallel with the portal vein and hepatic artery in the portal triad. The small bile ducts fuse into the left and right hepatic ducts. The canaliculi, small bile ducts plus the left and right hepatic ducts are classed as intrahepatic ducts.

Beyond the left and right branches, ducts are categorised as extrahepatic ducts and can be divided further into perihilar or distal ducts. The region where the left and right ducts leave the liver is called the hilum or hilar area. The left and right hepatic ducts join to form the common hepatic duct. If the bile acid is to be stored, it enters the gall bladders, a small pear-shaped gland below the liver, via the cystic ducts. The common hepatic duct and the cystic duct are termed the perihilar ducts. If the bile is to be expelled into the duodenum for absorption, it travels through the common bile duct, out the ampulla of Vater. The common bile duct is a distal duct (Castaing, 2008). In conclusion, the biliary system is a multi-staged, specialised funnelling system (**Figure 1.6**).

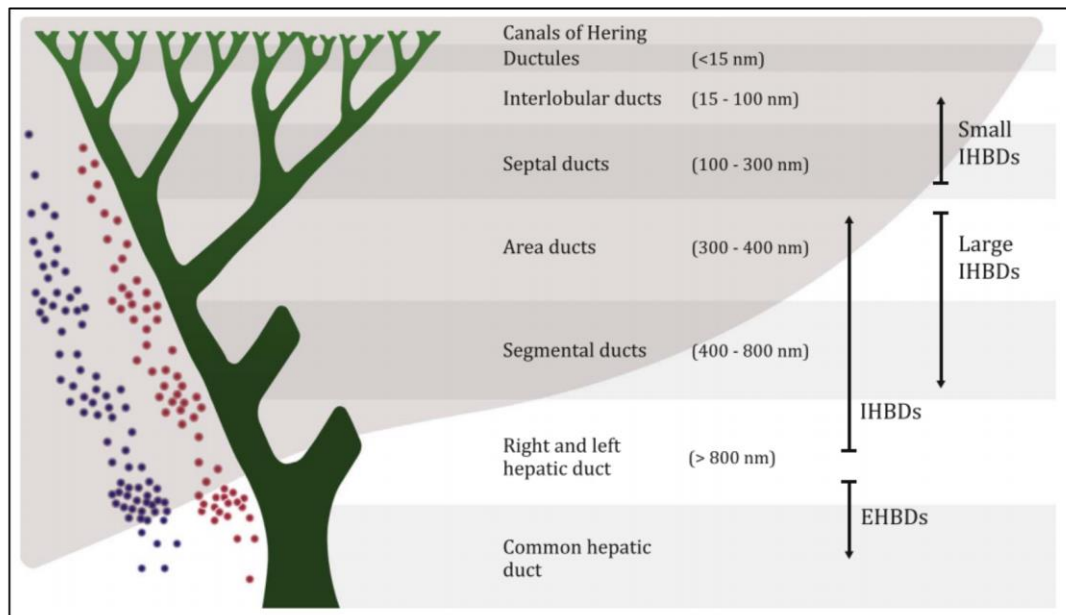


Figure 1.6: The biliary tree is a multi-staged, specialised funnelling system.

Figure from de Jong et al., 2018.

1.3.3 The Function of The Biliary System:

Bile is a yellow-green fluid that consists of bile salts, bile pigments (such as bilirubin), cholesterol, phospholipids, electrolytes and water. The salts emulsify lipids from the diet and bile is therefore important for the breakdown of foods and absorption of fat and fat-soluble vitamins, such as vitamin A, E, K and D (Hofmann and Hagey, 2008). The gallbladder releases bile upon meal digestion, and once the bile acids have entered the duodenum, they are actively and passively re-absorbed and returned to the liver. This loop is called enterohepatic circulation. Hepatocytes secrete the main elements of bile into the Canal of Hering. When this reaches the bile ducts, the cholangiocytes secrete HCO_3^- , via a Cl^- gradient, which gives bile an alkaline pH and aids in absorption. Cholangiocytes modify bile acid composition through re-absorption of bile acids, ions, glucose, and other components through modulating several channels, transporters and exchangers on the apical membrane, such as cystic fibrosis transmembrane conductance regulator (CFTR) and Aquaporins.

Cholangiocytes express many ion transporters, channels and exchangers for this function. (Tabibian et al., 2013).

1.3.4 Development of the Liver and Biliary System:

In embryogenesis, the liver tissue bud derives from the anterior foregut endoderm, which develops two protrusions, the cranial and caudal portions, that result in intrahepatic and extrahepatic bile duct formation, respectively (Roskams et al., 2008). The two portions merge at the hilum. The development of the biliary tree is intertwined with liver arterial vasculogenesis, at a protein signalling level (Fabris et al., 2008) and inactivation of biliary epithelium differentiation-associated transcription factors leads to bile duct, as well as hepatic artery, abnormalities.

There are liver-specific transcription factors that commit endoderm to hepatic fate. The pioneer transcription factor *FOXA* is expressed in all stages of liver development, then other transcription factors including the *GATA* TFs, *PROX1* and *PRH/HHEX* are expressed at very early liver specification stages, followed by hepatocyte nuclear factors (HNFs) at later stages of liver differentiation. Liver progenitor cells, or hepatoblasts, can differentiate into hepatocytes or cholangiocytes; the expression of *HNF4A* will lead the hepatoblast to differentiate to hepatocytes, the expression of *HNF1B* and *HNF6* results in cholangiocyte lineage (Tachmatzidi et al., 2021). The loss of *PRH/HHEX* TF expression leads to a loss of hepatic structure and the loss of HNF TF expression, thus *PRH/HHEX* is pivotal to liver development, and this is discussed in detail in section 1.4.4 (Hunter et al., 2007). The expression of these transcription factors is regulated through signalling pathways, such as fibroblast growth factor (FGF) and bone morphogenetic protein-4 (BMP-4) (Strazzabosco and Fabris, 2012) and transforming growth factor β (TGF β) gradient, where high

activation of TGF- β , Notch, BMP and FGF pathways commits a cell to biliary fate (Clotman and Lemaigre, 2006).

Notch signalling is critical for biliary development and maturation by activating the cholangiocyte transcription factors, HNF1 β and Sox9, and down regulating liver transcription factors, HNF1 α and HNF4. It is also important for intrahepatic tubule formation (Sparks et al., 2010). Wnt signalling also seems to be important throughout biliary development in canonical and non-canonical pathways. For example, Wnt ligand Wnt3a causes biliary differentiation in liver samples (Hussain et al., 2004). Additionally, common morphogenic signalling pathways such as Hedgehog are required to be shut down to enable hepatoblast differentiation in early embryogenesis (Hirosie et al., 2009). In parallel to cell differentiation, biliary tubule formation needs the proliferation of cells; this can be stimulated by several factors, namely oestrogens, insulin-like growth factor 1 (IGF1), interleukin-6 (IL-6) and VEGF (Strazzabosco and Fabris, 2012). Once biliary development is finalised, cholangiocytes enter a quiescent state, which is partly mediated through FOXA1 and FOXA2. FOXA1/2 represses IL-6, and without this repression, IL-6 leads to increased proliferation and increased portal fibrosis (Li et al., 2009). IL-6 acts as a biliary mitogenic by activating the (p44/p42) MAPK pathway (Park et al., 1999).

1.3.5 Non-malignant Disease of the Biliary System:

Cholangiocytes are subjected to many damaging substances including bile acids, toxins, pathogens and metabolites. Usually, the liver can repair damage, however, abnormal responses to hepatic injury, through genetic susceptibility or epigenetics, can lead to disease-promoting signalling. Disease of the cholangiocytes or cholangiopathies have commonalities such as biliary epithelium proliferation and differentiation, inflammatory signalling and

involvement of the immune response (Banales et al., 2018). Cholangitis is the inflammation of the hepatobiliary system and is characterised by the narrowing or obstruction of the ducts. Cholangitis syndromes can be classed into immune-related such as Primary Biliary Cholangitis (PBC), idiopathic such as Primary Sclerosing Cholangitis (PSC), secondary-sclerosing, and acute cholangitis (Mohammad, 2017). The only definitive treatment for PBC and PSC is transplantation, however there are drugs that suppress symptoms and block progression of the disease including anti-inflammatory drugs and bile (Purohit and Cappell, 2015).

1.3.6 Cholangiocarcinoma (CCA):

1.3.6.1 CCA Background:

Bile Duct Cancer, or Cholangiocarcinoma (CCA), refers to a group of malignancies that arise in the biliary system. CCA accounts for 15% of liver cancers and is the second most common primary liver cancer, after hepatocellular carcinoma (HCC). CCA is classed as a rare cancer and has an incidence of 0.3-6 per 100,000 persons, however those statistics have been increasing globally. In addition, it is a highly aggressive cancer, and despite progress to knowledge of CCA, 5-year survival is as low as 10% (Everhart and Ruhl, 2009) (Kamsa-Ard et al., 2015) (Spolverato et al., 2016) (Cambridge et al., 2021). It can be subdivided into classifications based on the location of the cancer. CCA located within the liver is referred to as intrahepatic CCA (iCCA), and when located outside the liver is referred to as extrahepatic CCA (eCCA). eCCA is further divided into perihilar CCA (pCCA) and distal CCA (dCCA). CCA can also be segregated by whether the tumour growth is mass forming (MF-CCA), periductal-infiltrating (PI-CCA) or intraductal-growing (IG-CCA) (**Figure 1.7**). These classifications are important as the different types of CCA have

varying incidence, biology, treatments and patient outcome (Banales et al., 2020).

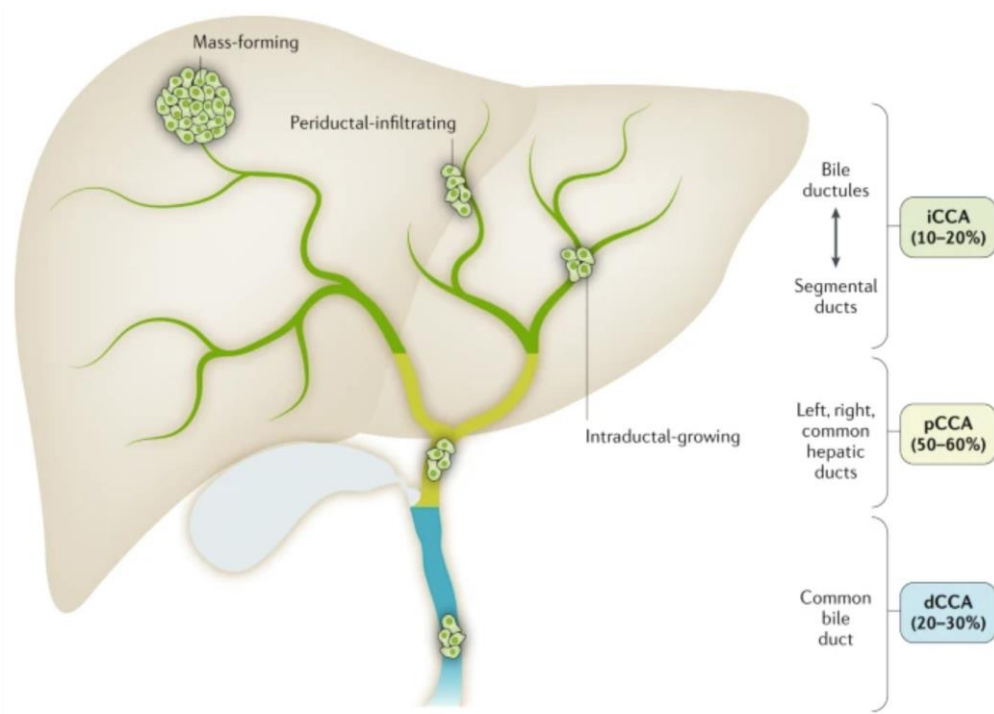


Figure 1.7: The Different Types of Cholangiocarcinoma.

CCA can be divided into classes intrahepatic (iCCA; in green), perihilar (pCCA; in yellow) and distal CCA (dCCA; in blue) depending on tumour location, and each has its own biology, patient outcomes and prevalence, 10-20%, 50-60% and 20-30%, respectively. Tumours can be mass-forming, periductal-infiltrating and intraductal-growing. Figure from (Banales et al., 2020)

1.3.6.2 CCA Risk factors and Pathogenesis:

CCA has many risk factors including cholangitis diseases, Primary Biliary Cholangitis and Primary Sclerosing Cholangitis. These diseases involve the dysregulation of the immune system. Additional risk factors include bacterial infection, acute cholangitis, as well as viral and parasitic infection, such as hepatitis B/C and liver fluke infection, increasing the chance of malignancy (Shin et al., 1996) (Prueksapanich et al., 2018). Inflammatory disease, namely irritable bowel disease and chronic pancreatitis, and metabolic disease (non-alcoholic fatty liver disease, diabetes, and obesity), are associated with CCA, as well as exposure to environmental toxins like tobacco and alcohol (Welzel et

al., 2011). The common denominator of all these risks is their strong relationship with inflammation or cholestasis. It is likely that these two processes, along with other growth factors and cytokines in the tumour microenvironment, are key mediators of CCA pathogenesis, and promote a number of intracellular pathways that ultimately lead to increased cell proliferation, growth, survival and resistance in CCA (Labib et al., 2019).

CCA is associated with high expression of inflammatory markers including IL-6 (Sugawara et al., 1998), TNF- α (Koromi et al., 2008) and COX-2 (Han et al., 2004). IL-6 is usually regulated by a negative feedback loop via SOCS3, however in CCA, this control is lost (Isomoto et al., 2007). In CCA, IL-6 is seen to: upregulate apoptotic inhibitor MCL-1 via STAT-5 (Kobayashi et al., 2005), decrease cell cycle inhibitor p21 by activating p38 MAPKs (Tadlock and Patel, 2001), and activate the Akt pathway through progranulin expression resulting in cell division, survival and migration (Frampton et al., 2012). TNF- α causes activation-induced cytidine deaminase (AID) gene expression in CCA. AID has mutagenic activity as the enzyme can exchange cytosine for uracil in DNA, therefore TNF- α directly links inflammation to mutagenesis and cholangiocarcinoma formation (Komori et al., 2008). COX-2, responsible for prostaglandin production in the inflammatory response, has increased expression in CCA, and COX-2 inhibitor, celecoxib, inhibits cell proliferation in CCA cell lines (Han et al., 2004).

Bile acids have a role in CCA tumourigenesis. It was found that specific conjugated bile acids (CBA) promoted CCA cells growth *in vitro* and in a mouse xenograft model, by IL-6 and COX-2 expression via NF- κ B activation (Dai et al., 2013). CBA also decrease farnesoid X receptor (FXR) gene expression; this receptor has tumour suppressor abilities (Dai et al., 2011) and regulates *HHEX* expression (Xing et al., 2009). Finally, CBA induce ERK and Akt signalling,

through sphingosine 1-phosphate receptor (S1R), to mediate CCA growth and invasion (Lui et al., 2014).

1.3.6.3 CCA Genetic and Epigenetic Alterations:

Integrative genomic approaches and sequencing studies have revealed that CCA has common genetic and epigenetic alterations within the transcriptomic landscape. Somatic alterations are seen in forms of mutation, homozygous deletion, amplification and gene fusion at oncogene and tumour suppressor genes (Nakamura et al., 2015) (Jusakul et al., 2017) (Ross et al., 2014) (Zou et al., 2014). Briefly, these genetic aberrations are found in genes that are associated with cell cycling (*CDKN1A*, *CDKN2A*, *CDKN2B*, *CCND1/3*, *RB1*, *CDK6*), DNA repair and genomic instability (*TP53*, *MDM2*, *BRCA1-2*), kinase-RAS signalling (*FGFR1-3*, *EGFR*, *ERBB1-3*, *KRAS*, *NRAS*, *BRAF*, *PIK3R1*, *PRKA*, *PTEN*, *STK11*, *TSC1-2*) and TGF β signalling (*TGFBR1-2*, *ACVR2A*, *SMAD4*). Additionally, there are common alterations to epigenetic mediators in CCA. These are factors that can promote changes to transcriptional programmes through histone modification and DNA methylation and include chromatin remodelling SWI/SNF complexes (*ARID-1A/-1B/-2*, *PBRM1*, *SMARCA-2/-4/-D1*), histone methyltransferases and demethylases (*MLL2-3*, *KMT2C*, *KDM4A-5D/-6A/-6B*), metabolic reprogramming and hypermethylation (*IDH1-2*), DNA demethylation (*TET1-3*) and histone deacetylase (HDAC6). A pool of non-coding RNA has also been associated with CCA tumourigenesis (Salati and Braconi, 2019).

1.3.6.4 CCA Signalling and Pathways:

Commonly dysregulated signalling pathways in CCA are usually pathways involved in embryonic liver organogenesis and/or liver regeneration. These include Notch signalling, the Wnt/B-catenin cascade and TGF- β signalling

pathways. These pathways will be briefly discussed below because they have been found to be dysregulated in CCA.

The Notch pathway is a conserved pathway known for its roles in embryonic development as well as cell survival, differentiation and apoptosis. There are four Notch receptors (Notch-1, -2, -3, -4) and ligands, Jagged-1 and -2 (JAG-1/-2) and delta like ligand 1 (DLL1). Both receptor and ligand are transmembrane proteins and are activated by cell-cell contacts. When stimulated, MMP proteases cleave the receptor whilst γ -secretase cleaves and releases the Notch intracellular domain (NICD). For Notch signalling, the NICD migrates to the nucleus to bind transcription factors and deliver cell-specific gene expression. Notch targets are generally mediators of proliferation, differentiation as well as metabolism (D'Souza et al., 2010). Oncogenic Notch signalling results in CCA formation and progression (Rauff et al., 2020). Notch-3 was identified as over-active in many models of CCA, and deletion of Notch-3 gene causes decreased CCA formation and tumour growth. Further investigation showed that tumour growth was fuelled through Notch-3 promoting PI3K/Akt activation (Guest et al., 2016). N3ICD overexpression promotes formation of progenitor-derived CCA, through increasing Notch signalling and the dysregulation of cyclin E expression. Cyclin E has been shown to promote DNA damage and genomic instability. This was shown by 70% of the CCA cells staining positive for DNA double strand breaks. (Zender et al., 2013). Notch-1 and Notch-3 are overexpressed in subsets of CCA. A Notch-2 inhibitor has been shown to reduce tumour formation and burden in cholangiocarcinoma-like and hepatocellular carcinoma-like tumours. Interestingly, a Notch1 inhibitor reduces HCC-like tumours but increases CCA-like tumours; this highlights a role of Notch1 in the favouring of biliary differentiation (Huntzicher et al., 2011)

Similarly to Notch, Wnt signalling is a conserved and complex pathway, implicated in a diverse range of physiological processes including cell fate, polarity, migration and others. Wnt signal transduction occurs when receptors from the Frizzled (FZD) family, along with co-receptors, such as low-density lipoprotein-related protein 5/6 (LRP5/6) are bound by Wnt glycoprotein ligands. The signalling can be canonical or non-canonical; here the canonical pathways will be discussed. Without ligand binding, B-catenin is normally degraded by a destruction complex of Axin, glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and protein phosphatase 2A (PP2A). Upon binding, this degradation is disrupted, and B-catenin translocates to the nucleus where it operates as a co-activator in gene expression. B-catenin has a range of binding partners, such as LEF/TCF DNA binding factors, and regulates genes associated with embryonic development and genes involved in tumorigenesis such as *CCND* and *MYC* (Komiya and Habas, 2008). Wnt/B-catenin pathways are significantly activated in CCA, and CCA is associated with high Wnt3a, Wnt5a and Wnt7b gene expression, as well as reduced membrane localisation but increase cytoplasm localisation of B-catenin. Blockage of B-catenin expression via siRNA inhibited CCA growth and decreased *CCND1* expression (Loilome et al., 2014). Thus, B-catenin is an important marker for CCA proliferation. Ligands Wnt7B and Wnt10A, plus FZD receptors, are overexpressed in CCA. Activated macrophages provide a source of Wnt ligands, and when xenograft mice models were given a drug that blocks the differentiation of monocytes to macrophages, the loss of this Wnt ligand pool reduced tumour growth (Boulter et al., 2015).

Transforming Growth Factor Beta or TGF- β signalling is important in human physiology and disease, and regulates cell differentiation, tissue homeostasis, cell proliferation, migration, cell death and metabolism, (David et al., 2018). The

pathway is initiated by TGF- β and BMP ligand binding to a spectrum of type I and type II TGF- β receptors and accessory co-receptors. Signalling can be blocked by antagonistic ligands. The type I receptors phosphorylate the type II receptors to mediate SMAD phosphorylation. The phospho-SMAD form trimers with common mediator SMAD4. The presence of pseudo receptors, inhibitory SMADs and Sloan-Kettering Institute or SKI proteins inhibit this process and offer regulation of the signal transduction. Activated SMAD complexes bind to target genes with guidance from context-defining transcription factors. SMAD uses a β -hairpin domain to bind DNA whilst other domains bind co-regulator proteins. CDK8/9-mediated phosphorylation of SMAD complexes recruit more binding proteins. Phosphorylation of SMAD by GSK-3B induces SMAD ubiquitination and proteasomal degradation, or alternative phosphorylation promotes the dissociation of SMAD from the DNA to halt gene expression. Many target genes have roles in developmental processes including SOX genes, *NANOG*, *OCT4*, *FOXA*, *GATA* and *MYC* (David et al., 2018). The TGF- β protein has dual functionality in cancer including CCA. Although, SMAD4 was seen to have a high mutational rate in CCA (Zou et al., 2014), the expression of SMAD4 in 42 resected CCA tumours was lost in 42% cases (Kang et al., 2002). TGF- β stimulation leads to EMT response in CCA (Sato et al., 2010) (Sritananuwat et al., 2017). Aberrant TGF- β signalling is provoked by hepatitis B infection that is involved in CCA progression (Lui et al., 2012).

Other signalling pathways involved in CCA include several well characterised pathways including the RAS-MAPK pathways, PI3K-AKT pathway, JAK-STAT signalling, and signalling through multiple tyrosine kinase receptors (Banales et al., 2020). For brevity the main steps for these signalling cascades are summarised in **Figure 1.8**.

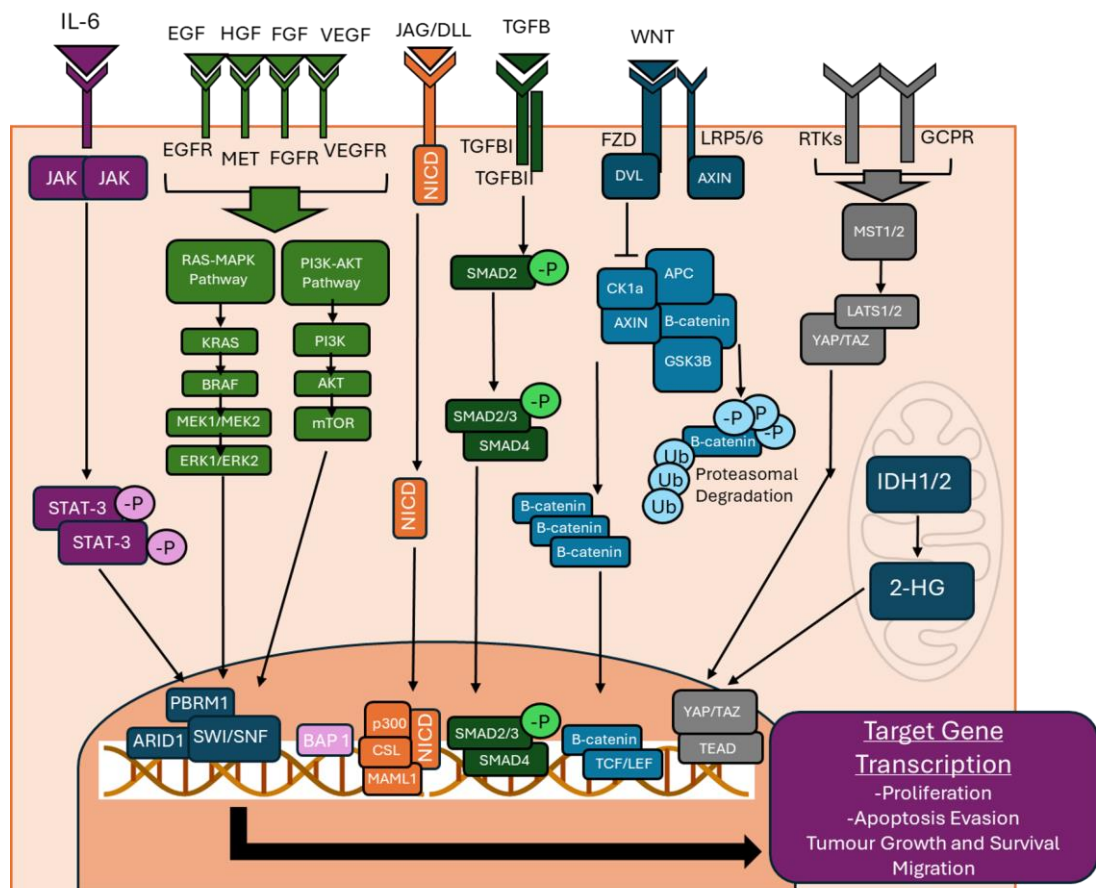


Figure 1.8: Signalling Pathways involved in CCA Development and Progression .
Adapted from Banales et al., 2020.

1.3.6.5 CCA Management and Treatment

The only curative treatment for CCA is surgery, which is effective in early disease. However, only approximately one third of patients qualify for resection; the silent nature of the cancer means the disease is usually diagnosed at late and metastatic stages and the tumour is inoperable (Banales et al., 2020). Chemotherapy is used as palliative treatment for CCA, with Gemcitabine combination with cisplatin as the first-line treatment (Valle et al., 2014). This treatment can be administered with or without immunotherapy in the clinic (Burris et al., 2024), and a fluoropyrimidine based treatment is employed if the primary line of treatment fails (Mansaur et al., 2015). There are options for radiotherapy, and loco-regional therapy (Park et al., 2011), but liver

transplantation is not recommended due to disappointing results, yet it can be highly beneficial to some groups of CCA patients (Mansaur et al., 2015). Nevertheless, some targeted therapies have become approved as they prove to have additional benefit in subsets of CCA patients harbouring specific genetic rearrangements or mutations.

Several tyrosine kinase inhibitors have been studied as anti-CCA therapies, including FGF receptor (FGFR) inhibition. Approximately 15% of patients with CCA have *FGFR2* abnormalities (Ross et al., 2014). Pemigatinib, a pan-FGFR inhibitor, has been recently FDA approved for use in FGFR2-fusion/rearrangement CCA (Abou-Alfa et al., 2024). The median progression-free survival was 6.9 months, and the median overall survival was 21.1 months. Other FDA-approved FGFR inhibitors are Futibatinib (Goyal et al., 2023) and Infigratinib (Javle et al., 2021). First generation neurotrophic receptor tyrosine kinases (NTRK) inhibitors, Larotrectinib and Entrectinib, have been trialled in CCA and have demonstrated rapid and durable responses and favourable safety profiles in patients with TRK fusion-positive cancers (Drlon et al., 2019). Human Epidermal Growth Factor Receptor, or HER2, blockade has been trialled for potential anti-CCA treatment. In phase I trials, Erlotinib in patients with biliary cancer responded with therapeutic benefit (Philips et al., 2006), however some other phase II trials have been unsuccessful (Peck et al., 2012). However, dual anti-HER2 regimen, Pertuzumab plus Trastuzumab was able to significantly improve prognosis of metastatic biliary tract cancers patients with *HER2* amplification or overexpression (Javle et al., 2021). Recently, Zanidatamab, a bispecific antibody targeting two distinct HER2 epitopes, demonstrated clinical benefit in patients with HER2-positive biliary tract cancer (Harding et al., 2023).

Isocitrate dehydrogenase inhibitors such as Ivosidenib have been approved for use in *IDH1* mutated CCA (Abou-Alfa et al., 2020). The drug is well tolerated, and progression-free survival was significantly improved with Ivosidenib compared with placebo. There are also commonly mutated genes in CCA directly or indirectly involving PI3K/AKT/mTOR pathway, thus mTOR inhibitors have been suggested for targeted therapy. Although mTOR inhibition through Everolimus showed promising results in preclinical investigation (Mollthiya et al., 2014), the drug gives varied results in the clinical setting (Kim et al., 2017) (Lau et al., 2018). Many other targeted therapies have been studied in CCA such as Platelet Derived Growth Factor Receptor (PDGFR) inhibitors, VEGFR inhibitors, anti-VEGFR monoclonal antibodies, B-Raf inhibitors, MAPK inhibitors and MET inhibitors (reviewed: Simile et al., 2019).

Immunotherapies are also available for CCA patients. Immune checkpoint inhibitor therapy allows T-cells to eliminate tumour cells by blocking the checkpoint PDL1 and PD-1 interaction that promotes T-cell inactivation (Shiravand et al., 2022). PDL1 inhibitor Durvalumab and CTLA-4 inhibitor Tremelimumab were assessed in combination with Gem-Cis in TOPAZ trials and showed promising efficacy in CCA patients (Oh et al., 2022). Gemcitabine-Cisplatin plus Durvalumab were further evaluated in phase III trial and it is currently the standard of care, as mentioned above, in patients with advanced biliary tract cancer (Burris et al., 2024). Clinical research investigating the effects of targeted therapy and immunotherapy combination is ongoing (Lui et al., 2023).

1.4 Proline Rich Homeodomain

(PRH/HHEX):

1.4.1 Background

Proline-Rich Homeodomain (PRH), also known by its gene name haematopoietically expressed homeobox (*HHEX*; in humans) (Crompton et al., 1992) (Bedford et al., 1993), belongs to a large family of proteins containing a ~60 amino acids structure, termed the homeodomain (HD) (McGinnis et al., 1984). The homeodomain contains within its N-terminal DNA binding regions and a type of helix loop helix-turn-helix motif that has the capacity to bind to specific DNA sequences. HD proteins have a conserved temporal and spatial expression in order to control a spectrum of biological processes (Bürglin & Affolter, 2016). In embryogenesis, they have roles in body patterning; they can act as master regulators by controlling tissue-specific transcription factors to determine cell fate. HD proteins are also vital for the tissue development and maintenance throughout life and in adulthood (Mark et al., 1997). Deregulation of HD protein activity can be lethal or result in various disease and disorders. PRH is classed as an NK-like homeotic gene, which means it contains an N-terminal TN/EH1/Octapeptide motif 5' to the central HD (Bürglin and Affolter, 2016). However, PRH is an orphan gene, so called as it is mapped on chromosome 10, outside of the gene clusters that HD proteins tend to be rearranged in; it is suggested that *HHEX* falls under its own class of NK-like proteins (Hromas et al., 1993). PRH was first discovered using degenerate homeobox-specific PCR primers in an avian haematopoietic cell line, and later in human cells, in attempt to find HD proteins that were responsible for haematopoietic lineage differentiation (Crompton et al., 1992) (Hroman et al.,

1993); PRH was later identified in murine cells and was later named haematopoietically expressed homeobox or HEX (Bedford et al., 1993), but its expression is not confined to the haematopoietic system. Despite this the human PRH gene is known as *HHEX*.

1.4.2 PRH in Early Development and Organogenesis

As with most homeodomain genes, the expression of *HHEX* is temporally and spatially regulated in embryogenesis and organ development (reviewed in: (Soufi et al., 2008) (Jackson et al., 2023)). The expression of *HHEX* occurs early in embryogenesis (Adjaye et al., 2000). *HHEX* expression during embryogenesis occurs at the anterior visceral endoderm (Thomas et al., 1998). The ablation of *HHEX* results in the loss of anterior-to-posterior patterning and impair the primitive streak (Stuckey et al., 2011). *HHEX* expression is essential in the development of many definitive mesoderm-derived and foregut-derived tissues including the forebrain, pancreas, liver, heart, the thymus and thyroid glands (Martinez-Barbera et al., 2000) (Bogue et al., 2000). Generation of null mutant *HHEX*^{-/-} in mouse embryonic stem cells caused multiple body truncations including liver and thyroid dysplasia, as well as forebrain defects; null mutation is not life compatible (Keng et al., 2000). *HHEX* null mice accumulate cardiac and vascular development defectives (Hallaq et al., 2004) (Martinez-Barbera et al., 2000) (Bogue et al., 2000). *HHEX* has roles in hepatic-pancreatic development, committing cells to their appropriate lineage (Yang et al., 2022) (Dumasia et al., 2021).

1.4.3 PRH in the Developed Adult:

HHEX is abundantly expressed in haematopoietic progenitor cells and is down regulated through cell differentiation, whilst *HHEX* overexpression in progenitor

cells prevents commitment toward differentiation phenotypes (Jayaraman et al., 2000). Expression of *HHEX* was showed to be important for haematopoietic progenitor cell self-renewal and response to genotoxic stress, which is important for stem cells maintenance through life and allow response to events requiring rapid blood production (Jackson et al., 2017). *HHEX* is crucial in lymphogenesis as it needed for B cell development (Bogue et al., 2003), whilst a loss of *HHEX* is required for T cell development and maturation (Mack et al., 2002). Recently, overexpression of constitutively active PRH protein was shown to reduce vascular smooth muscle cell proliferation and migration and could be beneficial in reducing intimal thickening of the vasculature (Wadey et al., 2017) (Reolizo et al., 2023).

1.4.4 PRH in liver and bile duct development

HHEX expression is required for normal liver organogenesis, with *HHEX* expression being detected in hepatocyte and bile duct cells and the loss of *HHEX* leading to the absence of the liver bud (Martinez-Barbera et al., 2000) (Keng et al., 2000) (Bogue et al., 2000). Hunter et al., used a conditional *HHEX* null mice system to observe PRH/*HHEX* function in hepatobiliary development. *HHEX* depletion in the hepatic endoderm resulted in liver hypoplasia and defective biliary tract formation. This was accompanied by the loss of liver-associated transcription factors *HNF4A* and *HNF6*, causing problems in hepatic epithelial development, and bile duct specific transcription factor *HNF1B* was downregulated in cholangiocytes. Therefore, *HHEX* is required for hepatic and biliary specific transcription factor expression and cell fate (Hunter et al., 2007). It was also observed that *HHEX* promotes pro-hepatic programmes of gene expression, including the induction of fibrinogens, apolipoproteins, and cytochromes (Kubo et al., 2010). The liver specific expression of *HHEX* is

induced by HNF3G and GATA4 transcription factors which directly binds to the *HHEX* promoter region (Denson et al., 2000).

1.4.5 PRH Structure

Human PRH is 270 amino acids in length, 30kDa in molecular mass, with its HD occurring between amino acids 137-197. Upstream of the HD is a proline-rich N-terminus (amino acids 1-136), and downstream is an acidic C-terminal region (198-270) (reviewed: Soufi et al., 2008). The PRH sequence is highly conserved in eukaryotes, with 75% overall sequence similarity between human and chicken PRH protein, and 94% identity between human and murine PRH protein (Crompton et al., 1992).

1.4.5.1 N-terminal:

The N-terminal domain has a high proportion of proline (20%), alanine (15%) and glycine (10%) residues (Crompton et al., 1992). Truncated N-terminal derivatives of PRH, with a predicted molecular weight of 19.5kDa, were observed to elute at 53kDa in gel-filtration chromatography, suggesting that the N-terminus can form dimers (Soufi et al., 2006). These dimers were found to be SDS-resistant, and residues 1-50 seem to be a critical interface for dimerisation. The N terminus carries no canonical or specific secondary structure motifs, and this portion of the polypeptide is extended and dynamic, and comparable to elastin (Soufi et al., 2006). The PRH N-terminal region serves as a protein-protein interaction interface with multiple partners including eukaryotic initiation factor 4E (eIF4E) (through residues 18-24), Groucho/Transducin-like enhancer (TLE) corepressor family members (through residues 32-38), Promyelocytic Leukaemic protein (PML) (through residues 50-115) and proteasome subunit C8 (Bess et al., 2003). It was inferred that N-terminus can function as a transcriptional repressor domain, independently from

the homeodomain, when PRH 1-141 was fused to a GAL4 DNA binding domain (DBD), it was able to repress the activity at thymidine kinase reporter (Guiral et al., 2000).

1.4.5.2 Homeodomain:

The HD is generally formed from three helices of which helix II and helix III form the helix-turn-helix with a loop between; this motif binds DNA at the major groove and the helices II and III contribute to binding specificity and recognition (Billeter, 1996). There is high conservation of the homeobox in PRH, with 97% of residues identical between chicken and human PRH protein. The murine PRH HD sequence only differs from the human sequence by one residue at residue 151 (Bedford et al., 1993). The homeodomain tends to bind at AT-rich sequences (Laughon, 1991). The PRH HD binds DNA specifically, at a variations of a consensus sequence 5'-(C/T)(C/T)ATTAA(A/G)-3' (Crompton et al., 1992). Interestingly, PRH has been shown to bind through 5'-TAAT-3' or the 5'-CAAG-3' core motifs, showing PRH has a more flexible specificity than other HD (Pellizzari et al., 2000). PRH HD differs from other NK homeo-proteins as it does not have an arginine at residue 5; arginine 5 is conserved in the homeodomain and has a key role in making contacts between the N-terminal arm of the HD and the DNA backbone (Gehring et al., 1994). This, and other differences in the PRH HD amino acid sequence, may lead to a lenient specificity for DNA binding (Soufi et al., 2008).

1.4.5.3 C-terminal domain:

The C-terminal portion has four times as many acidic residues compared to basic (Crompton et al., 1992). Highly acidic regions are common in transcription factors, and they are thought to offer a platform for regulation and specificity. Denson et al group identified PRH as a positive regulator of the sodium

dependent bile acid co-transporter (*NTCP*) in Hep G2 cells (Denson et al., 2000), and later it was shown that the C-terminal was required for this activation (Kasamatsu et al., 2004). Similarly, a rat HD and C-terminal region (PRH131-271) expressing constructs was able to stimulate the *NTCP* promoter as potently as full-length PRH, however HD only was unable to reproduce this activation; therefore, it was concluded that the C-terminus acidic region contributes to transcriptional activation of *NTCP* (Kasamatsu et al., 2004).

1.4.5.4 Full Length PRH and Oligomerisation:

Soufi et al showed that PRH could oligomerise to form higher order structures (Soufi et al., 2006). Endogenous, and overexpressed myc-tagged, PRH were detected as puncta in the nucleus and nuclear periphery in myelogenous leukaemia K562 cells. Additionally, when fibroblast-like COS-7 cells, which have negligible PRH expression, were infected with myc-tagged PRH, PRH was able to form monomer, dimer, trimer, tetramer and higher order oligomers up to an octamer, in cross-linking experiments. It was found that this oligomerisation occurs through a second interface to the N terminal-associated dimerization region PRH1-50, between residues 50-137 (Soufi et al., 2006). The oligomer forms of PRH may play an important role, as in sedimentation experiments, PRH oligomers were found to bind DNA. It is thought that these oligomeric complexes are able to bind with higher affinity at multiple sites (Soufi et al., 2006) (Williams et al., 2008). Chromatin immunoprecipitation (ChIP) experiments observing full-length, myc-tagged human PRH in CCLP, agree with previous findings, using avian PRH HD, that the HD binds an ATTA consensus sequence (Kitchen et al., 2020).

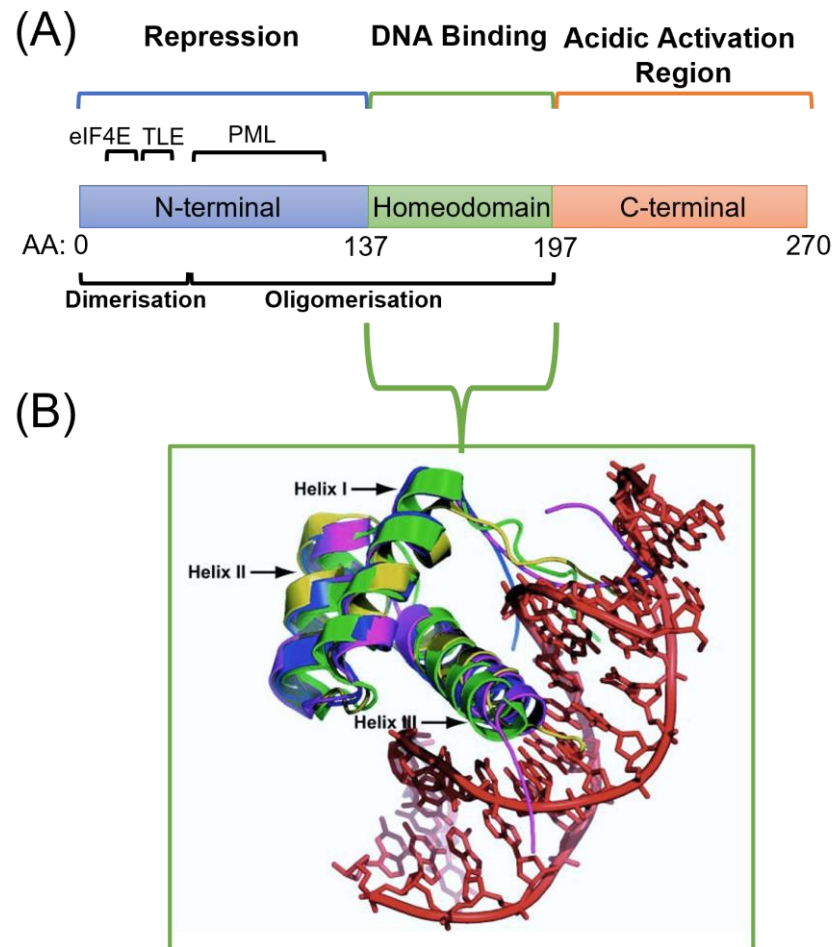


Figure 1.9: Features of the PRH Protein.

- (A) A schematic showing the PRH protein divided into three main domains: an N-terminal domain (blue), a homeodomain (green) and a C-terminal domain (red). The correlated function of each region of the protein is also illustrated, with known interacting proteins: eIF4E, TLE and PML.
- (B) A molecular model of PRH Homeodomain (HD) binding to DNA (Figure from Soufi et al., 2008). The image shows the alignment of HDs from different HD proteins, Engrailed (blue), Oct-2 (yellow), Msx-1 (green) and PRH (magenta) bound to DNA (red).

1.4.6 The Regulation of Gene Expression by PRH

PRH can both activate and repress genes through direct and indirect mechanisms. PRH can cause transcriptional repression through direct binding to a gene. For example, through its HD, PRH can bind and repress at the thyroglobulin promoter in thyroid cells (Pellizzari et al., 2000), and at the endothelial cell specific molecule 1, *ESM1*, promoter at an evolutionary conserved response element (Cong et al., 2006). PRH, like other homeotic protein Engrailed, can also repress transcription by directly binding at the TATA

box to sterically hinder TATA-box binding protein (TBP) and block transcriptional initiation *in vitro* (Guiral et al., 2001). However, direct interaction with TBP has not yet been observed *in vivo*. PRH is also able to bind to DNA interact with and recruit Groucho/transducing like enhancer of split (TLE) member TLE1, via its TN/Eh1 motif, to bring about repression (Swingler et al., 2004). Groucho/TLE members are a family of co-repressors that are recruited to promoters via DNA binding proteins and mediate transcriptional repression through direct interaction with histones or basal transcriptional machinery histones (Jennings & Ish-Horowicz, 2008).

Recently, PRH has been shown to interact with repression complex Polycomb repressive complex 2 (PCR2). Polycomb complexes modify histones to epigenetically regulate gene expression and have role in embryonic development in cell fate and differentiation (Di Croce and Helin, 2013). PRH was seen to precipitate with PCR2 subunit Suppressor of Zeste 17 or SUZ17, in immunoprecipitation experiments, in AML cell models (Shields et al., 2016). The loss of *HHEX* expression in this system re-activated some PCR2 targets and resulted in changes to H3K27 methylation status at some loci, however globally the levels of methylation were unchanged. In this tumour type PRH functions as an oncoprotein through effecting transcriptional repression.

PRH can also activate gene expression. PRH was found to transactivate the gene of the sodium dependent bile acid co-transporter, *NTCP*, by binding directly to an upstream element at positions -725 and -720, relative to the *NTCP* gene (Denson et al., 2000). As mentioned, this activation can be directly linked to the acidic C-terminus of the protein (Kasamatsu et al., 2004). Although PRH contributes to *NTCP* activation, it is not the primary activator. It is interesting to observe that PRH is unable to transactivate *NTCP* in COS-7 cells or non-

hepatic cells but is found in the nuclear compartment; it may be that other co-activators that facilitate the activation are liver-specific (Kasamatsu et al., 2004).

Given PRH is found as foci in the nucleus and can oligomerise (Soufi et al., 2006) it is possible that PRH has additional roles in regulating gene expression through genomic architecture level as well as local gene level regulation.

1.4.7 Regulation of the HHEX gene

PRH is a master regulator of transcription therefore it is subject to multiple levels of gene expression regulation. Sp1 and Sp3 bind to GC rich boxes at nucleotide positions -149 to -140 and -79 to -70 to the HHEX promoter to promote its transcription in both liver cancer cell lines (Kikkawa et al., 2001). GATA-1, GATA-2 and c-Myb can bind intronic enhancer elements to stimulate transcription (Sato et al., 2004). Liver specific transcription factors HNF3 and GATA-4 activate *HHEX* expression also by binding at the promoter region from -103/+22 (Denton et al., 2000). In the thyroid, Pax8 and TTF bind *HHEX* gene and promote expression of the PRH protein (Puppin et al., 2003). During embryogenesis, *HHEX* expression is regulated by liver organogenesis signalling pathways such as Wnt-B catenin signalling, TGF- β signalling, BMP signalling and FGF signalling pathways (Zorn et al., 1999) (Shin et al., 2007). For example, identification of a BMP-responsive element (BRE) in the mouse *HHEX* promoter led to the discovery of a BMP-responsive SMAD1/SMAD 4-mediated transcription of *HHEX* (Zhang et al., 2002). *HHEX* was identified as a novel Farnesoid X receptor-regulated target gene during adaptation of hepatocytes to chronic bile acid exposure, where FXR binds to an enhancer element in intron-1 (Xing et al., 2009).

1.4.8 Regulation of the PRH protein

The serine/threonine kinase Casein Kinase 2 or Protein Kinase CK2 can phosphorylate residues S163 and S177 of the PRH homeodomain (Soufi et al., 2009). This phosphorylation blocks the DNA binding capability of the homeodomain and the S163E/S177E PRH phosphomimic inhibits the repression of genes by PRH. Further studies show that the phosphorylation of PRH causes it to lose its nuclear localisation and alters its stability (Noy et al., 2012). PRH has also been shown to interact with the HC8 subunit of the proteasome *in vitro* and *in vivo* (Bess et al., 2003). In haematopoietic cells the inhibition of the proteasome was able to restore phospho-PRH proteins. Phospho-PRH is a substrate for proteasomal cleavage. The proteasome generates a stable PRH cleavage product (PRH Δ C). PRH Δ C is able to affect transcription by binding to the TLE co-repressor protein and sequestering it. This inhibits PRH-TLE complex formation and PRH- repression of the gene *VEGFR1* (Noy et al., 2012). In the regenerating liver, PRH has been shown to be regulated by Glypican 3 (GPC3) which binds to CD81 and decreases the binding of CD81 to PRH, resulting in PRH nuclear translocation and increased cell proliferation (Bhave et al., 2013).

1.4.9 PRH in Cancer

PRH is part of a niche group of transcription factors and kinases that can act as a tumour suppressor and an oncogene (Shen et al., 2018), depending on the cancer context (reviewed: Gaston et al., 2016). Not only abnormal expression status of PRH, but also differential subcellular localisation, can be correlated with cancer.

PRH as a tumour suppressor: PRH is a tumour suppressor in liver (hepatocellular carcinoma or HCC), prostate, breast and thyroid cancers (Gaston et al., 2016). When an *HHEX*-containing adenovirus was infected into mouse hepatoma cell line, Hepa1-6, growth was suppressed more than 50% and the ability of the cells to form colonies were largely decreased (Su et al., 2011). These Hepa1-6-HHEX cells showed increased expression in tumour suppressors, *TP53* and *RB1*, and decreased levels of proto-oncogenes, *JUN* and *BCL2*. Immunohistochemical staining showed that PRH had a higher expression in well-differentiated HCC, and that PRH correlates with the differentiation status of the cancer; it was concluded that PRH could act as a tumour suppressor in HCC cells (Su et al., 2011).

In breast cancer cells, the overexpression of PRH via an adenovirus vector caused a decrease in cell number with time and decreased tumour formations in xenograft models, whilst the knockdown of PRH expression via shRNA resulted in an increase to cell number over time in MCF-7 cells (Kershaw et al., 2017). PRH was also shown to inhibit the migration of breast cancer cells and prostate cells, where PRH KD resulted in increased migration and PRH overexpression decreased migration in scratch-wound and chemotaxis cell migration assays (Kershaw et al., 2014). PRH subcellular localisation has been implicated in cancer and there is an increase in nuclear-to-cytoplasmic shift of PRH in breast and thyroid cancers (Puppin et al., 2006) (D'Elia et al., 2002) (Siddiqui et al., 2017). As PRH is a tumour suppressor in breast and thyroid cancer, a reduction of nuclear PRH may mean it can no longer exert its tumour suppressing capabilities.

In prostate cells, PRH overexpression reduces cell migration and invasion. PRH is a repressor of Endoglin, TGF- β coreceptor; it was proposed in both the breast and prostate cancer cells that loss of PRH induced Endoglin-mediated

oncogenic signalling (Kershaw et al., 2014). Phosphorylated PRH via Casein Kinase 2 (CK2) decreases the PRH HD binding capabilities (Soufi et al., 2009). Higher levels of phospho-PRH are found in prostate cancer cell lines and prostate cancer tissue samples. PRH knockdown in PNT2-C2 cells increased cell migration, invasion and proliferation. Therefore, PRH is considered a tumour suppressor in prostate cancers (Siddiqui et al., 2017).

PRH as an oncoprotein: PRH functions as an oncoprotein in types of acute myeloid leukaemia (AML) and in bile duct cancer (CCA). It was identified that *HHEX* had a two to four-fold increase in expression in AML patients, relative to normal haematopoietic stem cells, and that loss of *HHEX* expression resulted in up-regulation of Polycomb complex PCR2-regulated tumour suppressors, p16INK4 (*CDKN2A*) and p19ARF (*CDKN2B*). It was concluded that oncogenic PRH could bind to the *CDKN2A* locus and recruit PRC2, which resulted in aberrant silencing via H3K27 methylation and epigenetic repression of these tumour suppressors in AML. Thus, leading to increased cell proliferation (Shields et al., 2016). PRH is oncogenic in cholangiocarcinoma, and this is described in detail below in section 1.5.

1.4.9.2 PRH Regulated Cancer-Associated Genes Targets:

PRH has a spectrum of cancer-associated targets which allow this protein to influence multiple cell processes such as: cell proliferation, cell differentiation, cell invasion, cell migration, metastasis, angiogenesis and inflammatory signalling, as seen in the table below (**Table 1.1**):

Target	Regulation	Function of Target	Source
<i>FLT1</i>	Repression	Vascular Endothelial Growth Factor Receptor 1 or VEGFR-1 ; angiogenesis, cell proliferation, cell migration and invasion.	Noy et al., 2010
<i>FLK1</i>	Repression	Vascular Endothelial Growth Factor Receptor 2 or VEGFR-2 ; angiogenesis, cell proliferation, cell migration and invasion.	Noy et al., 2010
<i>VEGFA</i>	Repression	Vascular endothelial growth factor A ; angiogenesis, cell proliferation, cell migration and invasion.	Noy et al., 2010
<i>ENG</i>	Activation	Endoglin Transforming Growth Factor β Co-Receptor ; angiogenesis, cell migration and invasion.	Kershaw et al., 2014
<i>ESM1</i>	Repression	Endothelial Cell-specific Molecule-1 ; cell adhesion, inflammation, angiogenesis, lymphangiogenesis, cell migration, cell invasion and metastasis.	Cong et al., 2006
<i>GCS</i>	Repression	Goosecoid Homeobox Transcription Factor ; dorsal-ventral patterning in embryonic development, cell differentiation and migration.	Brickman et al., 2000
<i>CDKN1B</i>	Repression	Cyclin Dependent Kinase Inhibitor 1B or p27kip1 ; cell cycle arrest and quiescence.	Kitchen et al., 2020
<i>CDKN2A</i>	Repression	Cyclin Dependent Kinase Inhibitor 2A or p16INK4A ; cell cycle arrest and quiescence.	Shields et al., 2016
<i>CDKN2B</i>	Repression	Cyclin Dependent Kinase Inhibitor 2B or p15INK4B ; cell cycle arrest and quiescence	Kitchen et al., 2020

Table 1.1: Cancer-associated PRH targets genes.
(Adapted from Gaston et al., 2016).

1.5 PRH in CCA

1.5.1 PRH is a CCA Oncoprotein

With PRH having roles in hepatic development and maintenance, it is not unexpected that if mis-regulated, PRH may result in liver malignancies. Through recent work of Kitchen et al., PRH has been discovered to be an oncoprotein in cholangiocarcinoma (Kitchen et al., 2020). This section describes in detail how PRH was shown to have a role as an oncoprotein in CCA.

Kitchen et al. first highlights that high PRH expression is found in CCA. Immunohistochemistry was performed on patient CCA tumour and adjacent border biliary epithelium tissue and staining for PRH expression. The staining was increased in 20/26 (77%) samples in a paired analysis, examining patients CCA versus border tissue, and in an unpaired analysis, PRH expression had a 20% higher median in CCA samples compared to border tissue samples. Additionally, when investigating various liver fluke associated Thai (HuCCA1 and RmCCA1) and non-liver fluke associated European (CCLP1 and CCSW1) CCA cell lines, all cell lines exhibited a high PRH expression compared to primary cholangiocytes, (also known as Biliary Epithelial Cells (BECs)), and an immortalised cholangiocyte cell line (AKN1). To determine whether PRH has roles in promoting CCA, a PRH knockdown (KD) was generated in CCLP cells using shRNA against *HHEX* and the shRNA KD cells were used in a mouse xenograft model. There was a significant reduction in tumour growth within the mice injected with the PRH KD cells compared to mice injected with control cells. Moreover, in the majority of mice injected with cells that are knocked down for PRH tumours were unable to form. Further studies using an EdU incorporation-based cell proliferation assay showed that PRH KD CCLP cells

had a reduction in proliferation rate to approximately half of the control cells. PRH overexpression (OE) using a myc-tagged PRH-overexpressing adenoviral vector, resulted in increased proliferation in CCA cell lines compared to cells infected with an empty adenovirus in CCLP and CCSW tumour cells. This increase in proliferation was also observed upon infections of primary cholangiocytes cells and immortalised cholangiocytes, BECs and AKN1, with the PRH expressing adenoviruses. Interestingly, PRH OE and KD both resulted in decreased caspase-3 enzymatic activity; however, this was not investigated further. A visible change to morphology, from the mesenchymal-like CCLP control to the epithelial-like CCLP PRH KD cells, revealed roles for PRH in epithelial-to-mesenchymal transition (EMT). Indeed, PRH KD in CCLP not only restored the expression of epithelial marker E-cadherin, and decreased the expression of mesenchymal marker Vimentin, but also decreased the number of migrating and invading cells in cell migration and invasion assays.

RNAseq data from PRH OE and KD cells highlighted PRH-regulated pathways such as EMT, TGF- β signalling, cell cycle-associated signalling, oestrogen response and Notch signalling; it also showed the differential expression of genes encoding a many biliary-specific transcription factors and proteins associated with biliary differentiation and bile acid transportation. Upon further investigation of Notch signalling and the generation of a knockdown Notch-3 CCLP cell line, it was shown that PRH forms a positive feedback loop with Notch-3 to activate multiple oncogenic pathways to drive CCA, both together and individually. A mechanism explaining the effect of PRH on signalling in CCA was proposed, whereby PRH drives *NOTCH3* gene expression as well as activation of Notch ligands and other genes in the Notch signalling pathway to form a positive feedback loop. The increased formation of the Notch3 ICD and PRH promote the expression of cycle cell activators (*CCND2*) and canonical

Wnt signalling members (*WNT11* *WNT16* *TCF7L1* and *DKK1*) but repress cell cycle inhibitors (p27 and p15) and E-cadherin. Overall, this drives cell cycle progression through CDK4/6 kinase activity.

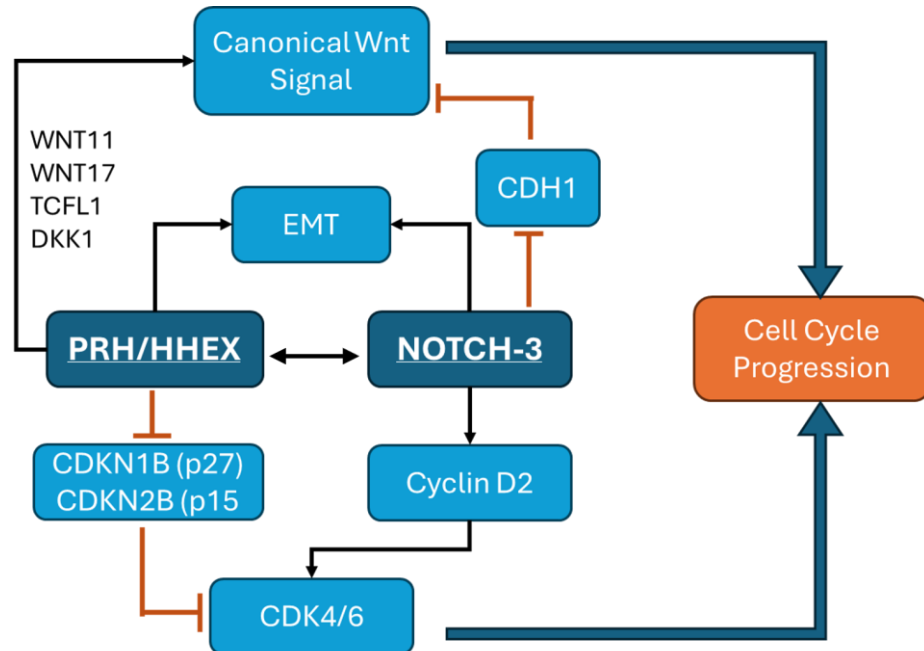


Figure 1.10: The PRH-Notch 3 Positive Feedback Loop that Drive CCA Cell Cycle Progression.
Adapted from Kitchen et al., 2020.

1.5.2 PRH effects on the Cell Cycle impact

Palbociclib sensitivity.

Palbociclib, a CDK4/6 inhibitor was able to significantly reduce the proliferation of CCLP cells (Kitchen et al., 2020). This decrease in proliferation was more obvious in CCLP cells overexpressing PRH in MTT cell viability assays because the elevated PRH expression drives cell proliferation. Therefore, it was suggested that PRH-high CCA cancers could be more sensitive to Palbociclib (Kitchen et al., 2020). Palbociclib is able to inhibit the cell cycle and apoptosis, and experiments showed that it can promote apoptosis of CCLP tumour cells (Lee, 2021 and PSJ personal communication).

1.5.3 Palbociclib

As mentioned previously, the dysregulation of cell cycle progression in cancer can occur through multiple mechanisms especially by promoting the Cyclin D-CDK4/6-p21 complex in cell cycle initiation (see section 1.1.2). Palbociclib (PD-0332991, Pfizer) is of the pyridopyrimidines drug class and acts as a potent inhibitor of CDK4/6 by binding the ATP active site (Fry et al., 2004). The half maximal inhibition of CDK4 occurs at 0.011 $\mu\text{mol/L}$ and occurs at 0.015 μmol for CDK6, and Palbociclib also targets a panel of CDKs and other protein kinases but at higher concentration (Fry et al., 2004). Palbociclib potently inhibits RB phosphorylation and causes G1 arrest in a wide range of cancer cell lines and in vivo (Fry et al., 2004). This preclinical study led to the use of Palbociclib in clinical trials. Initially, phase 1b studies were performed to assess safety of Palbociclib when taken with anti-hormone therapy, Letrozole, in oestrogen receptor-positive, HER2-negative, advanced breast cancer patients (Finn et al., 2009). Once tolerability was confirmed, Palbociclib was assessed through a series of clinical trials, the PALOMA trials, to evaluate its clinical use. It was show that the addition of the CDK4/6 inhibitor to Letrozole significantly improved progression free survival in patients (PALOMA-1) (Finn et al., 2015). A positive clinical effect of using Palbociclib was seen in the PALOMA-2 trial (Finn et al., 2016) and in the PALOMA-3 trials that investigated Palbociclib with Fulvestrant (Turner et al., 2018). The use of Palbociclib with aromatase inhibitors was approved by the National Institute for Health and Care Excellence (NICE) in November 2017 for HR+/HER2- locally advanced metastatic breast cancer under the name Ibrance (Palmieri et al., 2023).

The use of Palbociclib plus chemotherapy is being translated into other cancer types and has shown promising results in patients with soft tissue sarcoma

(Schuetze et al., 2024), head and neck squamous cell carcinoma (Ngamphaiboon et al., 2024), and advanced pancreatic ductal adenocarcinoma (PDAC) (Hidalgo et al., 2022). However, Palbociclib plus hormone therapy did not show clinical efficacy in prostate cancer (Palmbos et al., 2021). A substantial amount of pre-clinical work is being explore in diffuse pleural mesothelioma (DPM) (Costa et al., 2024), types of melanomas (Jagirdar et al., 2024), hepatocellular carcinoma (Cai et al., 2024) colorectal cancer (Lee et al., 2023) and leukaemia and lymphoma (Raetz et al., 2023). Most preclinical data investigate Palbociclib in combination with either another targeted therapy or chemotherapy to promote a greater anti-cancer activity, such as: mTOR inhibitor (Yamamoto et al., 2019), multi-target receptor tyrosine kinase mtRTK inhibitor Sunitinib (Moskovits et al., 2022), Oxaliplatin (Suppramote, 2022), BRAF and MEK inhibitors (Martin et al., 2018). In a basket trial Palbociclib was shown not to be of clinical use in biliary tract cancers as a monotherapy (Baghdadi et al., 2019).

1.6 Niclosamide, a potential CCA inhibitor identified through a repurposing drug screen

1.6.1 An Introduction to the CCA Drug Screen

As discussed, the range of therapeutic agents for CCA is limited. To broadly and rapidly investigate new CCA inhibitors, an off-patent repurposing drug screen was employed to test compounds that are clinically in use for treatment of other conditions for selective inhibition of CCA cell lines, CCLP and CCSW, over an

immortalised cholangiocyte cell line, AKN1. The screen used a library of 104 off-patent drugs selected from the British National Formulary, and were provided by Dr Farhat Khanim, University of Birmingham (Khanim et al., 2011). The cells were treated with drug at its maximum serum concentration and were incubated for an optimised 96 hours. Cell viability was measured through a MTT cell viability assay and results were normalised to the drug vehicle control. The experiment was repeated three times, and a p-value obtained through a paired tailed t-test plus Benjamini-Hochberg multiple correction analysis to identify compounds that showed significant decrease to CCA viability over AKN1 cells. From the screen, there were six compounds of interest that were able to promote a significant decrease to CCA cell viability over the immortalised cholangiocyte cell line: Ascorbic acid, Zinc acetate, Niclosamide, Mebendazole, Deferoxamine mesylate and Colchicine (Lee, 2021).

1.6.2 Niclosamide and its mechanisms of action

Discovered in the 1960s at Bayer chemotherapy research laboratories, Niclosamide, was originally found to be effective as a molluscicide (Andrews et al., 1983) and later found to be effective against human tapeworm infection (Pearson & Hewlett, 1985). In early studies, Niclosamide was found to cause decreases to oxygen uptake and glucose uptake, but simultaneously stimulate aerobic and anaerobic respiration in treated snails to eventually result in toxicity (Andrews et al., 1983). This is because Niclosamide is a salicylic acid derivative (Badraddin, 2005); see figure below. The chemical structure of salicylamides make this group of compounds potent mitochondrial uncouplers. The salicylic acid ring act as the H⁺-donating group, the anilide ring is highly hydrophobic, and the amide acts as an H⁺-withdrawing group. All these features are important for its uncoupling ability (Terada et al., 1988) (**Figure 1.9**). Replacing the

phenolic hydroxyl group of the salicylic acid moiety with a methyl group abolishes Niclosamide mitochondrial uncoupling capability (Kumar et al., 2018). This change in structure also resulted in a loss of Niclosamide anti-cell growth effect, providing evidence that mitochondrial uncoupling caused by Niclosamide results in its ability to reduce viability. From its discovery, Niclosamide has been deemed as a mitochondrial uncoupler however the range of mechanisms of actions of Niclosamide are yet to be completely understood.

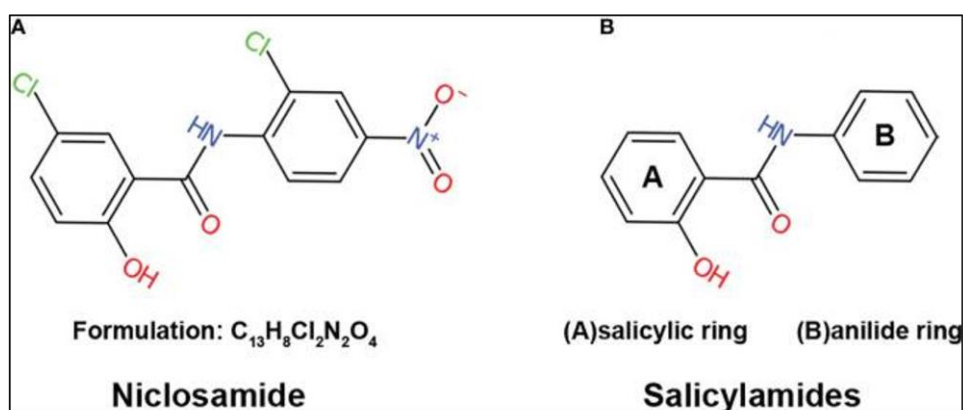


Figure 1.11: The Chemical Structure of Niclosamide, a Salicylamide Derivative. Niclosamide (A) is a salicylic acid derivative (B) and a potent mitochondrial uncouplers. The salicylic acid ring act as the H^+ -donating group, the anilide ring is highly hydrophobic, and the amide acts an H^+ -withdrawing group. All these features are important for its uncoupling ability. Figure from Badraddin, 2005.

1.6.3 Niclosamide in cancer

Niclosamide has been identified as a potential anti-cancer therapeutic in many repurposing screens, so much so it has been called the magic bullet (Jiang et al., 2022). Niclosamide displays many different mechanisms to decrease oncogenic signalling in a spectrum of cancer types, going beyond its role as a mitochondrial uncoupler.

Most of the literature that investigates Niclosamide as an anti-cancer therapeutic shows the drug is capable of causing mitochondrial uncoupling in the cancer cell and causes metabolic dysfunction-mediated growth inhibition or

apoptosis. A drug screen that identified Niclosamide as a potential inhibitor of multiple myeloma recorded a substantial uncoupling effect of Niclosamide in the cells and downstream formation of mitochondrial superoxide which paralleled with drug cytotoxicity (Khamin et al., 2011). Through screening, Niclosamide was identified as an agent that can promote mitochondrial fragmentation through its uncoupling abilities to activate both apoptotic and autophagic cell death pathways (Park et al., 2011). Similarly, the mitochondrial uncoupling induced by Niclosamide was seen to cause cell cycle arrest and reduce the ability of colon cancer cells to form clonal populations (Alasadi et al., 2018). Furthermore, the increase to cytotoxic metabolites, such as arachidonic acid, under Niclosamide treatment have been related to apoptosis (Kumar et al., 2018).

In addition to its primary mechanism of action in mitochondrial uncoupling, Niclosamide also demonstrates modulation of multiple oncogenic signalling pathways including Wnt/B-catenin, Notch, STAT3 signalling, NFkB signalling and the mTOR pathway. Niclosamide was identified as a compound able to inhibit Wnt/B-catenin signalling through multiple mechanisms including Frizzled-1 receptor internalisation (Chen et al., 2009), inhibition of LRP-6 co-receptor expression and phosphorylation (Lu et al., 2011) (Arend et al., 2014), and reduction in B-catenin activity and induction of B-catenin degradation via GSK-3 phosphorylation (Guo et al., 2022). A study in colorectal cancer cells proposed that Niclosamide directly binds to GSK-3B, which outcompetes the binding of GSK-3B to Axin-2, thus allows attenuation of canonical Wnt activity (Ahn et al., 2017). Niclosamide treatment was able to reduce Notch-1/-2/-3 and downstream target HEY-1 protein levels in colon cancer cell lines (Suliman et al., 2016) and was also seen to decrease gene expression of *NOTCH1* and *HES1* in hepatocellular carcinoma models (Zeyada et al., 2020). Niclosamide

was also identified in a drug screen as a potent inhibitor of STAT-3 phosphorylation (Ren et al., 2010). From this, Niclosamide has been used for its anti-STAT 3 activity in other cancer models that have acquired chemotherapy or radiotherapy resistance through STAT-3 activation in prostate cancer cells (Lui et al., 2011) and breast cancer cells (Lu et al., 2018). NF- κ B pathway was inhibited by Niclosamide, by attenuating upstream signal transduction mediators and preventing NF- κ B binding at its target gene, in acute myelogenous leukaemia cells (Jin et al., 2010). The blocking of NF- κ B signalling pathway was also described in multiple myeloma cells (Khanim et al., 2011) and glioblastoma cells (Wieland et al., 2013). Another drug screen identified Niclosamide as an inducer of autophagy and inhibitor of mTOR signalling in breast cancer cells (Baldi et al., 2009) (Fonseca et al., 2012). The activation of autophagy and deactivation of mTOR signalling by Niclosamide has been linked to lysosome dysfunction (Li et al., 2012) and oxidative stress (Chen et al., 2017).

1.6.4 Niclosamide and Palbociclib

From the six drug candidates identified in the repurposing drug screen, multiple drug combination experiments were performed to observe whether the drugs together could result in additional decrease to CCA cell viability. Additionally, Palbociclib was used in combination studies with the six drugs from the screen. The experiments were performed by Dr K. Y. Lee (Lee, 2021). CCLP, CCSW, KGU-100 and KGU-213 cells were treated with each drug as a single agent at its EC₅₀, or both drugs together at their EC₅₀, and cell viability was measured through MTT assay. Initial experiments indicated that Niclosamide and Palbociclib showed potential synergism across the four CCA cell lines.

1.7 Aim and Objectives

The research into new CCA therapeutics is needed urgently to improved patient outcome, which is currently very poor. PRH is well-documented as a factor in the development of the liver and biliary system and has recently been shown to acts as a oncoprotein in CCA. The investigation of downstream PRH oncogenic signalling has led to new therapeutic targets, and potential. Alongside this, a repurposing drug screen was conducted to highlight drugs that can target CCA cancer cell lines over an immortalised cholangiocyte cell line. The aim of this work is to continue the investigation of Niclosamide and Palbociclib, two drugs with potential anti-CCA activity, and evaluate the drugs as single agents and in combination. In addition, I aim to expand on research implementing PRH as a critical oncoprotein driving CCA, broadening the scope of targeting PRH in CCA.

Objectives:

1. Examine the effect of Niclosamide on the viability of CCA cell lines and Biliary Epithelial Cells (BECs) and investigate the mechanisms of Niclosamide action with a focus on the effect of Niclosamide on PRH expression.
2. Investigate the effect of Niclosamide and Palbociclib combination therapy on the viability of CCA cell lines and BECs, determine whether the combination is synergistic and reveal the mechanisms of any added benefit of using the drugs in combination.
3. To optimise, validate and characterise new siRNA- and shRNA-mediated PRH knockdown models, as well as a CRISPR PRH knockout model, to identify conserved PRH-responsive signalling pathways.

CHAPTER 2:

Materials and Methods

2.1 Cell Culture Protocols

2.1.1 Cell Line Information:

Cell identity was confirmed by STR profiling by Eurofins Genomic:

(A)

Client Sample Name	CCSW	RBE	Client Sample Name	TFK-1
Sample Code	CL00010651	CL00010658	Sample Code	CL00011894
D8S1179	11,13,14,15	13,14	D8S1179	14,16,17
D21S11	29,30,33	29,29	D21S11	29,31
D7S820	10,11,12,13	8,11	D7S820	7,8
CSF1PO	11,13	12,12	CSF1PO	10,11
D3S1358	15,16	14,15	D3S1358	16,17
TH01	9,3,9,3	6,6	TH01	9,9
D13S317	9,12	9,9	D13S317	11,11
D16S539	9,11,13	10,11	D16S539	10,13
D2S1338	17,17	17,24	D2S1338	22,22
D19S433	13,13	14,15,2	D19S433	14,14
vWA	15,17	20,20	vWA	17,19
TPOX	8,11	8,8	TPOX	8,8
D18S51	13,14	13,15	D18S51	16,17
AMEL	X,Y	X,X	AMEL	X,X
D5S818	11,12	11,12	D5S818	11,11
FGA	22,22	19,24	FGA	25,26
Database Name	CC-LP-1*	RBE	Database Name	KKU-M055

(B)

Client Sample Name	CCSW SVC 2	CCSW KD 2
Sample Code	CL00011891	CL00011892
D8S1179	11,13	11,13
D21S11	29,33	29,33
D7S820	10,11	10,11
CSF1PO	11,13	11,13
D3S1358	15,16	15,16
TH01	9,3,9,3	9,3,9,3
D13S317	9,12	9,12
D16S539	9,13	9,13
D2S1338	17,17	17,17
D19S433	13,13	13,13
vWA	15,17	15,17
TPOX	8,11	8,11
D18S51	13,14	13,14
AMEL	X,Y	X,Y
D5S818	11,12	11,12
FGA	22,22	22,22
Database Name	CC-LP-1	CC-LP-1

(C)

Client Sample Name	CCLP CTL 1	CCLP CTL 2	CCLP KO 1	CCLP KO 4
Sample Code	CL00011903	CL00011904	CL00011905	CL00011906
D8S1179	11,13	11,13	11,13	11,13
D21S11	29,33	29,33	29,33	29,33
D7S820	10,11	10,11	10,11	10,11
CSF1PO	11,13	11,13	11,13	11,13
D3S1358	15,16	15,16	15,16	15,16
TH01	9,3,9,3	9,3,9,3	9,3,9,3	9,3,9,3
D13S317	9,12	9,12	9,12	9,12
D16S539	9,13	9,13	9,13	9,13
D2S1338	17,17	17,17	17,17	17,17
D19S433	13,13	13,13	13,13	13,13
vWA	15,17	15,17	15,17	15,17
TPOX	8,11	8,11	8,11	8,11
D18S51	13,14	13,14	13,14	13,13
AMEL	X,Y	X,Y	X,Y	X,Y
D5S818	11,12	11,12	11,12	11,12
FGA	22,22	22,22	22,22	22,22
Database Name	CC-LP-1	CC-LP-1	CC-LP-1	CC-LP-1

Figure 2.1: STR Profiling confirms cell line identity.

Cell pellets were sent to Eurofins Genomic for STR profiling to confirm cell identity. The Client Samples Name is the name submitted and the Database Name is the correct identity.

(A) STR profiling of the wildtype CCA cell lines CCLP, RBE and KKU-M055.

(B) STR profiling of the shRNA CCLP PRH HKD and SVC control.

(C) STR profiling of the CCLP PRH KO clones and control clones.

Cell Line	Origins and Background (Source)	Documented Mutation (DEPMAP Portal)
CCLP1	Originated from a moderately differentiated intrahepatic CCA tumour, from a female American patient, pathology is not associated with fluke infection or hepatitis (Shimizu et al., 1992).	- <i>CTNNB1</i> (G34V) Likely Oncogenic: Gain of Function - <i>TP53</i> (R306*) Truncation: Predicted Loss-of-Function
RBE	Originated from tissue resections of an intrahepatic CCA adenocarcinoma tumour, from a female Japanese patient, pathology is not associated with fluke infection or hepatitis (Enjoji et al., 1997).	- <i>KRAS</i> (G12V) Oncogenic Gain-of-Function - <i>IDH1</i> (R132S) Likely Oncogenic: Switch-of Function
KKU-M055	Originated from poorly differentiated intrahepatic CCA tumour, from a male Thailand patient, associated with opisthorchiasis infection (Tepsiri et al., 2005).	- <i>MAP2K1</i> (K57T) Likely Oncogenic Gain-of-Function

Table 2.1: Cell Line Origins and Mutations

2.1.2 Cell Culture:

CCLP1, RBE, KKU-M055 wildtype cell lines and their modified derivative lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, D5796 Sigma), with 10% foetal bovine serum (FBS, F7524, Sigma). Cells were grown in tissue culture plastic flasks at 37°C and 5% CO₂ in a humidified incubator. Primary Biliary Cell Epithelial (BECs) cells were grown in Epithelial Cell Media (Innoprot, P60106) supplemented with 2% FBS and 1% Epithelial Growth Supplement, as per supplier's protocol. The medium is formulated to promote the proliferation and growth of human epithelial cells *in vitro*, by providing an optimally balanced

nutritional environment of amino acids, vitamins, hormones, growth factors and other components. BECs were grown to a maximum passage number of ten.

2.1.3 Cell Passaging:

Cells were split 1:10 when confluency reached 90%, normally every 3-4 days. For confluent flasks, complete media was removed, and cells were washed with X1 phosphate-buffer saline (PBS). Cells were harvested using X1 trypsin (T3924 Sigma) for 5 minutes at 37°C. The detached cells were collected using an equal volume of complete media, to neutralise the trypsin activity, and centrifuged at 500g for 5 minutes. The pellet was resuspended in fresh complete media and transferred to new flasks or experimental plates. Cells were routinely checked by microscopy for infection and samples were sent for mycoplasma testing monthly.

2.1.4 Cell Counting

To seed the appropriate number of cells, cells were counted before plating. A 10µL sample of cell suspension was mixed with 0.4% GIBCO Trypan Blue solution (Fisher Scientific, 1520061) at a 1:1 ratio before 10µL of sample was pipetted between the glass side and a haemocytometer. The live cells, or cells that had not been permeated and stained by the Trypan Blue dye, were counted in each 3x3 square. The readings were averaged and cells per mL was calculated (the average no. of cells per square $\times 10^4$ = cells/mL). Cells were also counted using an automated cell count (CellDrop™, DeNovix, Bioscience). The cells/mL value was used to create cell suspensions of the desired density.

2.1.5 Freezing Cells:

Cells were frozen, at early passages, to maintain stocks of cell lines. At 90% confluency, cells were collected via trypsinisation as described above and pelleted. The pellet was resuspended in freezing medium consisting of 80% complete media, 10% FBS and 10% DMSO (10213810, Fisher Scientific). Cells were diluted to approximately 2×10^6 cells in 1mL freezing media in 1mL cryovials. Cryovials were placed inside a Mr Frosty and transferred to a -80°C freezer. Cells were kept at -80°C for short-term use or relocated to -150°C for long-term storage.

2.1.6 Thawing Cells:

Cells from the -80°C or -150°C freezer were thawed at 37°C in a water bath. The thawed cell suspension was added to 4mL of pre-warmed complete media and centrifuged at 500g for 5 minutes to remove the DMSO. The pellet was resuspended in complete media and transferred to a T25. Cells were left until confluency was 90% before transferring to larger flasks.

2.1.7 Spheroid Formation:

Spheroids were generated to assess the ability of the drugs to influence cell viability in a complex 3D model. This protocol was supplied by Prof A. Grabowska (Pal et al., 2021) (Sueca-Comes, 2023). CCA cells were seeded at 1×10^3 cells per well in 200 μL complete media with 100 $\mu\text{g}/\text{mL}$ Matrigel® Basement Membrane Matrix (356234, Corning). The matrix is prepared from Engelbreth-Holm-Swarm (EHS) mouse tumour, and contains various extracellular matrix components, plus growth factors, to provide an environment for spheroid formation and development. Cells were centrifuged at 300g for 10 minutes on a round bottom, ultra-low attachment 96 well plate (7007, Fischer

Scientific) and left to grow at 37°C and 5% CO₂ for 7 days. Spheroids were not grown in the outer wells to avoid the effect of evaporation. Images of spheroid formation and growth were taken on a Nikon Plate Reading Widefield Microscope at X4 magnification

2.1.8 Spheroid Viability Assay using Presto Blue

To determine the effects of drugs and inhibitors in spheroid models, spheroids were left to grow for seven days, using the protocol described above, to form an established size around 500µm². The quantification of the spheroid size was performed with 2D images in ImageJ. On the seventh day, 100µL from each of the wells was carefully removed, to leave 100µL. Media containing vehicle control or drug were made at 2x concentration and 100µL was pipetted into the 100µL to make the desired 1x final concentration. Cell viability was measured over time, with readings being taken on day 0 (without drug), day 2, day 4, and day 6. Cell viability was measured through Presto Blue (A13261, Invitrogen). When readings were taken, 100µL of media was removed from the well, and 20µL of Presto Blue diluted 1:1 with complete media was dispensed in the well. The Presto Blue was dispersed around the well by gentle pipetting. Presto blue is a measure of metabolic activity where the blue, resazurin-based reagent is modified by the reducing environment of living cells into a highly fluorescent substrate. The reagent was left for an hour before reading on a FluorSTAR 96-well plate reader at wavelengths 544nm (Excit.) and 590nm (Emiss.) and gain 750. To control for background, outer wells were given 100µL media and 20µL of the diluted 1:1 Presto Blue in media. These background readings were subtracted from raw data and normalised to spheroid viability on day 0, where day 0 is 100% spheroid viability. Images were taken on days 0, 2, 4, and 6 for visual representation of spheroid viability on a Nikon Plate Reading Widefield

Microscope at X4 magnification. Presto Blue was used to assess spheroid viability because it is most representative of spheroid growth over time compared to other metabolism-based viability assays such as MTT that do not penetrate the 3D structure (see **Figure 3.25**); it is commonly used for 3D assays as it produces a fluorescent signal that correlates to cell number (Manlong et al., 2015).

2.1.9 Transient transfection using siRNA

To transiently silence genes, small interfering RNA or siRNA was used. SiRNA is duplex RNA synthesised to complement the target mRNA and cause degradation. It enters the cell and assembles into an RNA-Induced Silencing Complex (RISC) with Argonaute, a ribonuclease. The single stranded guide RNA leads the complex to the specific base pair sequence of the target gene where it binds and cleaves the target mRNA, resulting in its degradation. For targeting of HHEX, a pooled mixture of siRNA was used (SMARTpool, Horizon Discovery, M-011756-01-0050); this combined the effects of four siRNA for effective gene silencing. The target exon of each species is as follows: D-011756-01 targets exon 3/4 overlap, D-011756-02 targets exon 1/2 overlap, D-011756-03 targets exon 4 and D-011756-04 targets exon 3.

The transfection was mediated through RNAiMAX Lipofectamine, a lipid-based transfection agent optimised for small RNA delivery (13778075, Invitrogen). Reverse transfection was performed for effective silencing. A mixture of 2mL of warmed OptiMEM media, 2 μ L of siRNA (from 50 μ M stock), and 25 μ L of RNAiMAX were gently pipetted together, before transferring into a 10cm³ dish. Either a non-targeting siRNA control (siCtrl) or HHEX targeting siRNA (siHHEX/siPRH) was used. The siRNA containing media was incubated at room temperature for 30 minutes whilst the cells were harvested. Previously plated

cells in a T75 were harvested through trypsinisation, as described previously, and pelleted at 500g for 5 minutes. The pellet was resuspended in fresh complete media and 2×10^6 cells were counted for each condition (i.e. siCtrl and siHHEX). A cell suspension of 5mL complete media containing 2×10^6 cells was transferred to each media-siRNA mixture dish and incubated for 6-8hrs at 37°C. After 6-8hr, the cells became attached, and the transfection media was discarded and replaced with 10mL complete media. Replacing the media after 6-8hrs allows for the transfection to occur and the cells to remain viable as the RNAiMAX reagent is cytotoxic. The transfected cells were left 48hr post transfection before experiments were conducted.

2.1.10 Optimisation and creation of stable cell lines using shRNA

To generate stable knockdown cell lines, short hairpin RNA or shRNA was used. ShRNA is similar to siRNA through its mechanism of gene silencing described above, however the structure of shRNA slightly differs by having is two complementary sequences linked by a tight hairpin turn of around 4-11 nucleotides. For stable cell line generation, the shRNA is incorporated into a vector that can integrate into the genome for constant gene silencing. For HHEX gene silencing, the Hex (*HHEX*) Human shRNA Plasmid Kit was used (TR312464, OriGene), and the pHuSh-PRH-49 29mer construct was selected as it had previously shown the greatest gene silencing compared to other constructs (Kitchen et al., 2020). The sequence for the *HHEX*-targeting shRNA is CTGTGATCAGAGGCAAGATT. Alongside the *HHEX*-targeting plasmid, a scrambled vector control (TR30012, OriGene) was used to account for non-specific effects of the plasmid. The sequence of the shRNA in the scrambled

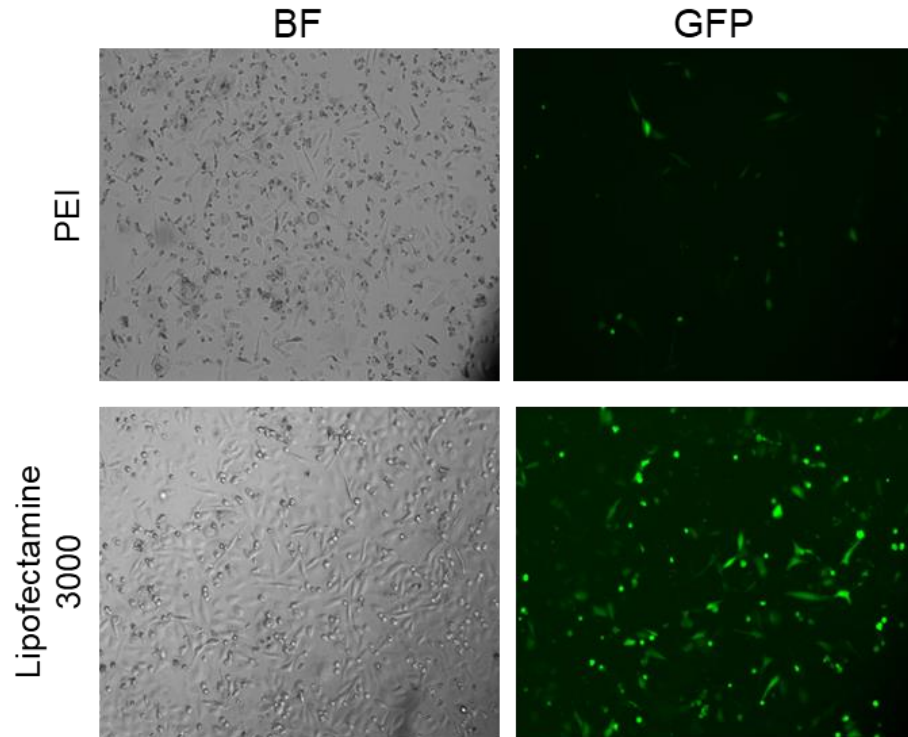


Figure 2.3: Optimisation of the CCLP SVC and PRH KD stable cell lines.

An eGFP plasmid was used to assess the success of transfections using different reagents in CCLP. PEI was used to generate the CCLP SVC and PRH KD previously, however, transfection efficiency was very poor with PEI in the CCLP cells. Lipofectamine was able to increase the transfection efficiency to around 22.5% therefore Lipofectamine was used for the CCLP SVC and PRH KD.

The transfection was optimised to give the following protocol. CCLP cells were plated at 5×10^5 cells per well in a 6 well plate with 2mL of complete media. After 12hr the cells had adhered and were at around 70% confluency, Lipofectamine 3000 was used according to manufacturer's protocol. A lipofectamine component, made from 125 μ L pre-warmed OptiMEM and 7.5 μ L Lipofectamine 3000, and a DNA component, made from 125 μ L pre-warmed OptiMEM, 2.5 μ g of plasmid and 5 μ L of P3000 reagent, were mixed via gentle pipetting and incubated at room temperature for 15 minutes. The 250 μ L of lipid-DNA complexes in OptiMEM were then added to cells on the 6-well plate. The transfection was left for 48hrs before selection.

Successfully transfected cells were selected through their acquired resistance to puromycin via the plasmid. Prior to selection, the optimum antibiotic concentration was determined. Cells were plated at 1×10^6 in a 6 well plate and subjected to Puromycin (SLS, P4512) at various concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/mL) for 5 days. The optimum concentration of puromycin for selection was defined as the minimum concentration to elicit 100% cell death over the 5 days. This was estimated to be 0.6mg/mL puromycin.

After the 48hrs of transfection, the media was removed and replaced with media containing 0.7mg/mL Puromycin. The media was replaced daily so that cell death would not affect clonal cell viability. After 5 days of selection, the antibiotic media was replaced with complete media. The cells were left to recover and when cells reached full confluency in the 6-well, cells were expanded into three T25 flasks. The cells were left to grow between 2-3 weeks after selection for enough cells to be placed in three T25 flasks. When at full confluency, pellets for protein and RNA were collected from two flasks, and the third kept for further culture into a T75.

2.1.11 Optimisation and creation of CCLP PRH

Knockout Cell Lines with CRISPR-Cas:

CRISPR-Cas gene editing was founded by Doudna and Charpentier (Jinek et al., 2012). The system has been researched and optimised for gene manipulation and is commonly used to generate 'knockout' models, called so as the gene has been rendered inactivated. In this system, a guide RNA (gRNA) is designed, which is complementary to a specific sequence in the target gene, and a Cas9 endonuclease enzyme is supplied, to generate double-strand breaks at the target gene. Protospacer Adjacent Motif or PAM sequences serve as a signal that allows Cas nuclease binding. PAM sequences are adjacent to

gRNA target sequences as the nuclease will only cleave the DNA if the PAM is present. Different Cas nucleases recognise different PAM sequences; Cas9 cleaves at 5'-NGG-3' where N is any nucleotide (reviewed; Ran et al., 2013).

2.1.11.1 HHEX KO Plasmids Generation:

The gRNA and Cas9n were delivered via plasmids. The plasmids were all derivatives of pX461, a gift from Feng Zhang (Addgene plasmid #48140), which encodes Cas9n (D10A nickase mutant), from *Streptococcus pyogenes*, followed by eGFP with an intervening 2A self-cleaving peptide sequence, all under a Chicken Beta actin promoter with CMV enhancer to facilitate expression of the Cas9n in transfected eukaryotic cells. See plasmid map from Addgene below (Figure 2.4).

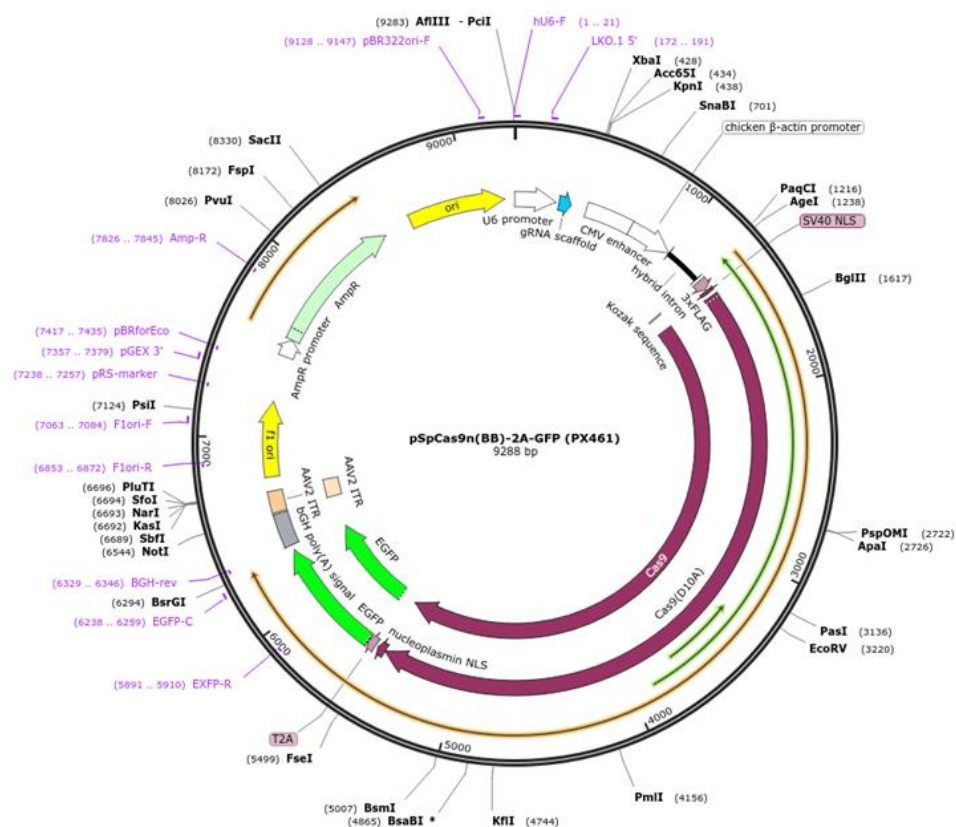


Figure 2.4: The plasmid map for pX461 with Cas9 D10A nickase mutant. The plasmid was generated by Zhang et al., and the fully annotated map was retrieved from Addgene.

Specific gRNA sequences were cloned into pX461 via two recognition sites for BbsI. Guides were designed and cloned, by Dr Christopher Roberts. Guides were designed with Benchling (<https://benchling.com>) using the “Design CRISPR Guides” tool with the following parameters: Paired guides; Guide Length: 20 nucleotides; Genome: GRCh38 (hg38, Homo sapiens); PAM: NGG (SpCas9, 3’ side); Pair Offset: 10 to 100 base pairs. The tool was supplied with the human genomic sequence of the *HHEX* locus (NC_000010.11 Chromosome 10 Reference GRCh38.p14 Primary Assembly; nucleotide positions 92689581 to 92695662). Positions proximal to the desired target loci (*HHEX* exon 1 and exon 2) were selected and possible guide pairs generated by the tool. A pair of suggested gRNAs for each target was chosen, with the most favourable On-Target Score (Doench et al. 2016) and the nick distance. Off-Target Score (Hsu et al., 2013) was considered less as the off-target errors with Cas9n are reduced. Exon 1 gRNA sequences target edits at the ATG start codon within exon 1, in a “PAM-out” orientation, with a distance between nick sites (“nick distance”) of 57 bp. Exon 2 gRNA sequences target edits to occur at the 3’ end of exon 2, in a “PAM-out” orientation, with a nick distance of 98 bp.

The following sequence were used:

Target	Sequence (5’—3’)	PAM	On-Target Score	Off-Target Score
Exon 1 Left	AGAGCTGCTGGCCCGCGCCG	CGG	54.6	81.2
Exon 1 Right	GCCATGCAGTACCCGCACCC	CGG	48.4	81.1
Exon 2 Left	GAATTTCTTCTCCAGCTCGA	TGG	52.5	42.3
Exon 2 Right	AGAGACAGGTGAGCTCGCGG	GGG	61.9	85.5

Table 2.2: HHEX target sequences for CRISPR knockout.

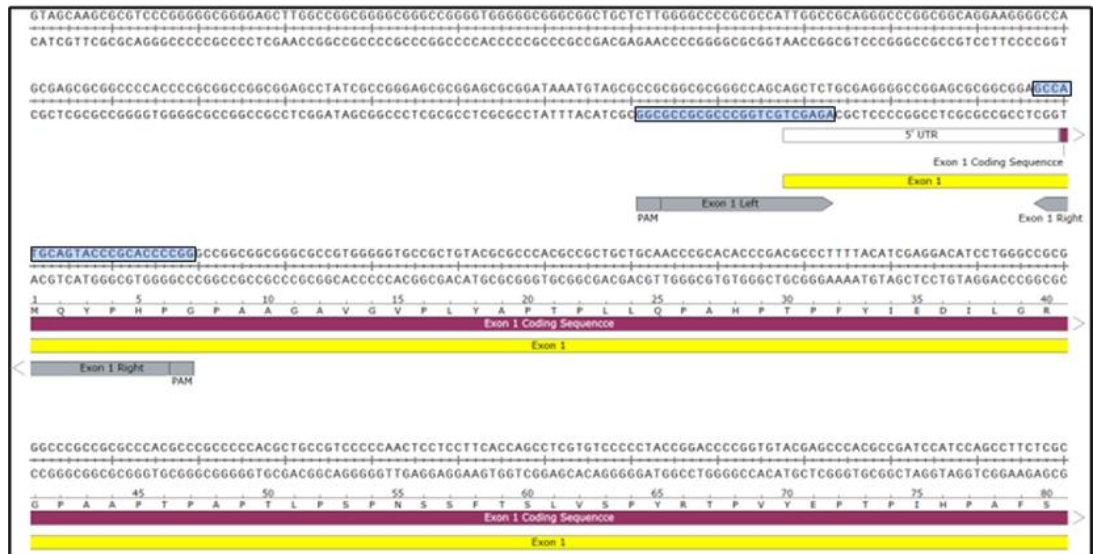
The following DNA Oligonucleotides were synthesised (Merck):

Pair		Sequence (5'—3')
Exon 1 Left	Exon 1 Left Top	CACCGAGAGCTGCTGGCCCGCGCCG
	Exon 1 Left Bottom	AAACCGGCGCGGGGCCAGCAGCTCT
Exon 1 Right	Exon 1 Right Top	CACCGGCCATGCAGTACCCGCACCC
	Exon 1 Right Bottom	AAACGGGTGCGGGTACTGCATGGC
Exon 2 Left	Exon 2 Left Top	CACCGGAATTTCTTCTCCAGCTCGA
	Exon 2 Left Bottom	AAACTCGAGCTGGAGAAGAAATTC
Exon 2 Right	Exon 2 Right Top	CACCGAGAGACAGGTGAGCTCGCGG
	Exon 2 Right Bottom	AAACCCGCGAGCTCACCTGTCTCT

Table 2.3: DNA oligonucleotide used for the gRNA.

The corresponding oligonucleotide pairs given above were synthesised (Merck), annealed together by heating to 95°C and gradually cooling. A T4 polynucleotide kinase was used to phosphorylate and create dsDNA oligonucleotides with 5'-CACCC-3' (top strand) and 5'-CAAAA-3' (bottom strand) overhangs on each pair to facilitate plasmid ligation. The pX461 plasmid was treated with the BbsI restriction enzyme and digested plasmid isolated by agarose gel electrophoresis, band extraction and purification (Monarch gel extraction kit, NEB). Ligation reactions were performed using a T4 DNA ligase to inset the annealed gRNA oligonucleotides into the digested plasmid and the ligation reactions were transformed into DH5α chemically competent *Escherichia coli* or *E. coli*. The bacteria were plated on LB agar plates containing antibiotic and resulting transformants were grown for DNA extraction and screened by sequencing (Source Bioscience) with the primer hU6-F (5'-GAGGGCCTATTTCCCATGATT -3'). The sequences of encoded sgRNAs in each plasmid were confirmed by Sanger sequencing and inspection of chromatograms. **Figure 2.5** shows the position of the guide and PAM sequences at each Exon.

At Exon 1:



At Exon 2:



Figure 2.5: The positions of guide plus PAM sequence for editing of HHEX Exon 1 and Exon 2.

The yellow and maroon features indicate the position of the exon whilst the grey and blue features indicate the position of the guide sequence plus PAM sequence, thus the sites of CRISPR editing.

2.1.11.2 HHEX KO Plasmids Transfection:

For transfection of the plasmids a Lipofectamine 3000 kit was used. CCLP cells were plated at 5×10^5 cells per well in a 6 well plate and left to adhere overnight. When the cells had grown to approximately 70% confluency, Lipofectamine 3000 was used to transfect the gRNA-Cas9n plasmids; this was performed as optimised previously for the shRNA transfection. A lipofectamine component, made from 125 μ L pre-warmed OptiMEM and 7.5 μ L Lipofectamine 3000, and a DNA component, made from 125 μ L pre-warmed OptiMEM, 2.0 μ g of plasmid and 5 μ L of P3000 reagent, were mixed via gentle pipetting and incubated at room temperature for 15 minutes. The 2.0 μ g of DNA was made from equal amounts, or 500ng each, of four plasmids used in the transfection. The 250 μ L of lipid-DNA complexes in OptiMEM were then added to cells on the 6-well plate. Alongside the production of KO clones, control clones were also generated by transfecting with 2.0 μ g of single guide RNA plasmid (exon 1 left gRNA plasmid), as a single-strand break can be repaired without introduction of indels with the Cas9n system. The transfection was left for 48 hours before inspecting for GFP signal and indication of successful transfection prior to harvesting. **Figure 2.6** below shows GFP positive cells from KO transfected CCLP:

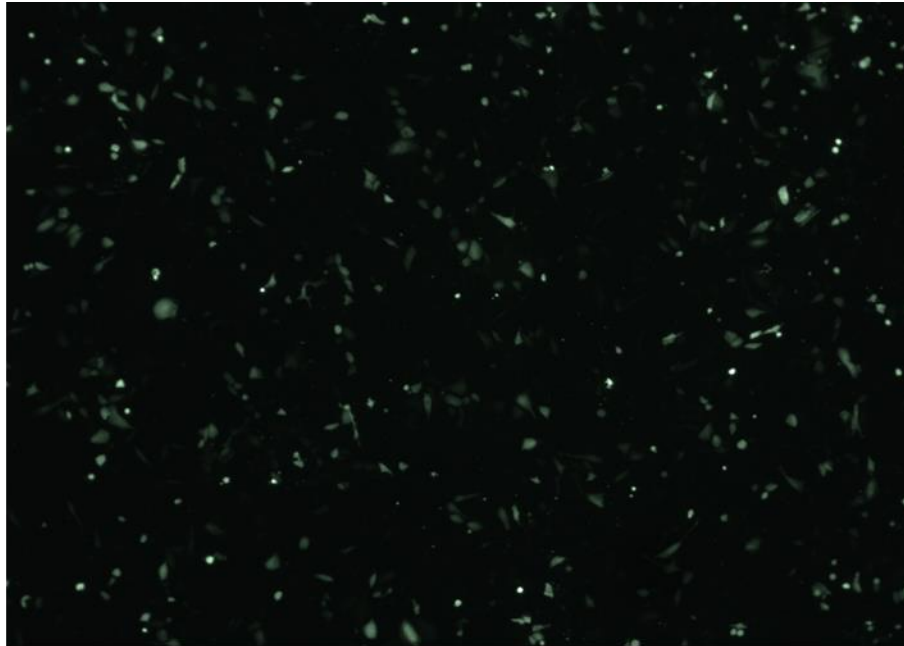


Figure 2.6: GFP Positive CCLP from the KO Transfection

2.1.11.3 Fluorescent Activated Cell Sorting of Clones

Post transfection, the cells were harvested and sent for fluorescent activated cell sorting (FACS) at the University QMC Flow Cytometry Units, in order to select the potentially edited cell lines, from the unedited cells, for further culture. The head of the facility, Dr David Onion optimised the Astrios Flow Cytometry System to gate for live and single cells, and then to filter cells on GFP signal. This is shown in the **Figure 2.7** below. As the number of GFP positive cells and the GFP signal from these cells was relatively low, the gating was set to collect cells that showed any level of GFP signal above background (R4). GFP positive cells were simultaneously collected and plated in a 96 well plate at one cell per well. The 96-well plate contained 100 μ L of filtered conditioned media. This media was taken from a flask of confluent CCLP cells, to promote cell viability and cell proliferation. A 96-well plate of potential KO clones was generated and a plate for the control clones was also generated.

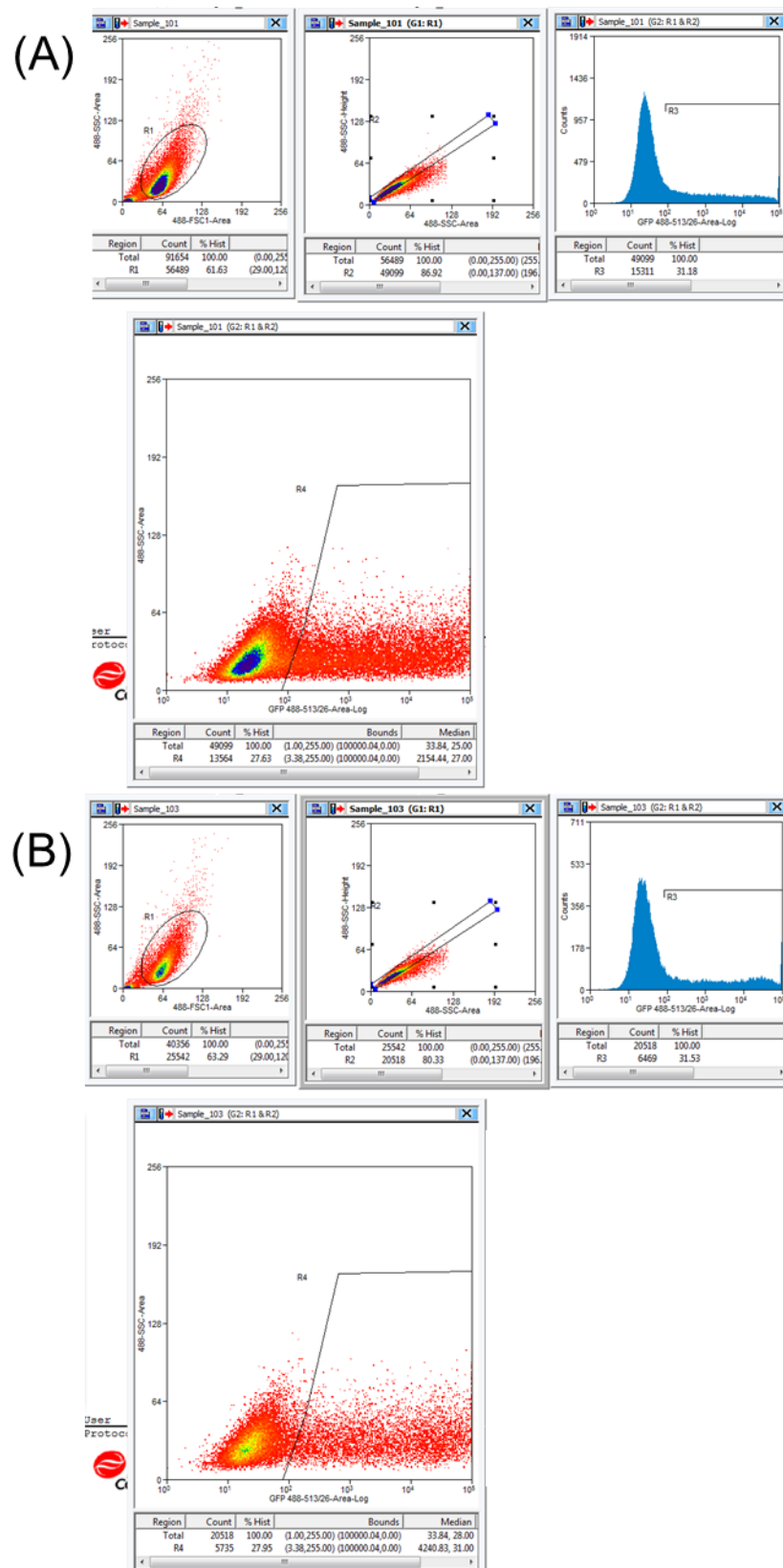


Figure 2.7: FACS Sorting of CCLP CON and KO Clones.

After 48 hours of transfection, CCLP CON and KO clones were harvested and sorted using an Astrios Flow Cytometry System, performed by Dr David Onion. Cells were gated primarily for live cells, then single cells, and were gated for GFP signal. GFP positive cells (R4) were simultaneously plated into the 96 well plate. The scatter plots show the sorting of (A) control clones and (B) potential HEX KO clones.

2.1.11.4 Clonal Expansion

Cells were left to grow in the incubator over a month with regular checking for growth. Once a single cell had expanded into a colony that was able to cover 50% of the well, cells were washed, trypsinised and replated in a 24-well plate. Once the cells were confluent in the 24-well plate, they were transferred to a 6-well plate. From a confluent well of a 6-well plate, cells were collected and replated in three wells of a 6-well plate. Once confluent, one well was collected to produce genomic DNA for genotyping, one well was collected to produce protein lysate, and the final well was maintained in cell culture.

2.1.11.5 DNA Extractions

Genomic DNA was isolated to use in PCR reactions to screen clones for PRH knockout edits, and was performed using QIAamp DNA Micro Kit (Qiagen, 56304) using the suggested protocol for "Isolation of Genomic DNA from Tissues". Briefly, pellets of cells were thawed on ice before adding 180µL of Buffer ATL at room temperature (22°C). A 20µL aliquot of Proteinase K was added and the samples were left to lyse for 30 minutes at 56°C in a ThermoMixer. A 200µL aliquot of Buffer AL was added and vortexed for 15 seconds, followed by 200µL of 100% ethanol for DNA precipitation. The sample was left to incubate at 22°C for 5 minutes. The samples were moved to a QIAamp MinElute column with a 2mL collection tube and centrifuged at 6,000g for 1 minute. The DNA was cleaned with 500µL Buffer AW1 and centrifugation at 6,000g for 1 minute, then cleaned with 500µL Buffer AW2 and centrifugation at 6,000g for 1 minute. The column was placed in a new collection tube and centrifuged at 20,000g for 3 minutes to dry the membrane, and rid of any ethanol before elution. The column was moved into a new Eppendorf tube and 20µL of TAE buffer was placed in the centre of the membrane. The gDNA was

eluted in the buffer by centrifugation for 1 minute at 20,000g. DNA concentration and quality was measured on a NanoDrop.

2.1.11.6 PCR Screen

Polymerase chain reaction experiments were used to amplify DNA samples to check for CRISPR edits. In PCR reactions, genomic DNA is denatured at 95°C to separate into single stranded DNA. Single stranded oligonucleotide constructs, or primers, then anneal to the single stranded DNA at their complementary sequence and at optimised annealing temperatures. Polymerase enzymes are then able to initiate primer extension and DNA replication by incorporation of nucleotides or NTPs to convert the single strand of DNA into newly synthesised double stranded DNA. The reaction is permitted at 72°C this is the optimal temperature for Taq polymerase activity. After cycles of the DNA denaturation, primer annealing and primer extension, the specific gene of interest is amplified at the site between the primers. For the PCR reaction, DNA samples were diluted to 10ng/1µL in RNase free water. Primers were diluted ten-fold from stock tubes (100µM) to create a 10µM working stock. The Taq polymerase with X10 Taq Buffer (NEB, M0273S) and the deoxynucleotide (dNTP) solution mix (New England, N0447S) were supplied ready to use. Master mixes for each primer pair were made from the following:

Component	Volume (µL)
10X Taq Buffer	5.0
dNTP	1.0
Forward Primer	1.0
Reverse Primer	1.0
DNA (100ng/µL)	2.0
Taq Polymerase	0.25
H ₂ O	39.75
Final Volume (per	/50

Table 2.4: Components of the PCR Master Mix for the KO Screen.

The master mix was made in excess of the number of DNA samples plus a negative control (50µL x (no. of DNA samples + H₂O control + 1)). A volume of 48µL was pipetted into each PCR tube followed by 2µL of appropriate gDNA dilution or H₂O for a negative control. The PCR tube was placed into a ThermoMixer; to allow the reaction to commence, the sample was set at the following temperature settings and underwent 35X cycles of the DNA denaturation, primer annealing and extension. After the cycles, the temperature slowly dropped to 4°C and samples were stored at -20°C:

		Repeated for 35 cycles				
Temp (°C)	95	95	55*	68	68	4
Time (mins)	3:00	0:30	0:30	1:10	5:00	∞

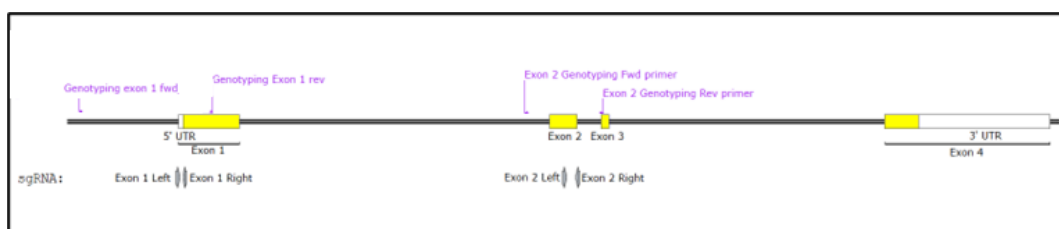
Table 2.5: The PCR Cycling Programme for the KO Screen.

* Annealing temperature changes with primer optimisation. A temperature of 55°C was used for amplification of HHEX exon 1 and exon 2 primers in the CRISPR KO screen. However, only the primer pair for exon 2 were used in the HHEX KO PCR screen as the primer pair for exon 1 were not able to perform successful PCR reactions due to high GC content of the HHEX genomic DNA in the exon1 region. The primer sequences are listed below:

Exon 1 FWD AACTCCAGCCATCCGAATCC
 Exon 1 REV GACACGAGGCTGGTGAAGG
 Exon 2 FWD TGTCGGTGGGAACGTGTTAGG
 Exon 2 REV ACCAGGTTTTGACCTACAGGAAAGAG

Table 2.6: The Sequences of Primers Used in the KO Screen.

The positions of the genotyping primers relative to the gene are shown below:



2.1.11.7 Preparation of Agarose Gels for DNA electrophoresis

PCR products were electrophoresed on agarose gels to determine the size of the PCR products and check for potential PRH knockout edits. The gel was mixed with ethidium bromide which intercalates with nucleic acids and emits a signal under UV exposure. A 1.5% agarose gel was prepared by dissolving 1.5g agarose (Sigma, A9539) in 100mL of 1X TAE buffer in a 250mL canonical flask. TAE buffer, or Tris-Acetate EDTA buffer, is composed of 40mM Tris-Acetate (Sigma, T1258) and 1mM EDTA (Sigma, E1644). The agarose and TAE buffer was microwaved in one-minute intervals in the flask until the agarose had dissolved to produce a clear homogeneous solution. The solution was cooled by running water over the outside of the flask, until the flask was cool enough to be handheld. Ethidium bromide (Promega, H5041) was added to the solution at 0.5µg/mL and mixed by swirling. The solution was poured into a cast with a comb at one end for sample loading. The gel was left for approximately 30 minutes at 22°C to solidify.

2.1.11.8 DNA Gel Electrophoresis

After the gel had set, the comb was removed, and the gel transferred into an electrophoresis tank. The TAE buffer was poured over the gel, so the level of buffer submerged the gel completely. From the 50µL of PCR product generated in 2.1.11.2, 16µL of DNA was mixed with 4µL of 5X loading buffer (NEB,

B7024S) and loaded into the gel along with DNA ladder (Bioline, BIO-33056). The gel was electrophoresed at 100V for around 45 minutes to allow for clear separation of the DNA ladder. The gel was loaded into a UV tray and imaged using a BioRad Gel Doc system.

2.2 Cell Phenotypic Protocols

2.2.1 Cumulative Growth Curve and Doubling time:

To observe increases in cell number over time, cells were studied in cumulative growth assays. For this, cells were seeded at 5×10^4 cells per well in a 6-well plate in 2mL of complete media. Cell number was counted every two days by harvesting by trypsinisation. The cells pellet was diluted in 1mL of media and counted via CellDrop which automatically provides the number of cells at cells per mL. This means the total number of cells in the flask was accounted for each timepoint. To calculate the growth rate, the following equation was applied (where $\ln$ = the natural log and no.=number):

$$\text{Growth Rate} = \ln(2) / \ln(\text{cell no. at day 10} / \text{cell no. at day 0}) \times \text{no. hour}$$

2.2.2 EdU Incorporation Proliferation Assay:

To measure cell proliferation rate, a Click-iT™ EdU Incorporation Assay was used. This assay uses a nucleoside analog EdU (5-ethynyl-2'-deoxyuridine) which is incorporated into DNA during S phase. An alkyne group present in EdU is covalently bound to the azide group in Horseradish Peroxidase (HRP) by a 'Click' reaction. This HRP metabolises a substrate to produce an optical density that can be measured. Therefore, the OD correlates to the amount of EdU incorporation, which reflects the proliferation rate. The kit was used according to manufacturer's protocol (C10337, Invitrogen). Cells were seeded at 5×10^3 or

1x10⁴ in triplicate, unless stated otherwise, in a 96-well plate and left to adhere overnight. Old media was replaced with 100µL of prewarmed complete media containing EdU, at an optimised concentration of 2µM, and incubated at 37°C for 2 hours. Wells without EdU were used as controls. After incubation with EdU, cells were fixed with 50µL per well formaldehyde-containing Click-iT™ EdU Fixative, for 5 minutes at room temperature. After fixing, the fixative was removed, and wells were washed with 200µL X1 Click-iT™ Wash buffer. 50µL of X1 Click-iT™ reaction cocktail (provided in manufacturer's protocol; provided below) was added directly to the wells and incubated for 30 minutes. A list of the component for X1 Click-iT™ Reaction Cocktail produced by the manufacturer's protocol:

Components	Per plate
Deionised Water	4.3mL
Click-iT™ EdU master mix (Component E)	1.1mL
HRP-azide (Component B)	25µL
Click-iT™ EdU reaction additive (Component F; diluted 1:10 in H ₂ O)	600µL

Table 2.7: The Components of the EdU X1 Click-iT Reaction Cocktail.

The reaction cocktail was removed after 30 minutes and incubated with a blocking buffer of 1.5% BSA in PBS for 5 minutes. After the incubation, the wells were washed three times with 200µL X1 Click-iT™ Wash buffer. One urea hydrogen peroxide buffer tablet and one OPD peroxidase substrate SIMGAFast™ OPD (P9187, Sigma) were dissolved in 20mL distilled water to create an activated OPD substrate; 100µL/well of substrate was incubated with the cells for 15 minutes or until a yellow colour was visible. The plate was read at absorbance 450nm. A sister plate with cells seeded at the same time as the EdU was stained with 200nM Calcein-AM for 30 minutes at 37°C and

fluorescent signal was measured on a FluoSTAR plate reading (Excit. 485nm; Emiss. 520; Gain 600). The EdU signal was divided by the calcein signal to account for differences in cell number. The average of the non-EdU containing control were subtracted from experimental measurements. Graphs and statistical analysis were performed on GraphPad Prism.

2.2.3 MTT Cell Viability Assay:

MTT Cell viability assays were used to measure cellular metabolism, and thus, can be used to infer cell number or viability. Cells were plated 1×10^4 per well, unless stated otherwise, in 96-well plates and left to adhere for at least 12 hours overnight. Cells were then treated as desired, with appropriate controls in place, for a desired duration. After incubation with drug, the MTT reagent, or tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide), was added at a concentration of 0.5mg/mL in pre-warmed complete media. Media was removed from the 96-well plate and replaced with 100 μ L filter-sterilised, MTT-containing media. The yellow MTT reagent was reduced to an insoluble purple formazan crystal by metabolically active cells after 2-3 hours. Media was carefully removed, without disturbing the crystals at the bottom of the wells, and crystals were solubilised using 150 μ L DMSO, to produce a homogenous purple solution. The OD of each well will be dependent on the cell's metabolic activity, and the amount of metabolic activity can be correlated with cell number. OD was read at 595nm. **Figure 2.8** shows that MTT signal correlates with cell number.

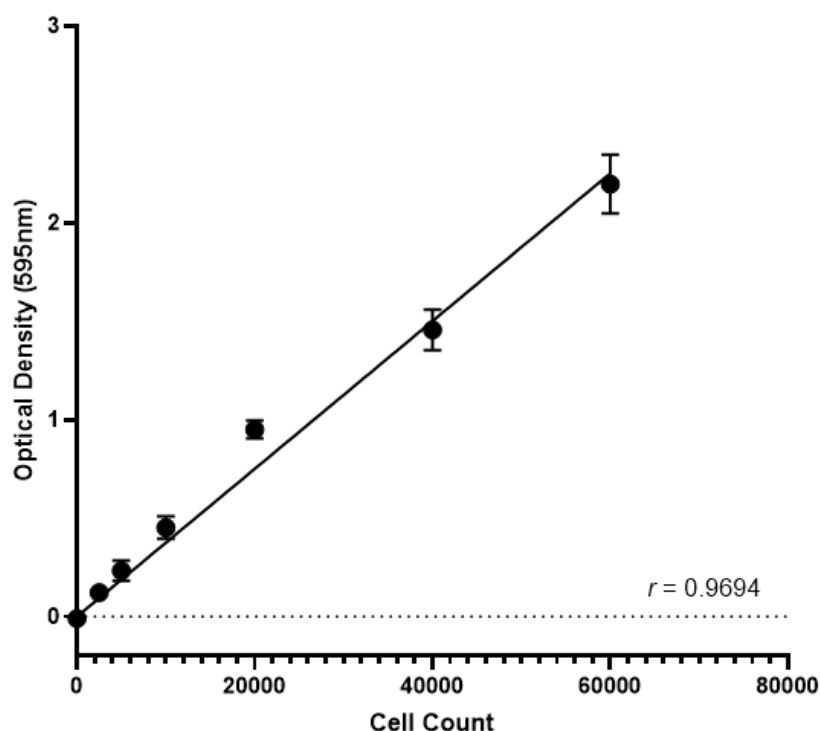


Figure 2.8: MTT Standard Curve.

MTT optical density measured at different cell number in CCLP cells

2.2.3.1 MTT Assay in Dose Response Curves:

MTT reagent was used as described above. After adhesion, the cells were treated with a dilution range of the drug of interest, often in a log₁₀ range to obtain complete dose response curves and assess drug potency and efficacy. The incubation of the drug was adjusted to the needs of the experiment. The raw data was manipulated by subtracting a background control, and normalising to a vehicle control. The background control were wells containing 100% DMSO-killed cells and represented 0% living cells, whilst the vehicle control were wells containing 1% DMSO in complete media and represented 100% living cells. The normalised data, as a percentage of the vehicle control, was plotted in GraphPad Prism to generate dose response curves. EC₅₀ values

were calculated through the analysis tool: non-linear regression (curve fit), dose-response inhibition, log(inhibitor) vs. response (three parameters).

2.2.3.2 Synergism Experimental Set Up:

To assess drugs in combinations, similar experiments were performed as to those in section 2.2.3.1 above, however cells were treated with a range of concentration of Drug 1, and separately, a range of concentration of Drug 2, and then treated with Drug 1 and Drug 2 together. For the cells treated with drug combination, the concentrations used were concentrations used for the testing of the drugs individually; this is important for downstream analysis. DMSO-treated cells, and vehicle control treated cells were measured alongside the experimental values for subtracting background and normalisation, respectively. Double the amount of DMSO was added for vehicle control, given that both drugs were reconstituted in DMSO in the combination treatment.

Each drug was used in its working concentration range; this can be found in the linear part of the sigmoidal curve generated in dose response assays. A proposed method by Chou and Talalay of obtaining a range of concentrations for synergism experiments is using a serial dilution determined by the EC50 value i.e. 0.125X, 0.25X, 0.5X, X, 2X, 4X, 8X, where X=EC50. For some drugs in this project, this method was used to determine the concentration range for synergism experiments (i.e. Niclosamide). However, for some inhibitors in this project (i.e. Palbociclib, Ribociclib), the working concentration range was adjusted and were different in each cell line. This is since the proposed method of using a serial dilution of the EC50 values was insufficient because it would generate concentrations that would not span a large enough range. Thus, the working concentrations for these inhibitors included concentrations where a reduction in cell viability is first seen in the dose response curves and

concentrations where cell viability is almost 100% reduced. The median concentration is based on the EC50 and the points between give equi-distance concentrations for a range of full coverage. For example, the table below shows the concentration of Niclosamide and Palbociclib used in each CCA cell line in combination experiments:

CCLP	1	2	3	4	5	6	7
NIC (nM)	62.5	125	250	500	1000	2000	4000
PAL (nM)	1	10	100	1000	5000	10000	15000
COM (nM)	62.5+ 1	125+ 10	250+ 100	500+ 1000	1000+ 5000	2000+ 10000	4000+ 15000

RBE	1	2	3	4	5	6	7
NIC (nM)	62.5	125	250	500	1000	2000	4000
PAL (nM)	31.5	125	500	2000	4000	8000	16000
COM (nM)	62.5+ 31.5	125+ 125	250+ 500	500+ 2000	1000+ 4000	2000+ 8000	4000+ 16000

KKU-M055	1	2	3	4	5	6	7
NIC (nM)	62.5	125	250	500	1000	2000	4000
PAL (nM)	500	1000	2000	4000	6000	8000	10000
COM (nM)	62.5+ 500	125+ 1000	250+ 2000	500+ 4000	1000+ 6000	2000+ 8000	4000+ 10000

Table 2.8: The Concentration of Niclosamide and Palbociclib used in Combination experiments for the Different CCA Cell Lines.

2.2.3.3 Synergism Calculations:

The method used to assess synergism between drugs was the Chou Talalay method. This is a dose-effect-based approach; it accounts for the amount of each drug needed to achieve a response as single agents and in combination. The calculation is derived from the Median-Effect equation (Figure Eq1). The Median-Effect equation (Chou, 1976, 1977) describes, in simple terms, the relationship between dose and effect. The relationship is influenced by m or the coefficient, which is a measure of the shape of the dose-response curve ($m=1$ signifies a hyperbolic curve; $m>1$ shows a sigmoidal curve; $m<1$ will indicate a flat sigmoidal curve). The equation can be rearranged (Eq2a, Eq2b), thus, for a given response, a dose can be calculated, and vice versa. When the Median-Effect equation is manipulated using logarithms, it forms the Median Effect Plot (Eq3). Equations are given below (**Figure 2.9**):

Eq1:	$fa/fu=(D/D_m)^m$
Eq2a:	$D=D_m[fa/(1-fa)]^{1/m}$
Eq2b:	$fa=1/[1+(D_m/D)^m]$
Eq3:	$\log(fa/fu)=m \log(D)-m \log(D_m)$

Figure 2.9: The Median Effect Equations and deviations used in the Chou Talalay Method.

Key: Fa= fraction affected; Fu= fraction unaffected; D= Dose; D_m= Median Dose (IC₅₀); m=coefficient, slope

Chou and Talalay proposed the concept of a Combination Index (CI) value) as a quantitative measure of synergism or antagonism. CI value is calculated from

dividing the amount of Drug 1, or (D)1, used in combination to achieve X% effect, by the amount of Drug 1, or (Dx)1, required to promote the same X% effect as a single agent. This is also calculated for Drug 2 and the fractions are added (Eq4). If less drug is needed to produce the same response in combination, (D)1 and (D)2, than as single agents, (Dx)1 and (Dx)2, this would make $CI < 1$; this means the drugs work synergistically. If more drug is required to create the same response in combination, compared to single agents, this would cause $CI > 1$ and the drugs would be classed as antagonistic. When $CI = 1$, drugs are additive. Chou Talalay describes further classifications of synergism (see below **Figure 2.10**).

Range of combination index	Description	Graded symbols	Graphic symbols
<0.1	very strong synergism	+++++	
0.1-0.3	strong synergism	++++	
0.3-0.7	synergism	+++	
0.7-0.85	moderate synergism	++	
0.85-0.90	slight synergism	+	
0.90-1.10	nearly additive	±	
1.10-1.20	slight antagonism	—	
1.20-1.45	moderate antagonism	--	
1.45-3.3	antagonism	---	
3.3-10	strong antagonism	----	
>10	very strong antagonism	-----	

Figure 2.10: Broad Terms for the Degree of Synergism, Additive or Antagonism described by Chou and Talalay.

Figure is taken from Chou, 2011 (American Journal of Cancer Research) and provides classes of synergism according to Combination Index value.

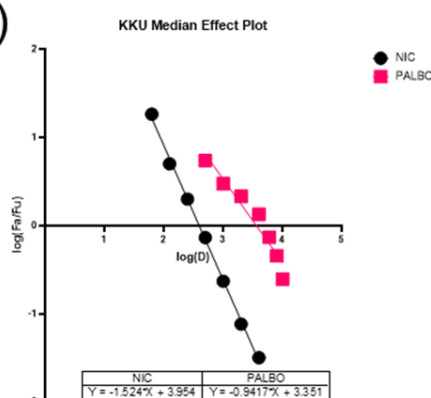
To calculate synergism, the raw data was used to calculate a line of best fit between the log of the drug concentration (logD) and the log of the response (log(Fa/Fu)). Here the Fraction affected (Fa) and the Fraction unaffected (Fu) are taken from the MTT cell viability data e.g. when the drugs in combination reduce cell viability by 22.27% in KLU-M055, Fa= 0.2227 and Fu= 1-0.2227 or

0.7773. This is called the Median Effect plot; as an example, the median effect plot used for calculating the synergism between Niclosamide and Palbociclib in the K KU-M055 cell line is shown in **Figure 2.11**. This linear equation allows correlation of concentration to response and provides the parameters m and D_m . Therefore, the response can be equated to any given concentration. Consider the K KU-M055 example, to reduce cell viability to 22.3% ($F_a=0.227$), 562.5nM of drug in combination is needed (62.5nM Niclosamide to 500nM Palbociclib), whereas 173nM of Niclosamide or 959nM of Palbociclib would be needed to reduce cell viability to 22.3% as single agents. The concentration of the drug used in combination, is divided by the amount of drug needed to produce the same response as a single drug. This is calculated for both Niclosamide and Palbociclib. These values are then added together to produce the Combination Index (CI). In the example, this makes the CI value 0.883 when $F_a=0.2227$. Synergism values are calculated like this for every combination data point tested, where the response to the drug in combination is known (i.e. $F_a=0.2227$) and the concentration is extrapolated. Calculations are summarised in **Figure 2.11**.

(A)

Drug (nM)		Fraction Inhibition (Fa)	Parameter			CI
Niclosamide	Palbociclib		m	Dm	r	
62.5		5.17				
125		16.65				
250		33.34				
500		57.79				
1000		81.04				
2000		92.91				
4000		96.92	1.5242	392.72	0.9984	
	500	15.48				
	1000	25.09				
	2000	31.79				
	4000	42.72				
	6000	57.60				
	8000	68.76				
	10000	80.20	0.9417	3617.03	0.9358	
62.5	+ 500	22.27				0.883
125	+ 1000	38.89				0.875
250	+ 2000	55.19				0.998
500	+ 4000	75.75				0.933
1000	+ 6000	87.64				0.912
2000	+ 8000	94.90				0.847
4000	+ 10000	98.35				0.733

(B)



(C)

When $F_a = 0.2227$

Combination = 62.5nM Niclosamide; 500nM Palbociclib

$$[D_p] = (D_m) [Fa/1-Fa]^{1/m}$$

Nic: $392.72 [0.2227/1-0.2227]^{1/1.5242} = 172.95\text{nM}$

Pal: $3617.03 [0.2227/1-0.2227]^{1/0.9417} = 959.13\text{nM}$

$$CI = \frac{(D)_1}{(D_1)_1} + \frac{(D)_2}{(D_2)_2}$$

$$CI = \frac{62.5}{172.95} + \frac{500}{959.13} \quad CI = 0.883$$

Figure 2.11: Calculating Synergism using the Chou Talalay Method Manually.

This is an example using the synergism between Niclosamide and Palbociclib in KKU-M055 cells.

- (A) The dose of drug or drugs used and its fraction inhibition. The experimental data were used to make *Median-Effect* Plots to retrieve the parameters m , D_m and r . The parameter m , D_m and r are the slope, the antilog of the X-intercept and the regression of the median-effect plot. These parameters were used to calculate Combination Index (CI) values.
- (B) The *Median-Effect* plot shows the log of the drug concentration ($\log D$) plotted against the log of the response ($\log(Fa/Fu)$). This provides a linear equation, from which m and D_m are derived, which correlates concentration to response.
- (C) To calculate Combination Index values, the concentrations of the drugs alone are calculated by substituting the D_m and m into the *Median-Effect* Equation. The concentration of drugs in combination is divided by the concentration of drugs as single agents; these values added are the CI value.

2.2.4 MitoTracker Staining:

MitoTracker Red probe (Invitrogen) is a membrane potential-sensitive dye that accumulates in the mitochondria, providing structural and functional information about this organelle. The intensity of the MitoTracker staining is dependent on its polarisation status, thus, healthy and active mitochondria with a polarised membrane show strong red staining, whereas dysfunctional mitochondria will show weak signal. MitoTracker® Red FM stock solution was made from reconstituting the 50µg lyophilised solid in 70µl DMSO. Cells were plated at 5×10^4 per well in a 24 well plate in 500µL complete media and left to adhere overnight. The following day, cells were incubated with media containing 200nM MitoTracker Red (as recommended by Invitrogen protocol 50-500nM), and either vehicle control or increasing concentrations of drug. Cells were incubated at 37°C for 1 hour. Prior to imaging, the MitoTracker-containing media was removed from the wells and cells were washed with warm x1 PBS twice, then left in x1 PBS for imaging. The cells were imaged using a Nikon live microscope (570nm excitation) at 20x magnification. The exposure was kept constant through acquisitions, so fluorescence can be compared between conditions.

2.2.4.1 MitoTracker Staining Image Analysis

To analyse the decay in fluorescence, images were imported to Image J. The image was duplicated and made into a binary image. In a binary image, if the pixel has measured MitoTracker staining, it becomes white, if no staining is detected, it becomes black. This created a mask where each particle would represent the localisation of the mitochondria. The mask was overlayed on the original image and used to analyse the intensity of MitoTracker staining over the localisation of the mitochondria. The images contained 30-50 cells per image, all of which were analysed together, and the intensity value was

averaged over the image. Finally, raw data was normalised to the vehicle control.

2.2.5 Transwell Migration Assay:

To assess the ability of cells to migrate in response to a growth serum gradient, cells were used in a Transwell Migration assay also known as a Boyden Chamber assay. Here, 1×10^6 cells were plated in T25 flask in complete media. Once the cells were attached, the cells were incubated in low serum (2% FBS) and 1mM hydroxyurea containing media for 24 hours. The aim of starving the cells and the addition of hydroxyurea was to inhibit their proliferation during the assay. After 24hrs incubation, the cells were trypsinised and harvested in the low serum and 1mM Hydroxyurea containing media. The transwell chambers were assembled by placing the 24-well ThinCert inserts (662610, Greiner Bio-one) into a 24 well plate and placing 750 μ L of complete media (10% serum) in the outer chamber. 2×10^5 cells were placed inside the insert in a total volume of 250 μ L low serum and 1mM hydroxyurea containing media. The cells were left for 48hrs at 37°C in an incubator to migrate through the 1 μ m pores via chemotaxis. After the incubation, the migrated cells were collected. Media in the insert was discarded whilst the media from the outer compartment was collected. Both the bottom of the insert and the contents of the outer well were washed in 0.5mL PBS before, adding 500 μ L of X1 Trypsin to collect any cells that had adhered to the underside of the insert or the well itself. This was collected with the media from inside the well and centrifuged at 500xg for 5 minutes. All the media was discarded, and the pellet was resuspended in media containing 3 μ M Calcein-AM (206700, Sigma Alrich) to make a cell suspension that was pipetted into a 96-well plate. Calcein-AM is a cell-permeable dye that is taken up by viable cells and metabolised by cell esterases to a fluorescent

calcein therefore the fluorescent signal correlates with viable cells that migrate cross the membrane. The cells were incubated at 37°C for 30 minutes whilst the calcein-AM was hydrolysed, and the fluorescence was then read on a FluoSTAR Plate Reader at 485nm (Excit.) and 520nm (Emiss.) with 600 Gain. The background fluorescence of calcein-AM containing media was measured, and subtracted from raw data, then the data was normalised to the experiment control.

2.2.6 Propidium Iodide DNA staining and Flow

Cytometry to measure changes in Cell Cycle

Progression

To observe the percentage of cells in each stage of the cell cycle, Propidium Iodide (PI) staining was used. PI is a nucleic acid- intercalating dye and its fluorescence correlates to the amount of DNA present in each cell. During G1 phase, the cell has one set of chromosomes, whilst during G2 phase, the cell has two sets of chromosomes, ready for cell division. Flow cytometry separates cells with low fluorescence (low PI staining and hence low DNA) (G1) from cells with high fluorescence (high PI staining and hence high DNA) (G2). Cell cycle analysis via Kaluza software algorithms that calculate the percentage of cells in G1 phase, S phase and G2, phase.

Cells were plated in a 6 well plate and treated with inhibitor if described. Cells were trypsinised and harvested as described previously, and 1×10^6 cells for each condition were counted and placed into an Eppendorf. The cells were washed twice in 1mL of ice-cold X1 PBS at 500xg for 5 minutes, then permeabilised and fixed with 0.5mL ice cold 70% ethanol. The cells were left to fix on ice with the ethanol for one hour. If the flow cytometry was not performed

on the day of fixing, the pellet was stored at 4°C until needed; the cells in ethanol can be kept for a week at 4°C. After fixation, the cells were centrifuged at 500xg for 5 minutes and the ethanol discarded. The cells were washed in 1mL of ice-cold PBS twice, before resuspending in 100µL of RNase solution. RNase solution was made from diluting PureLink RNase A (2201416, Invitrogen), in a ratio of 1:100 (0.2mg/mL), in X1 PBS. The cells were incubated with the RNase solution for 15 minutes at room temperature to eliminate RNA. The fixed cells were then stained with Propidium Iodide or PI (P4864, Sigma). The 1.0mg/mL stock was diluted 1:200, in X1 PBS, to give a 5µg/mL working stock. 400µL of the working stock was pipetted into 100µL of cell suspension and incubated for 15 minutes before analysing PI-stained cells on the CytoFlex Station Flow Cytometry machine.

2.2.7 Annexin and Propidium Iodide (PI) staining to measure apoptosis

To measure apoptosis, a FITC Annexin V Apoptosis Detection Kit was used (556547, BD Pharmingen TM). The kit stains cells with both Annexin V and PI, where Annexin V binds to exposed membrane phospholipid phosphatidylserine (PS) and PI can only enter cells with compromised membranes to stain DNA. As a result, cells can be analysed via Kaluza software into the following groups: live (PI-A-), early apoptotic (PI-A+), necrotic (PI+ A-), and late apoptotic (PI+A+) quartiles.

Cells were plated in a 6 well plate (and treated with inhibitor if described). Both cell debris in the media and attached cells harvested via trypsinisation were collected, and 1×10^6 cells for each condition were counted and placed into an Eppendorf. The cells were washed twice in 1mL of ice-cold X1 PBS at 500xg for 5 minutes, before resuspending in 100µL Annexin V binding buffer (diluted

1:10 in pure water) from the kit. Then 1µL of Annexin V and 1µL of PI were added to the 100µL cells in buffer, and the solution was mixed by pipetting. The cells were left to stain for 15 minutes in the dark, before the Eppendorf tubes were topped up with 300µL of Annexin V binding buffer. The samples were analysed for fluorescence from PI (Excit.: 535nm Emiss.: 610nm) and Annexin V-FITC (Excit.: 488nm Emiss.: 519nm) on the Flow Cytometer. Apoptosis analysis via flow cytometry was always performed on the day of harvesting the cells because freezing the pellet would destroy the dead cells and cell debris.

2.3 Quantitative PCR:

2.3.1. RNA Extraction

To extract RNA from cells an RNAeasy Mini Kit was used (74104, Qiagen). Between $1-3 \times 10^6$ cells were harvested by cell scraping in ice cold X1 PBS and centrifuged into a pellet at 500xg for 5 minutes. Pellets were kept at -80C until needed. Pellets were thawed on ice and lysed with 350µL of the RNAeasy kit RLT lysis buffer. The buffer contains a guanidine-thiocyanate which is highly denaturing and inactivated RNases to block RNA degradation. The solution was pipetted until the pellet was homogeneous in the lysis buffer. After lysis, 350µL of 70% ethanol was added to the 350µL lysis buffer. This is to provide conditions for optimal binding to the column. The 700µL was transferred to an RNAeasy spin column with a flow through tube. The column was centrifuged at 13000xg for 15 seconds at room temperature. The flow through was discarded. This caused the RNA to bind to the silica membrane. The RNA was washed by placing 700µL RW1 strong wash buffer into the column and centrifuging at 3000xg for 15 seconds. This was followed by 500µL RPE mild wash buffer which was added to the column and centrifuged at 13000xg for 15 seconds.

Another 500 μ L RPE was added to the column which was centrifuged at 13000xg for 2 minutes. The column was placed in a clean flow-through tube and centrifuged dry at 13000xg for 1 minute, before adding RNase-free water to the membrane and eluting the RNA into a clean Eppendorf tube. The RNA concentration and quality was measured through NanoDrop 2000 software, and the sample was either used to synthesise cDNA or stored at -80 until needed.

2.3.2. cDNA Synthesis

Complementary DNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) and according to manufacturer's protocol. A 2X Reverse Transcriptase Master Mix was made from: 2 μ L X10 RT Buffer, 0.8 μ L 25X dNTP Mix (100mM), 2 μ L 10X RT random primers, 1 μ L Multiscribe™ Reverse Transcriptase and 4.2 μ L nuclease-free water, to a final volume of 10 μ L. RNA was diluted to give 2 μ g in 10 μ L in a 0.2mL PCR tube, to which 10 μ L of X2 master mix was added. The 20 μ L was mixed by gentle pipetting and centrifuged to remove bubbles before loading into a thermal cycler. The samples were incubated at: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and cooled to 4°C before being transferred to -20°C for storage.

2.3.3 Quantitative Reverse Transcription PCR (RT-qPCR)

Reverse transcription polymerase chain reaction experiments allowed for the quantification of gene expression from cDNA samples. The theory is based on PCR reaction, previously described 2.11.1.2, however in qRT-PCR, a fluorescent dye, SYBR green, is incorporated into the newly synthesised DNA and the amplification of a product can be measured by the increase in

fluorescent signal. For qRT-PCR following production of the cDNA a, QuantiNova 2X SYBR green RT-PCR Master Mix (Qiagen, 208057) was used which consists of a ready-made mix of QuantiNova DNA Polymerase, QuantiNova SYBR Green RT-PCR Buffer and dNTP mix (dATP, dCTP, dGTP, dTTP). The primers are bought as lyophilised samples from Eurofins and reconstituted in RNase free H₂O to make 100µM stocks that are kept at -80°C. Stock solutions are further diluted to 10µM in water for working stocks used in qRT-PCR. It is usual that in measuring the gene expression of a gene of interest, a control, such as a housekeeping gene, is measured simultaneously for normalisation. For the qPCR experiment, a master mix of components is prepared for each primer pair:

Component	Volume (µL)
SYBR Green	22.5
FWD Primer	1.0
REV Primer	1.0
DNA (2µg/20µL) or H ₂ O	1.0
H ₂ O	19.5
Final Volume	/45µL

Table 2.9: The Components of the Master Mix used in qRT-PCR.

A

volume of 45µL Master Mix is required for each DNA sample. In addition, in all qRT-PCR experiments a no cDNA (H₂O only) control was always assembled; this is known as the non-template (NTC) control. The Master mix was made in excess and mixed by vortexing before aliquoting 44µL into PCR tubes, one of each DNA sample plus NTC. A volume of 1µL of cDNA or water was added to the appropriate tube and vortexed. The PCR tubes were centrifuged briefly to accumulate the volume at the bottom of the tube and 10µL of this mix was aliquoted into strip tubes so that the sample could be recorded in quadruplicate

replicates. The strip tubes were also briefly centrifuged and were then loaded into the RotorGene Q (QIAGEN) platform. The RotorGene was setup to a temperature profile of the following:

		Repeated for 45 cycles			Melt
Temp (°C)	95	95	Ta*	72	72-95
Time (mins)	10:00	0:10	0:15	0:10	90 sec (pre-melt) 5 sec (each 1-degree rise)

Table 2.10: The PCR Cycling Programme for qRT-PCR.

The output of the experiment includes cycling data and a melt curve. The cycling data can be used to generate Cycle Threshold values or Ct values, which refer to the number of amplifications cycles needed to produce a detectable PCR result. The melt curve plots fluorescence for a specific primer pair over a change in temperature to confirm that a single product has been made during the qRT-PCR run indicating that the specific product is amplified as expected.

2.3.4 qRT-PCR Analysis

Ct values gathered from the RotorGene output files were used to calculate relative gene expression using the Pfaffl method (Pfaffl., 2001). Briefly, the average Ct value amongst the four replicates of the control sample was calculated for each gene i.e. a housekeeper and a gene of interest. The individual Ct values were subtracted from the control average to calculate the change in Ct or ΔCt for both the control cDNA and the treatment cDNA. To account for the efficiency of the primers, the amplification factor was used to calculate the fold change in gene expression or $E^{\Delta Ct}$. The $E^{\Delta Ct}$ of the gene of interest was divided by the $E^{\Delta Ct}$ of the housekeeping gene; this calculates the relative gene expression. QPCR experiments were repeated three times and statistical significance was measured through an unpaired t-test.

2.3.5 Primer Bank:

Gene Name	Oligo Sequence
B-actin	F 5'-AAAGACCTGTACGCCAACAC-3' R 5'-GTCATACTCCTGCTTGCTGAT-3'
GAPDH	F 5' -TGATGACATCAAGAAGGTGGTGAAG-3' R: 5' -TCCTTGGAGGCCATGTGGGCCAT-3'
HHEX	F 5'-AAA CCT CTA CTC TGG AGC CC-3' R 5'-GGT CTG GTC GTT GGA GAA TC-3'
NOTCH-3	F 5'-CCTAGACCTGGTGGACAAG-3' R 5'-ACACAGTCGTAGCGGTTG-3'
CCND2	F 5'-GGACATCCAACCCTACATGC-3' R 5'-CGCACTTCTGTTCCTCACAG-3'

Table 2.11: The Sequences of Primers used for qRT-PCR.

2.4 RNA sequencing:

2.4.1 Sample preparation:

RNA samples were extracted from cell pellets using QIAGEN RNAeasy Mini Kit as described previously (section 2.3.1). The quality and concentration of the RNA eluted was measured by NanoDrop before RNA quality was further interrogated through an RNA integrity Number (RIN) test. An aliquot of sample (500ng/ 10µL water) was sent to the DeepSeq Next Generation Sequencing facility at the QMC, University of Nottingham. RIN scores were generated using the Agilent 4200 TapeStation system where a RIN score >9 qualified samples for RNAseq analysis. Samples for RNAseq were aliquoted from RNA stocks to provide 2000ng of RNA in 20µL of RNase-free water and sent to Novogene, Cambridge, on dry ice.

2.4.2 Novogene Library Preparation and Sequencing:

Novogene conduct quality checks on the samples including electrophoresis, Nanodrop and RIN test prior to sequencing. **Figure 2.12** shows that all samples sent for sequencing had high quality RNA.

Upon passing the in-house quality control tests, samples were used to generate a cDNA library. Briefly, mRNA is enriched by poly-A tail selection then fragmented into short fragments. The fragments are reverse transcribed into cDNA using random hexamer primers and the ends of the cDNA are repaired to create blunt ends for adapter ligation. The adapters allow fragments to bind to the sequencing flow cell and allow for amplification. The library is quality checked for concentration and fragment size distribution, so it is appropriate for sequencing. The sequencing is performed using the Illumina NovaSeq 6000 platform with a PE150 strategy. This means the fragment pair ends (PE) are read at a length of 150 bp. Bridge amplification creates clusters for sequencing, and the sequencing by synthesis reads nucleotide addition through fluorescent signals that indicate a base call. The sequences of base calls are computationally recorded for bioinformatic analysis.

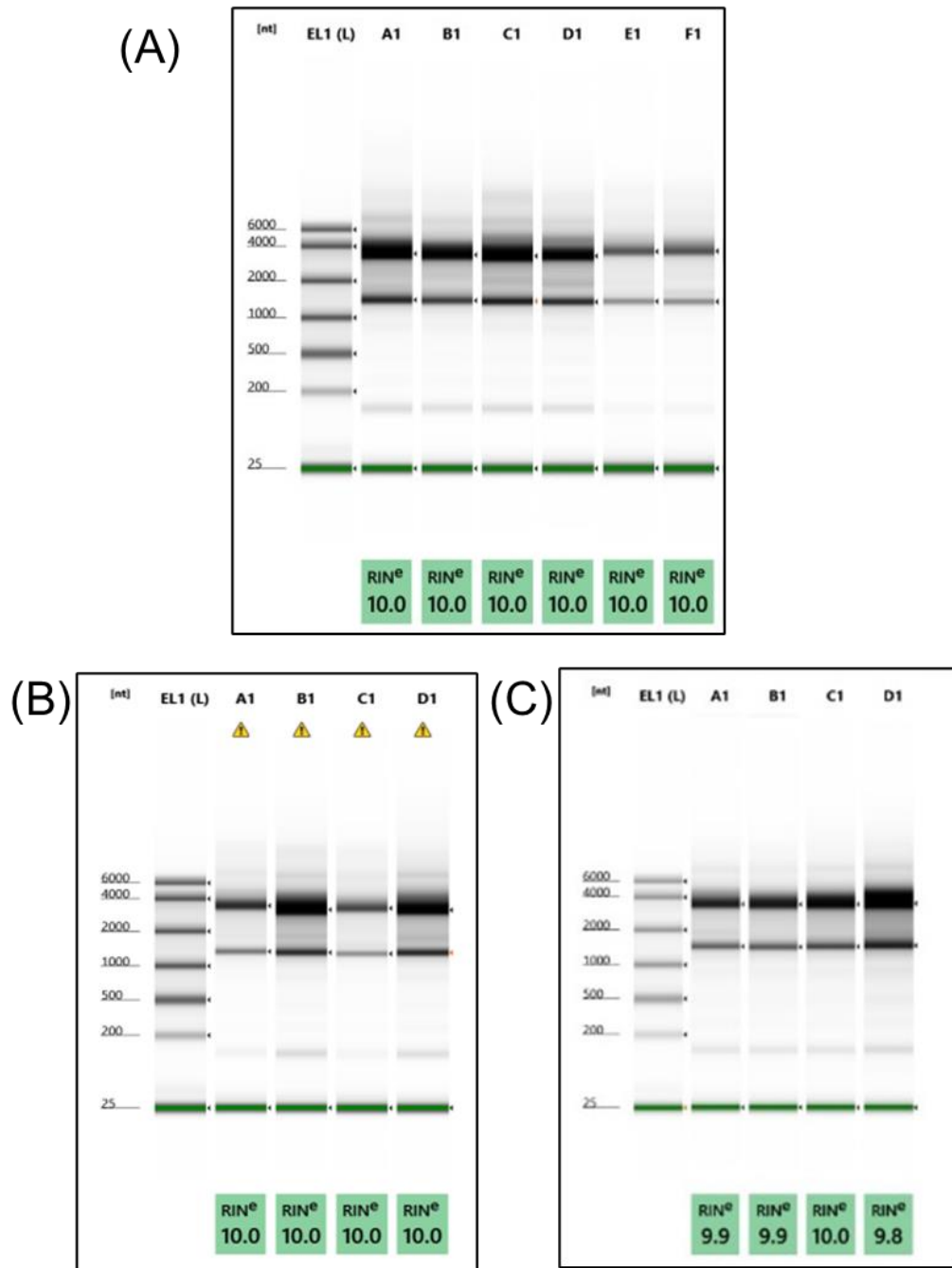


Figure 2.12: The RIN Score for CCLP KD and KO RNAseq data.

Prior to RNAseq, RNA samples were tested for quality via RIN test by the DeepSeq Lab QMC, University of Nottingham. A RIN score above 9 is considered acceptable.

- (A) The RIN scores for siRNA CCLP siCtrl 1 (A1) 2 (C1) 3 (E1) and siPRH 1 (B1) 2 (D1) 3 (F1).
 (B) The RIN scores for CCLP CON 1 (A1) and 2 (B1) and CCLP KO 1 (C1) and 4 (D1).
 (C) The RIN scores of CCLP SVC 1 (A1), SVC 2 (B1), PRH KD 1 (C1) and PRH KD (D1).

2.4.3 Novogene Bioinformatics Pipeline:

Original data from high-throughput sequencing platforms is transformed to sequenced reads by CASAVA base recognition (Base Calling). Raw data are stored in FASTQ (fq) format files, which contain sequences of reads and corresponding base quality. Before bioinformatics analysis, the low-quality bases and adapter sequences are trimmed, and the “clean” data is aligned to a reference genome. The genome used was Hg38 and the alignment tool used was HISAT2. After alignment the number of reads mapping to each gene is quantified using the FeatureCounts tool, generating raw count data. This data is manipulated through the DESeq2 tool to obtain differential gene expression analysis, where the counts are normalised through Fragment per Kilobase Million (FPKM) method. DESeq2 calculates the fold change, expressed as $\log_2FC$, for each gene and applies statistical testing to detect genes with significantly differential expression. DESeq2 provides a raw p-value, which is obtained through a Wald Test, and represents the likelihood of the differential gene expression being observed due to random change. A p-adjusted value is also calculated to account for false positive results, through the Benjamini-Hochberg procedure. The $\log_2$ of the fold change, normalised counts and p-adj values are used in downstream analysis.

2.4.4 Basic Bioinformatics Analysis:

For investigating overlapping sets of genes, Venn diagrams were created using the online tool Venny 2.0 (Oliveros, 2007) (<https://bioinfogp.cnb.csic.es/tools/venny/>). Volcano plots were made in GraphPad Prism application and Heatmaps were made in online tool SRPlot (Tang et al., 2023)

(https://www.bioinformatics.com.cn/plot_basic_cluster_heatmap_plot_024_en).

2.4.5 Gene Set Enrichment Analysis:

Gene Set Enrichment Analysis or GSEA is an application that identifies enriched pathways in the different phenotypes of an expression dataset. For this, the normalised counts for all the genes from the DESeq2 file are uploaded to GSEA along with phenotype labels. Analysis was performed with Hallmarks MSigDB and KEGG gene set databases. Parameters were kept to recommended values, apart from the “Permutation type” was set to gene_set when the number of replicates was two (i.e. for the shRNA PRH KD data and for the PRH KO data). This is because analysis with the permutation type set as phenotype_set would not provide thorough statistically testing. Similarly, the “Metric for ranking genes” was changed to log2_Ratio_of_Classes as recommended by GSEA user guide for GSEA with two biological repeats. The software ranks all genes in the DESeq2 list across the normalised counts where genes at the top of the list are enriched in one phenotype (i.e. PRH KD), and genes at the bottom of the list are enriched in the other phenotype (i.e. control). Once ranked, a desired gene set database will be compared to the list to observe whether the genes within the set are significantly enriched with another phenotype. This software quantifies this through an Enrichment Plot which generates an Enrichment Score (ES). The score is calculated by ‘walking down’ the ranked gene list; the running-sum is increased when a gene is encountered in the list, and the running-sum is decreased when there is no gene encountered. The maximum deviation from zero in the random walk is the ES value; the magnitude of the score is dependent upon how enriched the gene set is to the phenotype (Subramanian et al., 2005). The enriched pathways for each phenotype are

presented as a list from the most statistically enriched gene set running down. Each gene set is accompanied by its ES score, a normalised ES (NES), which accounts for the size of the gene set, a nominal p-value and an FDR q-value. The nominal p-value indicates whether the enrichment of the gene set is statistically significant when considering that single gene set, whereas the False Discovery Rate or FDR q-value corrects for multiple testing, accounting for the presence of false positive results in the analysis. An acceptable cut-off for FDR q-value is >0.25 . To create GSEA figures, the top ten enriched gene sets for each phenotype of the experiment were shown as a bar graph with NES plotted against gene set name. The GSEA programme generated the Enrichment plots which were also used.

2.5 Western Blot:

2.5.1 Whole Cell Extraction:

Cells were seeded in 6-well plates or flasks (T25, T75, T225) and left to adhere before treatment. After the cells had been treated as required, cells were washed twice with ice cold (4°C) X1 PBS, before collecting the cells in ice cold PBS with a cell scraper to create a cell suspension. The suspension was centrifuged at 500g for 5 minutes and the PBS removed. Cells were either left at -20°C or were lysed by resuspending in RIPA lysis buffer (see Buffers), supplemented with X1 Complete™ Protease Inhibitor Cocktail (11697498001, Roche), 1mM Phenylmethylsulfonyl fluoride (PMSF, 36978 Thermo Fischer Scientific) and 10mM Dithiothreitol (DTT, 20290; Thermo Fischer Scientific), and mixed forcefully via vortexing and pipetting. The lysate was subjected to 12 minutes sonication on ice, in a benchtop sonicator (Transsonic T310, Camlab), and centrifuged at 4°C for 10 minutes at 13000rpm to remove cell debris.

Supernatant was transferred to a new tube. A 2 μ L aliquot of the lysate was used for protein quantification. The rest of the sample was boiled in X1 Laemmli Loading Buffer (see Buffers) for 7 minutes at 95°C. Samples were left on ice for 1 minute to cool before storing at -80°C.

2.5.2 Protein Quantification:

Protein concentrations were determined through Bradford assays. BSA (2mg/mL) was serially diluted in distilled water at a ratio of 1:2, nine times. In triplicate, 10 μ L of each solution was plated in a clear 96-well plate. This created a standard curve to measure unknown concentrations of protein. The 2 μ L of protein lysate from the whole cell extraction described above, was diluted in 98 μ L distilled water (1:50), and plated at 10 μ L in triplicate alongside the standards, in the same 96-well plate. Bradford Protein Assay Dye Regent (5000006, BioRad) was diluted 1:5 in distilled water to provide a X1 working stock, and 200 μ L was added to the 10 μ L of either BSA serial dilution, diluted (1:50) protein lysate or distilled water control. Wells were mixed carefully, and optical density (OD) was measured in a plate reader at 620nm. A BSA standard curve was generated through the serial dilution and a linear equation was generated of BSA concentration to optical density in order to calculate relative protein concentration.

2.5.3 Western blotting -Gel assembly and

Electrophoresis:

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate proteins in a sample. For homemade gels, BioRad short glass slides and back glass sides were clamped and locked into a cassette with a sponge beneath them. Between the glass slides, 1mL of isopropanol was pipetted to check for

leaking. The protein gels are made of a 6% acrylamide stacking layer and a 10-12% acrylamide resolving layer of gel, depending on the desired separation. Once no leaking had been confirmed, the isopropanol was discarded and resolving layer was added until the space between the glass slides reached approximately 2cm from the top. A layer of isopropanol was immediately pipetted on the top to remove any bubbles. After 15 minutes, or once the resolving gel had solidified, the isopropanol was removed, and the stacking layer was added until it reached the top of the gel. A 1mm comb was insert in the top of the gel. After 15 minutes, the stacking layer had polymerised. Gels were either used straight away or wrapped in water-soaked blue paper towelling and clingfilm to be stored at 4°C and used within a week. The following components were used per gel:

Resolving Layer (10%/12%)		Stacking Layer (6%)	
Component	Volume	Component	Volume
ddH2O	3.8mL/3.4mL	ddH2O	2.9mL
40% Acrylamide	2mL/2.4mL	40% Acrylamide	0.75mL
1.5M Tris HCl pH 8.8	2mL	0.5M Tris HCl pH 8.8	1.25mL
10% SDS	80µL	10% SDS	50µL
10% APS	80µL	10% APS	50µL
TEMED	8µL	TEMED	5µL
/Final Volume= 8mL		/Final Volume= 5mL	

Table 2.12: The Components for making Polyacrylamide Gels for SDS-PAGE.

For gel electrophoresis, the western blotting tank was assembled by locking protein gels and buffer dams into an electrode chamber. The inside chamber

was filled with X1 running buffer (see Buffers) to check for leaking, and then the outer compartment was also filled with X1 running buffer.

For protein loading, protein lysates were boiled, for 5 minutes at 95°C, and cooled for one minute prior to loading either into homemade acrylamide gels or Mini-PROTEAN® TGX™ Precast Protein Gels (#4561033, BioRad). Equal amounts of protein, approximately 30µg unless stated otherwise, were loaded into each lane along with a protein ladder to assess protein size. The SDS-PAGE gel was run at 80V for 2 hours, or until the blue dye front was absent.

2.5.4 Protein Transfer:

Following SDS-PAGE proteins were transferred onto 0.45µM Nitrocellulose membrane (88018, Thermo Fischer Scientific) via Trans-Blot Turbo Transfer System (BioRad). The membrane and two sponge stacks were soaked in cold X1 transfer buffer (Buffers). The gel and membrane were sandwiched between the stacks in a cassette, air bubbles were removed by rolling over the stacks, and excess buffer poured away. The proteins were transferred on a Mixed Molecular Weights setting of 1.3A, and 25V, for 7 minutes. A High Molecular Weights setting of 1.3A, 25V for 10 minutes was applied for proteins with a molecular weight of >150kDa and a Low Molecular Weights setting of 1.3A, 25V for 5 minutes was applied for proteins with a molecular weight of <30kDa.

2.5.5 Protein Detection:

Once the proteins had been transferred to the membrane, the membrane was immediately incubated in blocking buffer to prevent non-specific binding; this was either 5% Bovine Serum Albumin (BSA) or 2% milk powder in TBS-T (see Buffers) for 1 hour at room temperature on a shaking incubator. The membrane was then incubated with primary antibody overnight at 4°C. Primary antibodies

were diluted in blocking buffer and were either kept refrigerated at 4°C or frozen at -20°C to be re-used. Primary antibodies were re-used 3-4 times (or until the bands were not clear). After primary antibody exposure, membranes were washed three times for 10 minutes with TBS-T, before incubating with secondary antibody. Secondary antibodies were either HRP-conjugated or fluorophore-conjugated. Information about both primary and secondary antibodies, and their dilutions, can be found in the Antibodies Table below. Membranes were washed three times for 10 minutes with TBS-T again before protein bands detected through an Odyssey® Fc Imaging System (Licor). For fluorescent antibodies, the membrane was placed into the system after the washes and excited with appropriate wavelengths for band detection. For HRP-conjugated antibodies, membranes were first incubated with SuperSignal™ West Pico PLUS Enhanced Chemiluminescence (ECL) solution (Thermo Scientific, 15669364). Here, 1mL of solution 1 was mixed with 1mL of solution 2, and incubated with the membrane for 1 minute in the dark. The chemiluminescence was detected by the Odyssey® system. The exposure time was adapted to each protein of interest. Protein expression was measured through band intensity on Image J software. Dividing the densitometry of the protein-of-interest band, by that of the loading control band (B-actin or B-tubulin), allowed the comparison of the relative expression between samples. If needed, membranes were incubated with stripping buffer (0.4M NaOH) under agitation for 15 minutes, followed by washing in TBS-T and blocking, to strip membranes for re-probing.

2.5.6 Antibodies:

Primary Antibody	Raised	Dilution	Code, Company
B-actin	Rabbit	1:1000 in 5% BSA	4970S, Cell Signalling
B-catenin	Rabbit	1:1000 in 5% BSA	8480S, Cell Signalling
B-tubulin	Rabbit	1:1000 in 5% BSA	2128S, Cell Signalling
Cleaved Caspase 3	Rabbit	1:1000 in 5% BSA	MAB835, BioTechne
Cyclin D1	Rabbit	1:1000 in 5% BSA	2978S, Cell Signalling
Cyclin D2	Rabbit	1:1000 in 5% BSA	3741S, Cell Signalling
HHEX 2018B	Rabbit	1:1000 in 5% BSA	FAB83771C, BioTechne
Notch-3	Rabbit	1:1000 in 2% milk	5276S Cell Signalling
P21	Rabbit	1:1000 in 5% BSA	2947S, Cell Signalling
Phospho-RB	Rabbit	1:1000 in 5% BSA	8516S, Cell Signalling
RB	Mouse	1:1000 in 5% BSA	9309S, Cell Signalling
Vimentin	Rabbit	1:1000 in 5% BSA	5741S, Cell Signalling
Ubiquitin	Mouse	1:1000 in 5% BSA	3936S, Cell Signalling
Secondary Antibody		Dilution	Code, Company
Anti-mouse IgG, HRP-conjugated	Horse	1:5000 in 5% BSA	7076S, Cell Signalling
Anti-rabbit IgG, HRP-conjugated	Goat	1:5000 in 5% BSA	7074S, Cell Signalling

Table 2.13: A list of Antibodies.

2.5.7 Buffers:

Buffer	Components
X1 RIPA Buffer	150mM NaCl 1% NP-40 50mM Sodium Deoxycholate 0.1% SDS

Supplements	50mM Tris HCl, pH 8.0 10mM DTT X1 Protease Cocktail Inhibitor 1mM PMSF
X5 Laemmli Protein Loading Dye	50% Glycerol 300mM Tris-HCl pH6.8 10% 2-mercaptoethanol 5mg/mL bromophenol blue 10% SDS
X1 SDS-PAGE Running Buffer	200mM Glycine 25mM TrisBase 0.1% SDS
X1 Western Blot Transfer Buffer	20% X5 BioRad Transfer Buffer 20% Ethanol 60% Distilled water
X1 TBS-T	20mM TrisBase 150mM NaCl 0.1% Tween 20
5% BSA Blocking Buffer	2.5g BSA fraction V 50mL DW

Table 2.14: A list of Western Blot Buffers.

2.6 Chemical Inventory:

Dimethyl Sulfoxide (Fisher Scientific, 10213810) was used to dissolve drugs, was used as dose response curve positive control and used to solubilise MTT crystals in MTT assay. DMSO was kept at room temperature or 22°C and kept air-tight to prevent oxidising. Oxidisation of DMSO affects its ability to dissolve substances.

Niclosamide: Niclosamide (MW: 327.199g/mol) (Merck-Millipore, 481909-1GM) was dissolved in DMSO to make 10mM and 1mM stocks for *in vitro* work.

Stocks were frozen at -20°C for short-term use (3 months) and at -80°C for long-term use. Niclosamide dissolved easily in DMSO and required little mixing via pipetting and vortex. The stock of Niclosamide powder was stored at 4°C.

MG132: Protein Stability Experiments were conducted using proteasome inhibitor, MG132. MG132 readymade solution (Sigma Aldrich, M7449). MG132 was used between 0.75µM-40µM in a variety of cell lines at 24hrs (MedChemExpress) and was optimised for use in CCA cell lines. CCA cell lines were incubated with 5µM MG132 for 24 hours.

Palbociclib: Palbociclib (447.53g/mol) (SelleckChem, S4482) was used in this work. Palbociclib is a highly insoluble, yellow powder. For solubilisation, DMSO was added to Palbociclib powder in an Eppendorf tube and vortexed violently for two minutes. The Palbociclib in the Eppendorf tube was then sonicated (Transsonic T310, Camlab) in a 50°C water bath three times. The powder was checked for complete solubilisation by centrifuging the samples to observe any precipitate; if precipitate was present, the warming and sonication continued until no precipitate remained. Stock was made at 10mM and 1mM for *in vitro* work and stored at -20°C for short-term use (3 months) and -80°C for long-term use. Palbociclib powder was kept at -20°C.

Ribociclib: Ribociclib aliquots were a gift from Prof S. Madhusudan. Ribociclib (SelleckChem, S7440) was dissolved in DMSO to make 10mM stocks that were kept at -20°C.

FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (Sigma Aldrich, C2920) was dissolved in DMSO to make 1mM aliquots that were kept at -20°C.

Staurosporine: Staurosporine (Merck, S6942) is a well-characterised apoptosis inducer and was used to study the apoptotic response in cells. Stock

concentration was 1mM and was diluted in media to the desired working concentrations. The vial was stored at -20°C.

2.7 Statistical Analysis:

Statistical analyses were performed using Microsoft Excel or GraphPad Prism. An unpaired t-test was used to assess statistically significant differences, unless stated otherwise, and experiments repeated to N=3 were declared significant if $p\text{-value} < 0.05$. The degree of significance is represented by asterisk: $*$ = $p < 0.05$, $**$ = $p < 0.01$, $***$ = $p < 0.001$, $****$ = $p < 0.0001$. The p-value will be described as one of these four categories or will be given as a number (e.g. $p=0.10$) to provide more information to the reader when needed. Error bars represent standard error of the mean (SEM), unless stated otherwise.

CHAPTER 3:

Investigating the effects of Niclosamide in CCA Cell Lines and Primary Biliary Epithelial Cells

3.1 Introduction

Previously, a drug screen using a drug-repurposing library of FDA-approved drugs (Khanim et al., 2011) was performed using CCA cell lines, CCLP and CCSW, as well as an immortalised cholangiocyte cell line, AKN1. This led to the identification of six drugs that were able to significantly and preferentially decrease CCLP and CCSW cell viability compared to AKN1 cell viability (Lee, 2021).

The primary aim of this chapter is to confirm that drug candidate, Niclosamide, can decrease cell viability in additional CCA cell lines, and to determine any effects in normal primary Biliary Epithelial Cells (BECs). As described previously (section 1.7.1), Niclosamide is a mitochondrial uncoupler (Khanim et al., 2011) (Jian et al., 2022) (Kumar et al., 2018) and has been shown to induce protein ubiquitination (Cheng et al., 2017) (Guo et al., 2022). A secondary aim is to investigate Niclosamide sensitivity in the CCA cell lines; this includes examining the effect of Niclosamide via known mechanisms, such as mitochondrial uncoupling and ubiquitination, and to examine effects on cell cycle progression and apoptosis in the CCA cell lines.

An additional aim is to determine whether knockdown of the PRH oncoprotein will alter the sensitivity of the CCLP cell line to Niclosamide treatment.

3.2 PRH Protein Expression across CCA

Cell Lines and BECs

PRH was previously shown to act as a CCA oncoprotein in a Notch-3 positive feedback loop that drives cell cycle progression (Kitchen et al., 2020). PRH and Notch-3 expression were shown to be high in CCA cell lines compared to BECs and immortalised cholangiocytes (Kitchen et al., 2020). To determine whether PRH and Notch-3 protein are both expressed at similar levels in three CCA cell types and to determine the levels of protein expression of these proteins in the BECs, prior to use of these cells in Niclosamide sensitivity experiments, western blotting experiments were performed. All CCA cell lines were STR profiled to confirm their identity by Eurofin Genomics (section 2.1.1).

Whole cell extracts were generated from CCLP, RBE and KKU-M055 CCA cells as well as from BECs. All three CCA cell lines were assessed for PRH and Notch-3 protein expression alongside B-actin loading control. **Figure 3.1** shows the expression of PRH, Notch-3 cleaved intracellular domain (ICD) and B-actin across the CCA cell lines and BECs. As the B-actin levels in the CCA cell lines are similar after loading 30µg of sample, this facilitates comparison of expression levels between these cell lines. Amongst the CCA cell lines, RBE has the highest observed PRH protein expression but very low Notch3 ICD levels, KKU-M055 cells have relatively low PRH expression and intermediate expression of Notch-3 ICD, CCLP cells have robust expression of both.

BECs show very low expression of PRH protein and undetectable levels of Notch-3 ICD, however there are also low B-actin protein levels compared to CCA cell lines indicating lower protein loading. Thus, the PRH levels in BECs and CCA are not comparable. However, the BEC expression data is similar to

published data showing PRH expression is low in normal cholangiocytes (Kitchen et al., 2020), therefore, the experiment was not repeated further. Overall, the CCA cell lines and BECs present different combinations of PRH and Notch-3 protein expression.

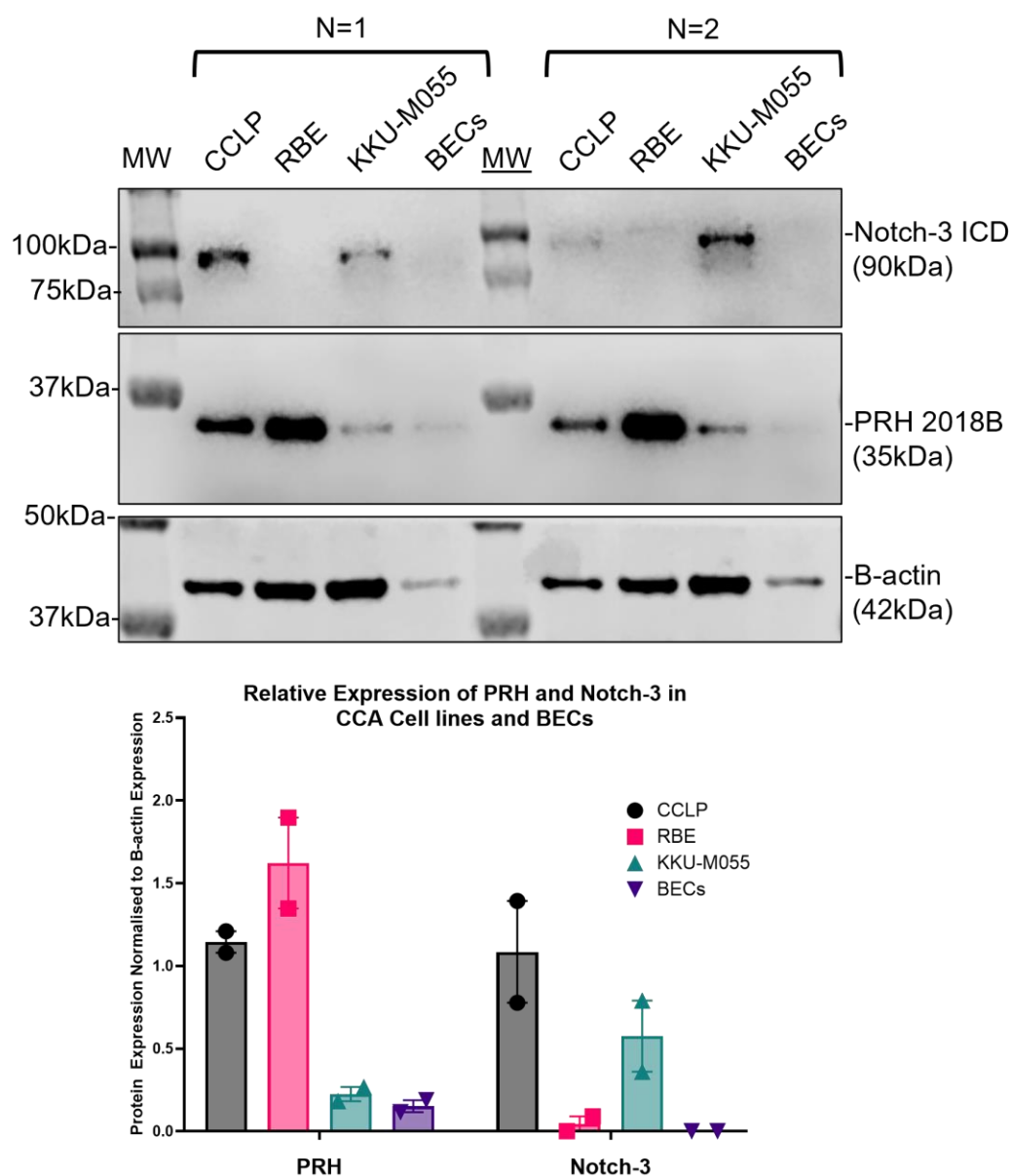


Figure 3.1: Protein Expression of PRH and Notch-3 in the STR Profiled CCA Cell Lines and BECs.

Two sets of whole cell extracts from CCA wildtype cell lines CCLP, RBE and KKU-M055, along with whole cell extracts from BECs were used in western blotting experiments to assess the expression of PRH and Notch-3. B-actin was used as a loading control (N=2).

3.3 The Effect of Niclosamide on CCA cell line and BEC viability:

To confirm that Niclosamide can reduce cell viability of CCA cell lines, and to compare the effects of Niclosamide on CCA cell lines and BECs, MTT dose-response assays were used to assess cell viability after 24 hours incubation with Niclosamide or vehicle control. The cell lines used were CCLP, RBE and KKKU-M055. The CCLP cells have mutated *TP53* mutated, the RBE harbour oncogenic KRAS (G12V) and gain-of-function IDH1 (R132S) and the KKKU-M055 cells have likely oncogenic gain-of-function MAP2K1 (K57T) (for cell origin details see section 2.1.1). Also, KKKU-M055 is a CCA cell line originated from a fluke-associated CCA patient (see section 2.1.1). These cell lines were compared for Niclosamide sensitivity because they have very different genetic backgrounds and different pathogenesis.

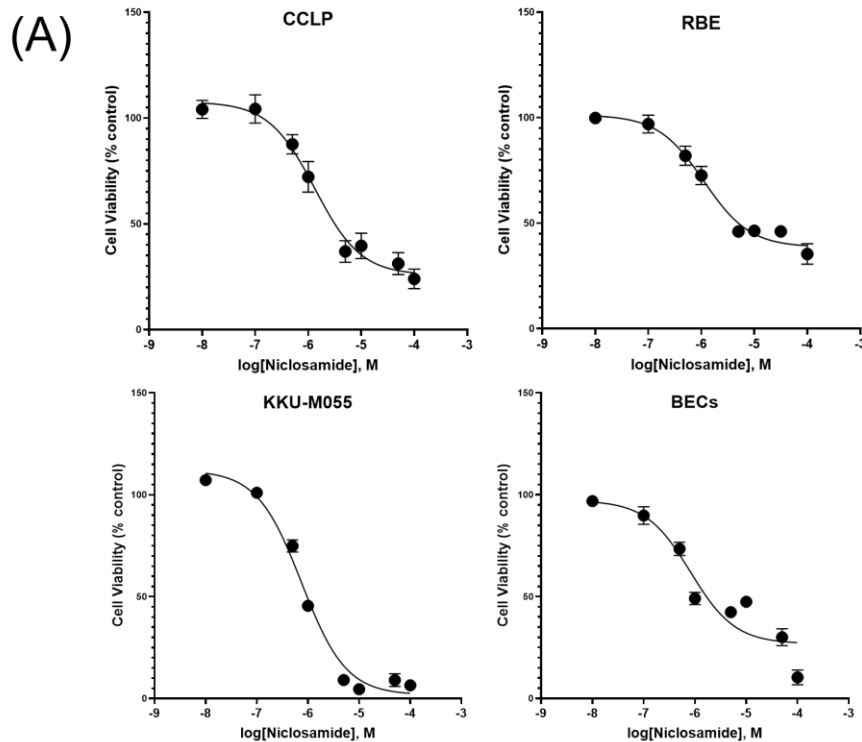
The MTT assay measures the metabolic activity of cells (Mosmann, 1983). MTT is a type of tetrazolium dye that upon reduction by cellular activity, forms an insoluble formazan salt. MTT reduction can be mediated via multiple metabolic enzymes such as oxidoreductase and dehydrogenase enzymes, as well as respiratory intermediates like NAD(P)H, which act as proton donors (Berridge et al., 2005). The water-insoluble salt can be solubilised in the organic compound DMSO to produce a homogeneous purple solution, which can subsequently be quantified by measuring the optical density (OD) of the solution. The amount of salt formed correlates to the amount of cellular metabolism, and thus, can be used to infer cell viability. To confirm that there is a linear relationship between cell number and MTT signal, increasing cell

number was plated and MTT readings after 24hrs were obtained. This is shown in the material and methods section 2.2.3.

For the experiments, cells were seeded at 1×10^4 cells per well in a 96-well plate in triplicate and left to adhere overnight. After adhesion, the media was removed and replaced with 100uL of either a vehicle control (media containing 1% DMSO) or a serial dilution of Niclosamide in media containing 1% DMSO. The peak serum concentration of Niclosamide in man has been reported to reach 182ng/mL or approximately 0.56 μ M (Schweizer et al., 2018). The stock concentration of Niclosamide was dissolved at 10mM in DMSO. The range of Niclosamide used was from 10nM-100 μ M to keep the DMSO content below 1%, preventing any effects of DMSO on cell viability (Forman et al., 1999). Cells were also treated with 100% DMSO to cause 0% cell viability and to act as a positive control for cell death. The drugs were incubated with the cells for 24hrs. After treatment, the drug-containing media was removed and replaced with media containing 5mg/mL MTT reagent. MTT was metabolised by cellular enzymes to an insoluble purple crystal over a 3-hour incubation. The crystals were then solubilised in 150uL DMSO to form a homogeneous purple solution and the OD was measured at 570nm on a plate reader. The positive control value (the background) was subtracted from the raw values and experimental data was normalised to vehicle control which represents 100% cell viability. Relative cell viability as a percentage was plotted against the log drug dose in GraphPad Prism and analysed to calculate *relative* EC50 values. The *relative* EC50 value is the effective concentration at which the drug has a 50% effect and is a measure of potency (Sebaugh, 2010). The *absolute* EC50 can also be calculated when the maximum concentrations of drug do not cause 0% viability (Sebaugh, 2010). The *absolute* EC50 is the concentration that provokes a response halfway between the vehicle control (100% viability) and the DMSO-

killed cells (0% viability), despite the maximum effect (Emax). The *absolute* EC50 reveals the dynamic range of the assay and can facilitate comparison of the cell lines.

Figure 3.2A shows the dose response curves at 24hrs for Niclosamide in the CCLP, RBE and KKU-M055 cell lines and BECs. Cell viability decreases with increasing dosage of Niclosamide in all cell lines, and in BECs. The *relative* and *absolute* EC50 values generated using GraphPad Prism are shown in the table with confidence intervals (**Figure 3.2B**). As the highest concentrations of Niclosamide do not reach 0% cell viability in CCLP, RBE or BEC cells, the EC50 values are relative to the maximum response to Niclosamide (Emax). The maximum response, or the lowest cell viability seen, in each graph is 26%, 39% and 27% in CCLP, RBE and BECs, respectively. Overall, this shows that KKU-M055 cells are the most sensitive to Niclosamide after 24 hours incubation as at the higher concentrations of Niclosamide, 0% cell viability is achieved. RBE cells are the most tolerant with the highest Emax. Whilst the CCLP cells and BECs have relatively moderate sensitivity to Niclosamide, as the cells are more tolerant to Niclosamide than KKU-M055 but are more sensitive to Niclosamide than RBE. The response to Niclosamide in the CCLP cells and BECs are similar.



(B)

Cell Line	Relative EC50 (μM)		Absolute EC50 (μM)
	Mean	CI	Mean
CCLP	1.33 [†]	0.79-2.30 [†]	3.48
RBE	1.11 [†]	0.71-1.79 [†]	5.73
KKKU-M055	0.75	0.63-0.90	1.15
BECs	0.82 [†]	0.49-1.49 [†]	3.71

Figure 3.2: Niclosamide Dose Response Experiments in CCA cell lines and BECs at 24hrs.

The CCA cell lines (CCLP, RBE, KKKU-M055) and BECs were plated at 1×10^4 cells per well in triplicate in a 96 well plate and treated with a range of Niclosamide concentrations (10nM-100μM) or vehicle control for 24hrs. After 24hrs treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Niclosamide concentration to create dose response curves in GraphPad Prism. EC50 values, plus 95% confidence intervals (CI), were calculated by the software.

- (A) Niclosamide dose response curves in CCLP, RBE, KKKU-M055 and BEC cells after 24hrs. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
- (B) Niclosamide sensitivity across the CCA cell lines and BECs. Relative EC50 values plus confidence intervals CI, and Absolute EC50 values (μM) were calculated using the GraphPad Prism software. † denotes dose response curves that did not reach Emax 0.

Cytotoxicity is the ability of a drug to induce cell death. As 24hrs is a relatively short timepoint and does not allow much time for the cells to proliferate, the effects of Niclosamide on cell viability at this timepoint are likely to be of a cytotoxic nature. Of note, the EC50 values for Niclosamide in each cell lines are above the maximum serum concentration of Niclosamide. Therefore, the effects of Niclosamide were investigated at 72 hours, to show if longer timepoints could highlight any cytostatic, or anti-proliferative, effects of Niclosamide.

At the 72hrs timepoint, the high concentrations of Niclosamide results in 0% cell viability. Thus, the dose response curves could be used to obtain accurate *relative* EC50 values for Niclosamide; therefore, no *absolute* EC50 values are given. Increasing the incubation to 72hrs results in a large reduction in *relative* EC50s across the cell lines from those obtained at 24hrs: CCLP 0.45 μ M, RBE 1.31 μ M and K KU-M055 0.39 μ M (**Figure 3.3**). Importantly, the EC50 concentration is less than the maximal serum concentration for CCLP and K KU-M055. The capacity of Niclosamide to further decrease cell viability at longer timepoint demonstrates that the drug has both cytotoxic and cytostatic effects on the cells.

Additionally, at this timepoint, all CCA cell lines have a significantly lower EC50 than that observed for the BECs, which have a EC50 of 2.55 μ M ($p < 0.05$) (**Figure 3.3C**). Therefore, Niclosamide is more effective in reducing the viability of CCA cells compared to BECs at this longer timepoint.

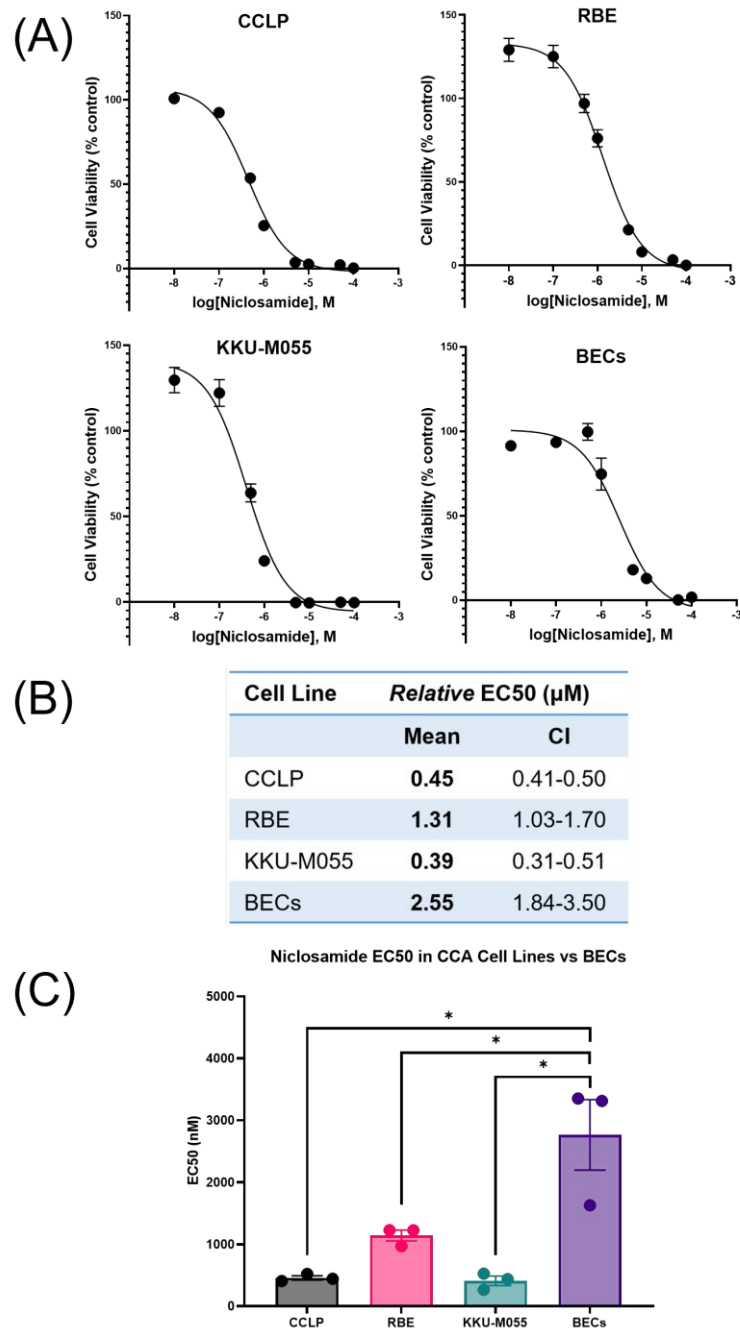


Figure 3.3: Niclosamide Dose Response Experiments in CCA cell lines and BECs at 72hrs.

The CCA cell lines (CCLP, RBE, KKU-M055) and BECs were plated at 1×10^4 cells per well in triplicate in a 96 well plate and treated with a range of Niclosamide concentrations (10nM-100μM) or vehicle control for 72hrs. After 72hrs treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Niclosamide concentration to create dose response curves in GraphPad Prism. EC50 values, plus 95% confidence intervals (CI), were calculated by the software.

- (A) Niclosamide dose response curves in CCLP, RBE, KKU-M055 and BEC cells after 72hrs. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
- (B) Niclosamide sensitivity across the CCA cell lines and BECs. Relative EC50 values plus confidence intervals CI (μM) were calculated using the GraphPad Prism software.
- (C) Comparison of Niclosamide sensitivity in CCA cell lines and BECs. An unpaired t-test was used to measure significant differences between CCA cell lines and BECs. Error bars represent SEM (*= $p < 0.05$).

3.3 The Effects of Niclosamide on CCA

Cell Mitochondrial Uncoupling

As discussed previously, Niclosamide is well documented as a mitochondrial uncoupler (Kumar et al., 2018). To observe any effect of Niclosamide on mitochondrial depolarisation in the CCA cell lines, MitoTracker staining was used. MitoTracker is a cell permeable probe that accumulates in the mitochondria when it reacts with specific thiol reactive chloromethyl groups on the mitochondrial membrane (Cottet-Rouselle et al., 2011). The MitoTracker Red probe (Invitrogen) is a membrane potential-sensitive dye; thus, the intensity of the mitochondrial staining is dependent on its polarisation status. Healthy and active mitochondria with a polarised membrane show strong red staining, whereas dysfunctional mitochondria will show weak signal. Therefore, this dye can provide structural and functional outputs.

For the experimental setup, cells lines and BECs were plated at 5×10^4 cells per well in a 24-well plate and left to adhere overnight. The next day, cells were incubated with media containing 200nM MitoTracker Red Dye, and simultaneously treated with vehicle control or different concentrations of Niclosamide (1 μ M, 2 μ M, 4 μ M or 8 μ M). The cells were incubated with the drugs and the dye for an hour as this allowed for sufficient mitochondrial staining and because Niclosamide is able to induce mitochondrial uncoupling within an hour in Seahorse metabolic activity experiments (Kumar et al., 2018). After staining, the cells were washed twice with warm PBS to remove excess stain and imaged. To allow for quantitative comparison all images were obtained with the same exposure times and red channel settings. Images were taken over three biological experimental replicates.

To quantify the change in MitoTracker staining, and therefore the change in mitochondrial membrane potential, the images of the stained cells were analysed using Image J as outlined in section 2.2.4. The intensity values were made relative to the values of the vehicle control in each repeat. **Figure 3.4** shows a set of representative images for each CCA cell line and BECs treated with MitoTracker and increasing concentrations of Niclosamide. **Figure 3.5** shows the quantification of relative MitoTracker fluorescence intensity across the conditions in the CCA cell lines and in BECs.

MitoTracker staining significantly decreased in intensity with the addition of Niclosamide in CCLP cells at all concentrations ($p < 0.001$). RBE cells showed a similar significant decrease to MitoTracker staining upon Niclosamide treatment ($p < 0.05$). KGU-M055 cells showed reduced MitoTracker staining with Niclosamide incubation which were significant at $1\mu\text{M}$, $2\mu\text{M}$ and $4\mu\text{M}$ ($p < 0.05$), however the relative pixel intensity did not decrease as much as in Niclosamide treated CCLP and RBE cells. BECs also showed significantly less MitoTracker staining when treated with Niclosamide at all concentrations ($p < 0.01$). Overall, acute treatment of Niclosamide reduced MitoTracker staining significantly in all cell types, showing it potentially induces mitochondrial uncoupling in these cells. The amount of mitochondrial uncoupling measured via MitoTracker staining was relatively similar between cell types, with the decrease to staining in KGU-M055 ranging slightly less than in the others.

Although it might be assumed that Niclosamide-sensitive cell types would show an increase in mitochondrial depolarisation, there appears to be no correlation between Niclosamide sensitivity and mitochondrial membrane depolarisation over the concentration range employed.

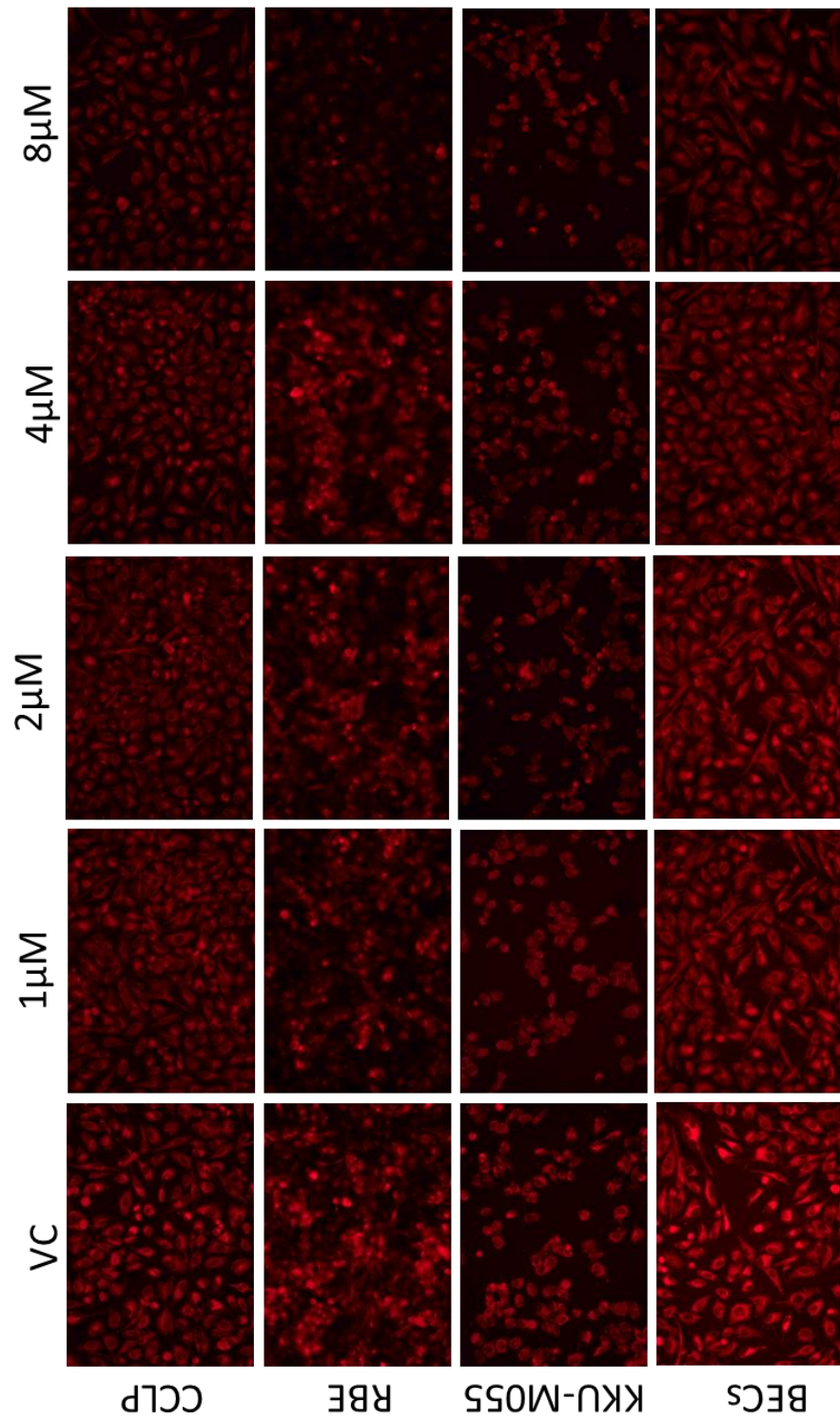


Figure 3.4: Representative Images of Mito Tracker Stained CCA Cell Lines with Increasing Concentration of Niclosamide. Cells were plated at 5×10^4 cells per well in a 24-well plate, then treated with MitoTracker and either vehicle control or Niclosamide at 1μM, 2μM, 4μM, or 8μM. Cells were washed in PBS before imaging fluorescence on a Nikon Live Brightfield Microscopy at X20 magnification. The fluorescent red signal reflects the mitochondrial membrane potential.

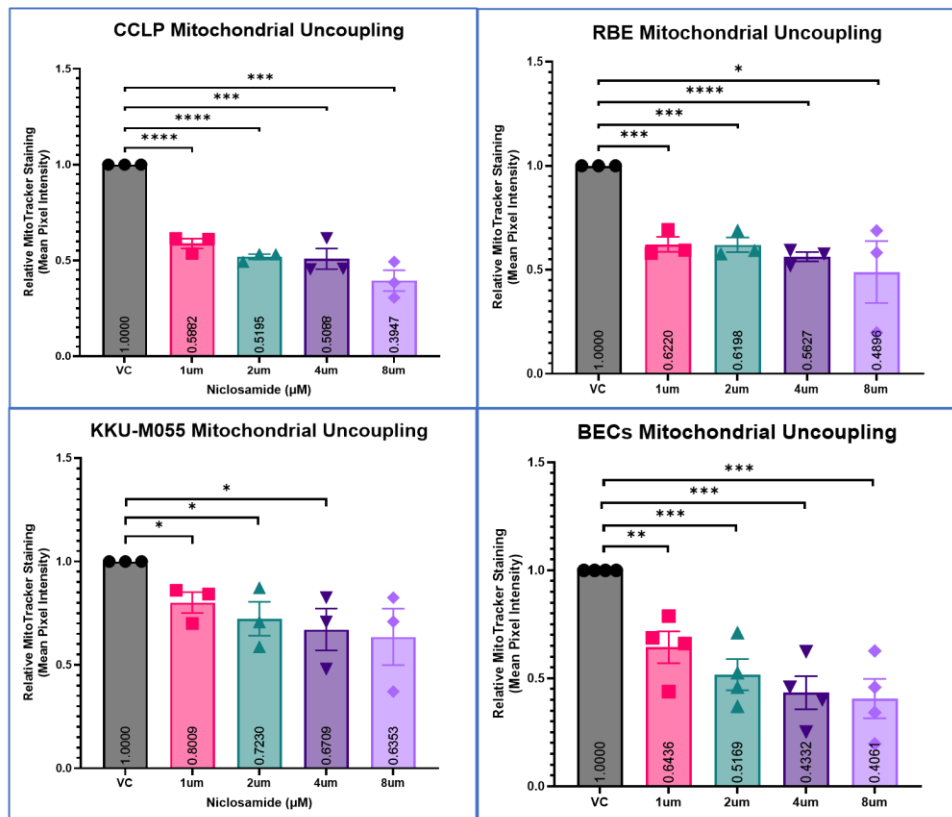


Figure 3.5: Analysis of MitoTracker Stained CCA Cell Lines and BECs with Increasing Concentration of Niclosamide.

CCA cell lines or BECs were treated with increasing concentrations of Niclosamide for 24 hours and mitochondrial uncoupling was measured through MitoTracker staining. MitoTracker fluorescence was quantified through Image J analysis. Mean pixel intensity was measured for the MitoTracker staining in each concentration and normalised to vehicle control across three experimental replicates. Error bars represent SEM (N=3) and statistical significance was measured through a two-tailed unpaired t-test (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001).

3.4 Niclosamide induces Ubiquitylation in

CCA Cell lines:

Niclosamide has been shown to induce total protein ubiquitylation (Cheng et al., 2017), thus, global ubiquitylation levels were investigated in Niclosamide treated CCA cell lines.

Lysates from vehicle control treated and 8 μ M Niclosamide treated CCA cells were used to assess ubiquitylation levels in western blot experiments. A high concentration of Niclosamide was used to ensure a large decrease in cell viability in all the cell lines after 24 hours of drug incubation. The protein lysates were separated via SDS-PAGE and transferred to nitrocellulose membranes before blotting for total ubiquitylated proteins and the housekeeper protein B-actin as a control. To quantify the global amount of ubiquitylation, the whole lane was measured by densitometry using Image J (from 250kDa-50kDa); the densitometry reading for the ubiquitin antibody was made relative to the densitometry reading for B-actin antibody and then the densitometry readings were normalised to the relative densitometry readings obtained for cells treated with vehicle control.

Figure 3.6 shows the effect of Niclosamide on protein ubiquitylation. There was a significant increase to ubiquitylated protein in Niclosamide treated CCLP cells ($p<0.05$) and KKU-M055 cells ($p<0.01$), however although there was also an increased level of ubiquitylated protein in RBE cells, the increase was not statistically significant over three repeats. Interestingly, the amount of ubiquitylation appears to correlate with Niclosamide sensitivity (**Figure 3.6C**).

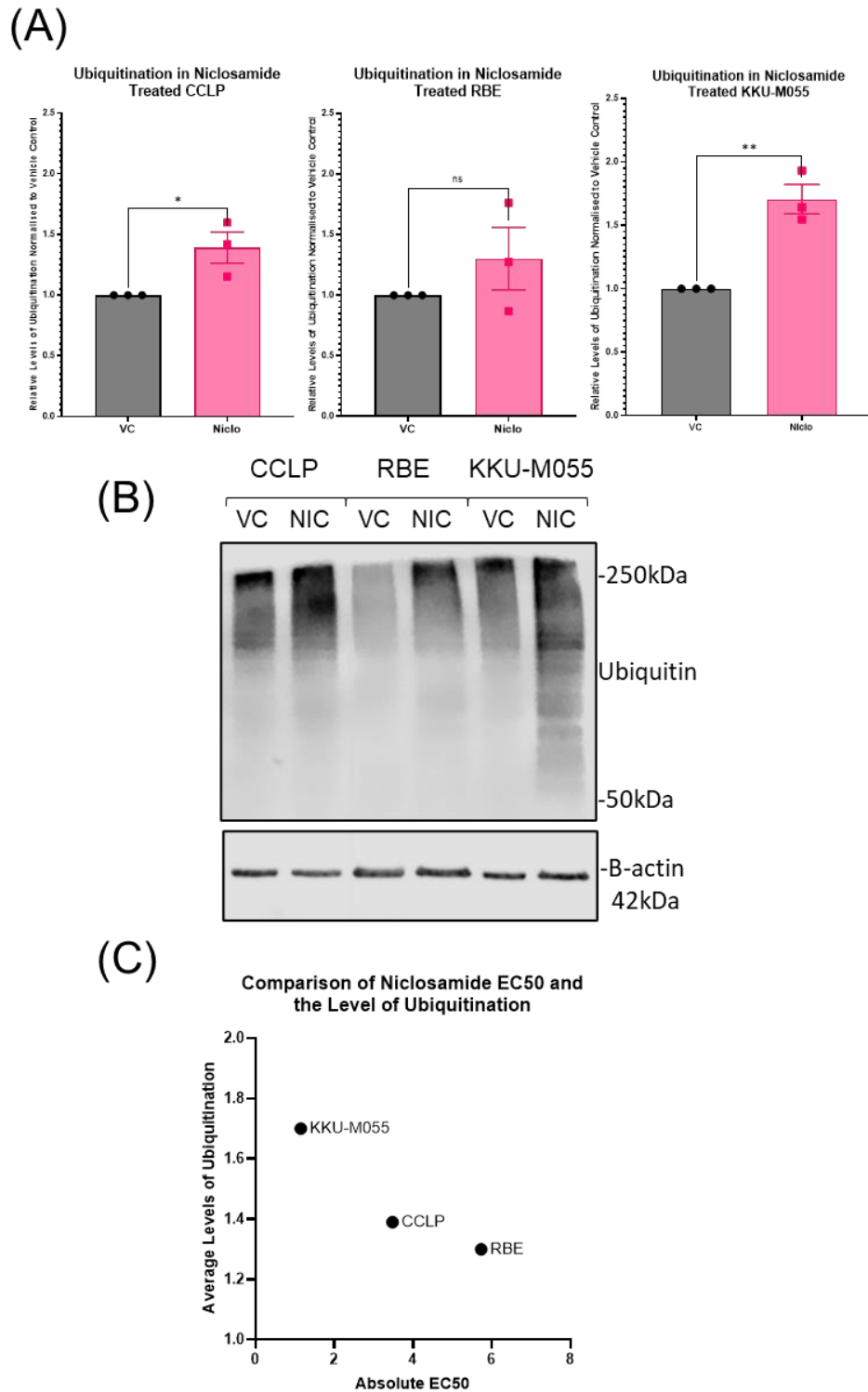


Figure 3.6: Ubiquitination Levels in Niclosamide Treated CCA Cell Lines.

Cells were treated with vehicle control (VC) or 8 μ M Niclosamide (NIC) for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30 μ g of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each cell lines, the level of Ubiquitination has been quantified by normalising to B-actin expression and relativising to vehicle control over three experimental replicates. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, **= $p < 0.01$).

(A) Relative Ubiquitination in Niclosamide treated CCA cell lines.

(B) A representative blot of Ubiquitination in Niclosamide treated CCA cell lines.

(C) A Comparison of the *absolute* EC50 values and the relative Ubiquitination Levels in Niclosamide Treated CCA Cell Lines.

3.5 The Effects of Niclosamide on Cell cycle activity and Apoptosis assessed in Phenotypic Experiments and Protein Expression.

In the literature, Niclosamide has been seen to cause cell cycle arrest and cell death in multiple cancer cell lines (reviewed: Li et al., 2014). To investigate how Niclosamide can reduce cell viability across the CCA cell lines, Niclosamide treated CCA cells were assessed for changes to cell cycle progression and apoptosis through flow cytometry methods and western blotting.

For the flow cytometry experiments, cells were plated at 2×10^6 cells in a T25 flask and left to adhere overnight. The next day, the media was removed and replaced with vehicle control or Niclosamide containing media. Again, a high concentration of Niclosamide ($3.2 \mu\text{M}$) in excess of the relative EC_{50} values for all the cell lines was chosen to ensure that more than 50% of the cells show reduced viability. After the 24hrs incubation, all cells were harvested and pelleted into samples for cell cycle and apoptosis analysis.

For the apoptosis assays, an Annexin V and Propidium Iodide (PI) staining kit was used to determine whether cells were alive or entering early apoptosis, necrosis, or late apoptosis. Live cells will not stain for either dye; early apoptotic cells will stain with Annexin V, as this protein binds to cells with exposed phospholipid phosphatidylserine (PS) which is associated with the plasma asymmetry in early apoptosis. PI cannot penetrate cells unless the membrane is compromised as it is for damaged or dead cells. Therefore, necrotic cells

stain positive for PI only, whilst cells in late apoptosis stain positive for both Annexin V and PI (section 2.2.7). Samples were run on a flow cytometer and the raw data analysis with Kaluza. All cells (vehicle control and Niclosamide) were subjected to the same gating so that only single viable cells were analysed, and further analysed based on PI and Annexin V stain intensity to separate cells into different groups: live (A-PI-), necrosis (A-PI +), early apoptosis (A+PI-) and late apoptosis (A+PI+).

Representative images of the raw data for the vehicle control and Niclosamide treated CCLP cells is shown in **Figure 3.7A** along with the analysis over three biological repeats shown in **Figure 3.7B and C**. Niclosamide statistically significantly increased the number of cells in late apoptosis to an average of 9.0% from 3.4% in the vehicle control ($p<0.05$) and decreases the number of live cells to 85.1% from 92.6% in the vehicle control ($p<0.05$), in CCLP cells. This confirms that Niclosamide has a cytotoxic effect in CCLP cells at 24 hours.

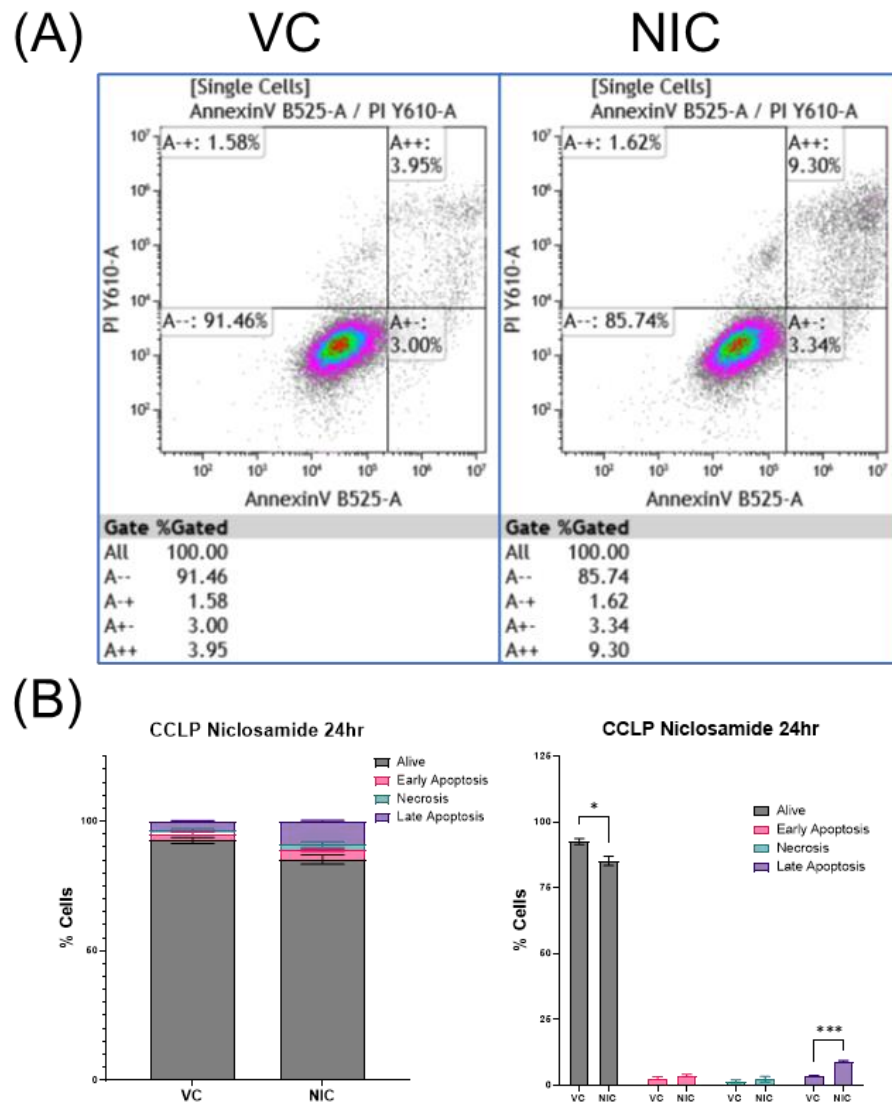


Figure 3.7: Apoptosis Analysis of Niclosamide Treated CCLP Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each stage of cell death: live, early apoptosis, necrosis and late apoptosis. Error bars represent SEM and two-tailed t-test was performed to record significant differences in changes between vehicle control and Niclosamide (N=3) (*= $p < 0.05$, ***= $p < 0.001$).

A) Representative images of apoptosis analysis in Kaluza for Niclosamide treated CCLP.

B) Apoptosis analysis of Niclosamide treated CCLP cells over three repeats.

Cells were also pelleted for cell cycle analysis though Propidium Iodide (PI) staining. PI is an intercalating DNA dye which allows quantification of cellular DNA content. When cells are in G1, they have lower DNA content, and when in G2, they have higher DNA content; G1 and G2 are identified through two peaks in flow cytometry where G2 has twice as much staining as G1. As PI can only enter cells when the cell membrane is permeable, ethanol is used as a dehydrating fixative to allow access to the dye. Also, as PI can stain RNA there is an RNA degradation step with RNase solution prior to staining. The vehicle control and Niclosamide treated CCLP samples were ran on a CytoFlex Station flow cytometry machine. The raw data was gated for viable and single cells on Kaluza software, and cell cycle analysis was performed. The algorithm defined cells in each stage of cell cycle: G1, S and G2 phase (for full PI stain protocol see section 2.2.6). Representative images are shown with cell cycle analysis over three experimental repeats.

Figure 3.8 shows the effect of Niclosamide on CCLP in flow cytometry experiments for assessment of changes in cell cycle progression. There is no change to the number of cells in each phase of the cell cycle with Niclosamide treatment showing that at this timepoint, Niclosamide does not cause cell cycle arrest in these cells.

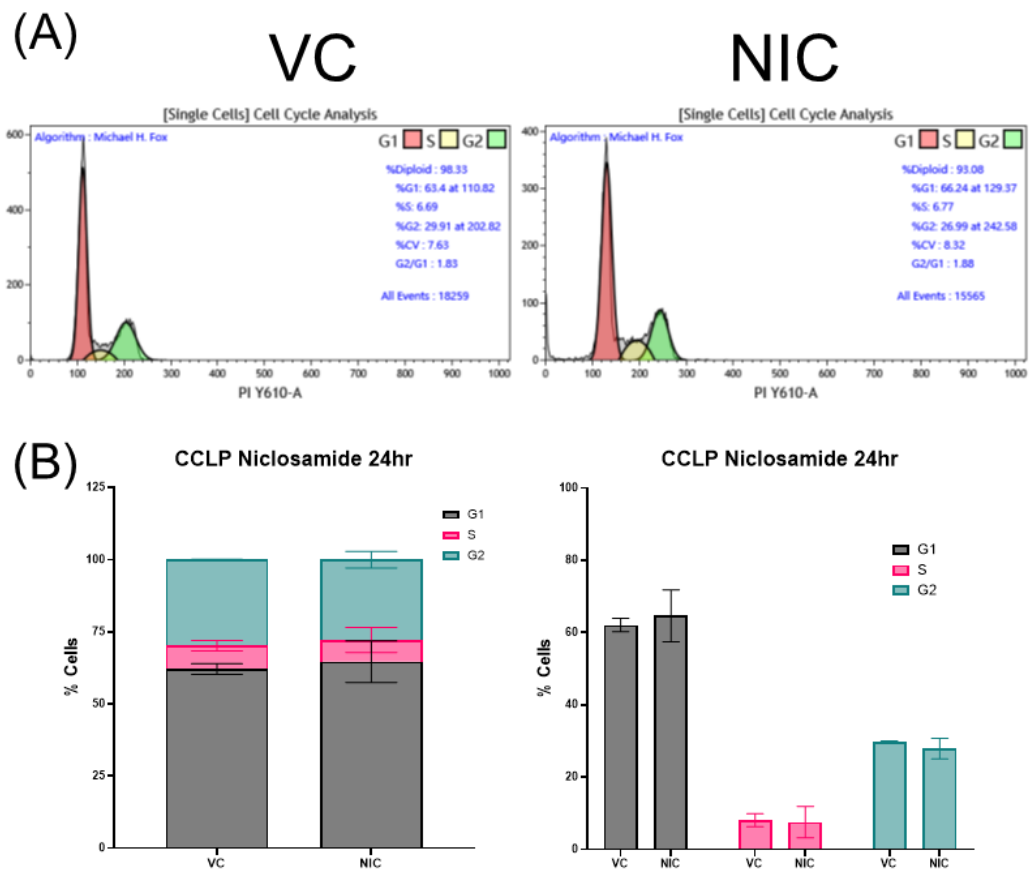


Figure 3.8: Cell Cycle Analysis in Niclosamide Treated CCLP Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green). Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3).

- A) Representative images of cel cycle analysis in Kaluza for Niclosamide treated CCLP.
 B) Cell Analysis analysis of Niclosamide treated CCLP cells over three repeats.

To support the phenotypic experiments, the effect of Niclosamide on various proteins associated with cell cycle and apoptosis were investigated using western blotting (**Figure 3.9**). Cells were plated at 2×10^6 cells in T25 flasks and treated with a vehicle control or a range of Niclosamide concentrations: 1 μ M, 2 μ M, 4 μ M, and 8 μ M. The drug was incubated with the cells for 24hrs before

both the detached and attached cells were collected via scraping, washed in cold PBS and made into lysates using RIPA buffer. Whole cell extracts were run on SDS PAGE gels, transferred to nitrocellulose membrane and probed with different antibodies in western blot experiments.

Caspase enzymes are key mediators of programmed cell death, and effector caspase 3 is activated upon cleavage, therefore cleaved caspase 3 is a marker of apoptosis initiation (Porter & Janicke, 1999). The effect of Niclosamide on the levels of these proteins in CCLP are shown in **Figure 3.9A**. The level of cleaved caspase 3 is significantly increased upon Niclosamide treatment at 2 μ M ($p<0.05$), 4 μ M ($p<0.05$) and 8 μ M ($p<0.05$) (Figure 3.17A).

Cyclin D1 and Cyclin D2 are proteins that modulate the transition of G1 to S phase and are regarded as cell cycle initiators (Hydbring et al., 2016). Protein p21, is known as a negative regulator of cell cycle through its ability to inhibit CDK-cyclin complexes but is also a potent modulator of apoptosis (Abbas et al., 2009). P21 can function as an oncoprotein instead of a cell cycle inhibitor in tumour cells that have mutated p53 activity (Abbas et al., 2009).

Cyclin D1 protein expression was reduced by Niclosamide in CCLP cells in a statistically significant manner; treatment of the cells with the lowest concentration of 1 μ M reduced protein expression to 10% of the vehicle control ($p<0.0001$), and Cyclin D1 expression in cells treated with above 1 μ M Niclosamide was undetectable (**Figure 3.9B**). Cyclin D2 also shows a statistically significant dose-dependent decrease in expression after Niclosamide treatment at every concentration ($p<0.01$) (**Figure 3.9C**). Interestingly, the decrease in Cyclin D2 expression is not as strong as Cyclin D1. The levels of p21 decrease with Niclosamide treatment in CCLP cells (**Figure 3.9D**), with a statistically significant reduction in protein expression at

2 μ M ($p<0.001$), 4 μ M ($p<0.0001$), and 8 μ M ($p<0.0001$). The change in protein expression is similar to the cyclin D proteins.

In conclusion, the increase in PI and Annexin V staining, as well as the increase in cleaved caspase 3 levels, shows a pro-apoptotic effect of Niclosamide in CCLP cells at 24 hours. Although there was no change to the cell cycle in flow cytometry experiments, Niclosamide treated CCLP cells do show statistically significant decreases in cell cycle associated proteins: Cyclin D1 and D2 and p21. An explanation for not observing changes in the cell cycle could be that the time it takes for Niclosamide to influence cell cycle phenotype is longer than the 24hr timepoint used. Indeed at 72 hours the relative EC50 is decreased from that at 24 hours, suggesting that Niclosamide promotes cell proliferation inhibition by 72 hours. Thus, CCLP cells respond to Niclosamide by undergoing apoptotic cell death by 24 hours and changing expression of proteins that are involved in the cell cycle. There is therefore the potential for additional inhibitory effects on cell division by 72 hours that are not detected in the PI staining and flow cytometry experiments (**Figure 3.9**).

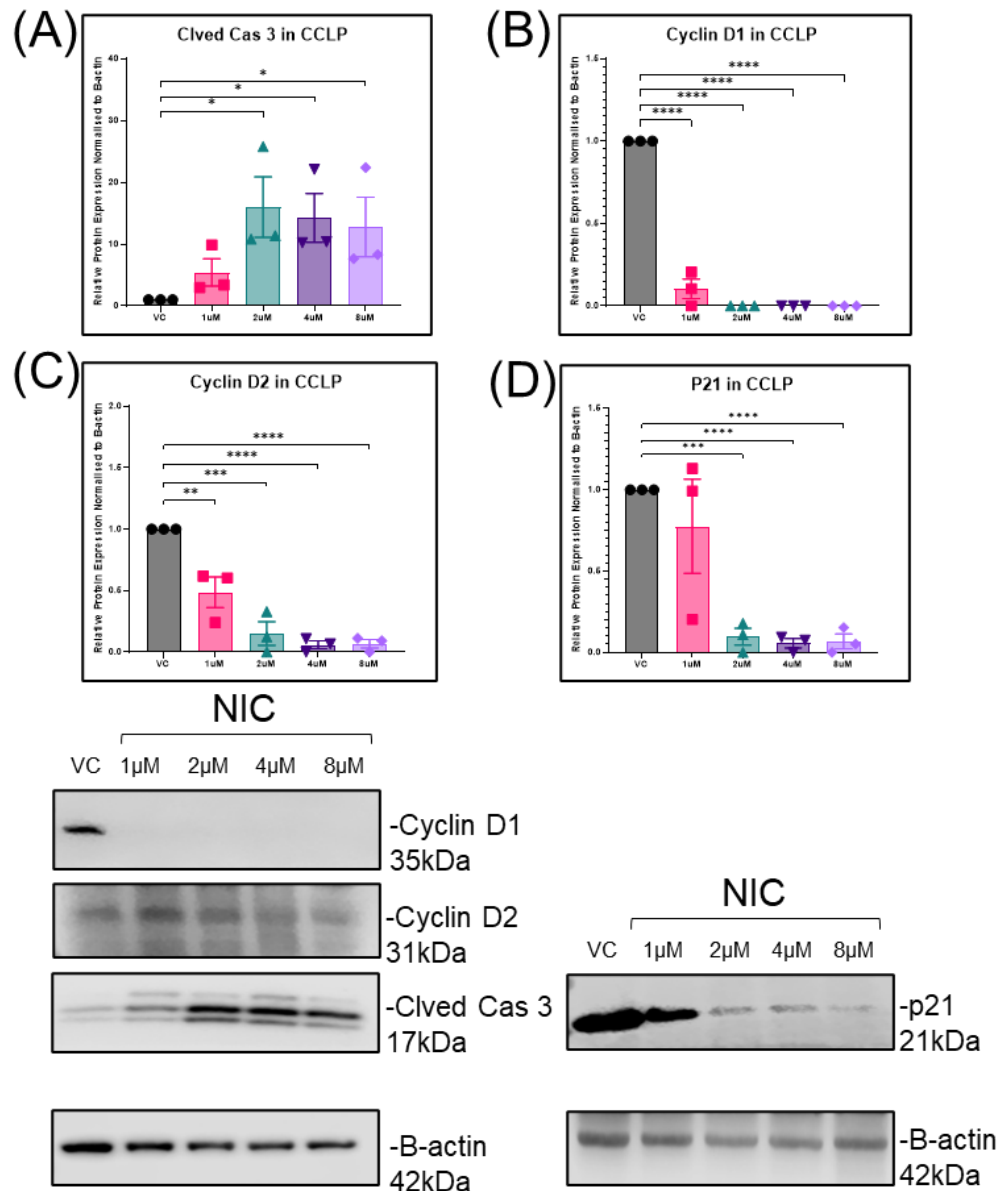


Figure 3.9: Expression of Cell Cycle and Apoptosis Associated Proteins in Niclosamide Treated CCLP Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, ****= $p < 0.0001$). The proteins probed for were (A) Cleaved Caspase 3 (B) Cyclin D1 (C) Cyclin D2 and (D) P21.

Previously PRH has been identified as a protein with roles in cell cycle and has been shown to affect the expression in regulation of cell cycle proteins such as Cyclin D proteins as well as cell cycle inhibitors such as p27 in CCLP (Topisirovic et al., 2003) (Kitchen et al., 2020). As Niclosamide is able to influence the expression or stability of cell cycle proteins, the lysates of the Niclosamide treated cells were examined for effects on PRH levels and on the levels of some PRH-regulated proteins including: Notch-3, B-catenin and Vimentin (Kitchen et al., 2020). These proteins are known to play an important role in the proliferation or migration of CCA tumour cells (Kitchen et al., 2020) (Lozano et al., 2023) (Saentaweessuk et al., 2018).

PRH protein expression is decreased in CCLP cells treated with Niclosamide (**Figure 3.10**), and this is significantly reduced when the Niclosamide concentration is 4 μ M ($p < 0.01$) and 8 μ M ($p < 0.01$) where PRH expression is 20% and 15% of the vehicle control, respectively. Surprisingly, Niclosamide treatment results in a decrease in Notch intracellular domain (ICD) at the lower Niclosamide concentrations 2 μ M and 4 μ M Niclosamide and a higher expression at 8 μ M in two out of three experimental repeats (**Figure 3.10B**). However, changes to Notch-3 protein expression are variable and not statistically significant across the different concentrations; the representative blot shown is the repeat that most closely resembles the average across the three repeats shown in the analysis. B-catenin expression is decreased with Niclosamide treatment in CCLP cells in a dose dependent manner with significant decreases shown at 4 μ M ($p < 0.05$), 8 μ M ($p < 0.01$) Niclosamide (**Figure 3.10C**). In all repeats, a secondary band below the canonical B-catenin band appears and increases in expression, with Niclosamide treatment. This is likely to be a result of B-catenin protein degradation or alternatively it could be a post-translational modification of the B-catenin protein induced by kinases,

such as Glycogen Synthase Kinase (GSK) and Casein Kinase 1(CK1), which are part of the B-catenin destruction complex (Gao et al., 2014). Vimentin protein expression showed a significant and reproducible increase in CCLP cells treated with low concentrations of Niclosamide, which then reduced to levels similar to the vehicle control with higher concentrations of Niclosamide **(Figure 3.10D)**. Overall, statistically significant dose responsive decreases were only seen for PRH and B-catenin proteins in CCLP cells treated with Niclosamide.

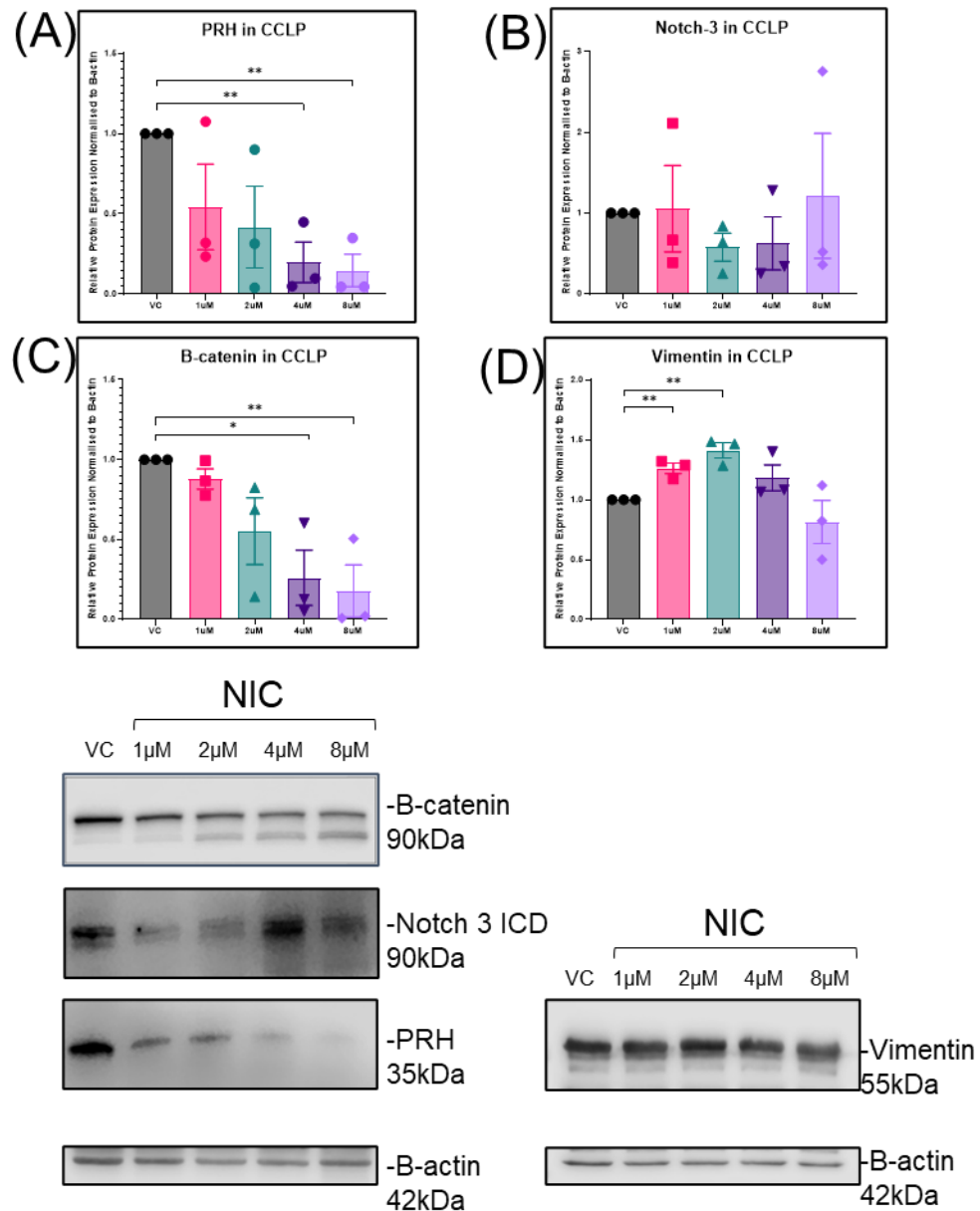


Figure 3.10: Protein Expression of PRH and PRH-regulated proteins in Niclosamide Treated CCLP Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (**=p<0.01). The proteins probed for were (A) PRH (B) Notch-3 (C) B-catenin and (D) Vimentin.

The same flow cytometry and western blotting experiments were performed in Niclosamide treated RBE cells. The effects of Niclosamide treatment on apoptosis in RBE cells are displayed in **Figure 3.11**, showing representative images and analysis of apoptosis experiments. In contrast to CCLP cells, Niclosamide has no effect on apoptosis. When the percentages of cells in the three different types of cell death (early apoptosis, necrosis and late apoptosis) are grouped together, there are still no significant differences to total cell death in Niclosamide treated RBE. Thus Niclosamide has little cytotoxic effect on RBE cells at this concentration at 24 hours.

Figure 3.12 displays the effect of Niclosamide on cell cycle in RBE cells. RBE cells treated with Niclosamide had significantly less cells in S phase compared to vehicle control ($p < 0.01$), however the number of cells in G1 phase and G2 phase did not change. Although the *relative* EC50 is similar for CCLP and RBE, the decrease in S phase cells in RBE treated cells reveals a major effect on cell cycle. This is in marked contrast with the effects of Niclosamide in CCLP which showed no significant effect on cell cycle at 24 hours.

Niclosamide treated RBE cell lysates were used in western blotting experiments where whole cell extracts were examined for changes in the same proteins investigated in CCLP cells. (**Figure 3.13**). As RBE cells do not express Cyclin D2, as indicated by the DepMap Database (Tsherniak et al., 2017), and cleaved caspase 3 proteins was undetectable in western blotting experiments (**Supplementary Figure 3.1 and 3.2**), these proteins were not examined here. Niclosamide treatment of RBE cells results in a dose dependent decrease to Cyclin D1 expression, and statistically significant decreases are observed at 4 μ M ($p < 0.05$) and 8 μ M ($p < 0.05$) (**Figure 3.13A**). Interestingly, the levels of p21 in RBE cells do not change with Niclosamide treatment to the same extent as in CCLP cells (compare **Figure 3.9D** with **Figure 3.13B**). The higher level of

p21 expression in RBE compared to CCLP (demonstrated in **Supplementary Figure 3.2**) might require higher doses of Niclosamide for decreases in p21 expression.

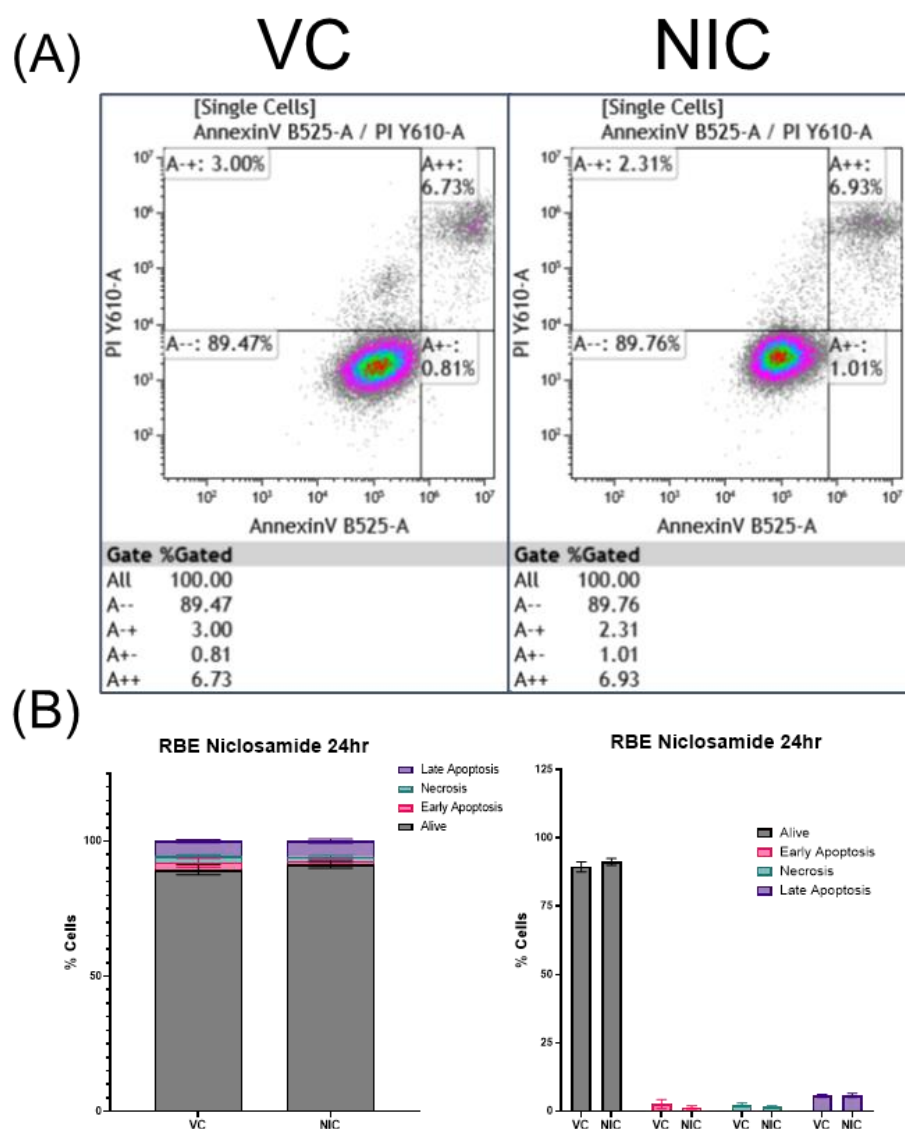


Figure 3.11: Apoptosis Analysis of Niclosamide Treated RBE Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each stage of cell death: live, early apoptosis, necrosis and late apoptosis. Error bars represent SEM and two-tailed t-test was performed to record significant differences in changes between vehicle control and Niclosamide (N=3) (*= $p < 0.05$, ***= $p < 0.001$).

A) Representative images of apoptosis analysis in Kaluza for Niclosamide treated RBE.

B) Apoptosis analysis of Niclosamide treated RBE cells over three repeats.

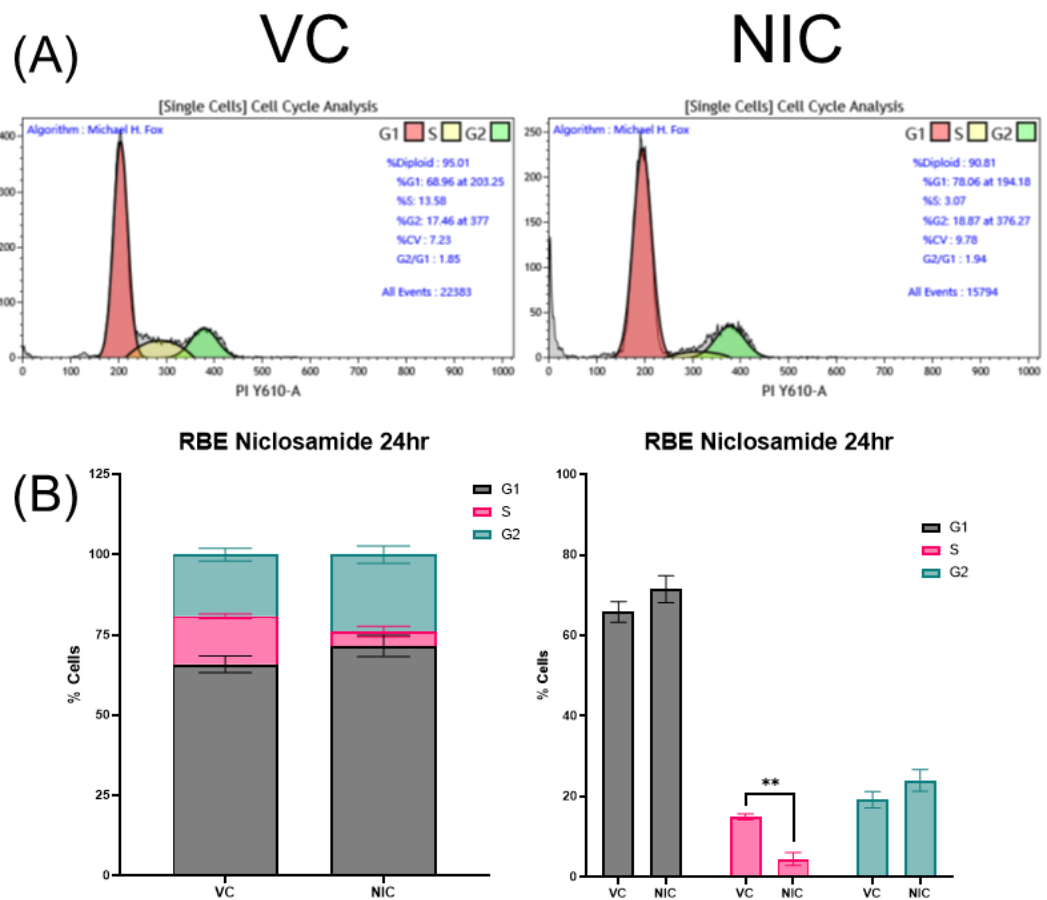


Figure 3.12: Cell Cycle Analysis in Niclosamide Treated RBE Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green). Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3).

A) Representative images of cel cycle analysis in Kaluza for Niclosamide treated RBE.

B) Cell Analysis analysis of Niclosamide treated RBE cells over three repeats.

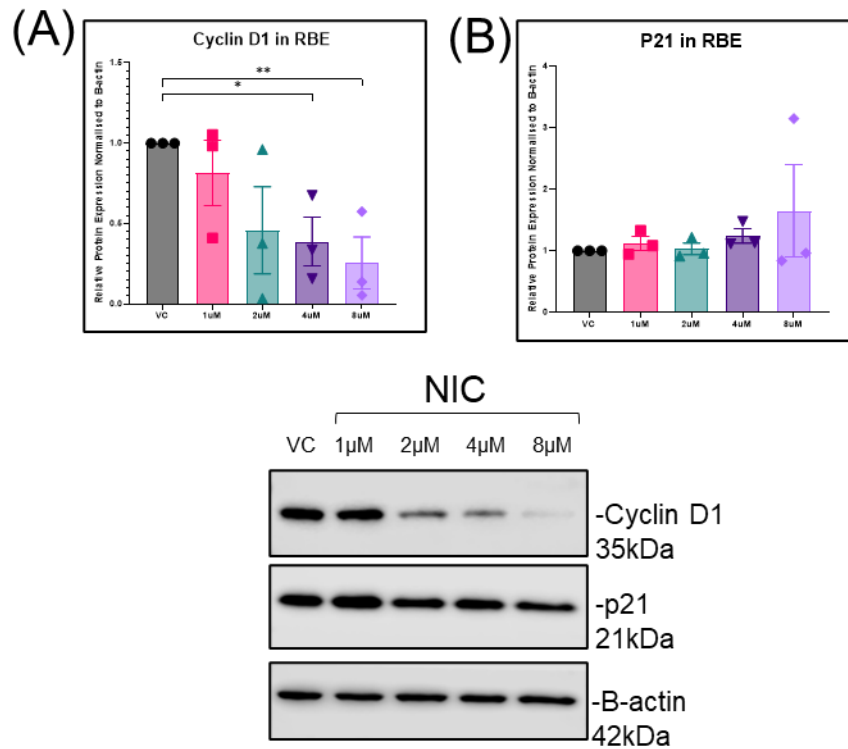


Figure 3.13: Expression of Cell Cycle and Apoptosis Associated Proteins in Niclosamide Treated RBE Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, **= $p < 0.01$). The proteins probed for were (A) Cyclin D1 (B) P21.

The lysates were also probed for PRH and the PRH-regulated proteins described above. In the Niclosamide-treated RBE cells, PRH protein expression was significantly reduced at all Niclosamide concentrations compared to vehicle control (**Figure 3.14A**). However, PRH is still expressed in cells treated (41%) with 8μM of Niclosamide. This level of PRH is much higher than that observed to be present in CCLP cells at the same concentrations of Niclosamide (compare **Figure 3.10A** and **Figure 3.14A**). Without the masking effect of Notch3-high cell lysate on the blot, Notch-3 ICD protein was able to be detected

after long exposure in RBE lysates. Notch-3 expression in RBE cells treated with Niclosamide is also significantly decreased at 2 μ M ($p<0.0001$), 4 μ M ($p<0.01$) and 8 μ M ($p<0.0001$) resulting in a 34%, 24% and 10% expression of the vehicle control, respectively. RBE shows a clear dose-dependent response to Niclosamide in Notch-3 expression (**Figure 3.14B**) which is markedly different from the effect of Niclosamide on Notch3 expression in CCLP cells (**Figure 3.9B**). Niclosamide decreased B-catenin expression in RBE cells, which was significant at 2 μ M ($p<0.05$) and 4 μ M ($p<0.001$) (**Figure 3.14C**). The reduction in B-catenin protein was similar to CCLP but the average B-catenin expression with 8 μ M Niclosamide was reduced to 18% of the control in CCLP cells (**Figure 3.9C**), whereas it was reduced to 52% of the control in RBE cells (**Figure 3.14C**). Also, there was no appearance of the potential secondary beta-catenin cleavage product as observed in CCLP cells. Vimentin protein expression showed no change in Niclosamide treated RBE cells compared to the vehicle control (**Figure 3.14D**). In general, statistically significant decreases were seen in PRH and B-catenin in Niclosamide treated RBE cells as seen in CCLP cells. However, Notch-3 expression was also significantly decreased by Niclosamide in RBE cells. The expression of Notch-3 in RBE is relatively lower than CCLP and it is possible that it requires smaller amounts of stimulus to cause a change in the protein level or it is easier to detect changes to protein expression. In summary, although PRH expression decreases in both cell lines with Niclosamide treatment PRH is relatively stable in RBE compared to CCLP cells. In contrast Notch 3 ICD is less stable with Niclosamide treatment in RBE compared to CCLP.

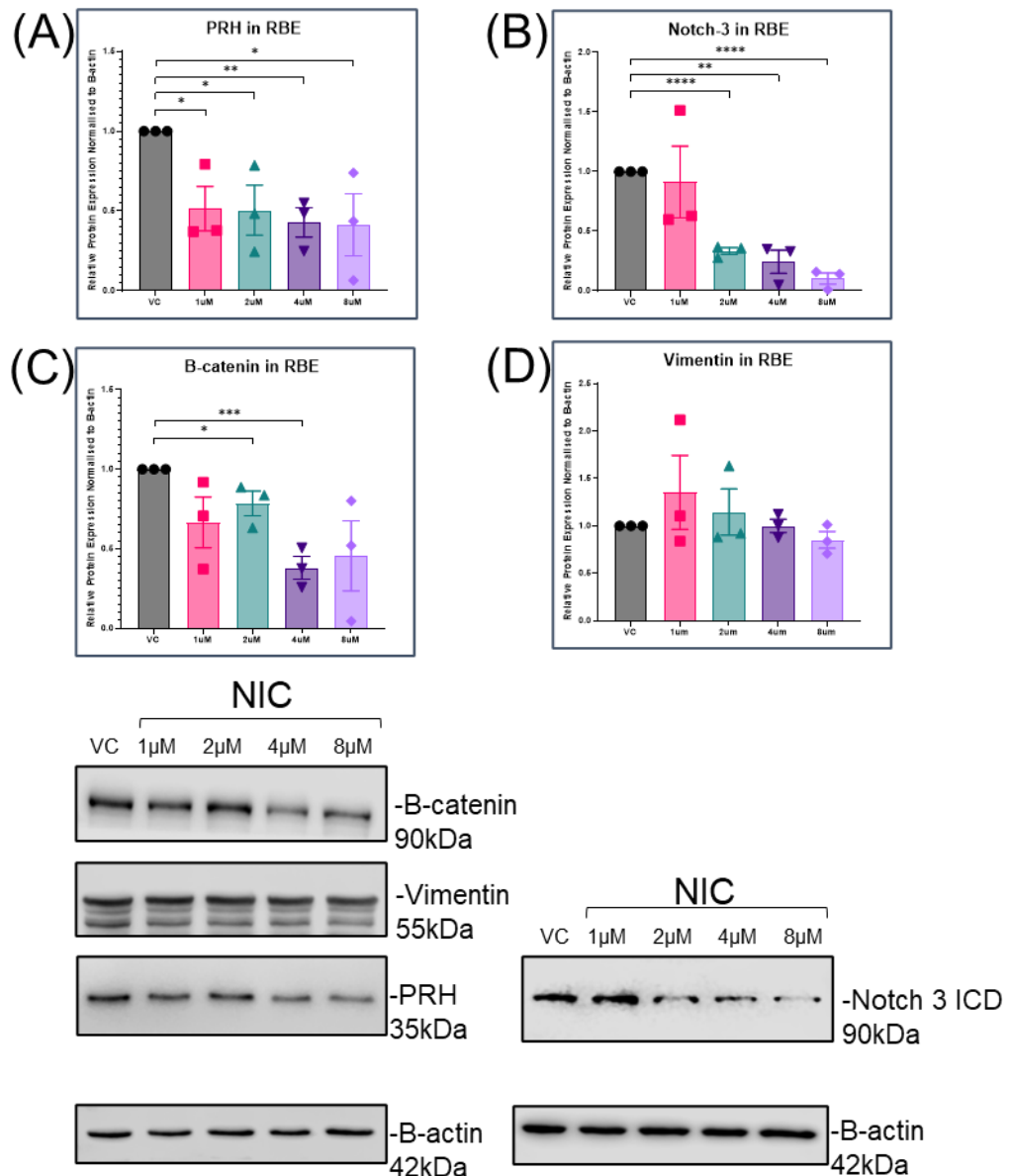


Figure 3.14: Protein Expression of PRH and PRH-regulated proteins in Niclosamide Treated RBE Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001). The proteins probed for were (A) PRH (B) Notch-3 (C) B-catenin and (D) Vimentin.

Vehicle control treated and Niclosamide treated KKU-M055 cells were also observed in flow cytometry and western blotting experiments. Niclosamide treatment of KKU-M055 cells resulted in a significant increase to cells in late apoptosis by 26% ($p<0.001$) and a reduction to live cells by 30.5% ($p<0.01$), compared to vehicle control see (**Figure 3.15**). This shows Niclosamide has a relatively strong cytotoxic effect on KKU-M055 cells compared to CCLP and RBE at 24 hours and this is reflected in its lower relative EC50.

Although in flow cytometry cell cycle experiments, KKU-M055 cells showed a 15% decrease in G2 ($p=0.07$) and a 14% increase to cells in G1 ($p=0.14$) upon Niclosamide treatment, these changes are not statistically significant (**Figure 3.16**). Thus the primary effect of Niclosamide at 24 hours is on apoptosis similar to that observed in CCLP cells.

Niclosamide treated KKU-M055 cells were used in western blotting as described previously (**Figure 3.17**). KKU-M055 cells do not express detectable levels of Cyclin D2 or p21 in western blotting experiments, thus the effects of Niclosamide on these proteins were not examined (**Supplementary Figure 3.3 and 3.4**). Similar to CCLP cells, KKU-M055 cells shows increased cleaved caspase 3 expression at 2 μ M ($p<0.05$), 4 μ M ($p<0.05$) and 8 μ M ($p<0.05$), and expression is increased greater than 50 fold more than vehicle control (**Figure 3.17A**). This dramatic increase in caspase 3 expression likely underlies the potent pro-apoptotic effects observed. Cyclin D1 expression is decreased in KKU-M055 cells (**Figure 3.17B**) showing a significant decrease to 25% expression of the vehicle control ($p<0.01$) at 1 μ M Niclosamide treatment and further reduction to 1.0% of the vehicle control, at 4 μ M and 8 μ M Niclosamide treatment ($p<0.0001$).

In summary, the significantly increased expression of cleaved caspase 3 correlates with the significant increase to PI and Annexin V staining in Niclosamide-treated KKU-M055 cells, showing Niclosamide is strongly cytotoxic in KKU-M055 cells. In cell cycle experiments, the Niclosamide-treated KKU-M055 cells show a tendency towards cell cycle arrest in some experimental repeats, however there were no statistically significant changes to the number of cells in each stage across multiple experimental repeats. Whilst a later time point may be required in flow cytometry experiments to reveal the effects on cell cycle more reproducibly, this is not feasible at these concentrations as the cytotoxic effects are so potent. As, KKU-M055 cells treated with Niclosamide showed a statistically significant decrease in Cyclin D1 expression this suggests that Niclosamide could also potentially have minor cell cycle effects at 24 hours.

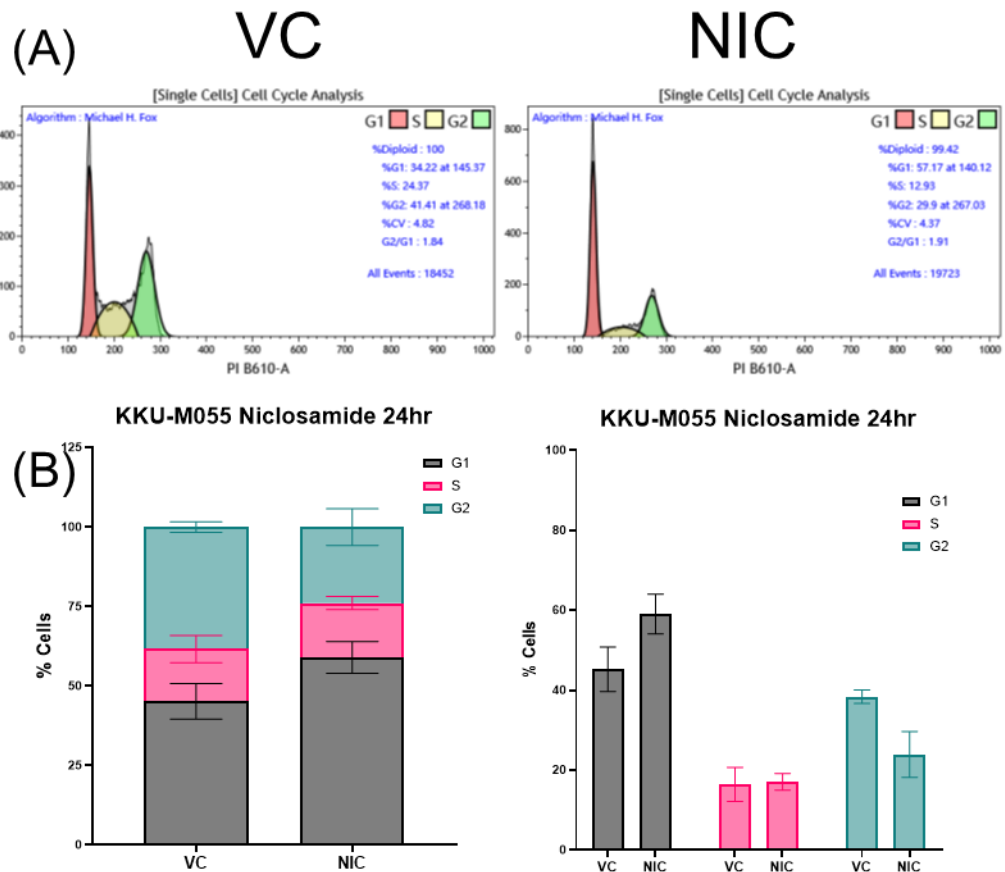


Figure 3.16: Cell Cycle Analysis in Niclosamide Treated KKU-M055 Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green). Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3).

A) Representative images of cel cycle analysis in Kaluza for Niclosamide treated KKU-M055.

B) Cell Analysis analysis of Niclosamide treated KKU-M055 cells over three repeats.

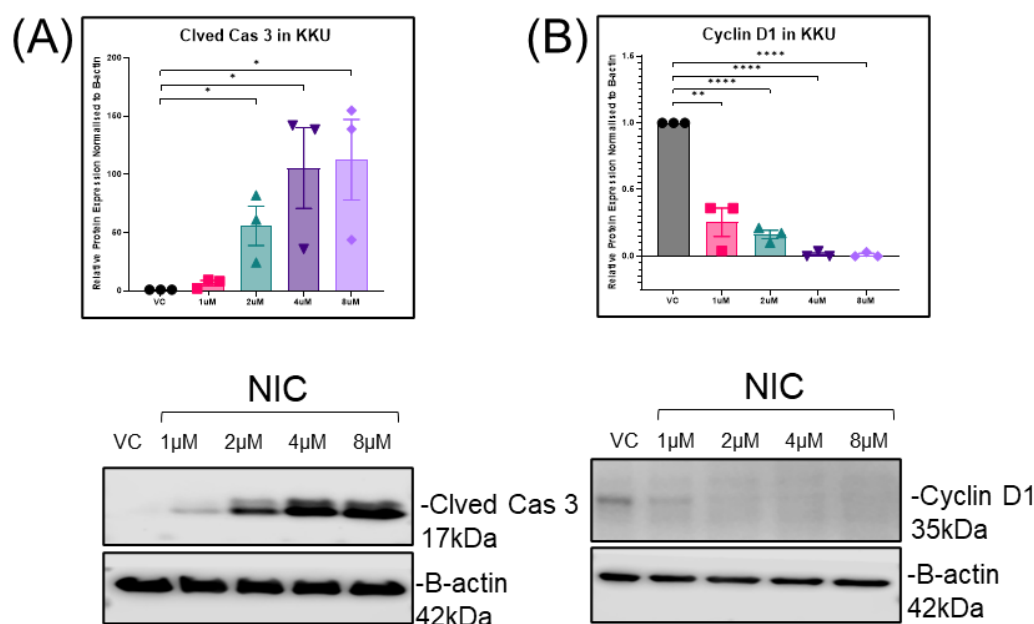


Figure 3.17: Expression of Cell Cycle and Apoptosis Associated Proteins in Niclosamide Treated K KU-M055 Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, **= $p < 0.01$, ****= $p < 0.0001$). The proteins probed for were (A) Cleaved Caspase 3 (B) Cyclin D1

Lysates generated from Niclosamide-treated K KU-M055 cells were also used in western blotting to examine PRH and PRH-regulated proteins (**Figure 3.18**). Niclosamide treated K KU-M055 cells showed the greatest reduction in PRH protein amongst the CCA cell lines; treatment at 1μM reduced protein expression to 25% of the vehicle control ($p < 0.01$), and treatment at 8μM Niclosamide caused a massive reduction (to 2% of the control) in the protein present ($p < 0.0001$) (**Figure 3.18A**). Notch-3 expression in K KU-M055 cells was variable (**Figure 3.18B**). Low concentrations of Niclosamide caused decreased expression of Notch-3; 1μM of Niclosamide saw expression fall to 43.2% of the vehicle control and 2μM of Niclosamide showed a statistically significant

decrease of expression that was 20.6% of the vehicle control ($p < 0.01$). Whilst higher concentrations result in a large increase in Notch-3 expression. The increase in Notch-3 expression was reproducible but variable in extent between the three experimental repeats and the variance precluded statistical significance. Nevertheless, the increase is apparent in all repeats. Although Notch-3 expression varied between repeats there are similarities between CCLP and KKKU-M055 cells in that lower concentrations decreased expression and higher concentrations increased expression. KKKU-M055 cells show a large decrease in B-catenin expression at all Niclosamide concentrations (**Figure 3.18C**). Vimentin expression in KKKU-M055 cells (**Figure 3.18D**), shows no significant change from levels in vehicle control treated cells. Overall, Niclosamide treatment results in significant decreases to PRH and B-catenin expression in KKKU-M055, whereas Notch-3 expression was variable, and like CCLP cells, it follows a biphasic trend.

It can be concluded that the effect of Niclosamide is predominantly cytotoxic in CCLP cells and KKKU055 cells whereas it is predominantly cytostatic in RBE cells. Moreover, Niclosamide treatment is able to significantly reduce PRH protein levels in all of the tested CCA cell lines.

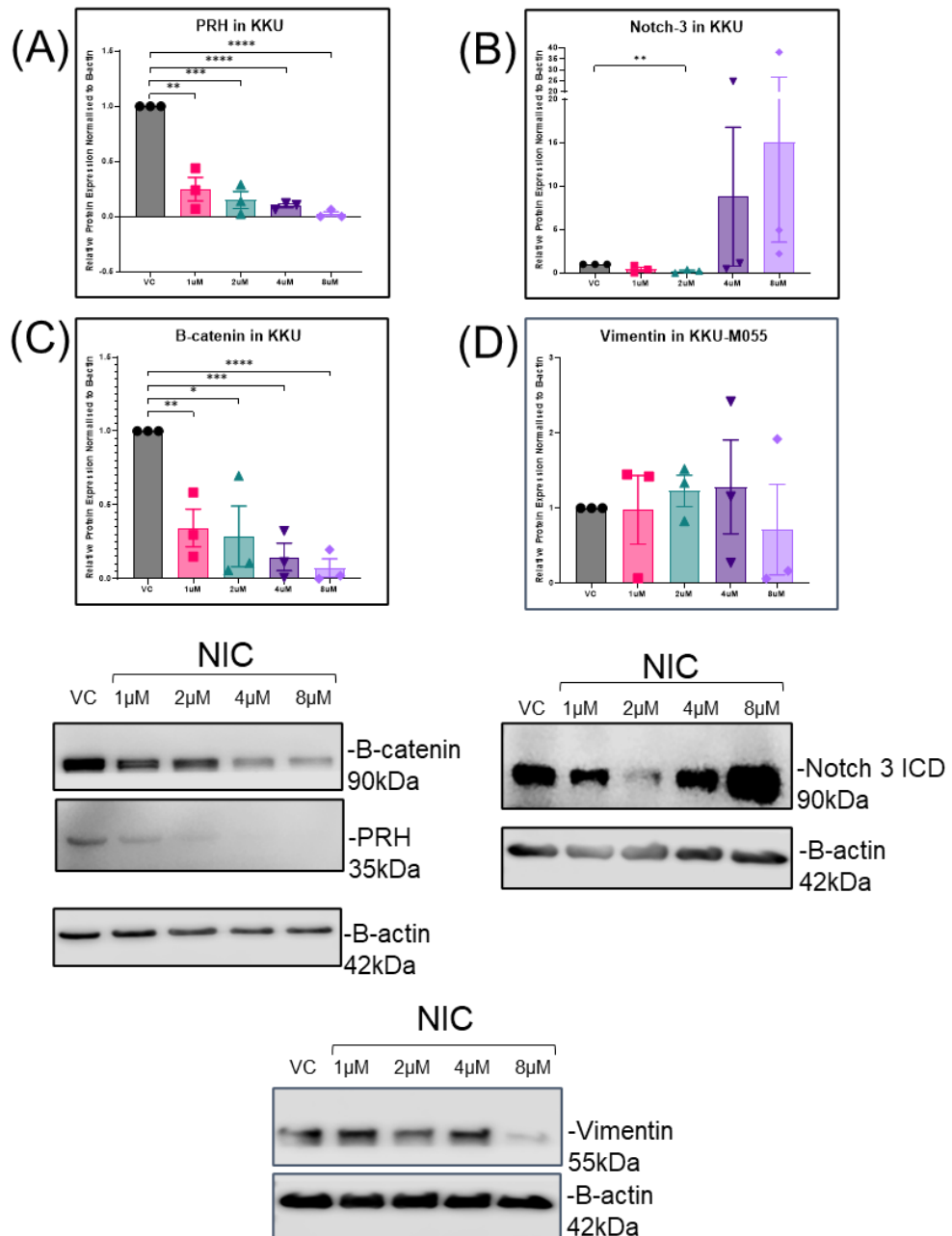


Figure 3.18: Protein Expression of PRH and PRH-associated proteins in Niclosamide Treated KKU-M055 Cells.

Cells were treated with vehicle control (VC) or Niclosamide (NIC) at concentrations 1μM, 2μM, 4μM and 8μM for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. For each protein, a representative blot is shown alongside the analysis of protein expression over three experimental replicates. For the analysis, the protein of interest was normalised to the expression of B-actin, and this was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$). The proteins probed for were (A) PRH (B) Notch-3 (C) B-catenin and (D) Vimentin.

3.6 Niclosamide does not alter PRH Gene Expression

To determine whether the effects of Niclosamide on PRH protein levels occur through changes in gene expression, mRNA was obtained from Niclosamide treated CCA cells and PRH mRNA levels were measured using qPCR experiments.

Cells were plated at 2×10^6 cells in a T25 flask and treated with vehicle control or Niclosamide at 8 μ M, known to have an effect on PRH protein levels in western blotting experiments and corresponding to the absolute EC₅₀ value for that cell line at 24 hours. Cells were collected after 24hrs via scraping in PBS and centrifugation to form a pellet. RNA was extracted from the pellets and used for cDNA synthesis, employing previously described methods (section 2.2.1). Equal amounts of cDNA were used in qPCR experiments (section 2.2.1) with *HHEX* and *B-actin* specific primers. The *HHEX* Ct values were normalised to *B-actin* Ct values, and relative *HHEX* expression in vehicle control treated cells were compared to the expression in Niclosamide treated cells. **Figure 3.19** shows the effect of Niclosamide on PRH gene expression in CCA cells.

Figure 3.19A shows that gene expression of PRH does not change with Niclosamide treatment after 24hrs, and that PRH mRNA levels do not differ from the vehicle control. To see if a longer timepoint was needed to reduce PRH gene expression, CCLP cells were treated with control or 0.45 μ M Niclosamide for 72hrs and the qPCR experiment was repeated. The longer timepoint did not result in a decrease in PRH mRNA expression (**Figure 3.19B**). RBE and KKM055 cells were also treated with Niclosamide at their absolute EC₅₀ concentrations (1.31 μ M and 0.39 μ M) over 72hrs (**Figure 3.19B**). The results

were similar to those seen with CCLP cells, with no significant change to PRH mRNA with Niclosamide treatment. It was concluded that Niclosamide treatment does not down regulate PRH expression at the transcriptional level.

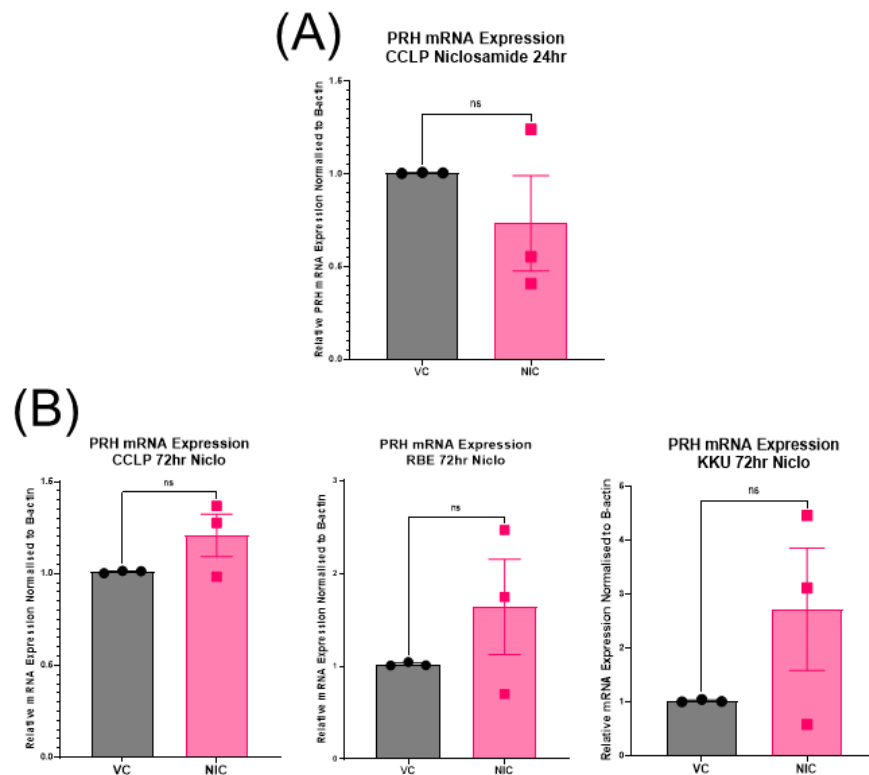


Figure 3.19: Expression of PRH mRNA in Niclosamide treated CCA Cell Lines. Vehicle control (VC) or Niclosamide (NIC) treated CCA cells were collected for RNA extraction and cDNA synthesis for qRT-PCR experiments to assess the gene expression of PRH. PRH primers were used to quantify PRH mRNA levels and B-actin primers were used to calculate relative expression of PRH mRNA. This was further normalised to the vehicle control. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3).
 (A) PRH mRNA expression in CCLP cells treated with EC50 Niclosamide for 24hrs.
 (B) PRH mRNA expression in all CCA cell lines treated with EC50 Niclosamide for 72hrs.

3.7 Investigating the effects of Proteasome

Inhibition on PRH expression

Whilst cell cycle proteins like Cyclin D1 are well known to be regulated by ubiquitylation and proteasomal protein degradation (Alao, 2007), the stability of the PRH protein has not been investigated in CCA cells. To examine whether PRH is degraded by the proteasome in CCA cells, and during Niclosamide treatment, cells were treated with MG132. MG132 is a potent proteasome inhibitor that reversibly blocks the enzymatic activity of the 26S proteasome complex (Lee and Goldbery, 1998).

A preliminary experiment was performed to determine the concentration of MG132 to be used for 24 hours without killing all the cells. In the literature, MG132 used at 5 μ M is able prevent degradation of p53 and p27, which are strongly ubiquitylated and are canonical substrates of the proteasome (Wang et al., 2019). A dose response curve was performed in CCLP and RBE cells to assess cell viability in the presence of the MG132 inhibitor. **Figure 3.20** shows that 5 μ M MG132 decreases cell viability to 50% in both CCLP and RBE cells. This shows that although MG132 at 5 μ M is having a toxic effect on the cells, (likely as proteasome inhibition causes cell cycle arrest and apoptosis (MacLaren et al., 2001)), this concentration would leave enough cells to harvest for protein samples.

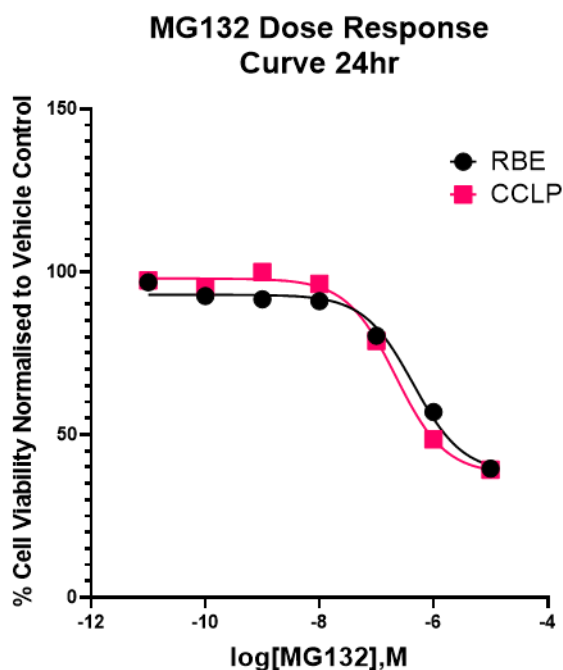


Figure 3.20: Dose Response Curve of MG132 in CCLP and RBE cells. MG132 dose response curves were measured for CCLP and RBE over 24hrs. Cells were plated at 1×10^4 cells per well in triplicate in a 96 well plate and were treated with a range of MG132 (10pM-10 μ M) or vehicle control. Cell viability was measured through MTT assays and values were normalised to vehicle control. Experiments were performed once to aid in optimising MG132 concentration for further experiments.

Experiments were therefore performed by plating 2×10^6 cells for each CCA cell line, into four T25 flasks and leaving the cells to adhere overnight. Cells were simultaneously given either vehicle control, or 8 μ M Niclosamide with or without 5 μ M MG132. After the CCA cell lines were treated with the four conditions for 24hrs (VC, VC+MG132, NIC, NIC+MG132), the cells were collected via scraping in PBS and pelleted. Pellets were lysed with RIPA buffer plus supplements as previously described (section 5.3) and lysates were electrophoresed before transferring to a membrane for immunoblotting. Membranes were probed with anti-PRH antibody and B-actin antibody; PRH protein expression was normalised to B-actin protein expression. The normalised PRH protein expression was then divided by the vehicle control to show PRH protein levels relative to vehicle control. **Figure 3.21** shows the levels of PRH protein with Niclosamide and/or MG132 treatment.

In CCLP cells, Niclosamide treated cells show significantly decreased PRH expression compared to vehicle control ($p<0.01$) (**Figure 3.21A**). The addition of MG132 alone caused PRH to increase in two repeats out of three, suggesting that it is likely degraded by the proteasome in these cells. The expression of PRH was significantly higher in CCLP cells treated with Niclosamide and MG132 than the CCLP cells treated with Niclosamide only, showing that the inhibition of the proteasome allowed partial rescue of the PRH protein from Niclosamide-induced degradation.

In RBE cells, PRH expression was significantly decreased with Niclosamide treatment ($p<0.001$) but MG132 treatment caused no difference in PRH expression (**Figure 3.21B**). MG132 with Niclosamide treatment increased the level of PRH compared to Niclosamide alone, however this data does not fall within statistical significance due to variance between repeats ($p=0.06$). However, the trend in this cell line is similar to CCLP cells where addition of MG132 partially reduces PRH degradation.

In KKU-M055 cells, Niclosamide potently reduced PRH expression ($p<0.0001$) (**Figure 3.21C**). Treatment with MG132 alone increased PRH expression in every repeat but the variance in the data precluded statistical significance ($p=0.12$). When cells were treated with Niclosamide and MG132, PRH protein was increased on average 4x fold compared to Niclosamide alone, but again the difference is not statistically significant ($p=0.13$). It is possible also that further optimisation of the Niclosamide and MG132 concentrations used is needed for each cell line to allow maximal Niclosamide induced PRH degradation and maximal inhibition of that degradation. In conclusion, MG132 treatment significantly increases PRH levels in Niclosamide treated CCLP and there is a trend for this in RBE and KKU-M055 cells. The addition of MG132 inhibits PRH degradation both in the presence of Niclosamide and in the

absence of Niclosamide in all cell lines. Further repeats are required to reach statistical significance in RBE and KKU-M055 cells.

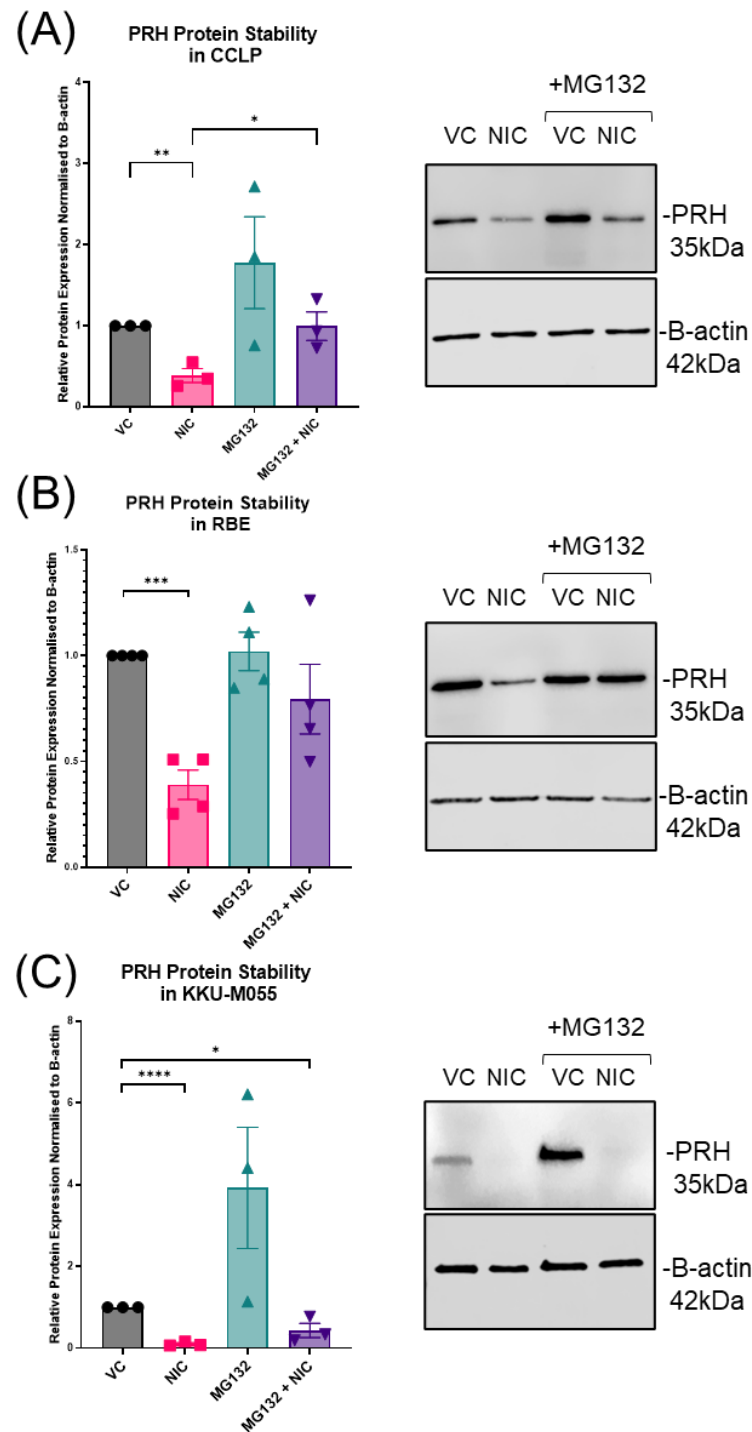


Figure 3.21: PRH Protein Expression in Niclosamide Treated CCA Cells with and without MG132.

Cells treated with vehicle control (VC) or 8µM Niclosamide (NIC), with and without the presence of 5µM MG132, were harvested for protein lysates. Lysates were used in western blotting to probe for PRH protein. PRH protein expression was normalised to B-actin and relativised to vehicle control. Assays were performed to N=3. Error bars represent SEM, and a two-tailed unpaired t-test was used to measure significant changes. The results are summarised in a graph which is supplemented by a representative lot of the experiment in (A) CCLP (B) RBE and (C) KKU-M055.

3.8 The Effect of Niclosamide on Cell Viability in a PRH KD Model

3.8.1 Generation and Validation of PRH KD shRNA

CCLP cells

We showed that PRH protein expression is reduced by Niclosamide treatment in section 3.5 above. To understand the role of PRH in CCLP Niclosamide sensitivity, stable PRH knockdown cells (PRH KD) were generated using short hairpin-RNA (shRNA). Short hairpin RNA silences gene expression through RNA interference, where the gene is transcribed but the mRNA is degraded, or blocked for translation, resulting in reduced protein expression. In RNA interference, the shRNA is cleaved by Dicer and bound to by Argonaute to produce RNA induced silencing complexes (RISC) or RNA induced transcriptional silencing (RITS) complexes. The complementary sequence of the shRNA guide strand binds to the target mRNA sequence which is subsequently cleaved (Moore et al., 2010). To make a stable knockdown cell line, the shRNA is initially delivered by a plasmid, via transient transfection, however, the shRNA expression can become permanent if integrated into the genome. In this case, the shRNA is permanently expressed despite cell replication and division. Cells that successfully integrate the plasmid, can be selected for via antibiotic resistance if the plasmid carries a resistance gene.

Details of optimisation and a full protocol are described in section 2.1.10. Briefly, CCLP cells were seeded at 5×10^5 per well in a six well plate and were transfected with shRNA PRH plasmid, or a scrambled vector control plasmid, using Lipofectamine 3000 Kit. After the transfection, cells were incubated for 48

hours before selecting with 0.7mg/mL Puromycin. After five days of selection, the antibiotic media was replaced with complete media. The poly-clonal population of cells was left to recover, and when the cells reached full confluency, the cells were further expanded. This resulted in two control cell lines, SVC 1 and SVC 2, and two PRH knockdown cell lines, PRH KD 1 and PRH KD 2.

RNA was extracted from cell pellets and converted into cDNA with Reverse Transcriptase kits (see section 2.3 for details). In qRT-PCR relative gene expression was measured using *HHEX*/PRH primers and B-actin primers, where PRH expression was normalised to B-actin. The relative PRH expression levels in SVC 1 and SVC 2 were then averaged and the KD 1 and KD 2 compared to the average expression in the controls. **Figure 3.22** shows that, compared to the SVC 1 and 2, both PRH KD 1 and 2 have a significant decrease in expression of PRH mRNA. The PRH KD 2 has the greater decrease in PRH mRNA with expression dropping to 32% of the SVC controls, whereas PRH KD 1 expressed 65% of PRH mRNA compared to controls.

The SVC and PRH KD cell lines were also examined by western blotting for PRH expression. For western blotting approximately 3×10^6 SVC and PRH KD cells were harvested and centrifuged into cell pellets that were treated with RIPA buffer to produce whole cell extracts. Extracts were electrophoresed by SDS-PAGE and then transferred to a membrane to be probed with an anti-PRH 2018B rabbit monoclonal antibody and B-actin antibody. Expression of B-actin protein was used as a loading control. In PRH KD 2 expression of PRH protein decreased to 15% of the SVC controls, while PRH KD 1 expression of PRH protein was reduced to 38%, with reference to the SVC controls. CCLP SVC 2 and PRH KD 2 were used in subsequent experiments as they showed greater PRH knockdown. Also, as the PRH KD cell lines were able to maintain

knockdown status beyond 20 passages, they could be considered a stable KD cell line.

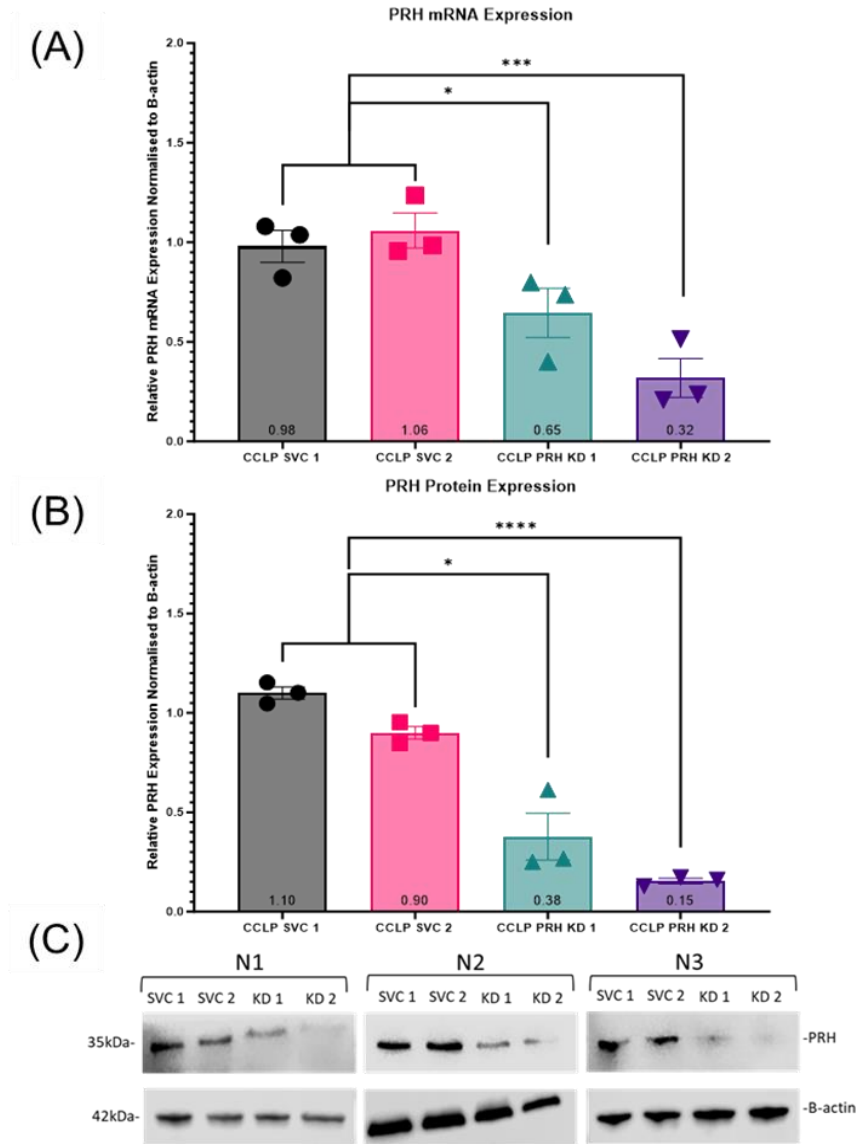


Figure 3.22: Validation of PRH knockdown in shRNA expressing CCLP

Cells were transfected, as detailed in section 2.1.7, and two sets of pellets for RNA and protein samples were taken from each SVC and KD cell line at low passage.

- (A) PRH mRNA expression in CCLP PRH KD 1 and 2 compared to a CCLP SVC 1 and 2, measured through qPCR. Ct values were normalised to B-actin mRNA expression, and further normalised to the average value for both SVC 1 and SVC 2. Error bars represent SEM and statistical significance was measured through two-tailed t-test (N3; *= $p < 0.05$, ***= $p < 0.001$).
- (B) PRH protein expression in CCLP PRH KD 1 and 2 compared to a CCLP SVC 1 and 2, measured through western blot. Band densitometry values were normalised to B-actin protein expression, and further normalised to the average SVC value. Error bars represent SEM and statistical significance was measured through two-tailed t-test (N3; *= $p < 0.05$, ****= $p < 0.0001$).
- (C) Western blot showing PRH protein expression in the CCLP SVC and PRH KD pair 1 and 2. Each lane has 30ug protein and was probed with PRH (2018B) and B-actin antibodies. N=3 each blot shows a different biological repeat.

3.8.2 Niclosamide sensitivity in shRNA PRH KD

CCLP

As PRH influences cell proliferation and cell viability (Kitchen et al., 2020), the shRNA cell lines were used to examine whether PRH downregulation can affect Niclosamide response. Assays were performed as outlined earlier in wildtype CCA cell lines (Figure 4.1). Experiments were performed three times unless stated otherwise. The results for Niclosamide sensitivity in the shRNA PRH KD and control are shown in **Figure 3.23**. The figures show the dose response curves at 24hrs and 72hrs timepoints, the *relative* and *absolute* EC50 values and a ratio-paired t-test between EC50 values between control and PRH low models to measure statistical significance.

CCLP SVC 2 and PRH KD 2 cell viability decreases with increasing dose of Niclosamide over 24hrs and 72hrs. After 24hrs incubation, PRH KD cell viability seems to decrease less than SVC at the highest concentrations; (**Figure 3.23A**). The average Emax value for SVC is 25%, whereas it is 32% for PRH KD, however, this is not statistically significant ($p=0.34$). The *relative* EC50 values for SVC and PRH KD cells are 0.992 μ M and 1.219 μ M, respectively, and the *absolute* EC50 values for SVC and PRH KD are 1.899 μ M and 2.343 μ M. However again, there is no statistically significant difference between SVC and PRH KD *absolute* EC50 values at 24hrs ($p=0.16$). After 72hrs incubation, *relative* EC50 values are decreased further, where SVC has a value of 0.54 μ M and KD has a value of 0.81 μ M. Although relatively small, the difference in the EC50 for SVC and PRH KD was reproducible and statistically significant at 72 hours ($p<0.05$). Thus, the PRH KD showed more resistance to Niclosamide at 72hrs. As a significant difference was only recorded at the longer timepoint,

PRH KD resistance may stem from the mixture of both cytotoxic and cytostatic effects of Niclosamide.

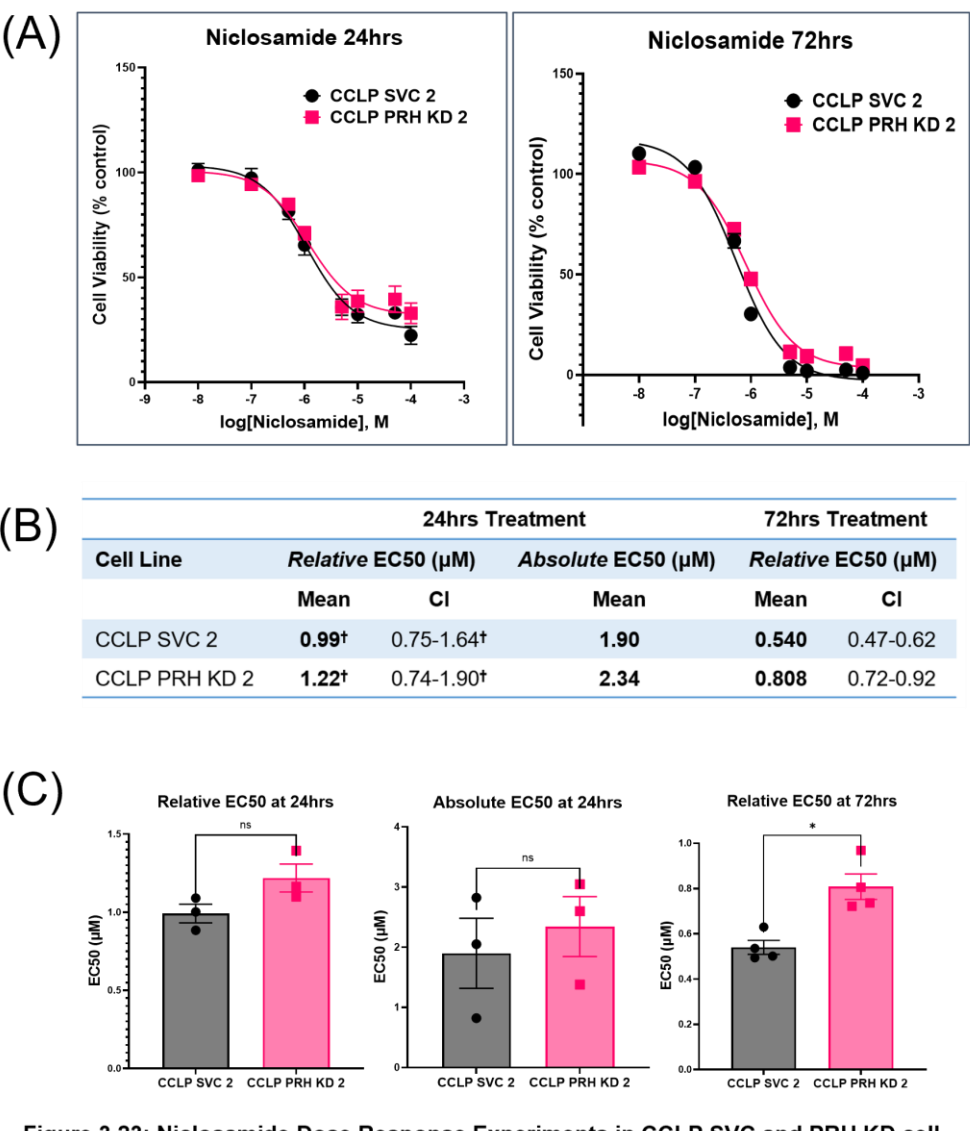


Figure 3.23: Niclosamide Dose Response Experiments in CCLP SVC and PRH KD cell lines at 24hrs and 72hrs.
 The CCLP SVC 2 and PRH KD 2 cell lines were plated at 1x10⁴ cells per well in triplicate in a 96 well plate and treated with a range of Niclosamide concentrations (10nM-100μM) or vehicle control for 24hrs or 72hrs. After treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Niclosamide concentration to create dose response curves in GraphPad Prism. EC50 values, plus 95% confidence intervals (CI), were calculated by the software.
 (A) Niclosamide Dose Response Curves in CCLP SVC and PRH KD cells after 24hrs and 72hrs. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
 (B) Niclosamide sensitivity across the CCLP SVC and PRH KD cell lines after 24hrs and 72hrs treatment. Relative EC50 values, plus confidence intervals CI, and absolute EC50 values (μM) were calculated using the GraphPad Prism software. † denotes dose response curves that did not reach Emax 0.
 (C) Comparison of Niclosamide sensitivity between CCLP SVC and PRH KD with a ratios paired t-test. Error bars represent SEM (*=p<0.05).

Overall, there is a statistically significantly decreased sensitivity in shRNA PRH KD cell lines compared to SVC at 72 hours. This indicates that the PRH KD cell line is less sensitive to Niclosamide when the drug can elicit both cytostatic and cytotoxic effects.

When cells are grown as a monolayer in cell culture, they may lose some of their oncogenic cellular characteristics and therefore the translation of drugs to more physiologically relevant models such as spheroids may provide more of an appropriate prediction of how the drugs may behave in a patient. Matrigel is an extracellular matrix (ECM) extract from mouse sarcoma cells which contains ECM components like laminin, collagen and other structural protein, as well as growth factors which is used to provide some of the features of an ECM for the growth of spheroids (Herrmann et al., 2014). Spheroids are commonly used in 3D drug testing because the spheroid model recapitulates the cell-cell interaction, the effect of the extracellular matrix and various nutrient/O₂/cell waste gradients, found in solid tumours (Lee & Kim, 2021).

Optimised protocols from the Grabowska laboratory (Pal et al., 2020) (Dr M. Sueca Comes Ph.D thesis) were used to obtain spheroids in all three CCA cell lines CCLP, RBE and KKV-M055 cells. Briefly, cells were harvested and centrifuged at 300xg at 10°C for 10 minutes in a round bottomed ultra-low attachment 96-well plate with 0.1mg/mL Matrigel (Corning) in 200µL complete media. The cells were left to grow over time and images were taken at day 1, day 3, day 5, day 7.

Figure 3.24 shows how the three CCA cell lines behave in 3D spheroid culture. CCLP cells form a canonical spheroid with clear borders, and the individual cells are distinguishable. The CCLP spheroid grows exponentially over the seven days (**Figure 3.24; Column 1; Day 1-7**). The RBE cells form spheroids however

they are small, and they fail to grow (**Figure 3.24; Column 2; Day 1-7**). This would make the RBE spheroids a poor model to test drugs as any effect of the drug on spheroid growth would not be highlighted. KCU-M055 cells do not form a typical spheroid with a border, but rather form an accumulation of cells, similar to that seen in colony formation assays (**Figure 3.24; Column 3; Day 1-7**). There is a lot of space between cells in the structure and consequently this compromises some of the interesting characteristics of spheroid models such as cell-cell contact and nutrient/O₂/waste gradients.

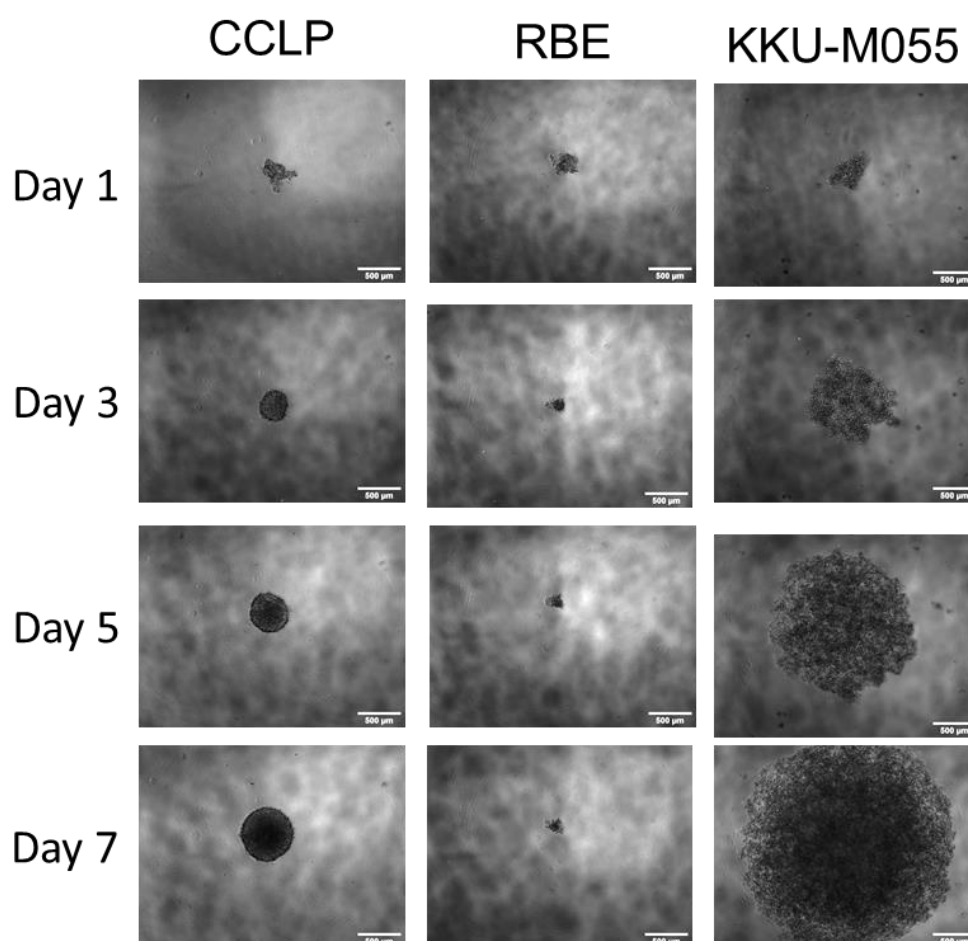


Figure 3.24: Obtaining 3D Spheroid Models of the CCA Cell Lines.

Protocols from A. Grabowska laboratory were used to observe CCLP, RBE and KCU-M055 cells as 3D spheroid models. Cells were seeded at 1×10^3 cells per well in an ultra-low attachment bottom round 96-well plate with 0.1mg/mL Matrigel in 200uL. The plate was centrifuged at 300xg for 10 minutes. Cells were left to form spheroids and images were taken on a Nikon brightfield microscope at days 1, day 3, days 5, and days 7. Representative images were taken for each cell lines.

As CCLP wildtype cells were able to form spheroids the CCLP SVC and PRH KD cells were next grown as spheroids to determine whether the difference in sensitivity to Niclosamide is maintained in a 3D tumour model.

SVC and PRH KD spheroids were generated and grown for five days before adding a range of Niclosamide concentrations and measuring viability over time using a presto blue assay. The concentrations used were extrapolated from the previous work; thus, spheroids were incubated with a vehicle control or 1 μ M, 2 μ M, 4 μ M, or 8 μ M of Niclosamide. At the time of adding the drugs, 100 μ L of media was removed from the well without disturbing the spheroid, and media containing 2X drug was added to the well. The media was gently mixed before leaving for 72hrs. After the treatment, 100 μ L of media as removed from the well and 20 μ L of (1:1) presto blue was added. Presto Blue is a commonly used metabolism-based viability assay for 3D assays involving a resazurin-based reagent that is reduced by cellular activity to a fluorescent signal that correlates to cell number (Manlong et al., 2015). **Figure 3.25** shows that Presto Blue is better able to measure cell viability in spheroids than MTT assays.

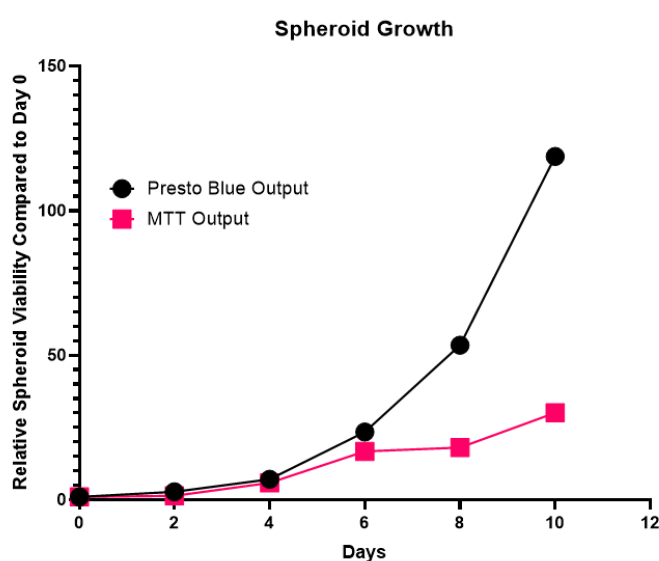


Figure 3.25: CCLP Spheroid Viability over time measured through MTT and Presto Blue Assay. Spheroids were generated from CCLP WT cell lines and viability was measured by Presto Blue and MTT assay every two days for 10 days to show spheroid growth. Measurements were started from day 0, when the spheroid was generated, and all data was made relative to the assay signal on day 0. The experiment was performed N1.

Presto Blue measurements increase exponentially over time, reflecting spheroid growth, whereas MTT shows a linear increase. This indicates that the MTT reagent cannot penetrate the spheroid to provide an accurate reading, and that MTT assays are inappropriate to measure spheroid viability. Thus, Presto Blue was used in spheroid experiments to measure cell number. The spheroids were incubated with the presto blue for an hour, in which the reagent was metabolised to a fluorescent signal that corresponded to spheroid viability. In the cell viability assays, wells with media only were also measured for a background control. The background was subtracted from experimental values and were normalised to wells treated with vehicle control. The experiment was repeated three times. Viability was measured at day 0, day 2, day 4 and day 6.

Figure 3.26 show the spheroid viability at each timepoint for each concentration and the data is normalised to the average presto blue reading at day 0. When spheroids were incubated with vehicle control, the SVC and KD spheroid viability increases with time, due to the un-inhibited exponential growth of the cells. Spheroids treated with a concentration of Niclosamide that is 2µM or more show decreased viability compared to day 0, showing Niclosamide is cytotoxic at high concentrations. However, treatment of spheroids at 1µM do not increase neither do they decrease from day 0, showing a cytostatic effect of the drug.

To compare the sensitivity of SVC and KD spheroids to Niclosamide, the data was further normalised to the vehicle control each day. In **Figure 5.36B**, SVC spheroids show a decrease in viability compared to KD spheroids which is dose- and time-dependent. After two days incubation, treatment with 4µM or 8µM Nic causes a significant decrease in viability ($p < 0.01$, $p < 0.001$). After four days treatment, 2µM, 4µM and 8µM of Niclosamide result in significant decreases in SVC viability compared to KD ($p < 0.05$, $p < 0.01$, $p < 0.05$). After six days, both type of spheroids die at the higher concentrations, but SVC is significantly more

sensitive at 2 μ M Niclosamide compared to KD ($p < 0.0001$). In conclusion, the PRH down-regulated spheroids are less sensitive than the SVC control spheroids to Niclosamide.

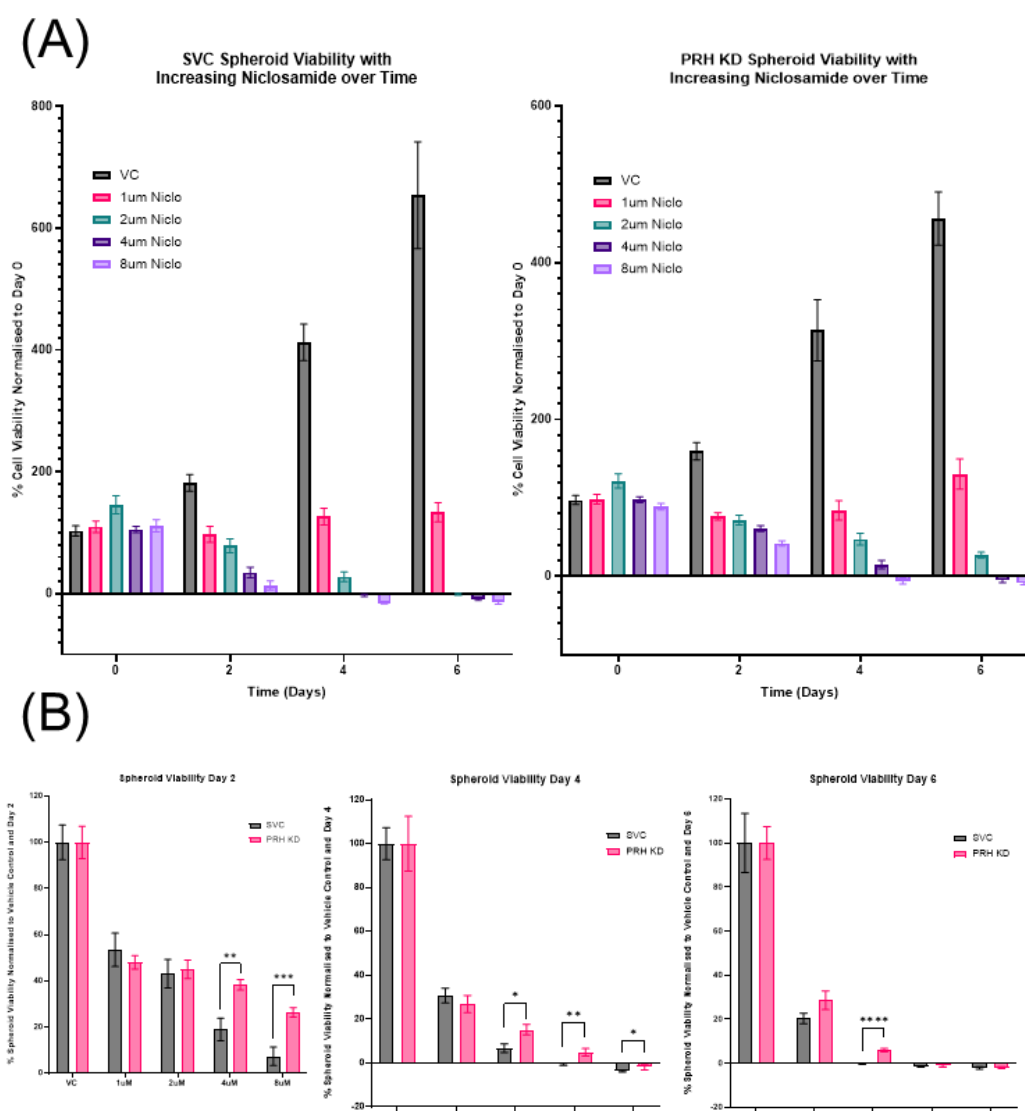


Figure 3.26: CCLP SVC and PRH KD Spheroid Viability with Increasing Niclosamide Concentration over Time.

Spheroids were generated from CCLP SVC 2 and CCLP PRH KD 2 cell lines and left to grow for five days before treating with vehicle control or a range of Niclosamide 1 μ M, 2 μ M, 4 μ M or 8 μ M. Viability was measured at day 0, day 2, day 4 and day 6 through a presto blue assay.

- (A) Spheroid viability in CCLP SVC and PRH KD spheroids with increasing Niclosamide concentration over time. The data was normalised to vehicle control on day 0. Error bars represent SEM.
- (B) Spheroid viability in CCLP SVC and PRH KD spheroids with increasing Niclosamide at each time point where the data is normalised to the vehicle control at that data point. Error bars represent SEM and significant was calculated through two-tailed paired t-test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$) (N=3).

3.8.3 The Effect of Niclosamide on CCLP SVC and PRH KD in Cell Cycle Progression and Apoptosis

To further investigate the decreased sensitivity seen in the CCLP shRNA PRH KD cell lines compared to the CCLP SVC cell lines, Niclosamide treated CCLP SVC and PRH KD cells grown in 2D were assessed for changes to cell cycle and apoptosis through flow cytometry and protein expression. For flow cytometry experiments, CCLP SVC and PRH KD cells were treated with vehicle control or high Niclosamide concentration (3.2 μ M) for 24 hours, then harvested for apoptosis and cell cycle samples. For protein lysates, CCLP SVC and PRH KD were treated with vehicle control or 1 μ M, 2 μ M, 4 μ M or 8 μ M Niclosamide for 24 hours. Experiments were conducted as described for the CCA wildtype cell lines.

Figure 3.27 illustrates representative images of the cell cycle analysis, whilst **Figure 3.28** shows a summary of the analysed data. Similar to results seen with the CCLP wildtype, there is little change in the cell cycle in flow cytometry experiments upon Niclosamide treatment in CCLP SVC cells at 24 hours. However, surprisingly, there is a significant inhibition of the G1 phase in Niclosamide treatment compared to the vehicle control in the CCLP PRH knockdown line (**Figure 3.28B**).

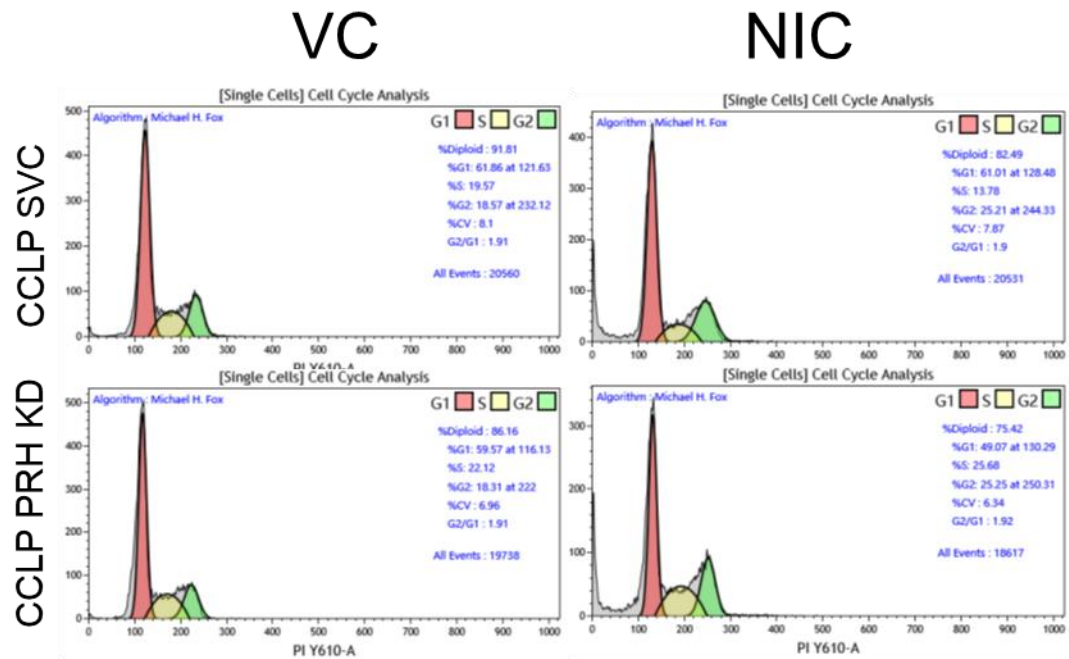


Figure 3.27: Representative Images showing Cell Cycle Analysis in Kaluza for Niclosamide Treated CCLP SVC and PRH KD cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green).

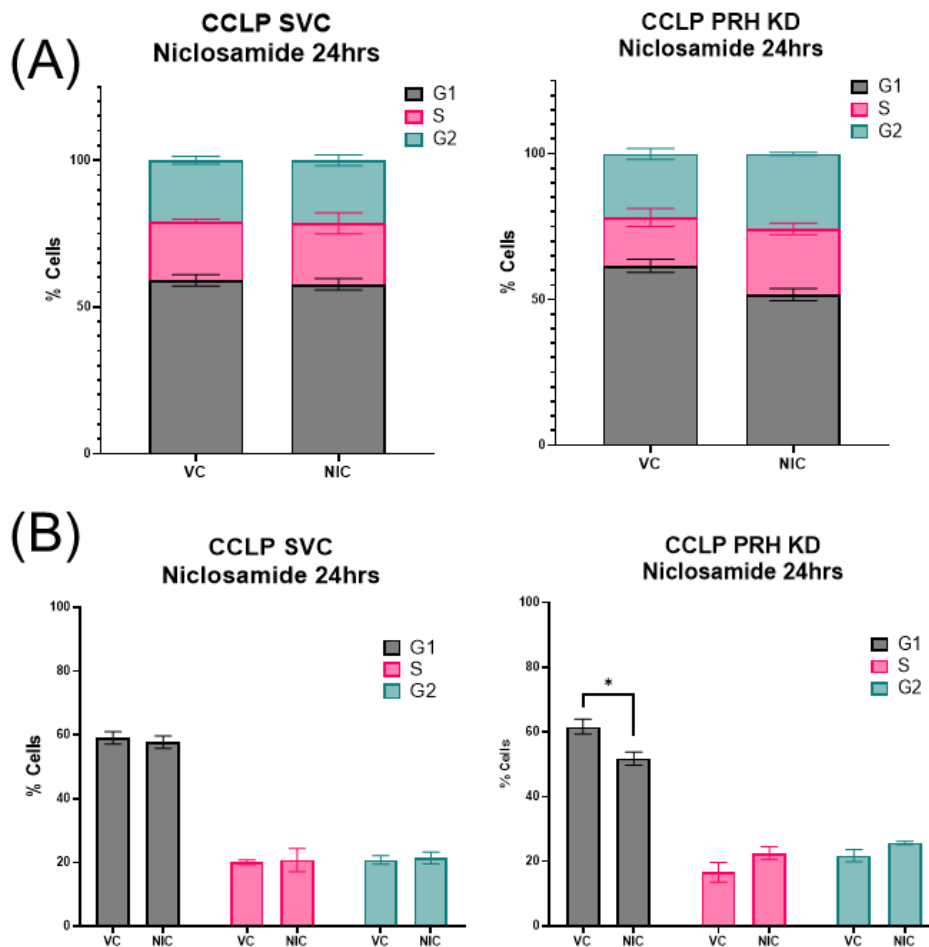


Figure 3.28: Cell Cycle Analysis in Niclosamide Treated CCLP SVC and PRH KD Cells.

Cells were treated with vehicle control (VC) or 3.2 μ M Niclosamide (NIC) 24hrs before harvesting for cell cycle experiments. Stained cells were run on a flow cytometer and data was analysis in Kaluza software. The experiment was repeated to three biological replicates. The analysis shows the proportion of the cell population in each stage of the cell cycle, G1, S and G2, for each treatment in each CCA cell line. Error bars represent SEM and two-tailed t-test was performed to record significant differences in changes between vehicle control and Niclosamide (N=3) (*=p<0.05).

(A) Overall cell cycle analysis of vehicle control or Niclosamide treated CCLP SVC and PRH KD cells.

(B) Statistical significance in cell cycle analysis between vehicle control and Niclosamide treated CCLP SVC and PRH KD cells.

Representative images and analysis of the experimental repeats for CCLP SVC and CCLP PRH KD for apoptotic cell death is summarised in **Figure 3.29** and **Figure 3.30**, respectively. Niclosamide treatment caused a significant decrease in live cells ($p<0.05$), as well as significant increases to both early apoptotic cells ($p<0.05$), and late apoptotic cells ($p<0.05$), in CCLP SVC cells. Although similar results were also observed in PRH KD cell line the effects of Niclosamide on apoptosis were less pronounced; the percentage of cells over the three cell death groups totalled to 13% in Niclosamide treated control cell but only 8% in Niclosamide treated PRH KD cells. **Figure 3.31B** shows that cleaved caspase 3 expression is initiated at lower concentrations of Niclosamide in SVC controls, and its expression is increased to a greater extent compared to PRH KD cells. The lower pro-apoptotic response and lower levels of cleaved caspase 3 expression in PRH KD cells shows that PRH KD cells are less apoptotic than control cells in response to Niclosamide treatment at 24 hours.

Figure 3.31A shows the results of western blotting for B-catenin in CCLP SVC and PRH KD cells with and without Niclosamide treatment. Upon Niclosamide treatment, B-catenin is significantly decreased compared to vehicle control in SVC cells, but not in PRH KD cells. Previously, B-catenin was shown to significantly decrease due to Niclosamide treatment in all CCA wildtype cell lines. Similarly, in the literature, B-catenin is shown to be ubiquitylated and degraded in response to Niclosamide (Kang et al., 2023). Thus, it is credible to hypothesise that B-catenin protein is degraded in response to Niclosamide in the CCA cells. The lack of B-catenin degradation in PRH KD cells, highlight differences in the two CCLP cell line biology and may be one reason PRH KD cells are less sensitive to Niclosamide treatment.

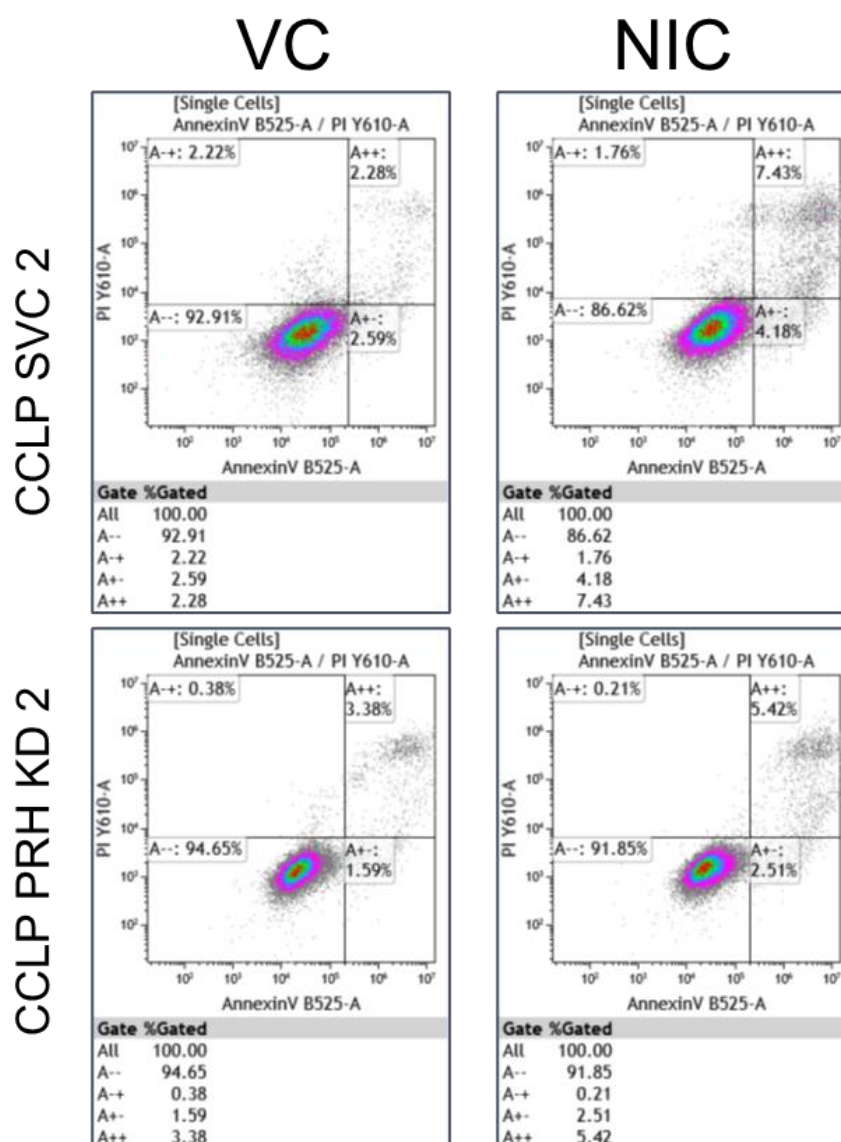


Figure 3.29: Representative Images showing Apoptosis Analysis in Kaluza for Niclosamide Treated CCLP SVC and PRH KD cells.

Cells were treated with vehicle control (VC) or 3.2µM Niclosamide (NIC) for 24hrs before harvesting for cell cycle experiments. Approximately 1x10⁶ cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each stage of cell death: live, early apoptosis, necrosis and late apoptosis.

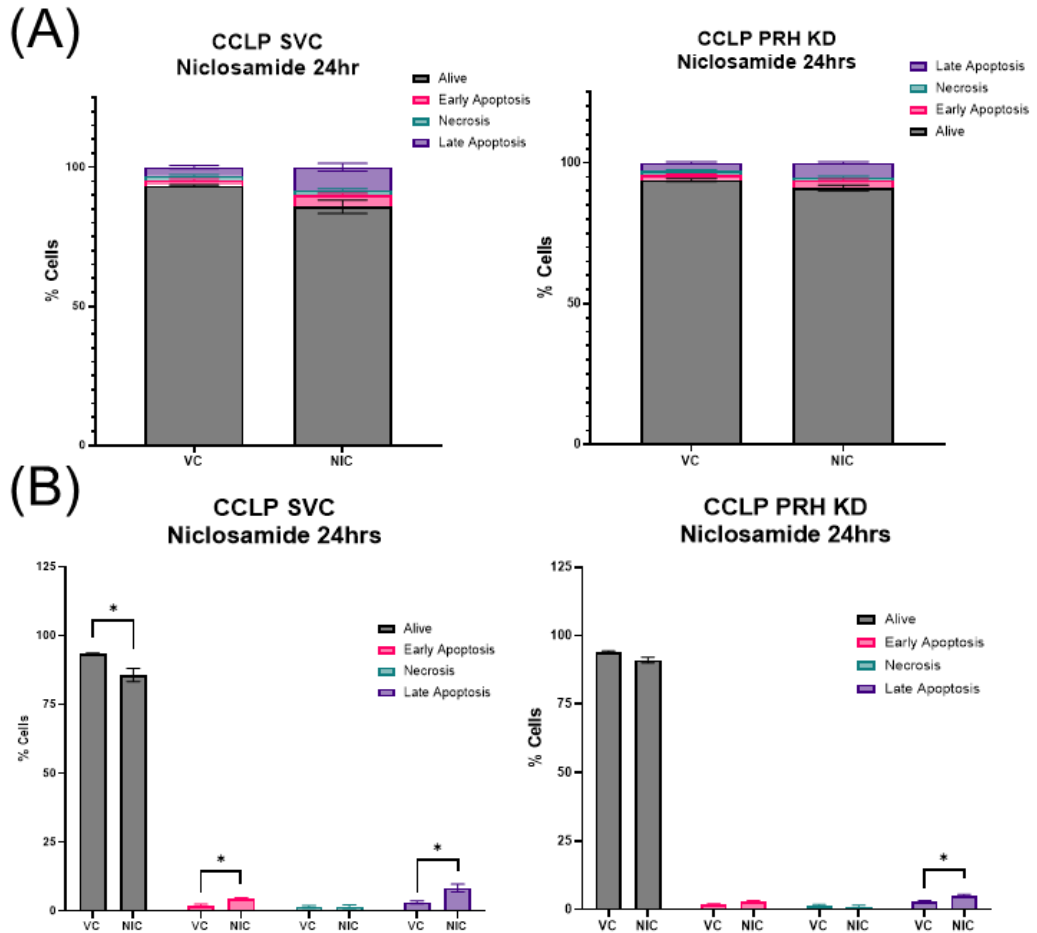


Figure 3.30: Apoptosis Analysis of Niclosamide Treated CCLP SVC and PRH KD Cells.

Cells were treated with vehicle control (VC) or 3.2μM Niclosamide (NIC) 24hrs before harvesting for apoptosis experiments. The stained cells were run on a flow cytometer and data was analysis in Kaluza software The analysis shows the proportion of the cell population in each stage of cell death: Live, Early Apoptosis, Necrosis and Late Apoptosis. Error bars represent SEM and two-tailed t-test was performed to record significant differences in changes between vehicle control and Niclosamide (N=3) (*=p<0.05).

(A) Overall apoptosis analysis of vehicle control or Niclosamide treated CCLP SVC and PRH KD cells.

(B) Statistical significance in apoptosis analysis between vehicle control and Niclosamide treated CCLP SVC and PRH KD cells.

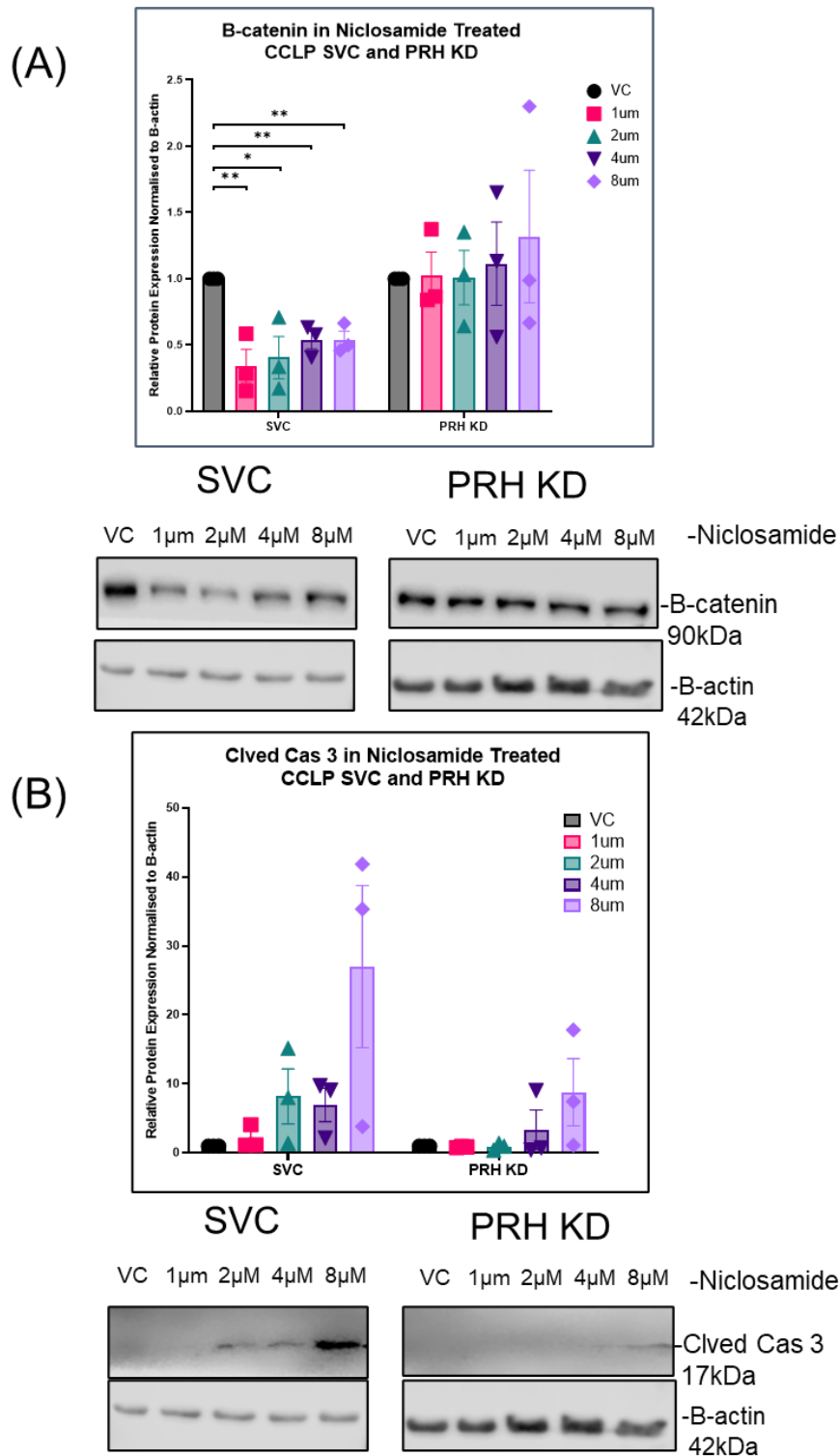


Figure 4.31: B-catenin and Cleaved Caspase 3 Levels in Niclosamide Treated CCLP SVC and PRH KD Cell Lines.

Cells were treated with vehicle control (VC) Niclosamide (NIC) for 24hrs before harvesting for protein samples. Cells were lysed with RIPA buffer and 30μg of protein were electrophoresed in SDS PAGE before transferring to membrane for antibody probing. Protein expression was calculated by normalising to B-actin expression and relativising to vehicle control over three experimental replicates. Error bars represent SEM and two-tailed t-test was performed to record significant differences (N=3) (*= $p < 0.05$, **= $p < 0.01$)

(A) Relative expression of B-catenin in Niclosamide treated CCLP SVC and PRH KD cell lines supplemented with a representative blot.

(B) Relative expression of Cleaved Caspase 3 in Niclosamide treated CCLP SVC and PRH KD cell lines supplemented with a representative blot.

3.9 Discussion

CCA cell line viability is inhibited by Niclosamide

Niclosamide dose response curves were performed in STR profiled CCA cell lines, CCLP, RBE and K KU-M055. After 24 hours incubation, the cells showed a 50% reduction of cell viability when Niclosamide was used in the 1µM-5µM concentration range. With longer Niclosamide incubation times (72 hours), the EC₅₀ was significantly reduced to the 0.1µM-1µM range. The Niclosamide EC₅₀ values calculated from these cell viability assays after 72 hours treatment in these cell lines, are in the same range as EC₅₀ values for Niclosamide sensitivity reported in a range of cancer cell lines (Ren et al., 2010). Even though the CCA cell lines have different mutational landscapes, different morphology and different patient profile (K KU-M055 is fluke-associated), the response to Niclosamide is similar, especially after 72 hours when all cell lines start to show cytostatic effects. Alongside this, other CCA cell lines K KU-100, K KU-213 and CCSW were shown to be sensitive to Niclosamide in the Jayaraman laboratory (Lee, 2021), suggesting Niclosamide is effective in a range of CCA tumours. Interestingly, the RBE cells were more resistant than the other cell lines. This may be linked to the *IDH1* mutation which causes altered cell metabolism in RBE and may have made the cells less sensitive to mitochondrial uncouplers. CCLP has mutated *TP53* and p53^{-/-} cells have been shown to have increased sensitivity to Niclosamide (Kumar et al., 2018), therefore this may account for some of the sensitivity CCLP show towards Niclosamide. However, as K KU-M055 is the most sensitive cell line to Niclosamide, and harbours a different mutation, *MAP2K1* or MEK1, highlighted in the DepMap Portal (Tsherniak et al., 2017), it is likely that Niclosamide, due

to its unspecific mechanisms of action, is non discriminative and reduces CCA cell viability despite mutational background.

Niclosamide has less effect in BECs

Importantly whilst BECs are sensitive to Niclosamide at 24 hours of treatment, with an EC50 value similar to that of the CCLP cells, incubation with the drug for 72 hours does not promote further increase in sensitivity. This differential response may become of clinical significance if it can be shown that Niclosamide can selectively target CCA cells over the normal tissue. One caveat is that the difference in sensitivity between CCA cells and normal cholangiocytes is small and only observed with longer drug exposure. A paper published during this study also examined CCA cell lines in Niclosamide dose response experiments (Kulhawatsiri et al., 2023), where five CCA cell lines of the KKU group (liver fluke associated CCA) were tested for Niclosamide sensitivity against a human cholangiocyte cell line MMNK1 which is more representative of an untransformed cell line. The EC50 values in the paper were similar to values obtained for KKU-M055 cell within this work. In addition, they report the selective inhibition of CCA cell lines over the MMNK1 cell line by Niclosamide, agreeing with the results found in this chapter.

Niclosamide affects mitochondrial membrane polarisation and global protein ubiquitination in all CCA cells

Although Niclosamide is classically an anti-helminth drug, its repurposed employment as an anti-cancer drug has been well-documented (reviewed: Li et al., 2014). Many studies have shown Niclosamide can cause mitochondrial uncoupling in cancer cells (Khanim et al., 2011) (Alasadi et al., 2018) (Kumar et al., 2018), thus mitochondrial depolarisation was examined in the Niclosamide treated CCA cells with MitoTracker staining. Niclosamide was able to promote

significant levels of uncoupling, within an hour of treatment, at concentrations as low as 1 μ M. The effect of Niclosamide on CCA cell mitochondrial depolarisation was reported in the KKU cell lines with 48 hours Niclosamide treatment (Kulhawatsiri et al., 2023). The level of mitochondrial uncoupling between the CCA cancer cells and the BECs did not significantly differ. Although it might be assumed that the Niclosamide-sensitive cell types will show increased mitochondrial uncoupling with Niclosamide treatment, there is no correlation between sensitivity and MitoTracker staining in our studies. In Kumar et al., although p53^{-/-} cells showed significant resistance to Niclosamide treatment in cell viability assays, p53^{-/-} and p53^{+/-} cells showed no difference in rates of mitochondrial uncoupling in Seahorse experiments (Kumar et al., 2018). The authors saw that the differential response was due to an indirect effect of the mitochondrial uncoupling on toxic metabolite formation and clearance. Therefore, downstream effects on metabolism may also be responsible for sensitivity in the CCA cells

Niclosamide has been reported to induce global ubiquitylation (Cheng et al., 2017) and the ubiquitylation of specific targets, such as B-catenin (Kang et al., 2023). The levels of global protein ubiquitylation were investigated in Niclosamide treated CCA through western blotting with a ubiquitin antibody, as Universal levels of ubiquitylation were increased in all the CCA cell lines and the levels of ubiquitylation appear to correlate with Niclosamide sensitivity. Ubiquitylation is important in protein stability and cellular homeostasis and is a pivotal regulatory mechanism in the control of cell cycle, cell growth and apoptosis and autophagy (Zhang et al., 2004). For example, ubiquitylation regulates the turnover of cyclin proteins, CDKs and CDKIs (Zou & Lin, 2021). Similarly, ubiquitylation promotes apoptotic signalling through engaging p53 pathway, inappropriate degradation of proteins, and activation of NF κ B

pathway to determine cell survival or cell death (Orlowski, 1999). Thus, it is likely that the Niclosamide induced ubiquitylation of proteins could be causing a decrease in cell viability in CCA cells.

The effects of Niclosamide on apoptosis are cell line specific

Niclosamide promoted the cleavage of caspase 3 in a dose-dependent manner in CCLP and KKU-M055 cells, but not in RBE cells. Similarly, Niclosamide induced a significant increase to the amount of Annexin V/PI-stained cells in flow cytometry experiments in CCLP and KKU-M055 cells, but not in RBE cells. As previously discussed, this may be due to the *IDH1* mutation in RBE cells that may have altered RBE cell metabolism to reduce the effect of Niclosamide on apoptotic signalling and cell death. IDH1 mutation has been previously seen to induce resistance of apoptosis through mitochondrial stress and hyper-succinylation, via succinate dehydrogenase inhibition, in non-CCA cells (Li et al., 2015). The amount of Niclosamide-induced apoptosis correlates with the amount of Niclosamide-induced protein ubiquitylation.

Interestingly, there was no statistically significant difference in cell cycle progression in Niclosamide treated in CCLP and KKU-M055 cells compared to vehicle controls in flow cytometry experiments. This suggests that any reduction in cell viability at 24 hours treatment, is caused by Niclosamide cytotoxicity rather than an anti-proliferative effect.

Cell cycle arrest of Niclosamide does not occur at 24 hours but there are changes to the level of expression of cell cycle proteins

Niclosamide has been shown to induce apoptosis and cell cycle arrest in multiple cell cancer types, and in *in vitro* and *in vivo* models (Khanim et al., 2011) (Li et al., 2014) (Ye et al., 2014) (Chai et al., 2020) (Kang et al., 2023). In these studies, the use of Niclosamide around the 0.5µM-5µM range is sufficient

to elicit significant aberrations in cell cycle and promote apoptosis but these are observed at 48 hours and 72 hours drug incubation. Thus, to examine cell cycle arrest by Niclosamide in the different cell types tested it is likely that a longer timepoint for Niclosamide incubation was needed. Additionally, alternative proliferation assays such as EdU incorporation assays could also provide more information about the effect Niclosamide has on CCA cell division.

Despite the lack of change in cell cycle, Niclosamide caused a major decrease in cell cycle markers Cyclin D proteins and p21 in all CCA cell lines. A reduction of Cyclin D1 in Niclosamide treated cells has been previously reported (Kang et al., 2023) (Tomizawa et al., 2013) (Arend et al., 2014), and similar to the work in this chapter, the reduction of Cyclin D1 requires low drug concentrations for a potent effect on Cyclin D1 expression. In Arend et al., the decrease in Cyclin D1 is linked to a Niclosamide-induced down-regulation of Wnt signalling. However, Cyclin D1 protein expression is absent before the loss of Wnt mediators LRP and B-catenin (Arend et al., 2014).

B-catenin was also investigated in Niclosamide treated CCA cells, as B-catenin had been previously reported to undergo Niclosamide-induced ubiquitylation and subsequent degradation through the proteasomal pathway (Kang et al., 2023). Niclosamide was able to promote a dose dependent decrease to B-catenin across the CCA cell lines. As total ubiquitylation is increased in Niclosamide treated CCA cells in this project, this could explain the decrease in B-catenin. When comparing the decrease in B-catenin protein in response to Niclosamide, to the decrease in Cyclin D1, the decrease in Cyclin D1 occurs at lower concentrations and to a much greater effect. It is likely that other mechanisms, including the downregulation of Wnt signalling, are also responsible for the loss of Cyclin D1. Cyclin D1 is commonly ubiquitylated and degraded by the proteasome for its regulation through the cell cycle (Alao,

2007). Therefore, Cyclin D1 protein level may decrease through a Niclosamide-induced global protein ubiquitylation and proteasomal degradation mechanism.

Niclosamide also promotes a dose dependent decrease to PRH protein expression in all of the CCA cells tested here. As PRH functions as an oncoprotein in CCA via Wnt/B-catenin signalling and promoting Cyclin D2 expression, the decrease in expression to PRH, B-catenin and Cyclin Ds is beneficial (Kitchen et al., 2020). The expression of *HHEX* was unchanged with short-term and long-term Niclosamide treatment in all CCA cell lines, indicating that the decrease to PRH in Niclosamide treatment is occurring at a protein level. As it had been previously reported that Niclosamide can cause protein degradation (Kang et al., 2023), Niclosamide was used to treat CCA cells alongside a proteasomal inhibitor, MG132, to observe whether the inhibition of the proteasome could rescue Niclosamide-induced PRH degradation. Although the results were not conclusive MG132 was able to increase PRH protein levels from the levels in Niclosamide treated cells and was also able to increase PRH protein levels in untreated cells in at least two cell lines, CCLP and KKU-M055. Previous findings show PRH is an interactor of the proteasome in K562 haematopoietic cells (Bess et al., 2003) and it can be processed by the proteasome rather than being simply degraded (Noy et al., 2012). To further confirm the hypothesis that PRH is proteasomally regulated in CCA, the MG132 concentration and Niclosamide concentration would require optimisation for each CCA cell line to show a statistically significant rescue of PRH protein. For example, in RBE cells, 8µM Niclosamide did not decrease PRH protein levels sufficiently to show significant rescue by MG132; in the KKU-M055 cells, 8µM Niclosamide decreased PRH but the concentration of MG132 was insufficient to reproducibly restore its level. To examine whether PRH is directed to the proteasome by Niclosamide-induced ubiquitylation, co-immunoprecipitation

experiments with a tagged ubiquitin protein could be performed to observe whether PRH is modified by ubiquitin and whether this modification increases under Niclosamide treatment.

Niclosamide treatment causes reduction in a range of protein expressions in a dose dependent manner as well as increased protein ubiquitylation. Therefore, the decrease in cell cycle proteins is possibly a result of global protein degradation and not specific cell cycle arrest. Additionally, in the western blotting experiments, Notch-3 and Vimentin expression did not change in response to Niclosamide treatment. Full length Notch-3 and Vimentin are large proteins found on the plasma membrane or in the cytoskeleton (Zhou et al., 2022) (Patteson et al., 2020). Thus, these proteins may not show the rapid down regulation observed for PRH, B-catenin and the cyclin D proteins by a potential Niclosamide-induced ubiquitin-directed proteosomal degradation.

Niclosamide sensitivity is greater in CCLP control cells

A decrease in PRH protein is a consequence of Niclosamide treatment, and we wondered whether PRH knockdown would affect Niclosamide sensitivity. Interestingly we showed that Niclosamide sensitivity was significantly reduced in the PRH KD cells after 72 hours, demonstrating that the PRH KD cell line is less sensitive to Niclosamide at that time point when the drug can elicit both cytostatic and cytotoxic effects. This was further investigated in 3D spheroids where the differential response to Niclosamide between SVC and PRH KD spheroids was confirmed over a temporal study.

In phenotypic experiments, Niclosamide induced apoptosis in both cell lines, however the amount of apoptosis in the SVC cells was greater. In CCLP SVC cells, high levels of cleaved caspase 3 protein expression were initiated with a low concentration of drug, and to a greater extent, compared to PRH KD cells;

this increase in pro-apoptotic cleavage protein agrees with the apoptosis flow cytometry results. Similar to the CCLP wildtype cell line, there were no biologically important changes to SVC and PRH KD cells in cell cycle progression with Niclosamide treatment at 24 hours. Interestingly, the level of B-catenin is significantly decreased in SVC controls with increasing Niclosamide concentration, but in PRH KD cells the level of B-catenin is lower, and this does not change with increasing Niclosamide concentration. The increased B-catenin stability suggests that B-catenin turnover is decreased in the PRH KD cells. Lower protein turnover and decreases in apoptotic proteins could account for the decreased sensitivity to Niclosamide in the PRH KD cells.

To conclude, Niclosamide induces mitochondrial uncoupling, ubiquitylation and subsequent protein degradation in CCA cell lines. The accumulation of cellular stress and imbalance in protein homeostasis, brought about by Niclosamide, signals for downstream apoptosis and promotes the cleavage of caspase 3 (at 24 hours), whilst the degradation of cell cycle proteins results in cell cycle arrest after longer timepoints (post 24 hours). With this model, the potency of Niclosamide is dependent on the ability of the cell to promote ubiquitylation and is also dependent on the ability of the cell to survive the downstream apoptotic and anti-proliferative signals which ultimately is dependent on each cell genetic and metabolic makeup.

CHAPTER 4:

Investigating the effect of
Niclosamide and Palbociclib
Combinations on CCA viability

4.1 Introduction:

Cycle inhibitors have recently been utilised in pre-clinical studies as a strategy for CCA treatment (Arsenijevic et al., 2023). In previously published work, the cell cycle inhibitor Palbociclib was shown to reduce cell viability of CCA cell (Kitchen et al., 2020). However, when Palbociclib was used clinically in a basket trial to assess efficacy against *CDKN2A* loss or mutated biliary cancers, it was not found to give benefit in a small study with 10 patients (Baghdadi et al., 2019). Combination therapies are now being investigated as better treatments for CCA (Zeng et al., 2023), and to improve Palbociclib efficacy (Alvarez & Malumbres, 2020). Combinations of drugs have been used to provide additive benefit in multiple type of cancer (Mokhtari et al., 2017). The use of drug combinations allows for many favourable outcomes including improving therapeutic efficacy, decreasing dosage to avoid toxicity, overcoming drug resistance, and improving the on-target effects of a drug (Mokhtari et al., 2017). Combination therapy can also give rise to synergistic effects. Synergism is when two drugs promote an effect greater than the sum of the effect from the individual drugs combined (reviewed: Duarte & Vale, 2022). Initial experiments in the Jayaraman laboratory suggested that Palbociclib and Niclosamide may have a greater effect on reducing cell viability when used in combination (Lee, 2021). In the previous results chapter, Niclosamide was shown to be an inhibitor of CCA cell viability that induces mitochondrial uncoupling, protein ubiquitination and cell apoptosis. The aims of this chapter are to investigate whether there is synergism between Niclosamide and Palbociclib in 2D and 3D CCA models and to investigate any changes in cell cycle progression and apoptosis that occur during combination treatment.

4.2 The Effect of Palbociclib on CCA Cell Line and BEC Viability

Initial cell viability experiments were performed to confirm previous findings which indicated that Palbociclib is a potent inhibitor of CCA cell viability (Kitchen et al., 2020) (Lee, 2021). The effect of Palbociclib was investigated by performing cell viability assays with increasing Palbociclib concentration in the three CCA cell lines, CCLP, RBE and KKU-M055. This was repeated in primary BECs to assess whether Palbociclib is more potent in the CCA cell lines compared to primary cholangiocytes.

The experiments were performed by seeding 1×10^4 cells in 96 well plates and, after adhering overnight, subjecting the cells to a serial dilution of Palbociclib or a vehicle control. The range of Palbociclib used was from 100pM to 100 μ M to produce complete cell viability curves with increasing dose (dose response curves). The drug was incubated with the cells for either 24hr or 72hrs before measuring cell viability using an MTT assay (described in section 2.2.3). A 100% DMSO background control was subtracted from experimental MTT cell viability values for the different Palbociclib concentrations and MTT readings were made a percentage of the vehicle control value, as described previously (section 3.1). The *relative* EC50 concentration (the concentration at which 50% cell viability was achieved) was calculated using Graph Prism software. Three independent biological repeats of the experiment were performed each in triplicate. **Figure 4.1** shows the dose response curves and EC50 values, plus confidence intervals, for Palbociclib in CCLP, RBE, KKU-M055 cells and BECs after 24hrs of drug treatment. All three CCA cell lines were sensitive to Palbociclib. CCLP cells show the highest EC50 value of 12.9 μ M, followed by RBE cells that show

an EC50 value of 8.9 μ M and KKU-M055 show the lowest EC50 value of 5.3 μ M.

In contrast, the BECs are insensitive to Palbociclib with an EC50 >50 μ M.

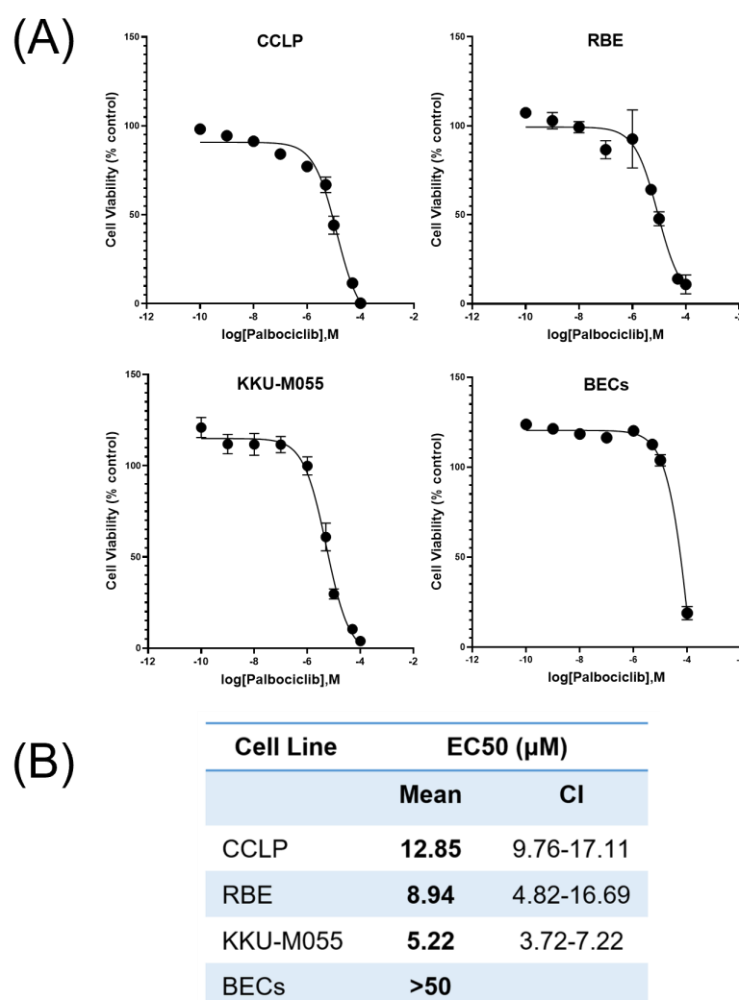


Figure 4.1: Palbociclib Dose Response Experiments in CCA cell lines and BECs at 24hrs.

CCLP, RBE, KKU-M055 and BECs were plated at 1×10^4 cells per well in triplicate in a 96 well plate and treated with a range of Palbociclib concentrations (100pM-100 μ M) or vehicle control for 24hrs. After 24hrs treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Palbociclib concentration to create dose response curves in GraphPad Prism. EC50 values, plus 95% confidence intervals (CI), were calculated by the software.

- (A) Palbociclib dose response curves in CCLP, RBE, KKU-M055 and BEC cells after 24hrs. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
- (B) Palbociclib sensitivity across the CCA cell lines and BECs. Relative EC50 values plus confidence intervals CI (μ M) were calculated using the GraphPad Prism software.

The mean maximum serum concentration of Palbociclib was found to be 81.8 μ g/L, which equates to 182.8nM (Royer et al., 2021). Similarly, another study investigating a range of dose weights and regimes recorded Cmax values

with a range of 41.4-185.5ng/mL, or 91.8nM-414.5nM, with the higher concentrations found in patients with multiple doses and/or greater doses (Tamura et al., 2016). As the EC50 values for CCA cells reported above were above the maximum serum concentration range, Palbociclib dose response curves were also measured after 72 hours incubation. **Figure 4.2** shows the dose response curves and EC50 values, plus confidence intervals, for Palbociclib in the CCA cell lines and BECs after 72 hours. The EC50 values decrease in CCLP (from 12.9µM to 1.5µM) and RBE (from 8.9µM to 2.6µM) cells with the longer Palbociclib incubation, however the decrease to EC50 in the KKKU-M055 cells was not as great (from 5.3µM to 4.9µM). Somewhat surprisingly, the EC50 value for Palbociclib in the BECs remained relatively high at 31.9µM. These results indicate that there may be in a significant therapeutic window for Palbociclib in CCA, where it could target the cancer cells whilst not influencing the normal tissue (**Figure 4.2C**).

The EC50 values reported in CCA cell lines are in the micromolar range and similar range to reported values for cell lines such as breast cancer (MCF-7, MDA-MB-468) and melanoma (M229, M238), ovarian (A2780), and prostate (Du-145, Ca-2B, PC-3) (Tadesse et al., 2017). Of note, in the experiments reported above, treatment with the 99.98% Palbociclib compound (SelleckChem) resulted in a 10x difference in EC50 values from the nanomolar range EC50 reported previously in CCLP cells (Kitchen et al., 2020) and (Lee, 2021). One possible reason for this difference in drug potency is that the Palbociclib used currently may be less soluble than the Palbociclib compound used in the original experiments. The protocol for Palbociclib solubilisation described in materials and methods (section 2.6) took extensive optimisation and involved changing solvent, and several physical methods to dissolve the powder. Several different Palbociclib compounds from different manufacturer

sources were found to have poor solubility. Thus, differences in solubility are a possible source of the discrepancy between current and reported EC₅₀ values.

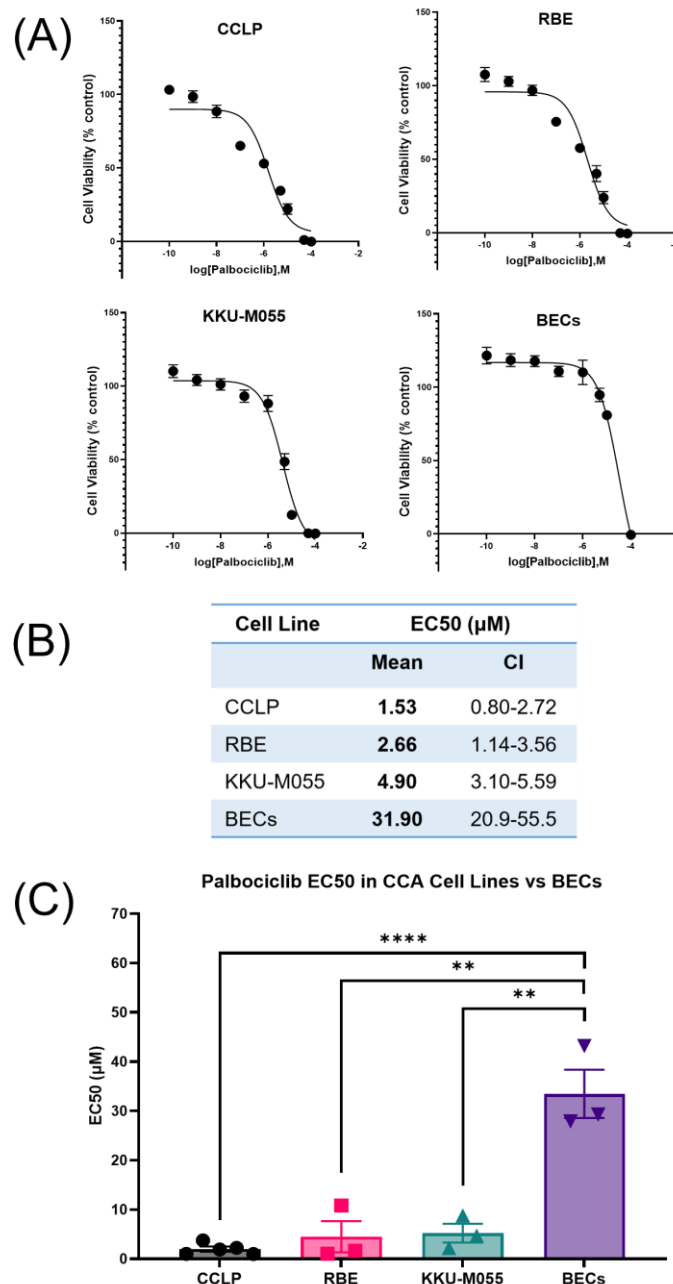


Figure 4.2: Sensitivity of CCA cell lines and BECs to Palbociclib at 72hrs.

CCLP, RBE, KKKU-M055 and BECs were plated at 1×10^4 cells per well in triplicate in a 96 well plate and treated with a range of Palbociclib concentrations (100pM-100μM) or vehicle control for 72hrs. After 72hrs treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Palbociclib concentration to create dose response curves in GraphPad Prism. EC₅₀ values, plus 95% confidence intervals (CI), were calculated by the software.

- (A) Palbociclib dose response curves in CCLP, RBE, KKKU-M055 and BEC cells after 72hrs. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
- (B) Palbociclib sensitivity across the CCA cell lines and BECs. Relative EC₅₀ values plus confidence intervals CI (μM) were calculated using the GraphPad Prism software.
- (C) Comparison of Palbociclib sensitivity in CCA cell lines and BECs. An unpaired t-test was used to measure significant differences between CCA cell lines and BECs. Error bars represent SEM (**=p<0.01, ****=p<0.0001).

It was previously reported that Palbociclib sensitivity in CCLP cells is influenced by PRH expression, with Ad-PRH infected PRH-high CCLP cells being more sensitive to Palbociclib than Ad-Empty control cells (Kitchen et al., 2020) (Lee, 2021). To determine whether PRH knockdown has the opposite effect Palbociclib dose response curves were obtained using the CCLP shRNA SVC and PRH KD cells produced in this work (see Chapter 3), with experiments performed as described for the wildtype cell lines, and Palbociclib sensitivity was assessed by MTT assay after 24 hours and 72 hours drug incubation. The dose response curves after 24 hours and 72 hours treatment are shown in **Figure 4.3A**, along with the EC₅₀ values in **Figure 4.3B**. After 24 hours of exposure to Palbociclib, there was little decrease in cell viability, (**Figure 4.3A**), just as was previously observed for wildtype CCLP cells (**Figure 4.1A**). Moreover, there was no difference in the response to Palbociclib between the SVC and PRH KD cells at 24 hours (**Figure 4.3A** left panel). However, after 72 hours treatment, the PRH KD cells showed a 5-fold higher EC₅₀ value compared to SVC cells (SVC EC₅₀= 1.09µM, PRH KD EC₅₀= 5.26µM) (p<0.05). (**Figure 4.3B** right panel).

In conclusion, Palbociclib shows selective inhibition of CCA cell lines over BECs, and this sensitivity is influenced by PRH protein expression. However, although cell viability is decreased when using 100nM Palbociclib in the CCLP and RBE cells, the EC₅₀ values range outside of the reported maximum serum concentration range. Therefore, combination studies with Palbociclib were investigated, to maintain Palbociclib potency whilst reducing the dosage of Palbociclib needed.

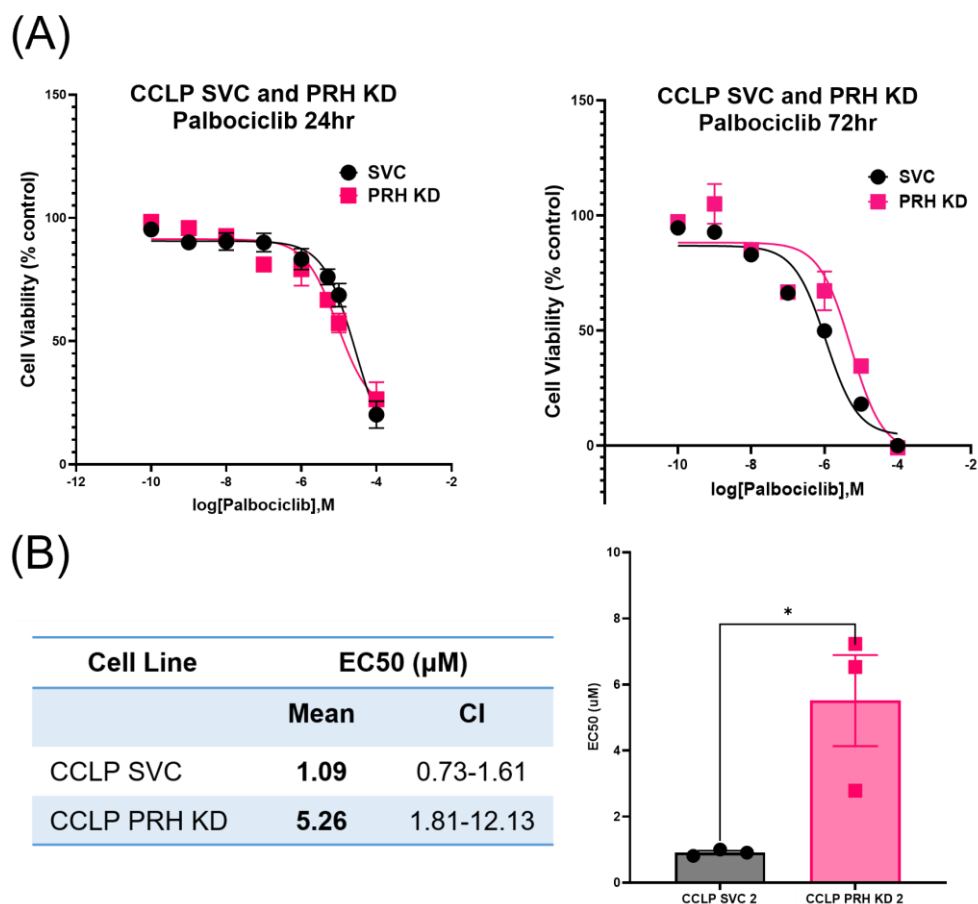


Figure 4.3: Palbociclib Dose Response Experiments in CCLP SVC and PRH KD cell lines. CCLP SVC 2 and PRH KD 2 cells were plated at 1×10^4 cells per well in triplicate in a 96 well plate and treated with a range of Palbociclib concentrations (100pM-100 μ M) or vehicle control for 24hrs or 72hrs. After treatment, cell viability was measured through MTT assay. Values were normalised to the vehicle control and relative cell viability was plotted against log Palbociclib concentration to create dose response curves in GraphPad Prism. EC50 values, plus 95% confidence intervals (CI), were calculated by the software.

- (A) Palbociclib dose response curves in CCLP SVC 2 and PRH KD cell lines after 24hrs and 72hrs treatment. Assays were performed three times. Error bars represent SEM and when error bars cannot be seen, they are smaller than the symbols (N=3).
- (B) Palbociclib sensitivity in the CCLP SVC versus the CCLP PRH KD cells. Relative EC50 values plus confidence intervals CI (μ M) were calculated using the GraphPad Prism software. A ratio paired t-test was used to measure significant differences between CCLP SVC and PRH KD EC50 values. Error bars represent SEM (*= $p < 0.05$).

4.3 Investigating Niclosamide and Palbociclib

Synergism in CCA cell lines.

Previous work showed that Niclosamide and Palbociclib in combination were able to inhibit CCA cell viability and preliminary work suggested that this might occur in a synergistic manner (Lee, 2021).

The method used in this thesis to assess possible synergism between Niclosamide and Palbociclib is the Chou-Talalay method (Chou, 1976, 1977). The Chou and Talalay method measures synergism by calculating a Combination Index (CI) value. CI values below 1 indicate synergism, CI values equal to 1 indicate additivity, and CI values above 1 indicate antagonism. To see full methods on synergism calculations, see section 2.2.3.2-3

It was established in the previous chapter that Niclosamide has both cytotoxic and cytostatic effects at 72 hours and therefore its effects on cell viability are more potent at this time point. Since Niclosamide and Palbociclib both decrease cell viability effectively at 72 hours, this was the timepoint chosen for the combination experiments. As recommended with the Chou-Talalay method, each drug was used in its working concentration range. This is referring to the linear part of the sigmoidal curve generated in dose response assays. If the drug is used outside of the linear working range, the effect of synergism when used in combination will not be shown (i.e. the drug is either having no effect on the cell or it has caused a 100% reduction in cell viability as a single agent). A proposed method by Chou and Talalay of obtaining a range of concentrations for synergism experiments is using a serial dilution determined by the EC₅₀ value i.e. 0.125X, 0.25X, 0.5X, X, 2X, 4X, 8X, where X=EC₅₀. The EC₅₀ values for Niclosamide in the CCA cell lines are 0.45µM, 1.31µM, and 0.39µM in CCLP,

RBE and KKU-M055 cells, respectively. As these values are within proximity, the working concentrations for Niclosamide were kept the same across the cell lines. The range of Niclosamide concentrations used was: 62.5nM, 125nM, 250nM, 500nM, 1µM, 2µM and 4µM. These doses would result in cell viability values that span where Niclosamide has a linear working effect and where the cell viability will be between 0% and 100% (**Figure 4.1**). Regarding Palbociclib, the EC50 values were more varied between the cell lines. The EC50 values were 1.54µM, 2.66µM and 4.90µM in CCLP, RBE and KKU-M055 cells, respectively. Therefore, the working concentration range was adjusted for each cell line. The working concentrations included concentrations where a reduction in cell viability is first seen in the dose response curves and concentrations where cell viability is almost 100% reduced. The median concentration is based on the EC50 and the points between give equi-distance concentrations for a range of full coverage. Thus, the concentrations for each of the cell lines were the following: CCLP (1nM, 10nM, 100nM, 1µM, 5µM, 10µM, 15µM), RBE (31.5nM, 125nM, 500nM, 2µM, 4µM, 8µM, 16µM), and KKU-M055 (500nM, 1µM, 2µM, 4µM, 6µM, 8µM, 10µM):

Cell Line	1	2	3	4	5	6	7
CCLP	1nM	10nM	100nM	1µM	5µM	10µM	15µM
RBE	31.5nM	125nM	500nM	2µM	4µM	8µM	16µM
KKU-M055	500nM	1µM	2µM	4µM	6µM	8µM	10µM

Although this experimental design uses non-constant ratios, the parameters m , the coefficient or the measure of the slope of the graph, and D_m , the dose at which there is a median response or EC50, can be obtained for each single drug so the CI values for each combination can be calculated (see section 2.2.3.3 for full explanation). The CCA cells were plated at 1×10^4 cells per well

in a 96-well plate, left to adhere overnight, and treated with the dilution series of Niclosamide, the dilution series of Palbociclib or the dilution series of both drugs in combination. Cells were incubated with the treatment for 72hrs before measuring cell viability using an MTT assay. The experiment was performed three times in triplicate.

Figure 4.4 shows the effect of Niclosamide (Black) and Palbociclib (Magenta) plus their combination (Teal) on the viability of CCLP, RBE and KKU-M055 cells compared to vehicle control. At each point along the x-axis is the cell viability data when cells are treated with Niclosamide at concentration *A*, Palbociclib at concentration *B*, and when both drugs are used at concentrations *A* and *B* simultaneously. There are seven different data points which test different ratios of Niclosamide and Palbociclib. The concentrations used in each of the data points are shown beneath the x-axis, note that these are different between cell lines.

The combination of drugs decreases cell viability beyond that seen with either drug alone. The decrease in cell viability caused by the drugs in combination, compared to the drugs alone, is statistically significant at multiple data points. For example, the reduction in cell viability is greater in combination than with either drug alone, when using the drugs at their approximate EC50 values; specifically, 0.5µM:1µM in CCLP cells, 0.5µM:2µM in RBE cells, or 0.5µM:4µM in KKU-M055 cells, cell viability is statistically significantly decreased compared to each drug alone ($p < 0.05$, $p < 0.01$).

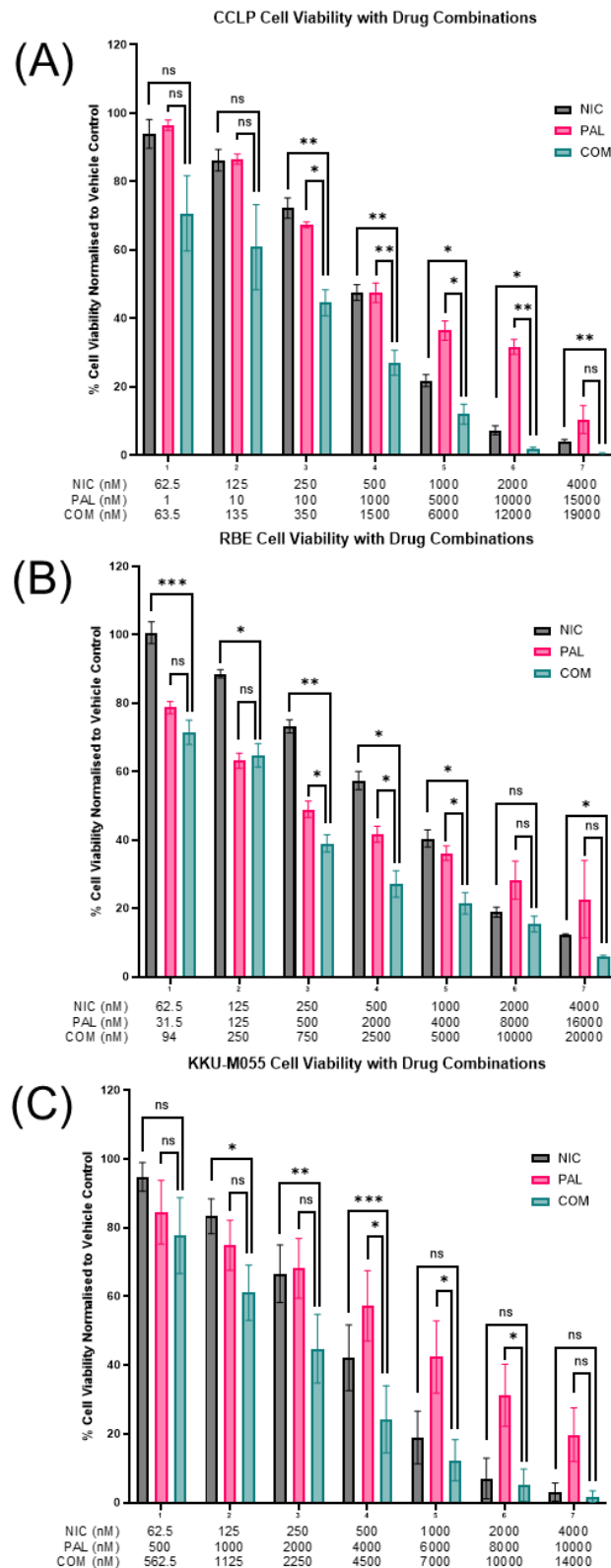


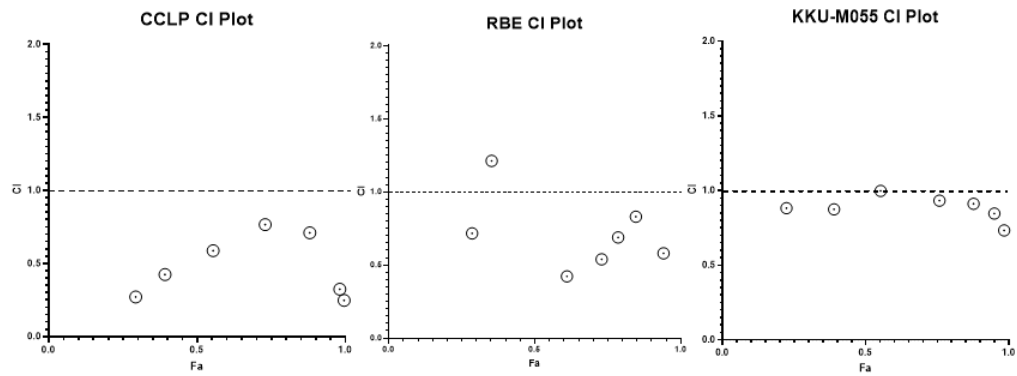
Figure 4.4: Cell Viability in Niclosamide and Palbociclib Treated CCA Cell Lines.

Cells were plated in triplicate at 1×10^4 cells per well in a 96-well plate and left to adhere overnight. Cells were treated with a range of Niclosamide or Palbociclib or both in combination; specific concentrations are given for each cell line underneath the graph. Cells were incubated with the drug treatment for 72 hours then cell viability was measured with MTT assay. Background values were subtracted from experimental values, and then normalised to vehicle control. The experiment was repeated to three experimental replicates. Error bars represent SEM and two-tailed paired t-test was performed to record significant differences between response to single drugs and drugs in combination ($N=3$). The experiment was performed in (A) CCLP (B) RBE and (C) KKU-M055 cells.

To calculate synergism values, the raw data was used to calculate a line of best fit between the log of the drug concentration (logD) and the log of the response ($\log(Fa/Fu)$) in a Median Effect plot (Chou, 1976, 1977). The Fa is the fraction affected and the Fu is the fraction unaffected; these values are from the MTT data. A linear equation allows the responses to be equated to any given concentration and allows the calculation of the Combination Index (CI). Synergism values were calculated for every combination data point tested. For full details on synergism calculations see section 2.2.3.3 in the methodology.

Figure 4.5A shows the Combination Index plot for the combination of Niclosamide and Palbociclib in CCLP, RBE and K KU-M055 cells. Here combination index (CI) value is recorded against response (Fa), for each combination data point tested. **Figure 4.5B** reviews the CI values with their respective response (Fa). Niclosamide and Palbociclib show synergism in all three cell lines as defined by $CI < 1$. CCLP cells show the most potent synergism (lowest CI values) across the different responses recorded with synergism ranging from strong to moderate. RBE cells tended to become more synergistic as $Fa > 0.5$. In K KU-M055 cells, the drug combination is also synergistic but less so than in CCLP cells.

(A)



(B)

Fa	CI
0.293	0.271
0.391	0.425
0.554	0.588
0.729	0.766
0.880	0.710
0.981	0.324
0.995	0.248

Fa	CI
0.285	0.716
0.352	1.212
0.610	0.422
0.729	0.539
0.785	0.689
0.846	0.830
0.940	0.580

Fa	CI
0.223	0.883
0.389	0.875
0.552	0.998
0.758	0.933
0.876	0.912
0.949	0.847
0.983	0.733

Figure 4.5: Combination Index (CI) Plots for Combination Treated CCA Cell Lines.

Cell viability data from Figure 4.4 was used to make a median response plots between drug concentration and response. From these graphs, combination index (CI) values were calculated and plotted against the corresponding response (Fa) values in a CI plot. Synergism occurs when $CI < 1$, additivity occurs when $CI = 1$ and antagonism occurs when $CI > 1$.

- (A) Combination Index Plot that shows the degree of synergism for every combination data point tested.
- (B) A summary of Combination Index (CI) values against the degree of response that was measured (Fa), where if $Fa = 1$ cell viability would be 0.

4.4 Investigating Niclosamide and Palbociclib Combination in Breast Cancer Cells

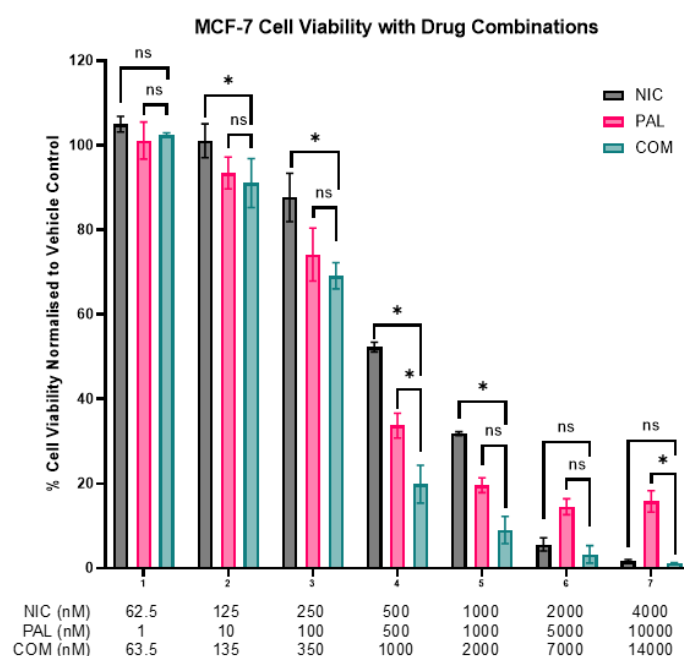
Palbociclib in combination with endocrine therapy is the first line of treatment for patient with HR (Hormone Receptor)+/ HER2- (Human Epidermal Growth Factor Receptor 2) metastatic breast cancer (Wang et al., 2024). Therefore, it is also of clinical interest to assess the effectiveness of Niclosamide and Palbociclib in combination in hormone receptor positive breast cancer cells.

Synergism experiments with Niclosamide and Palbociclib were conducted in MCF-7 cells, a luminal (hormone receptor positive) carcinoma cell line from a breast cancer metastasis (Soule et al., 1973). Working concentrations of the two drugs were optimised for this cell line and were subsequently used at a range of 62.5nm-4000nm and 1nm-10000nm, respectively. These ranges were the same to concentrations of Niclosamide and Palbociclib used in CCA cell lines facilitating comparisons. MCF-7 cells were plated at 1×10^4 cells per well, left to adhere overnight and treated with a concentration range of Niclosamide, Palbociclib or a combination of both drugs. Cell viability was measured through MTT assay and synergism was calculated using Chou-Talalay method as described above (section 4.3).

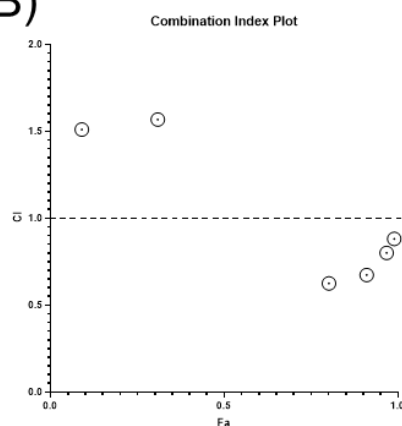
Figure 4.6 shows that MCF-7 cell viability is reduced further with the drugs in combination at most data points, compared to single agents, and this difference is statistically significant at the 500nm:500nm ratio. **Figure 4.6B** shows that Niclosamide and Palbociclib are synergistic or slightly synergistic, at higher concentrations or when $F_a > 0.8$. However, at lower concentrations the CI increases beyond 1.5 showing antagonism. The CI values from the MCF-7 synergism experiments show both antagonism and synergism and are therefore

much more varied than those obtained from the CCA cell lines. Thus, although the combination is synergistic in MCF7 cells the synergism appears to be concentration dependent.

(A)



(B)



(C)

NIC (nM)	PAL (nM)	Fa	CI
125	10	0.089	1.511
250	100	0.309	1.568
500	500	0.801	0.626
1000	1000	0.909	0.674
2000	5000	0.967	0.801
4000	10000	0.989	0.881

Figure 4.6: Niclosamide and Palbociclib are Synergistic in MCF-7 WT in High Concentrations.

Cells were plated at 1×10^4 cells per well in a 96-well plate and left to adhere overnight. Cells were treated with a range of Niclosamide or Palbociclib or both in combination; specific concentrations are given underneath the graph. Cells were incubated with the drug treatment for 72 hours then cell viability was measured with MTT assay. Background values were subtracted from experimental values, and then normalised to vehicle control. The experiment was repeated to three experimental replicates.

- (A) Cell viability in CCLP cells treated with a range of Niclosamide, or Palbociclib, or both in combination. Error bars represent SEM and two-tailed paired t-test was performed to record significant differences between response to single drugs and drugs in combination (N=3).
- (B) A Combination Index Plot that shows the degree of synergism for every combination data point tested.
- (C) A summary of Combination Index (CI) values against the concentrations used and the degree of response that was measured (Fa), where if Fa=1 cell viability would be 0.

4.5 Can Niclosamide Synergise with an Alternate CDK4/6 Inhibitor?

Niclosamide has been used in combination with multiple small molecule targeted drugs to provide a potent anti-cancer therapy, however, there have not been any research into combining Niclosamide with a CDK inhibitor at the time of this work (Ren et al., 2022). Although, Niclosamide has been observed in combination with chemotherapy agents that can cause cell cycle arrest indirectly, such as Gemcitabine, where there has been further benefit to using the drugs in combination (Kang et al., 2023). To determine whether it is Palbociclib specifically that synergises with Niclosamide or whether another cell cycle inhibitor would synergise with Niclosamide, experiments were performed using the alternative CDK4/6 inhibitor, Ribociclib, in place of Palbociclib.

Like Palbociclib, Ribociclib is also an approved drug for first-line treatment of hormone-receptor positive, HER2 negative metastatic breast cancer (Wang et al., 2024). It also blocks the formation of cyclin D- CDK4/6 complexes, reducing retinoblastoma phosphorylation and inducing G1 arrest. For synergism experiments, Ribociclib was used over the same concentration range used for Palbociclib as Ribociclib resulted in a decrease in cell viability similar to Palbociclib in the CCLP cells. This means that the dilution range for Ribociclib is the same as for Palbociclib and this facilitates comparing the CDK4/6 inhibitors in these experiments. CCLP cells were plated at 1×10^4 cells per well in a 96-well plate, left to adhere overnight, and treated with the dilution range of Niclosamide described above, the linear dilution range for Ribociclib and both drugs together at the same dilution range as each drug separately. Cells were

incubated with the treatment for 72hrs before measuring cell viability with an MTT assay. This experiment was performed three times in triplicate.

Figure 4.7A shows the effect of the drugs as single agents and in combination on CCLP cell viability. The combination of Niclosamide and Ribociclib decreases cell viability further than either drug alone at all time points and the decrease is statistically significant when Niclosamide and Ribociclib are used at a ratio of 1 μ M:5 μ M and 2 μ M:10 μ M ($p < 0.05$ and $p < 0.01$). As in the previous synergism experiments, the raw data was used to calculate a line of best fit between the log of the drug concentration and the log of the response. This allowed the calculation of a response for any given concentration. **Figure 4.7B** shows the Combination Index plot where the degree of synergism, indicated by CI value, is mapped against response (F_a), for each combination data point tested. **Figure 4.7C** summarises the CI values with their according concentration for each drug and response (F_a). At all data points, the behaviour of the drugs is classified as synergistic, with CI values between 0.3-0.7 broadly defined as synergistic and values between 0.1-0.3 defined as strongly synergistic. As both Niclosamide and Palbociclib or Ribociclib synergised to a similar extent, it is likely that Niclosamide can synergise with multiple CDK4/6 inhibitors.

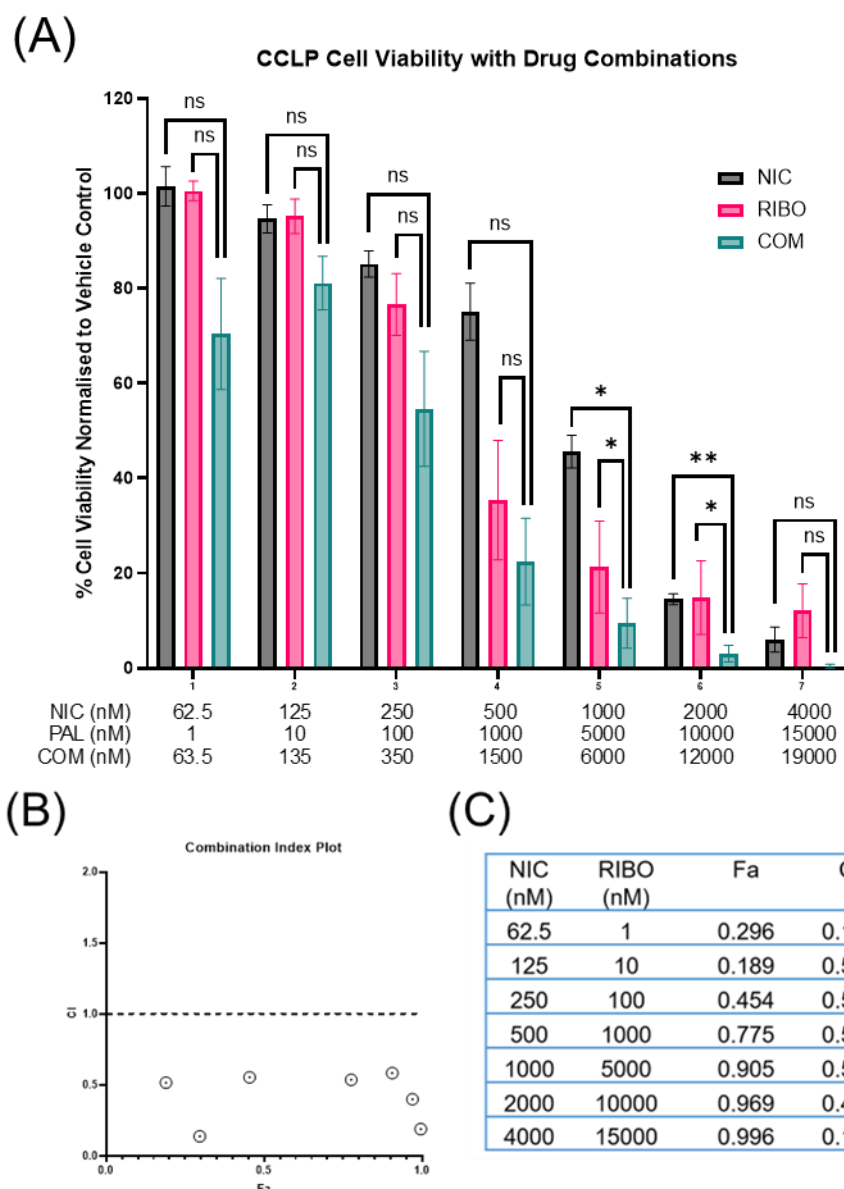


Figure 4.7: Niclosamide and Ribociclib are Synergistic in CCLP WT.

Cells were plated at 1×10^4 cells per well in a 96-well plate and left to adhere overnight. Cells were treated with a range of Niclosamide or Ribociclib or both in combination; specific concentrations are given for each cell line underneath the graph. Cells were incubated with the drug treatment for 72 hours then cell viability was measured with MTT assay. Background values were subtracted from experimental values, and then normalised to vehicle control. The experiment was repeated to three experimental replicates.

- (A) Cell viability in CCLP cells treated with a range of Niclosamide, or Ribociclib, or both in combination. Error bars represent SEM and two-tailed paired t-test was performed to record significant differences between response to single drugs and drugs in combination (N=3).
- (B) A Combination Index Plot that shows the degree of synergism for every combination data point tested.
- (C) A summary of Combination Index (CI) values against the concentrations used and the degree of response that was measured (Fa), where if Fa=1 cell viability would be 0.

4.6 Can Palbociclib Synergise with an Alternate Mitochondrial Uncoupler?

Having established that synergism occurs between Niclosamide and both Ribociclib or Palbociclib, we wondered whether there might be a synergistic response if an alternative mitochondrial membrane depolarising agent such as FCCP was combined with Palbociclib. Although, Palbociclib has not been reported to synergise with mitochondrial uncouplers in the literature, Palbociclib has been shown to increase mitochondrial biogenesis, and can synergise with anti-cell metabolism drugs such as mTOR inhibitors (Uzhachenko et al., 2021).

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, or FCCP, is a well-established mitochondrial membrane uncoupler. It is an ionophore that transports hydrogen ions across the mitochondrial membrane to disrupt the ion gradient and ATP synthesis (Demine et al., 2019). It is commonly used in the Seahorse Metabolism Assay to measure maximal respiratory capacity (Plitzko et al., 2018).

The FCCP working concentration range was optimised and used at 0.5 μ M, 1 μ M, 2 μ M, 4 μ M, 6 μ M, 8 μ M, and 10 μ M in CCLP cells. This is a much higher range of concentration compared to the working range of concentrations optimised for Niclosamide. The range of FCCP concentrations was combined with the same Palbociclib working concentrations used previously (1nM- 15 μ M). CCLP cells were treated for 72hrs with the drugs as single agents, and in combination, and cell survival was measured using an MTT cell viability assay. **Figure 4.8A** shows the effect of FCCP and Palbociclib plus their combination on CCLP cell viability. In combination, FCCP and Palbociclib decrease cell viability to a greater extent than either drug alone at all time points. The

Combination Index plot shows that all data points have a CI<1 apart from one (CI=1.053, Fa=0.702) (**Figure 4.8B-C**). Thus, synergism occurs between FCCP and Palbociclib in CCLP cells.

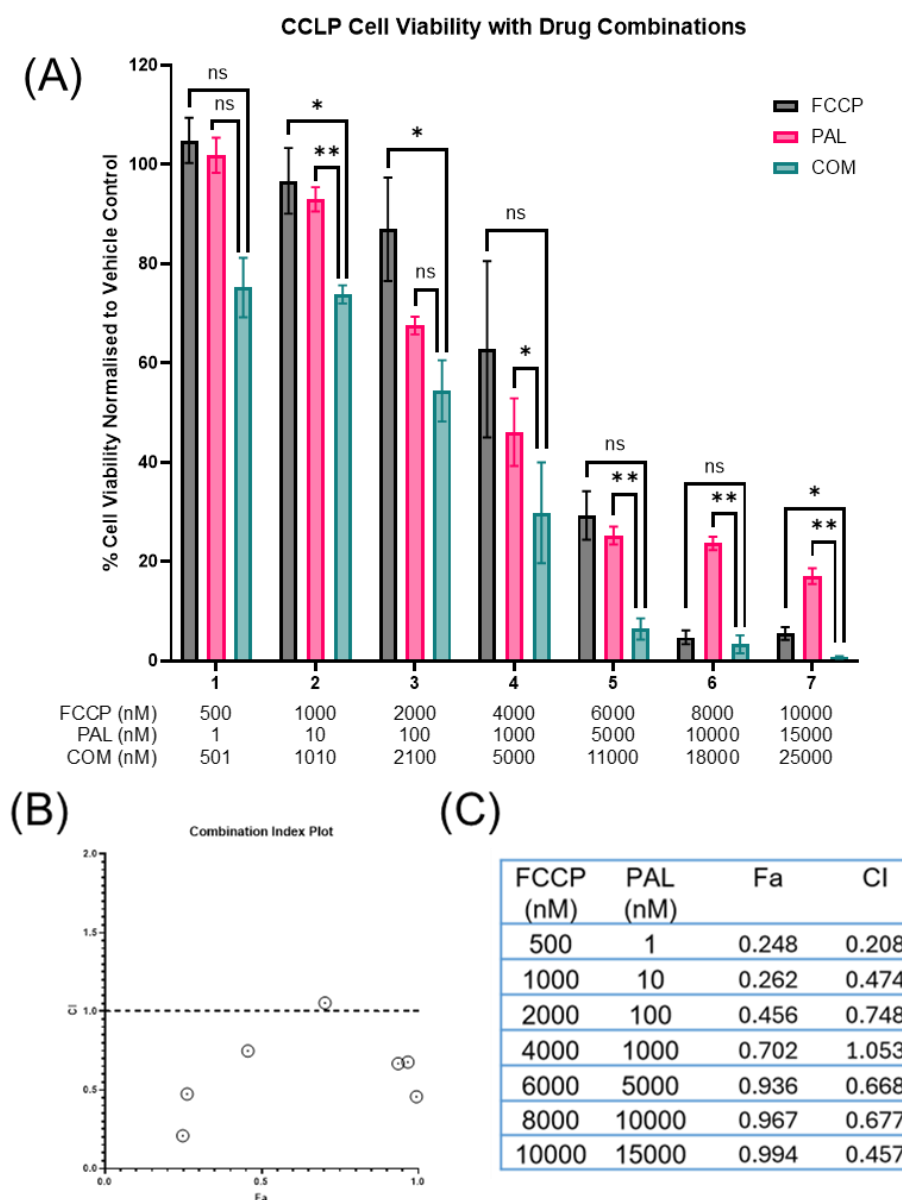


Figure 4.8: FCCP and Palbociclib are Synergistic in CCLP WT.

Cells were plated at 1×10^4 cells per well in a 96-well plate and left to adhere overnight. Cells were treated with a range of FCCP or Palbociclib or both in combination; specific concentrations are given for each cell line underneath the graph. Cells were incubated with the drug treatment for 72 hours then cell viability was measured with MTT assay. Background values were subtracted from experimental values, and then normalised to vehicle control. The experiment was repeated to three biological replicates.

(A) Cell viability in CCLP cells treated with a range of FCCP, or Palbociclib, or both in combination. Error bars represent SEM and two-tailed paired t-test was performed to record significant differences between response to single drugs and drugs in combination (N=3).

(B) A Combination Index Plot that shows the degree of synergism for every combination data point tested.

(C) A summary of Combination Index (CI) values against the concentrations used and the degree of response that was measured (Fa), where if Fa=1 cell viability would be 0.

Interestingly, the degree of synergism between Niclosamide and Palbociclib, Niclosamide and Ribociclib, and FCCP and Palbociclib are very similar. **Figure 4.9** shows the CI index plots for each of these combinations. FCCP and Ribociclib are highlighted to distinguish the different combinations. The trend in the plots is comparable, with the CI values showing strong synergism at the lowest Fa values and the highest Fa values, whilst CI values seem to increase in the mid-range. Niclosamide and Ribociclib have lower CI values in general and therefore display marginally stronger synergism amongst all the combinations. When comparing the combination of Niclosamide or FCCP with Palbociclib, the CI is less potent ($CI=1$) when FCCP is combined with Palbociclib. This suggests that mitochondrial uncoupling is not solely responsible for the potent Niclosamide and Palbociclib synergism.

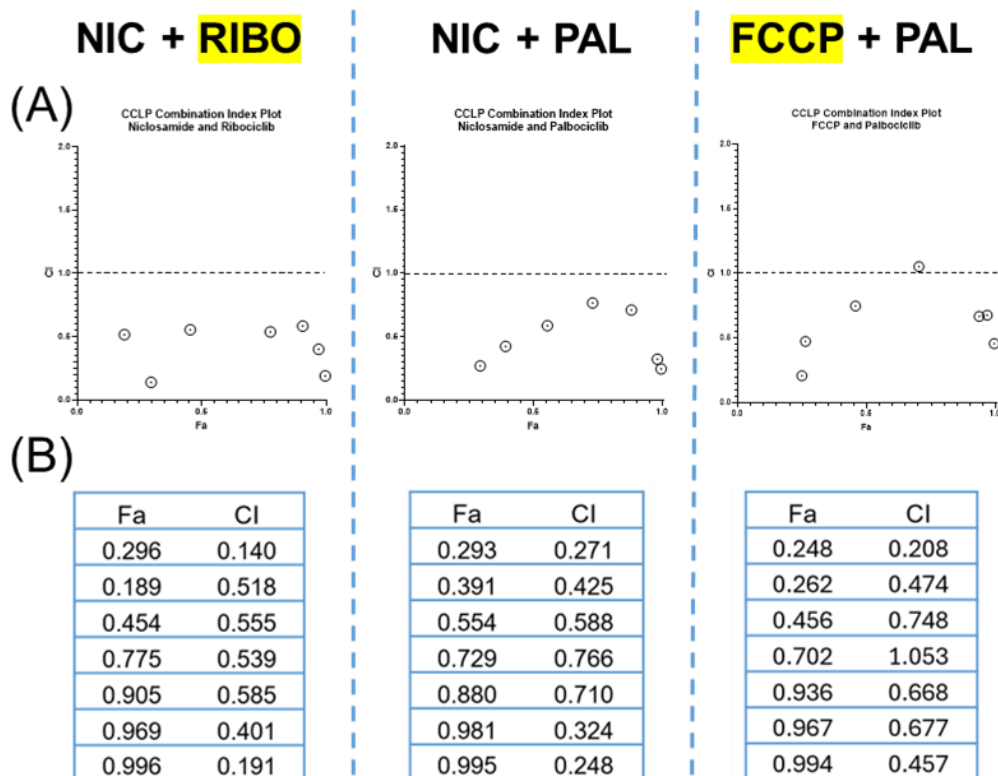


Figure 4.9: Comparing the Synergism between Niclosamide with Palbociclib, Niclosamide with Ribociclib, and FCCP and Palbociclib in CCLP WT.

The three drug combination, Niclosamide with Palbociclib, Niclosamide with Ribociclib, and FCCP and Palbociclib were investigated in synergism experiments in CCLP cells that have been described previously (N=3). Raw data was used to make a line of best fit between drug concentration and response, which allows estimation of concentrations required for any given response. Concentrations of drug needed to reduce cell viability, at a range of percentages, were extrapolated from the line of best fit, for both drugs on their own and drugs in combination. Estimated CI values were calculated from the difference in concentration from the drugs in combination and the drugs as single agents and plotted against the corresponding response (Fa) values.

(A) Combination Index Plots that shows the estimated CI values over a range of response values (Fa).

(B) A summary of Combination Index (CI) values against the degree of response that was measured (Fa), where if Fa=1 cell viability would be 0.

4.7 Investigating the Effect of Niclosamide and Palbociclib Combination on Cell Cycle Progression and Apoptosis

The previous data showed that Niclosamide and Palbociclib have a synergistic effect in combination experiments. To better understand the mechanisms resulting in the synergy observed, CCA cells treated with a synergistic concentration of Niclosamide and Palbociclib were investigated using flow cytometry methods, and western blotting, to determine whether the combination of drugs was promoting cell cycle arrest or inducing apoptosis more than single agent alone.

CCA cells were seeded at approximately 2×10^6 cells in four T75 flasks in complete media and left to adhere overnight. The next day, when the cells had reached around 33% confluency by microscopy inspection, the media was removed and replaced with media containing the drugs. For CCLP cells, Niclosamide was used at $0.5 \mu\text{M}$ and Palbociclib $1 \mu\text{M}$. For RBE, Niclosamide was used at $0.5 \mu\text{M}$ and Palbociclib $2 \mu\text{M}$. For KKKU-M055 cells, a concentration of $0.5 \mu\text{M}$ Niclosamide and $4 \mu\text{M}$ for Palbociclib was used. These concentrations were used as they have been shown here to decrease CCA cell viability further in combination compared to either drug alone (**Figure 4.3**). These concentrations are synergistic CI= 0.766 in CCLP cells, CI=0.539 in RBE cells, and CI=0.933 in KKKU-M055 cells (**Figure 4.4**). Additionally, at these concentrations, there would be an adequate fraction of cells remaining after the 72 hrs of treatment to harvest for phenotypic experiments and protein expression analysis.

4.7A CCLP Phenotypic and Protein Expression Data:

To observe any effect the drug combination may have induced on cell cycle progression, PI staining and flow cytometry were performed as previously described (section 2.2.6). The data was analysed using Kaluza software, where the gating was kept the same for each of the four conditions in each repeat: vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) and Combination (COM). The percentage of cells in each stage of the cell cycle was recorded across the conditions, and the experiment was performed three times independently. A paired t-test was performed between the conditions to observe any statistically significant changes to cell cycle progression when the drugs were in combination, compared to vehicle control or drugs as single agents.

Figure 4.10 shows representative images of the flow cytometry experiments, and **Figure 4.11** shows the average effect of the drugs as single agents and in combination on cell cycle progression in CCLP cells after 72 hrs of treatment. In CCLP cells Palbociclib alone has a major effect on cell cycle causing accumulation of cells in G1 ($p < 0.01$) and G2 ($p < 0.01$). Niclosamide alone showed no statistically significant effects over the three repeats. Niclosamide and Palbociclib in combination also cause a major increase to the percentage of cells in G1 ($p < 0.0001$), and major decrease to the percentage of cells in G2 ($p < 0.01$), compared to vehicle control. The differences in the number of cells in G1 and G2 is also statistically significant between the combination and Niclosamide treatment ($p < 0.05$ not shown in graph), however the differences are not statistically significant compared to Palbociclib treated ($p = 0.14$ not shown in graph). Thus, combination treatment has little additional effect over Palbociclib treatment alone.

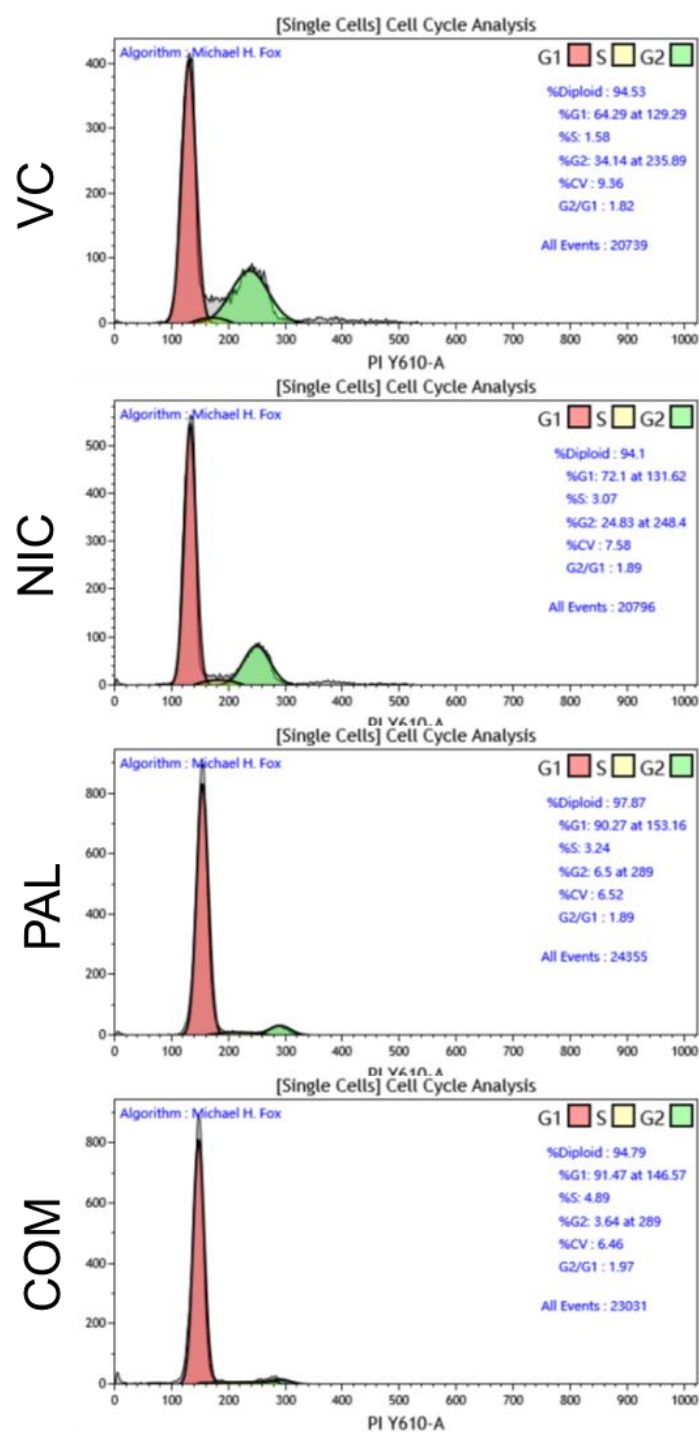


Figure 4.10: Representative Images showing Cell Cycle Analysis in Kaluza for Combination Treated CCLP Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green).

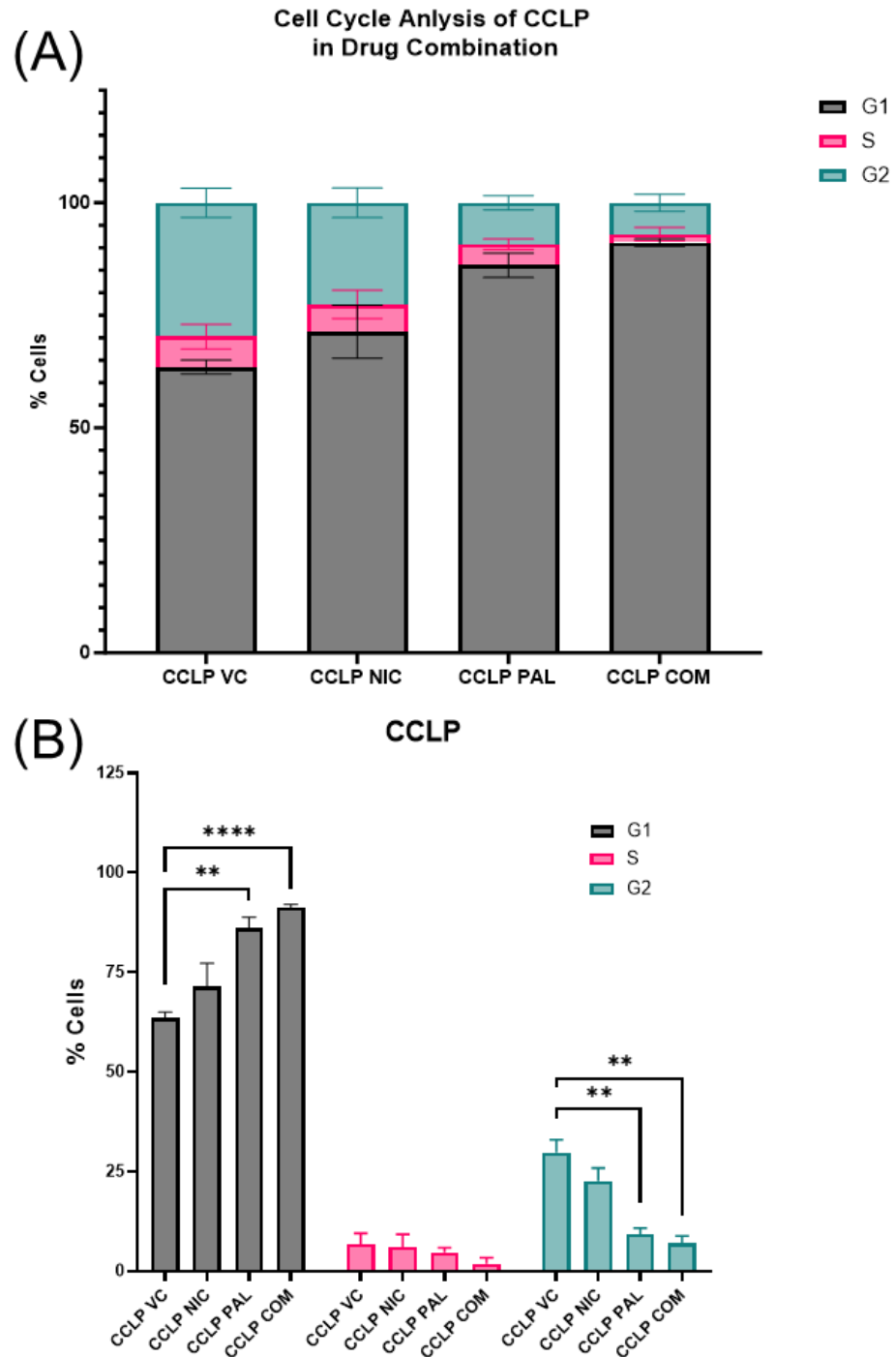


Figure 4.11: Cell Cycle Analysis in Combination Treated CCLP Cells. Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. The analysis shows the proportion of the cell population in each stage of the cell cycle, G1, S and G2, for each treatment. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).

(A) An overview of the changes to cell cycle in single and combination treated CCLP.

(B) The statistical significance between vehicle control and the treatments.

Alongside the cell cycle experiments, cells were pelleted for western blotting analysis of cell cycle related proteins. Approximately 2×10^6 cells were washed and frozen at the 72 hour timepoint and analysed by western blotting as described previously (section 2.5). Beta actin was used as a loading control for all treatment conditions. The same blot was reprobed for several proteins and therefore the loading control is the same for these proteins. The density of all the different protein bands was quantified using Image J and the values were normalised to vehicle control treated cells. Statistical significance was measured through an unpaired t-test between conditions, and the quantification of the effect on protein expression are shown in graphs adjacent to the corresponding western blots. **Figure 4.12** shows representative western blots and analysis of proteins associated with cell cycle in CCLP cells treated with either vehicle control, Niclosamide, Palbociclib or both in combination for 72hrs. These proteins are phospho-RB (**4.12A**), Cyclin D1 (**4.12B**), Cyclin D2 (**4.12C**) and p-21 (**4.12D**).

Palbociclib is known to decrease phosphorylation of retinoblastoma protein (reviewed: Wang et al., 2024). After 72hrs treatment with Palbociclib, p-RB expression was reduced in CCLP cells relative to the vehicle control ($p < 0.0001$). Although phospho-RB appeared to show some reduction in expression with Niclosamide treatment, this was not statistically significant over three repeats. In combination, the drugs promoted a major decrease in p-RB (relative to the beta actin loading control) compared to vehicle control ($p < 0.0001$), and more than Palbociclib as a single agent ($p < 0.01$), but not statistically significant to Niclosamide treated CCLP ($p = 0.27$) (**Figure 4.12A**). Usually, the level of total RB is observed when assessing the effect of a drug on p-RB. However, RB antibodies were unable to detect total RB clearly as a smear of proteins were seen (**Supplementary Figure 4.1**). Two different total RB antibodies were

utilised yet both antibodies yielded the same smeared bands (**Supplementary Figure 4.2**). Therefore, in these experiments the level of p-RB was assessed against the B-actin loading control; this is not uncommon in the Palbociclib literature as the treatment of cells with Palbociclib for more than 24 hours results in a decrease in total RB as well as p-RB (Maskey et al., 2021). However, for completeness the RB protein results for all the CCA cell line data are shown in **Supplementary Figure 4.1**.

The levels of Cyclin D1 and Cyclin D2 were also assessed (**Figure 4.12B-C**). The level of Cyclin D1 in Palbociclib treated CCLP cells appears to increase however, overall, the level of expression compared to vehicle control is not statistically significantly different ($p=0.30$). Niclosamide treatment decreased Cyclin D1 expression ($p<0.05$), compared to vehicle control; this agrees with previous work showing decreased Cyclin D1 protein expression with Niclosamide after 24 hours of treatment (see section 3.5). Combination treated cells show a major reduction in Cyclin D1 protein levels compared to vehicle control ($p<0.05$), however it is similar to the decreased levels of Cyclin D1 observed in Niclosamide treated cells and not further reduced by the combination ($p=0.89$). The level of Cyclin D2 was also reduced by Niclosamide treatment ($p<0.05$), and its expression was increased by Palbociclib treatment ($p<0.05$). In combination experiments the Cyclin D2 expression results were inconsistent, but in 2/3 experiments Cyclin D2 expression is similar to control levels, suggesting that the combination has opposing effects on Cyclin D2 expression resulting in similar levels to untreated cells.

After 72 hours of Niclosamide treatment, the expression of cell cycle inhibitor p21 is strongly decreased ($p<0.05$) (**Figure 4.12D**), however Palbociclib treatment results in no overall change compared to vehicle control across the three independent repeats. Both drugs in combination resulted in a substantial

decrease in p21 compared to vehicle control ($p < 0.001$) however this is not statistically significant to Niclosamide treated CCLP ($p = 0.29$).

From these data, it can be concluded that specific cell cycle proteins p-Rb, Cyclin D1 and p21 are down-regulated to a greater extent by the combination treatment than by individual treatments. Although cell cycle was not more affected by combination treatment than single agent Palbociclib, the decreased expression of these proteins in the combination treatment than observed for either single agent will likely contribute to the synergism observed in the combination treatment.

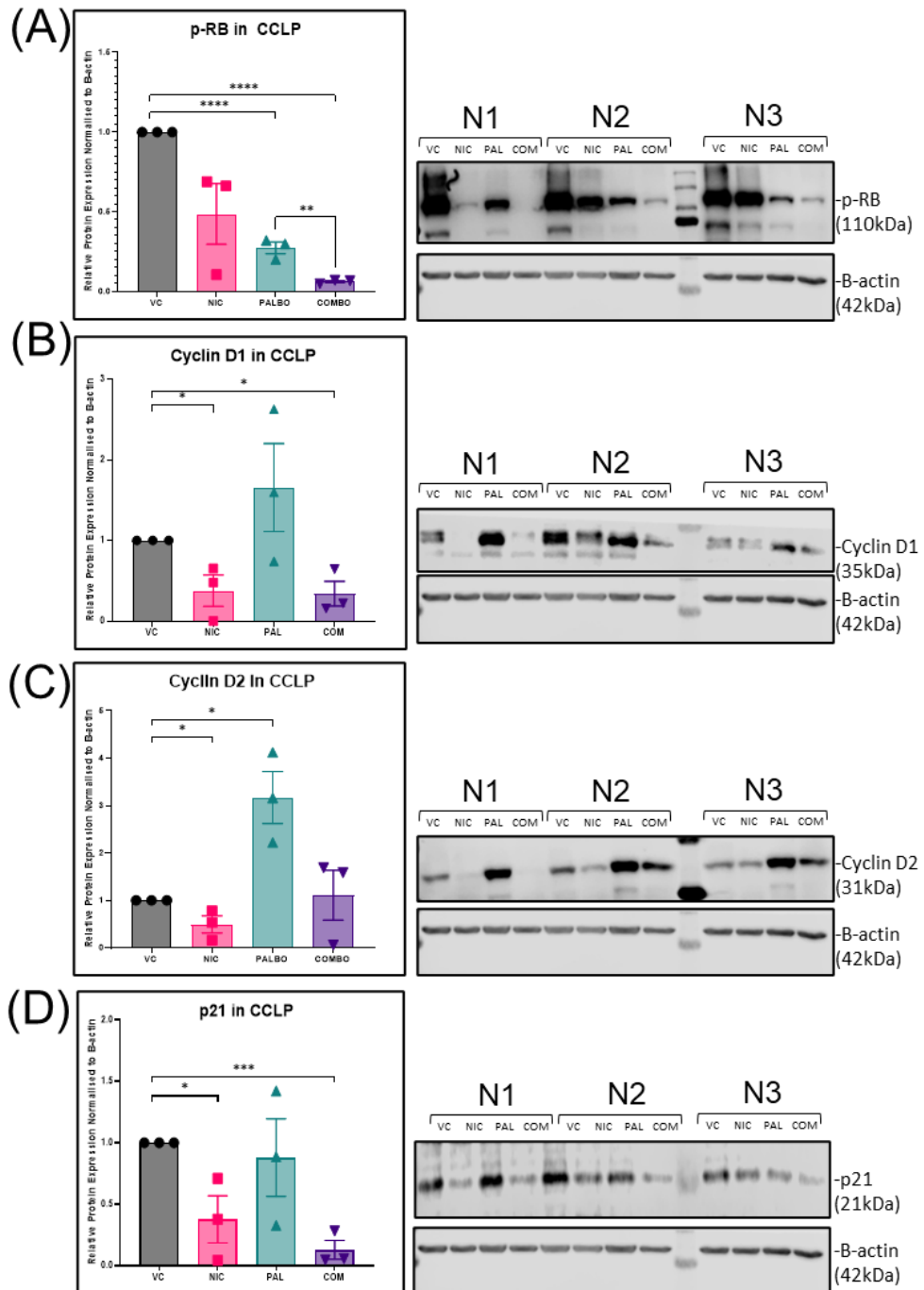


Figure 4.12: Western Blot Analysis of Cell Cycle Protein in Combination Treated CCLP Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM, and significance was verified by a two-tailed unpaired t-test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ ****= $p < 0.0001$). The proteins investigated were (A) p-RB (B) Cyclin D1 (C) Cyclin D2 (D) p21

PRH expression was examined in the same protein extracts described above using western blotting (**Figure 4.13A**). Interestingly, single agent Palbociclib treatment decreased PRH by over 50% compared to vehicle control ($p < 0.01$). In combination, PRH protein levels are dramatically decreased compared to vehicle control ($p < 0.0001$). However, the decrease in PRH expression with combination treatment compared to treatment with single agent Palbociclib is not statistically significant, although there is a trend towards there being less PRH expression with the combination than Palbociclib alone in two out of three repeats. Unexpectedly, Niclosamide treatment for 72 hours did not result in a statistically significant change to PRH expression compared to vehicle control. This is unexpected as when investigating PRH levels in Niclosamide treatment at 24 hours, PRH protein level was decreased (see section 3.5; **Figure 3.10**). A possible explanation for this is that for the combination experiments, a much lower concentration of Niclosamide is used.

The subcellular localisation of B-catenin protein is known to be regulated by PRH expression (Kitchen et al., 2020) and Niclosamide was shown to decrease B-catenin protein expression at 24 hours (section 3.5) so this protein was also investigated in combination experiments. There are no significant changes in B-catenin expression between the different conditions across the three repeats (**Figure 4.13B**). Similar to PRH, this may be due to the much lower Niclosamide concentration used in the combination studies.

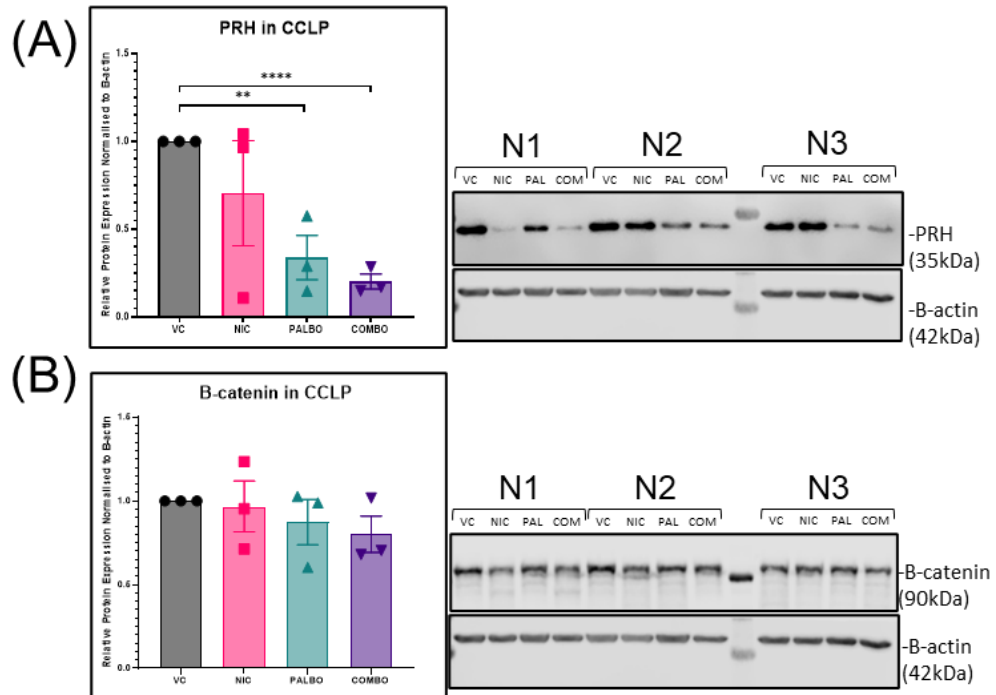


Figure 4.13: Western Blot Analysis of PRH and B-catenin in Combination Treated CCLP Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM, and significance was verified by a two-tailed unpaired t-test (**= $p < 0.01$, ****= $p < 0.0001$). The proteins investigated were (A) PRH (B) B-catenin.

To determine the effects of the drug treatments on cell death, in parallel with the cell cycle experiments, the treated cells were also stained for PI and Annexin V as previously described (section 2.2.7). Stained cells were then used in flow cytometry on a CytoFlex Station. An example of the raw data from the Kaluza software is shown in **Figure 4.14**. The experiment was repeated three times in triplicate, and the data from the three repeats is shown in **Figure 4.15**. Only the drugs in combination caused a statistically significant, but relatively minor, reduction the number of live cells ($p < 0.05$) and corresponding increase in the number of early and late apoptotic cells ($p < 0.05$) (**Figure 4.15B**). When the drugs are used as single agents, although there is a trend towards increased

apoptosis and increased cell death overall, the data does not reach statistical significance, even when the different stages and type of cell death are grouped together to assess overall cell death (**Figure 4.15C**).

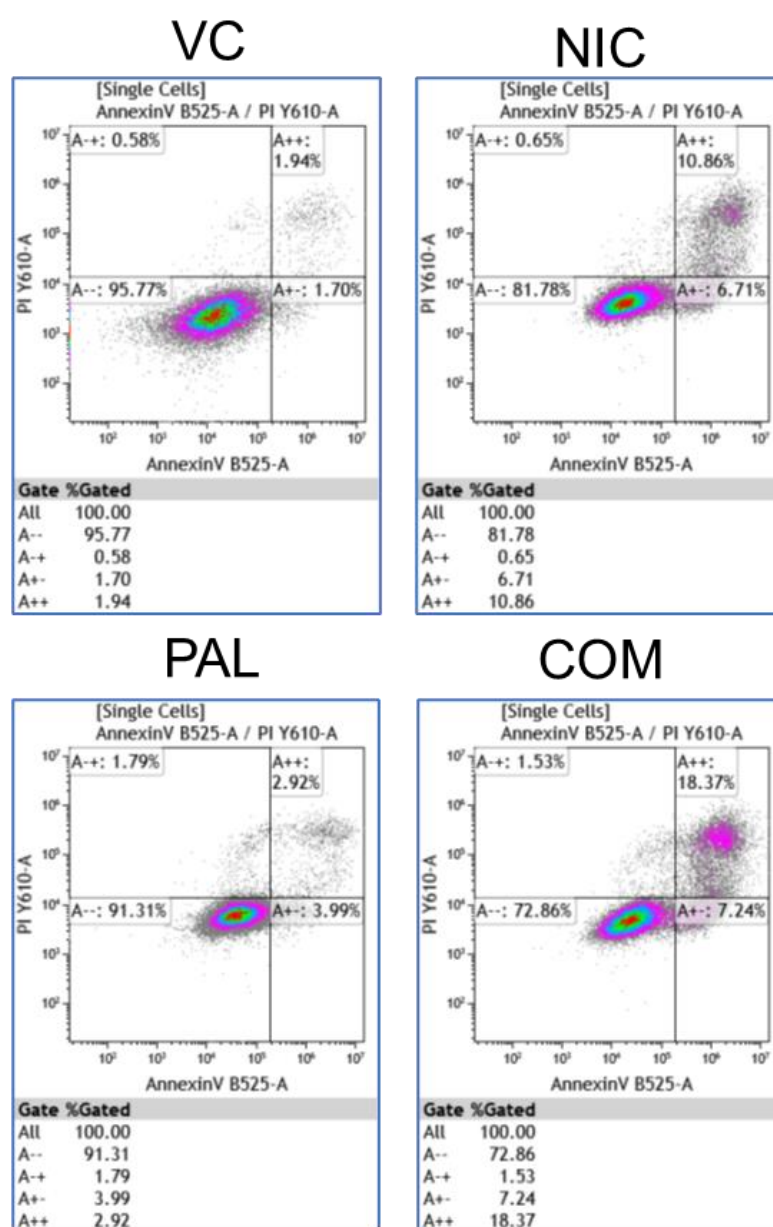


Figure 4.14: Representative Images showing Apoptosis Analysis in Kaluza for Combination Treated CCLP Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell apoptosis: Live (A--), Early Apoptosis (A+-), Necrosis (A-+) and Late Apoptosis (A++).

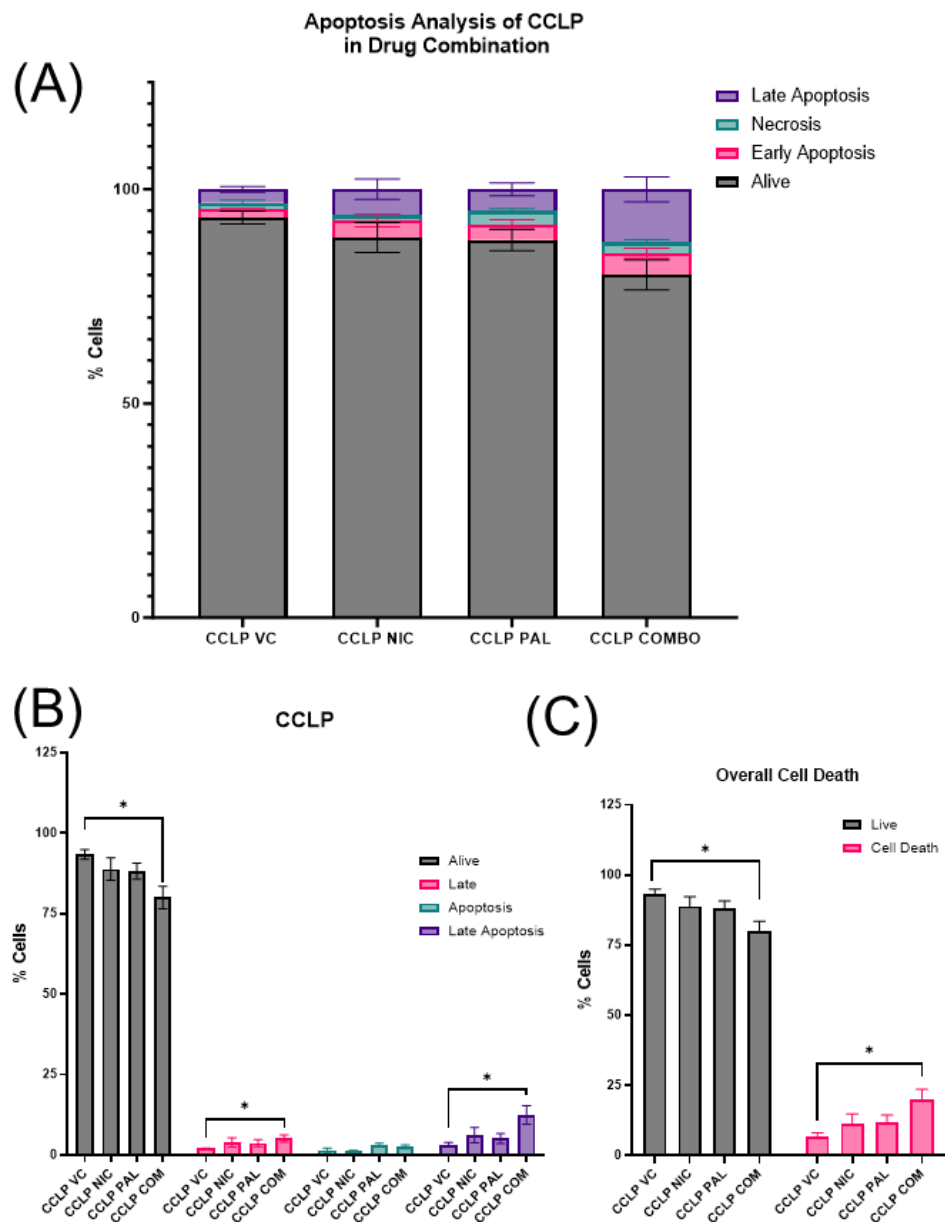


Figure 4.15: Apoptosis Analysis in Combination Treated CCLP Cells.
 CCLP cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for apoptosis experiments. The analysis shows the proportion of the cell population in each stage of cell death: Live, Early Apoptosis, Necrosis and Late Apoptosis. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).
 (A) An overview of the changes to cell cycle in single and combination treated CCLP.
 (B) The statistical significance between vehicle control and the treatments.
 (C) The statistical significance between vehicle control and the treatments when early apoptosis, necrosis and late apoptosis are grouped as one into cell death.

In summary, combination treatment is able to substantially decrease p-RB, p21, and PRH protein expression further than single agent treatment. Also, treatment of CCLP cells reduces the Palbociclib-induced increase to Cyclin D proteins. The change in expression observed in these cell cycle proteins likely underpins the major decrease in cell cycle progression caused by combination treatment. However, the cell cycle arrest is not statistically significantly increased above that observed for Palbociclib alone in the experiments. Apoptosis is also increased by combination treatment more than single agent treatment. Thus, combination in CCLP is likely to be synergistic due to a combination of elevated alterations in cell cycle and apoptosis.

4.7B RBE Phenotypic and Protein Expression Data:

Cell cycle progression and apoptosis analysis by flow cytometry and the western blotting experiments were also performed in RBE cells. **Figure 4.16** shows representative images of the flow cytometry cell cycle data and **Figure 4.17** shows the effect of the drugs as single agents, and in combination, on cell cycle progression over three independent repeats. At 72 hours of treatment, Niclosamide alone did not cause changes to cell cycle progression in RBE cells. However, as expected, Palbociclib treatment resulted in a major increase to the percentage of cells in G1 ($p<0.05$) and a major decrease to the percentage of cells in G2 ($p<0.001$) compared to vehicle control. In combination, there was a substantial increase in cells in G1 ($p<0.01$) and substantial decrease in cells in G2 ($p<0.0001$) compared to vehicle control. The increase in cells in G1 and decrease in cells in G2 is also statistically significant compared to single agent Niclosamide-treated RBE ($p<0.05$), however it is not statistically significant compared to single agent Palbociclib treated RBE cells. Thus, although Palbociclib is exerting cell cycle inhibition in this experiment, this is not greatly improved by the combination of drugs.

Figure 4.18 shows representative western blots in RBE treated cells. The expression of p-RB was reduced by both drugs as single agents (**Figure 4.18A**). Niclosamide treatment reduced p-RB expression ($p<0.01$). Palbociclib treatment also reduced p-RB expression ($p<0.05$), interestingly, this decrease was not as great a reduction as that observed in the Niclosamide treated RBE cells. In combination, p-RB expression was decreased compared to vehicle control ($p<0.0001$) and when compared to single agent Palbociclib ($p<0.05$) but not compared to single agent Niclosamide. These changes to p-RB expression are similar to those seen in the CCLP cells with the drug combination resulting

in the lowest p-RB level across the conditions. However, when comparing single drug treatments, p-RB expression was reduced more by Niclosamide in the RBE cells, and p-RB expression was reduced more by Palbociclib in the CCLP cells.

Cyclin D1 expression was decreased in Niclosamide treated RBE cells compared to vehicle control ($p < 0.01$) (**Figure 4.18B**). The levels of Cyclin D1 in Palbociclib treated RBE are similar to vehicle control. Combination treated cells have a reduced expression of Cyclin D1 compared to vehicle control ($p < 0.01$), however those levels are no different from the levels of cyclin D1 expressed in Niclosamide alone treated RBE cells. Therefore, the trend in Cyclin D1 expression in RBE across the different drug treatments is similar to that seen in CCLP cells.

Expression of p21 is decreased by Niclosamide treatment compared to control ($p < 0.01$) (**Figure 4.18C**). Treatment with Palbociclib had variable effects on p21 protein expression, however, over three repeats, the average expression is similar to that of the vehicle control. The levels of p21 in combination treated cells are between the level of p21 in the Niclosamide alone, and the Palbociclib alone, treated cells. The average expression across the three repeats is lower than the vehicle control, however this is not statistically significant. In contrast, with the lack of effect of the combination on p21 in RBE cells combination treated CCLP cells showed further decrease to p21 expression compared to Niclosamide treatment alone.

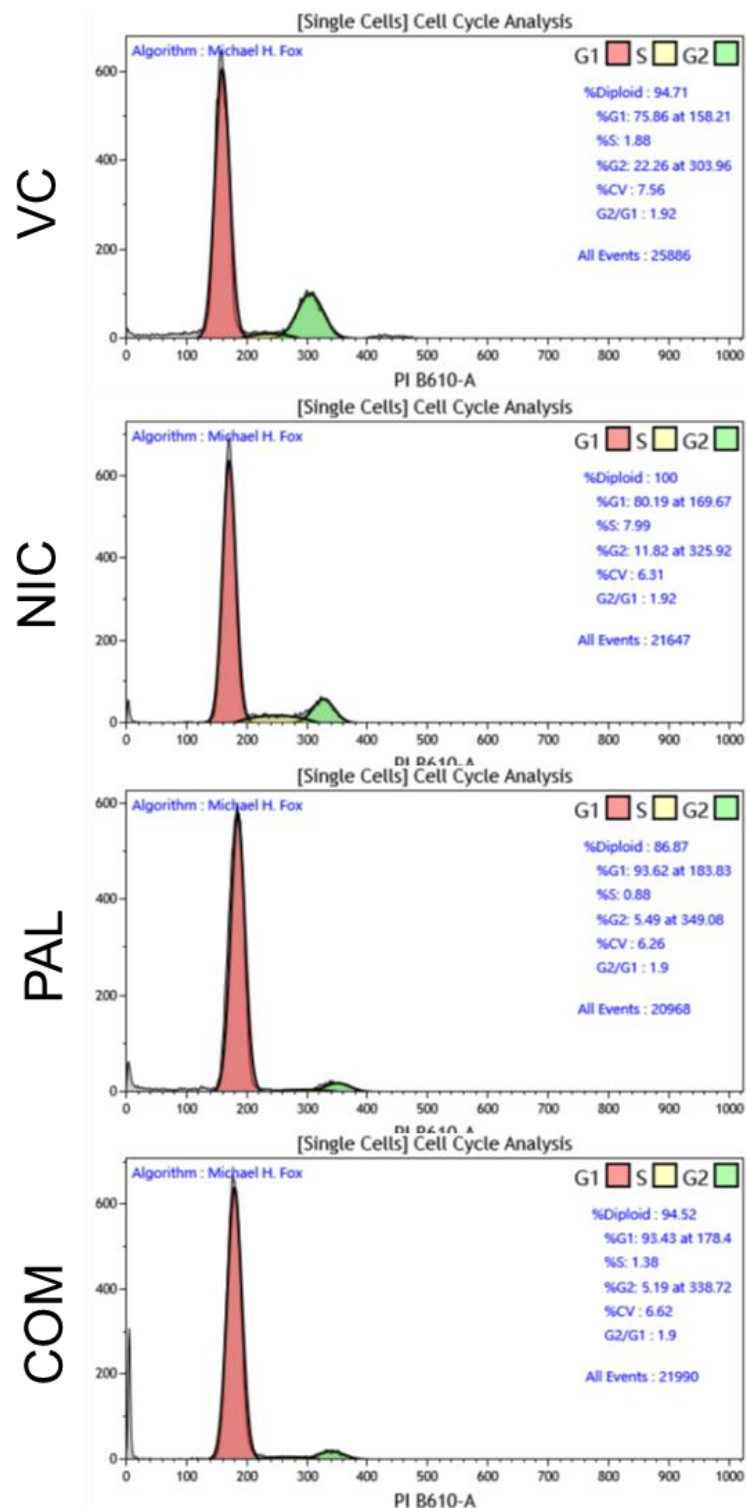


Figure 4.16: Representative Images showing Cell Cycle Analysis in Kaluza for Combination Treated RBE Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green).

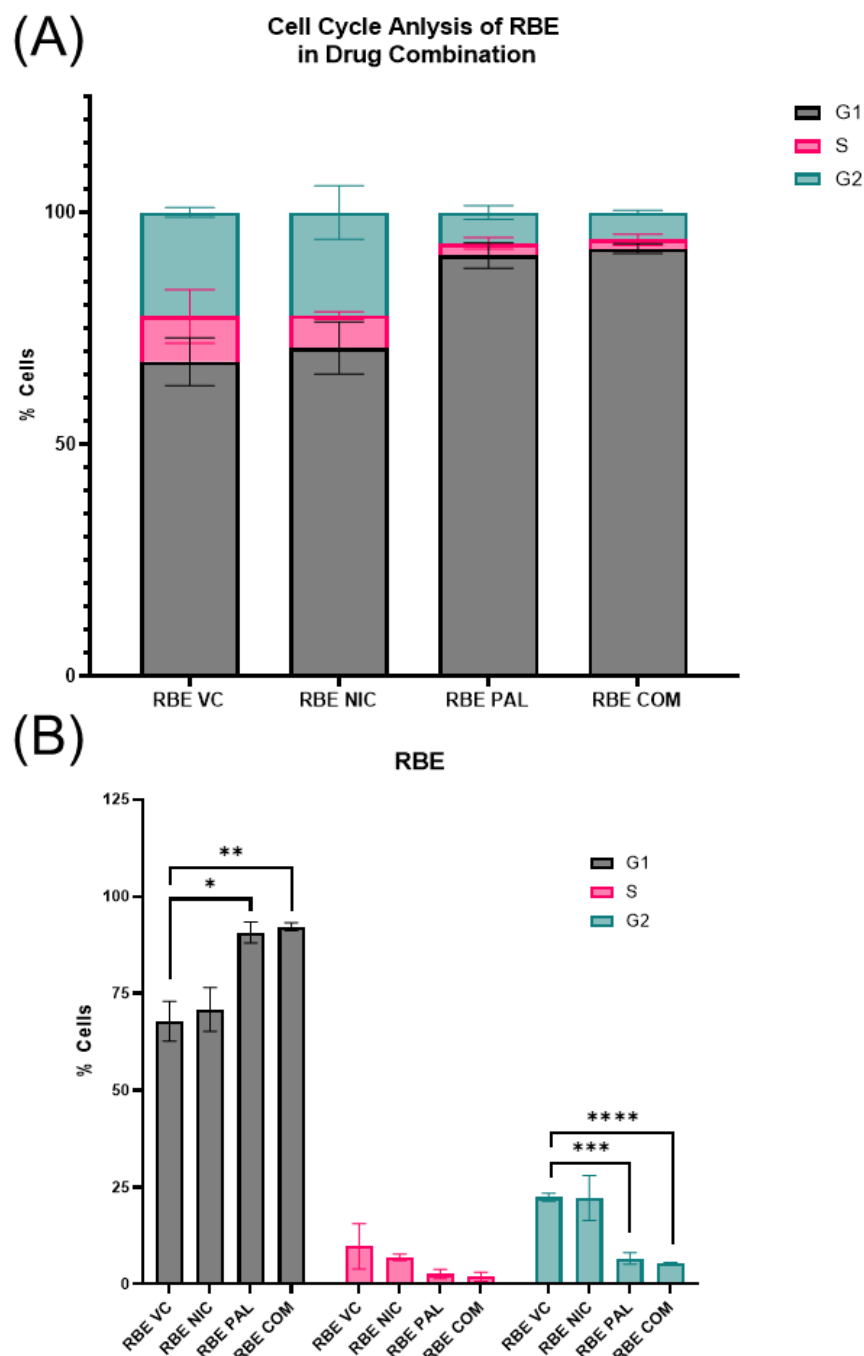


Figure 4.17: Cell Cycle Analysis in Combination Treated RBE Cells. Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. The analysis shows the proportion of the cell population in each stage of the cell cycle, G1, S and G2, for each treatment. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).

(A) An overview of the changes to cell cycle in single and combination treated RBE.

(B) The statistical significance between vehicle control and the treatments.

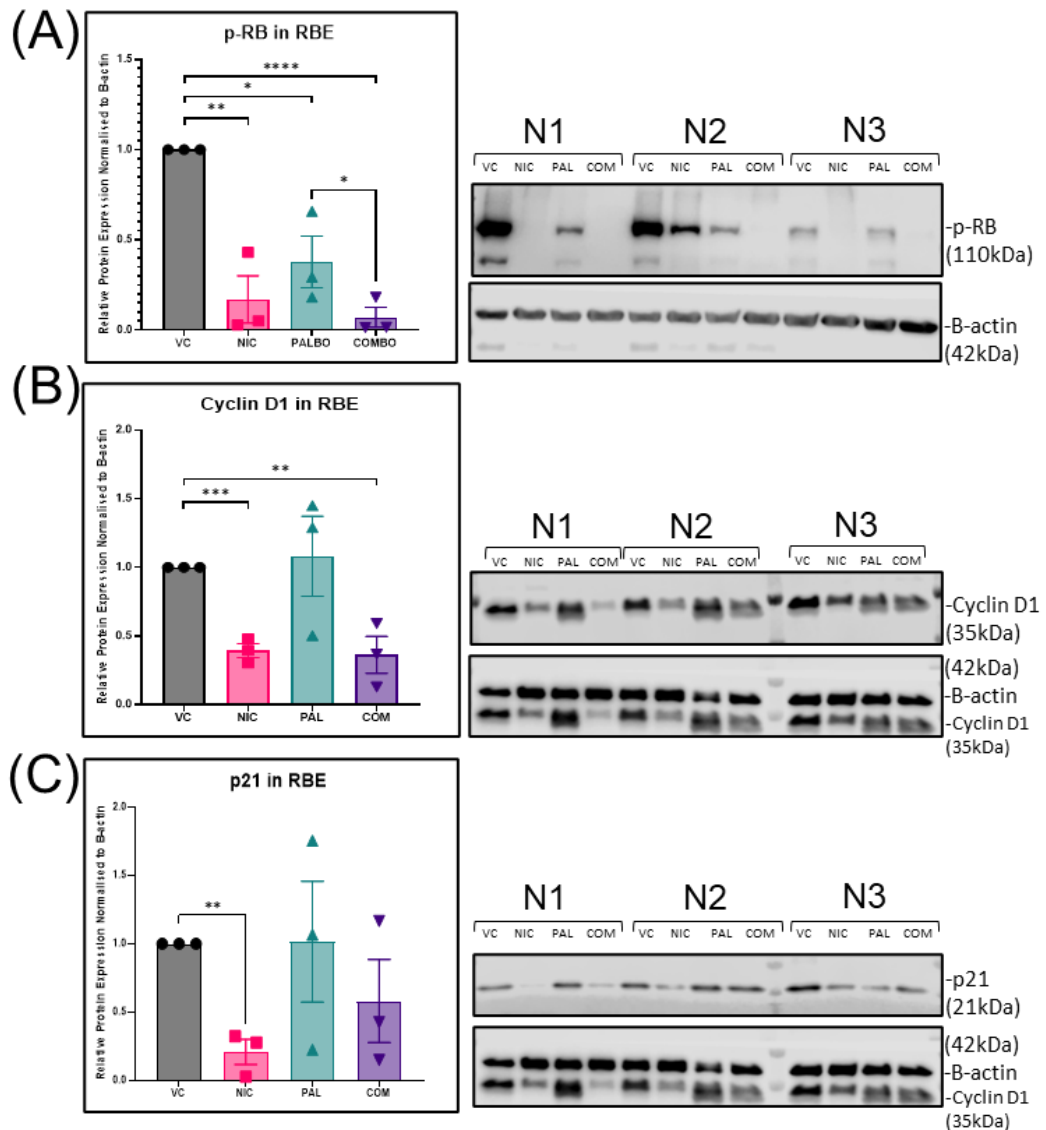


Figure 4.18 : Western Blot Analysis of Cell Cycle Protein in Combination Treated RBE Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM and significance was verified by a two-tailed unpaired t-test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ ****= $p < 0.0001$). The proteins investigated were (A) p-RB (B) Cyclin D1 (C) p21

The expression of PRH in Niclosamide treated RBE shows a ~60% decrease compared to vehicle control at 72 hours of treatment ($p<0.01$) (**Figure 4.19A**). This agrees with previous data showing a significant decrease to PRH expression with Niclosamide treatment after 24 hours. Although Palbociclib treatment also appear to decrease PRH expression in these cells this is not statistically significant ($p=0.23$). The combination of drugs reduces PRH levels compared to vehicle control ($p<0.01$), but this is not statistically significantly different from single agent treated RBE cells. Therefore, Niclosamide appears to exert a greater effect on PRH protein levels than Palbociclib in RBE cells. The expression of B-catenin in the drug-treated RBE cells under all treatments remained similar to the vehicle control (**Figure 4.19B**), similar to CCLP cells.

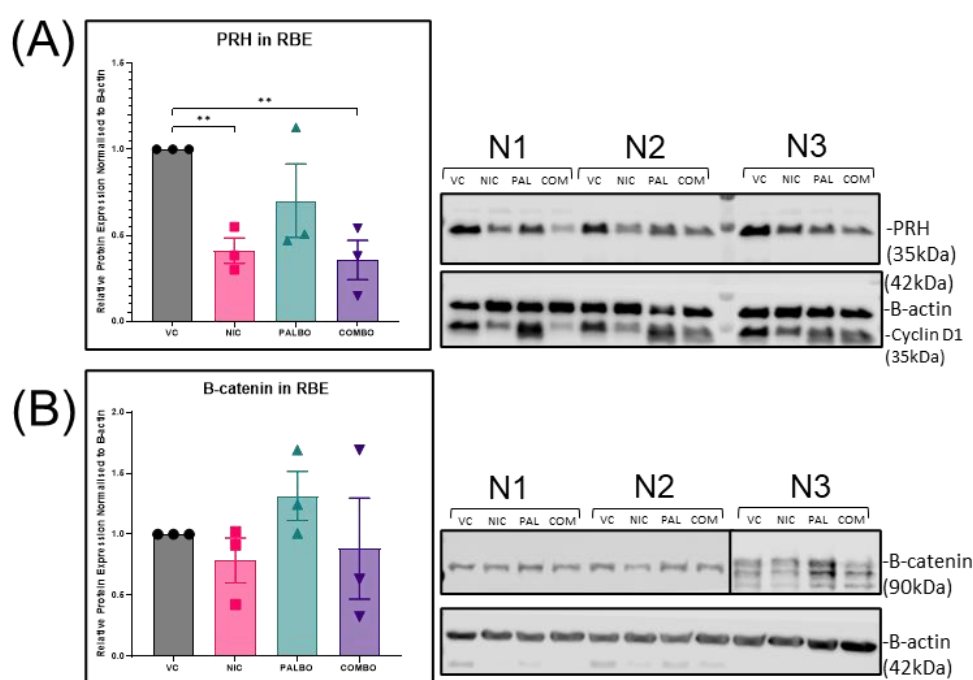


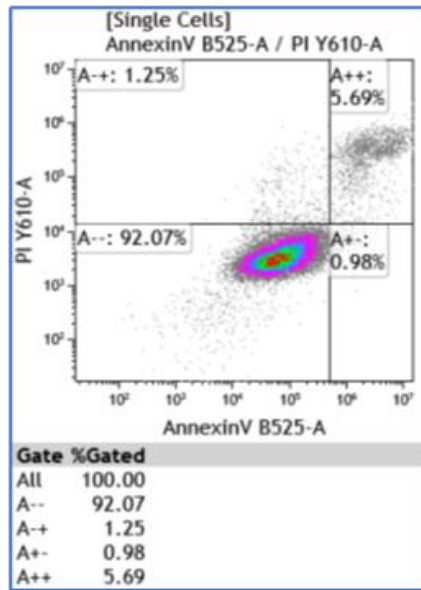
Figure 4.19: Western Blot Analysis of PRH and B-catenin in Combination Treated RBE Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM, and significance was verified by a two-tailed unpaired t-test ($**=p<0.01$). The proteins investigated were (A) PRH (B) B-catenin. Note there are multiple bands seen in the B-catenin western blot third repeat due to the need to increased exposure when detecting the protein bands as the bands were fainter in this repeat for unknown reasons.

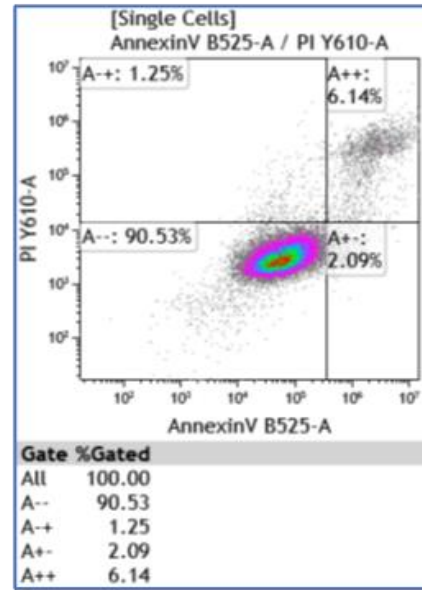
The results of the apoptosis analysis with drug treatment are shown in **Figure 4.20** and analysis of three repeats is shown in **Figure 4.21**. At the concentrations of drug tested, there is no statistically significant effect on the levels of apoptosis in RBE cells when they were treated with Niclosamide alone or Palbociclib alone or both in combination. When total cell death is analysed again there is no statistically significant effect (**Figure 4.21C**).

To summarise, treatment with Niclosamide as a single agent in RBE cells decreases the levels of p-RB, Cyclin D1 and p21 and PRH proteins, whilst Palbociclib alone also causes a reduction to p-RB and PRH levels. Whilst the drugs in combination are able to further reduce p-RB expression, they did not further reduce the expression of Cyclin D1 and p21 more than Niclosamide treatment alone. However, cell cycle arrest is predominantly caused by Palbociclib, and the combination does not increase this more than Palbociclib alone. Interestingly, the levels of apoptosis did not change with any drug treatments in RBE cells.

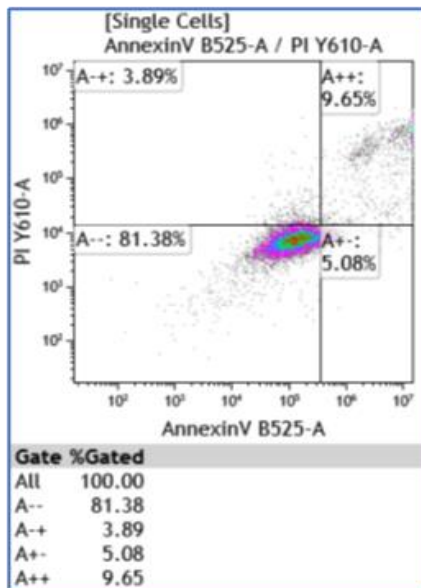
VC



NIC



PAL



COM

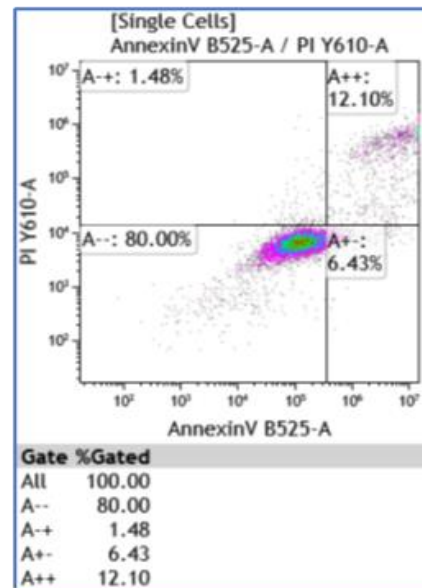


Figure 4.20: Representative Images showing Apoptosis Analysis in Kaluza for Combination Treated RBE Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell apoptosis: Live (A--), Early Apoptosis (A+-), Necrosis (A+-) and Late Apoptosis (A++).

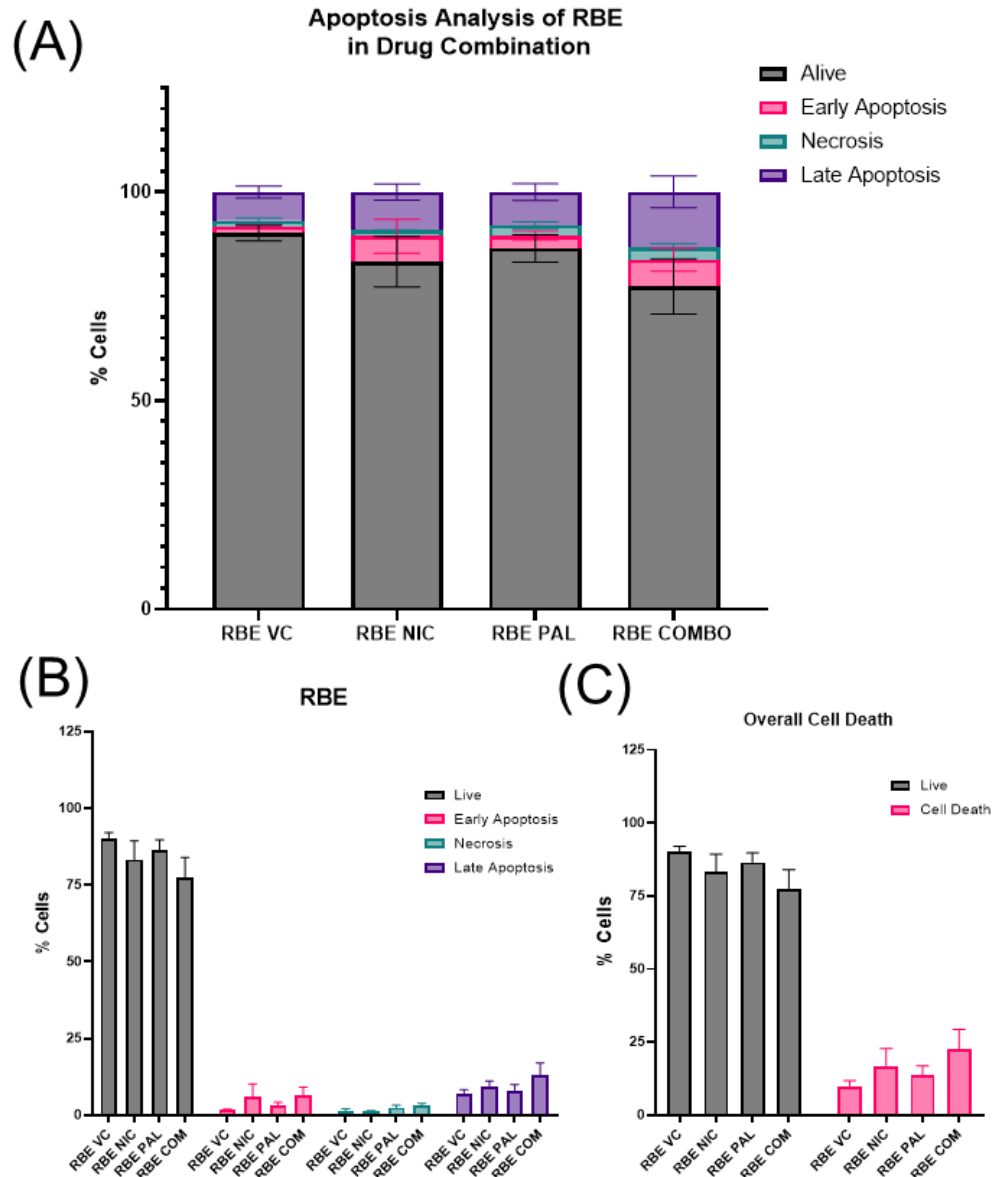


Figure 4.21: Apoptosis Analysis in Combination Treated RBE Cells.

CCLP cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for apoptosis experiments. The analysis shows the proportion of the cell population in each stage of cell death: Live, Early Apoptosis, Necrosis and Late Apoptosis. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).

(A) An overview of the changes to cell cycle in single and combination treated RBE.

(B) The statistical significance between vehicle control and the treatments.

(C) The statistical significance between vehicle control and the treatments when early apoptosis, necrosis and late apoptosis are grouped as one into cell death.

4.7C KKU-M055 Phenotypic and Protein Expression Data:

Niclosamide-, Palbociclib- and combination-treated KKU-M055 cells were also investigated in cell cycle and apoptosis experiments and western blotting experiments.

KKU-M055 cells treated with Niclosamide for 72 hours (see **Figure 4.22** for representative example of data and **Figure 4.23** for analysis) showed a decrease in the number of cells in G2 compared to vehicle control ($p < 0.05$). Although this was accompanied by an increase to the number of cells in G1, this was just outside statistical significance ($p = 0.059$). There was no difference in the percentage of cells in S phase in Niclosamide treated KKU-M055 compared to vehicle control. Palbociclib treatment increased the number of KKU-M055 cells in G1 ($p < 0.01$), and simultaneously decreased the number of cells in S phase ($p < 0.05$) and G2 ($p < 0.01$). Treatment of KKU-M055 with drug combination has a similar effect to Palbociclib treatment alone, showing an increase in the number of cells in G1 compared to vehicle control ($p < 0.01$) and a major decrease in the number of cells in S phase ($p < 0.05$) and G2 phase ($p < 0.001$). The increase to numbers of cells in G1 in combination treatment are significantly different to Niclosamide treatment ($p < 0.05$), but the combination treatment is not statistically significantly different compared to single agent Palbociclib treatment ($p = 0.07$). In addition, the decrease to the number of cells in G2 was decreased compared to Niclosamide treatment ($p < 0.01$), but not significantly decreased with Palbociclib ($p = 0.11$).

The expression of p-RB is reduced by Niclosamide in every western blot repeat, however due to high levels of variance in the data this change is not statistically significant ($p = 0.15$) (**Figure 4.24**). Palbociclib treated KKU-M055 cells have decreased p-RB expression ($p < 0.01$) compared to vehicle control, and

combination treated KKU-M055, p-RB is also decreased in expression compared to vehicle control ($p < 0.0001$). However, the p-RB expression in combination treated KKU-M055 cells is not significantly different from cells treated with Palbociclib alone ($p = 0.21$).

Cyclin D1 expression decreases with Niclosamide treatment compared to control samples ($p < 0.05$). With Palbociclib treatment, the expression of Cyclin D1 increases in the three experimental repeats, but this is not statistically significant due to variance in the repeats ($p = 0.10$). In combination, Cyclin D1 expression is similar to the expression levels observed for Palbociclib treated KKU-M005 cells. Thus, at synergistic concentrations, Niclosamide is unable to decrease the expression of cyclin D1 as the influence of Palbociclib on cyclin D1 expression is far greater.

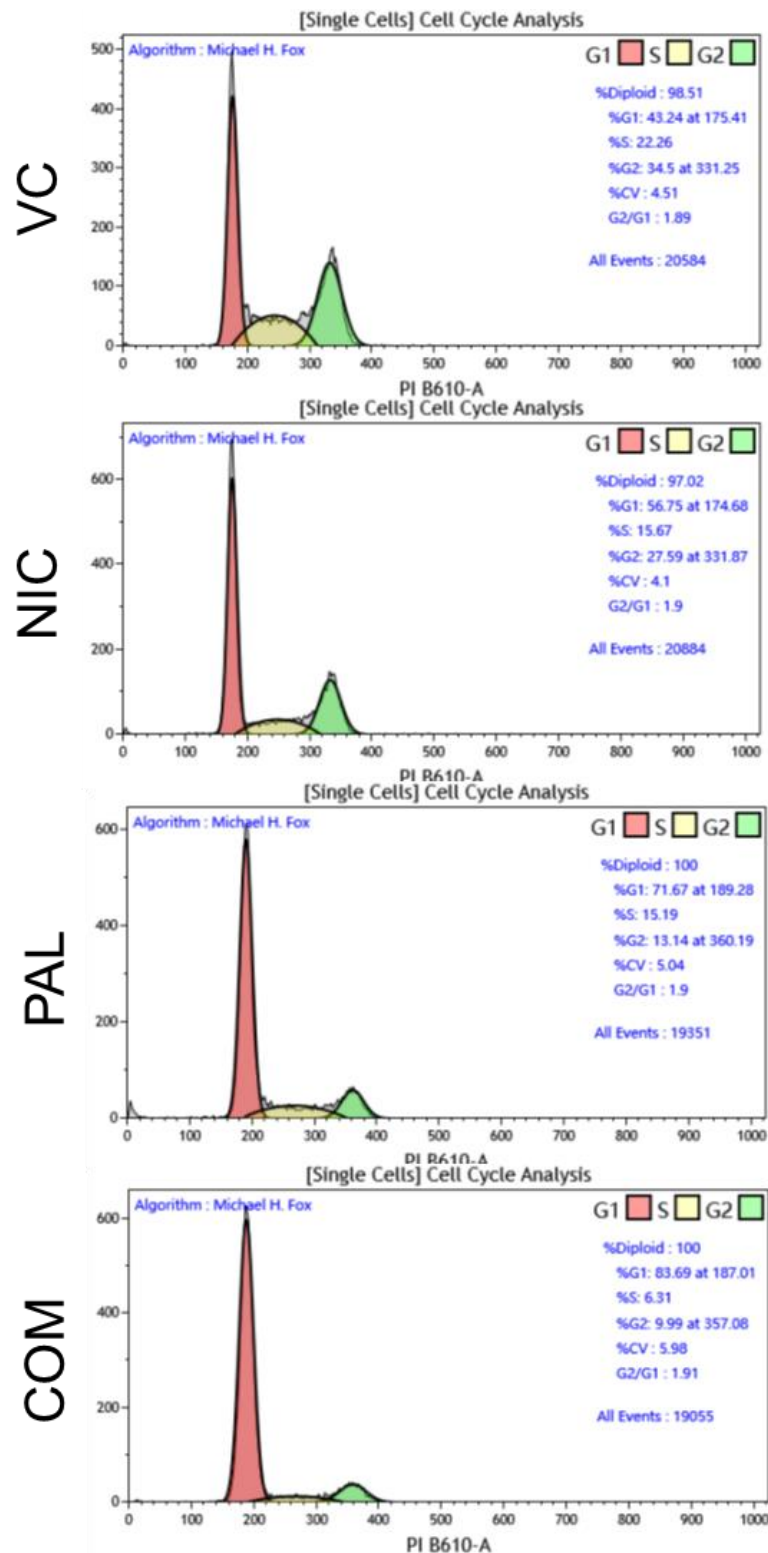


Figure 4.22: Representative Images showing Cell Cycle Analysis in Kaluza for Combination Treated KKKU-M055 Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained and fixed with 70% ethanol, before further washing, RNase treatment and PI staining. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell cycle: G1 (red), S (yellow), and G2 (green).

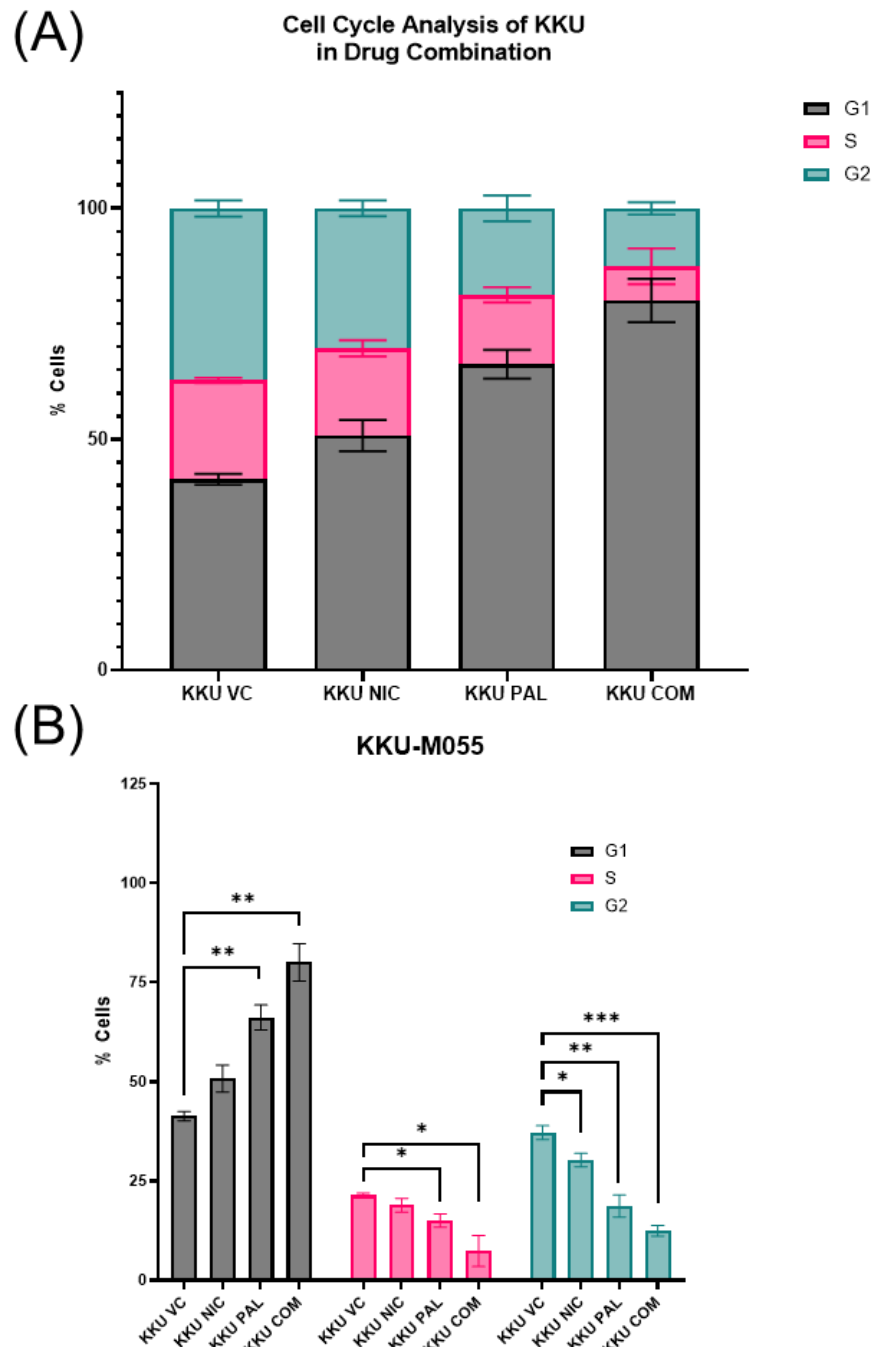


Figure 4.23: Cell Cycle Analysis in Combination Treated KKU-M055 Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. The analysis shows the proportion of the cell population in each stage of the cell cycle, G1, S and G2, for each treatment. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).

(A) An overview of the changes to cell cycle in single and combination treated KKU-M055.

(B) The statistical significance between vehicle control and the treatments.

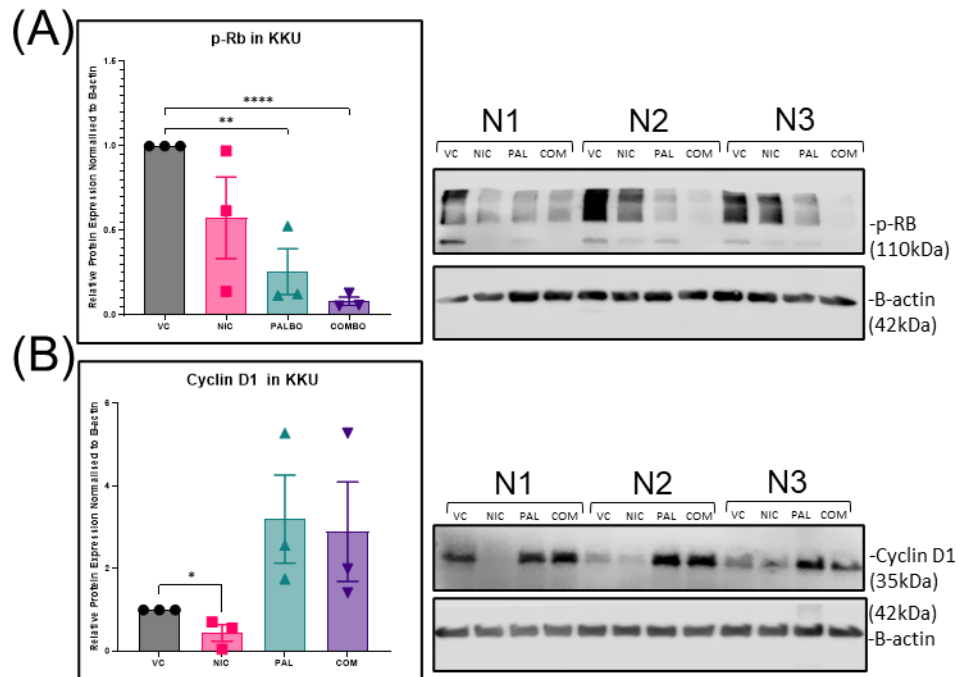


Figure 4.24: Western Blot Analysis of Cell Cycle Protein in Combination Treated KKU-M055 Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM, and significance was verified by a two-tailed unpaired t-test (*= $p < 0.05$, **= $p < 0.01$, ****= $p < 0.0001$). The proteins investigated were (A) p-RB (B) Cyclin D1.

Figure 4.25 shows the effects of the drugs as single agents and as a combination treatment on PRH and B-catenin expression in KKU-M055 cells. In Niclosamide treated KKU-M055 cells, PRH expression was not consistently altered from that of the vehicle control. There is variance in the data, and this may be because the expression of PRH protein in KKU-M055 cells is relatively low compared to CCLP and RBE cells. Strikingly, Palbociclib treatment results in a significant decrease in PRH levels compared to control ($p < 0.05$). In the combination treated KKU-M055 cells, PRH levels are also low compared to vehicle control ($p = 0.08$), but they did not decrease further than observed for

Palbociclib treatment cells. B-catenin expression showed no significant differences and no concordance between repeats.

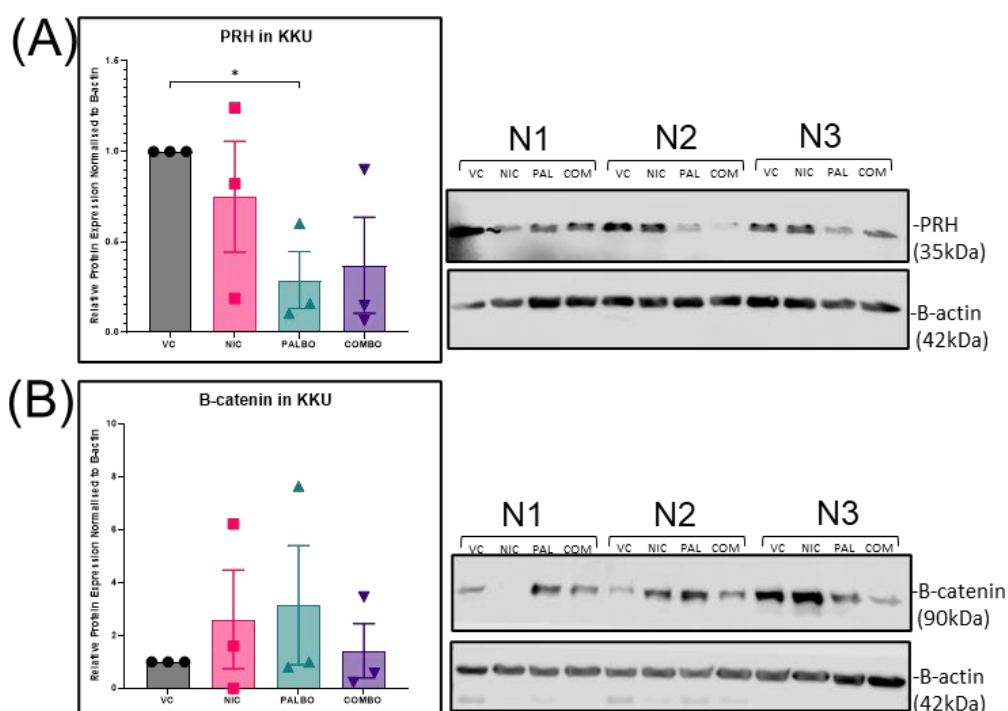


Figure 4.25: Western Blot Analysis of PRH and B-catenin in Combination Treated KKU-M055 Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates, shown in the western blot images (right). Band density was measured through Image J software and expression was normalised to B-actin expression and relativised to vehicle control. Error bars represent SEM, and significance was verified by a two-tailed unpaired t-test ($*=p<0.05$). The proteins investigated were (A) PRH (B) B-catenin.

In apoptosis assays KKU-M055 cells treated with Niclosamide alone for 72 hours showed a decrease in live cells ($p<0.05$) and an increase to late apoptotic cells ($p<0.05$) in apoptosis staining followed by flow cytometry. Treatment of KKU-M055 cells with Palbociclib alone also resulted in a decrease in the number of live cells compared to vehicle control ($p<0.05$) and an increase to the number of cells in cell death overall ($p<0.05$) (**Figure 4.26 and 4.27C**). Niclosamide and Palbociclib together significantly reduced the percentage of

live cells ($p < 0.05$) and increased the percentage of cells in late apoptosis ($p < 0.05$), compared to control sample. The combination treatment however did not alter cell death more than treating with Niclosamide alone.

To summarise p-RB and cyclin D1 undergo a significant decrease in expression when K KU-M055 cells are treated with Niclosamide. Palbociclib also significantly decreases p-RB expression, and it also stabilises cyclin D1 protein. In combination treatment, whilst p-RB is further significantly decreased over vehicle control, Cyclin D1 expression remains relatively higher than vehicle control. Cell cycle arrest is brought about by Niclosamide alone and also by Palbociclib alone and the combination results in a potent cell cycle arrest, which is only significantly different to Niclosamide only treated cells. However, it is likely that further repeats of these experiments would result in significance difference between Palbociclib treated and combination treated cell. The levels of apoptosis in combination treated cells were the same as levels of apoptosis in Niclosamide treated cells, thus Palbociclib did not cause cell death in K KU-M055 cells.

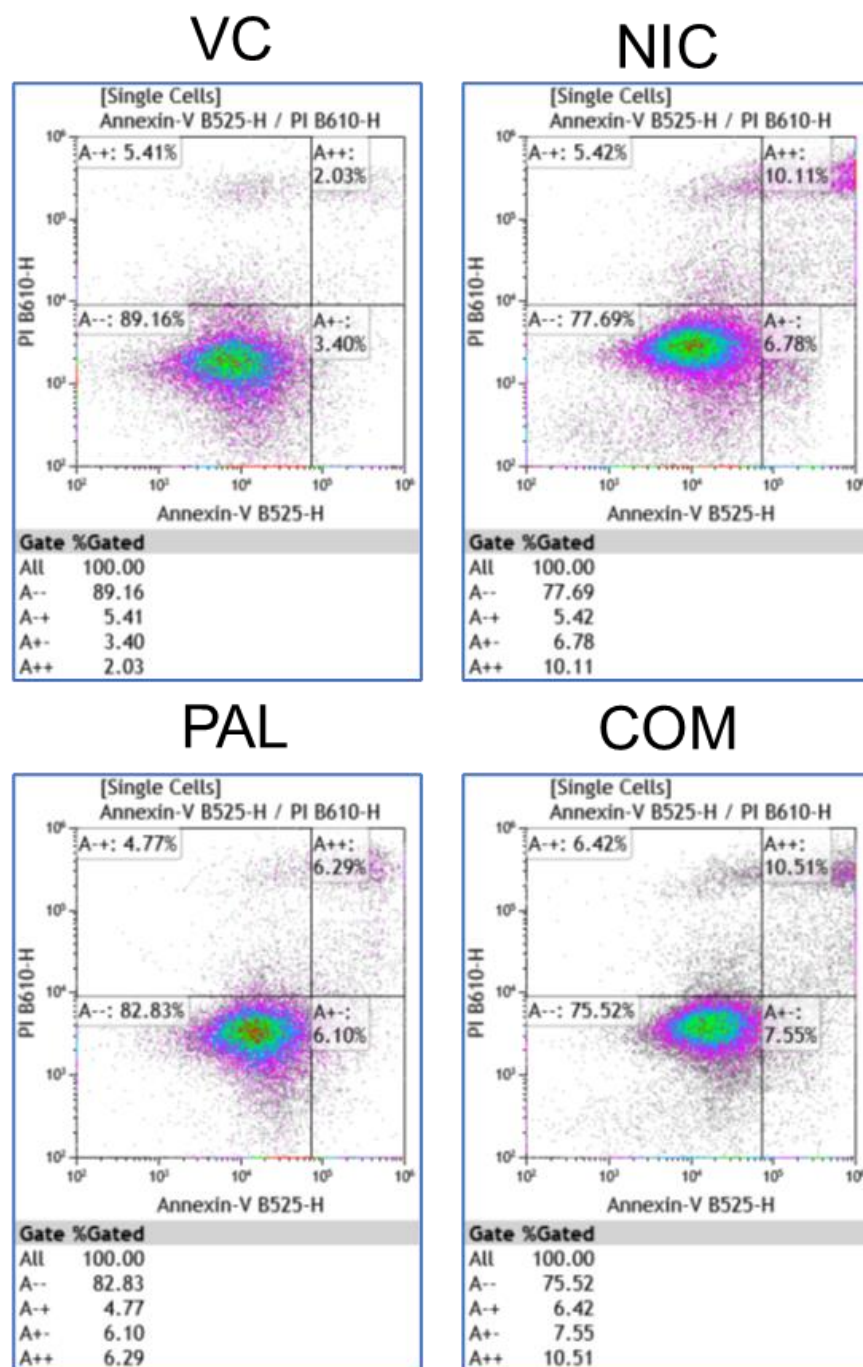


Figure 4.26: Representative Images showing Apoptosis Analysis in Kaluza for Combination Treated KKKU-M055 Cells

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for cell cycle experiments. Approximately 1×10^6 cells were washed and stained with Annexin V and PI. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each phase of cell apoptosis: Live (A--), Early Apoptosis (A+-), Necrosis (A-+) and Late Apoptosis (A++).

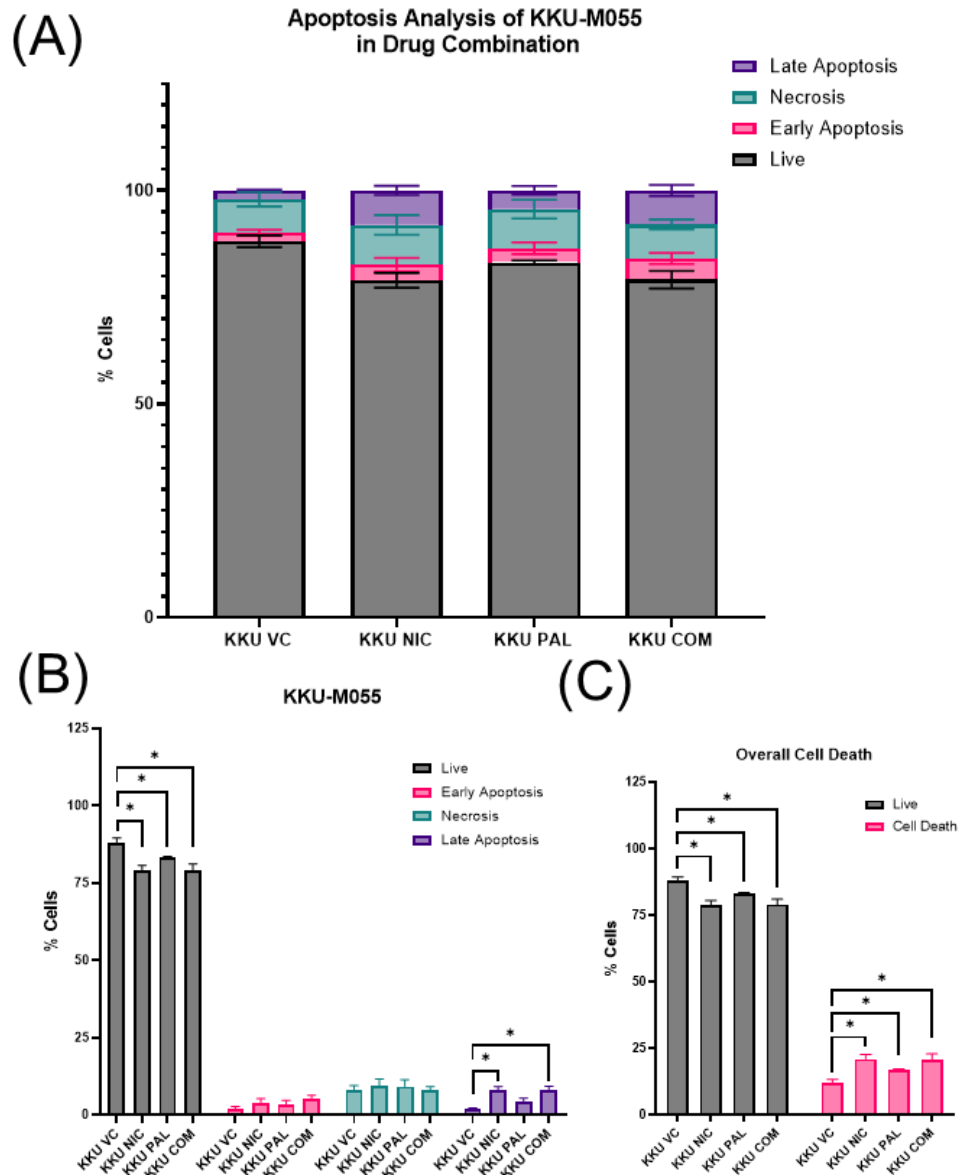


Figure 4.27: Apoptosis Analysis in Combination Treated KKU-M055 Cells. KKU-M055 cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for apoptosis experiments. The analysis shows the proportion of the cell population in each stage of cell death: Live, Early Apoptosis, Necrosis and Late Apoptosis. Error bars represent SEM and two-tailed unpaired t-test was performed to record significant differences (N=3).

(A) An overview of the changes to cell cycle in single and combination treated KKU-M055.

(B) The statistical significance between vehicle control and the treatments.

(C) The statistical significance between vehicle control and the treatments when early apoptosis, necrosis and late apoptosis are grouped as one into cell death.

4.7D Comparison of the Effect of Combination Treatment in

CCA Cell Lines:

A summary of the effect of single agent and combination treatment on cell cycle progression and apoptosis through flow cytometry methods is shown in **Figure 4.28**, and a summary of the effect of single agent and combination treatment on protein expression is shown in **Figure 4.29**. This allows comparison between the effects of single agents and combination treatment to observe any statistical significance in using the combination.

Compared to vehicle control, Palbociclib and combination treatment caused substantial increase to the percentage of cells in G1 (**Figure 4.28A**) and a substantial decrease to the percentage of cell in G2 (**Figure 4.28B**). This occurs in all CCA cell lines and is indicative of cell cycle arrest. Although slightly more cell cycle arrest is seen in combination treated CCLP and KKU-M055 cells, this is not statistically significant to Palbociclib only treated cells, although experimental repeats are likely to increase significance in KKU-M055 cells. Niclosamide was only seen to significantly decrease the percentage of cells in G2 in KKU-M055 compared to vehicle control ($p < 0.05$). Cell cycle arrest, seen through a decrease in G2 cells and an increase in G1 cells, is strongly increased in combination treated cells compared to Niclosamide treated cells ($p < 0.05$). Thus, cell cycle arrest is further induced with combination treatment but there is little difference to Palbociclib treatment alone.

Compared to vehicle control, combination treatment increased the level of apoptosis in CCLP and RBE cells however this was only statistically significant in CCLP cells ($p < 0.05$) (**Figure 4.28C**).

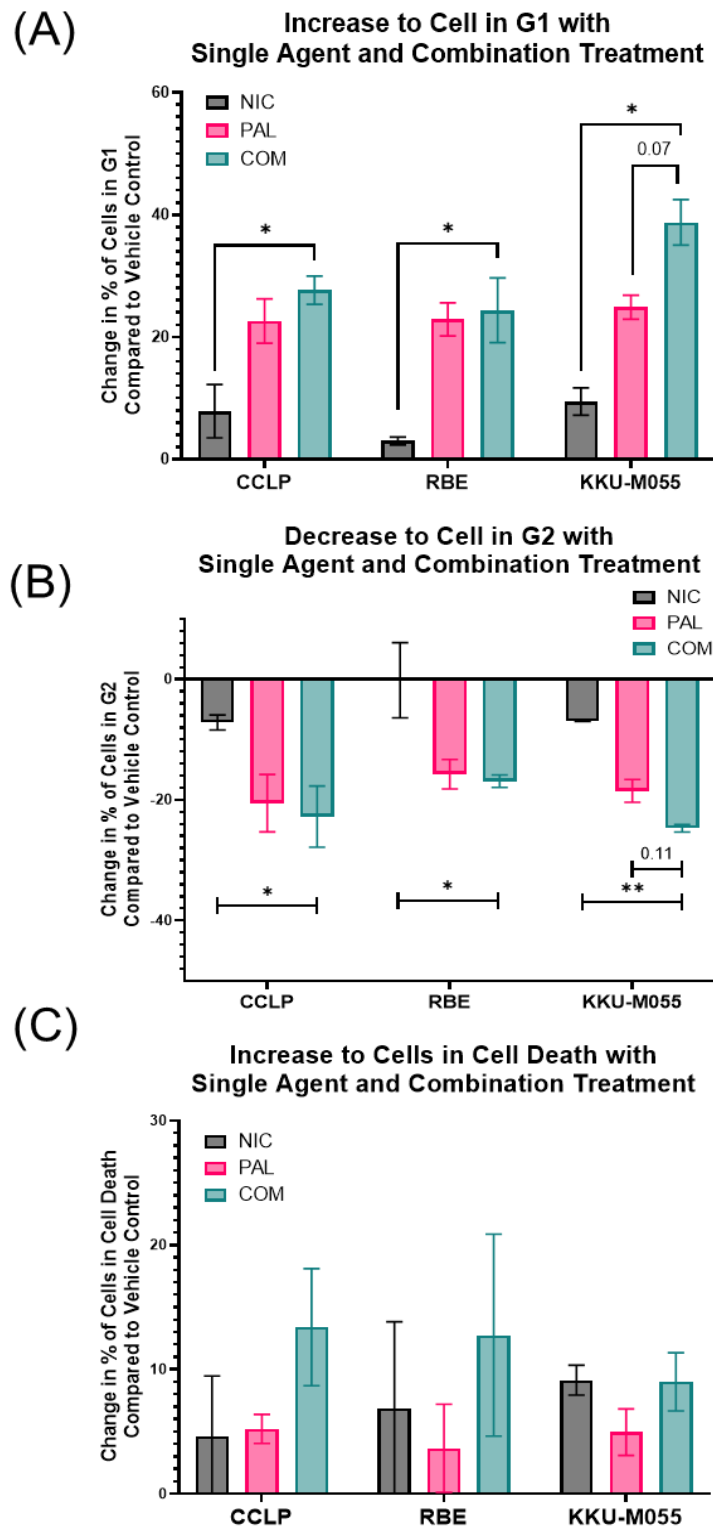


Figure 4.28: Assessing Changes to Cell Cycle Progression and Apoptosis across the Combination Treated CCA Cell Lines.

A summary of data taken from the cell cycle and apoptosis flow cytometry experiments to assess patterns across the CCA cell lines. Graphs show the difference in percentage of cell compared to vehicle control. Error bars present SEM, significance was measured through an unpaired t-test (*= $p < 0.05$) (N=3).

- (A) The increase in the percentage of cell in G1 due to Niclosamide, Palbociclib or Combination treatment across CCA cells.
- (B) The decrease in the percentage of cells in G2 due to Niclosamide, Palbociclib or Combination treatment across CCA cells.
- (C) The increase in percentage of cell in cell death due to Niclosamide, Palbociclib or combination treatment across the CCA cell lines.

Levels of apoptosis in combination treated KKU-M055 were the same as Niclosamide treated KKU-M055, thus no additive effect from Palbociclib was observed. It is likely that apoptosis is not the main cause of synergism but may occur in some CCA cell lines and this may promote stronger synergism, such as that seen in CCLP cells.

The level of p-RB follows a similar pattern amongst the drug treated CCA cell lines, whereby each drug as a single agent causes a decrease in p-RB protein and the combination of both Niclosamide and Palbociclib causes a substantial decrease in p-RB protein (**Figure 4.29A**). In CCLP and RBE cells, the further decreased caused by combination treatment shows a major decrease in p-RB compared to Palbociclib treatment alone ($p < 0.05$).

The levels of PRH expression follow a very similar pattern to that of p-RB, where combination treatment promotes the strongest repression of PRH protein (**Figure 4.29B**). However, the decrease in PRH is not statistically significant compared to Niclosamide treatment alone or Palbociclib treatment alone, generally.

Niclosamide causes a decrease in Cyclin D1 in all CCA cell lines, whilst Palbociclib promotes an increase in Cyclin D1 in all CCA cell lines (**Figure 4.29C**). The level of Cyclin D1 varies amongst the combination treated CCA cell lines, with CCLP and RBE showing levels similar to Niclosamide treated cells, and KKU-M055 showing levels similar to Palbociclib treated cells. It is likely that the effect of Palbociclib on Cyclin D1 expression in the KKU-M055 cells is stronger than the effect of Niclosamide.

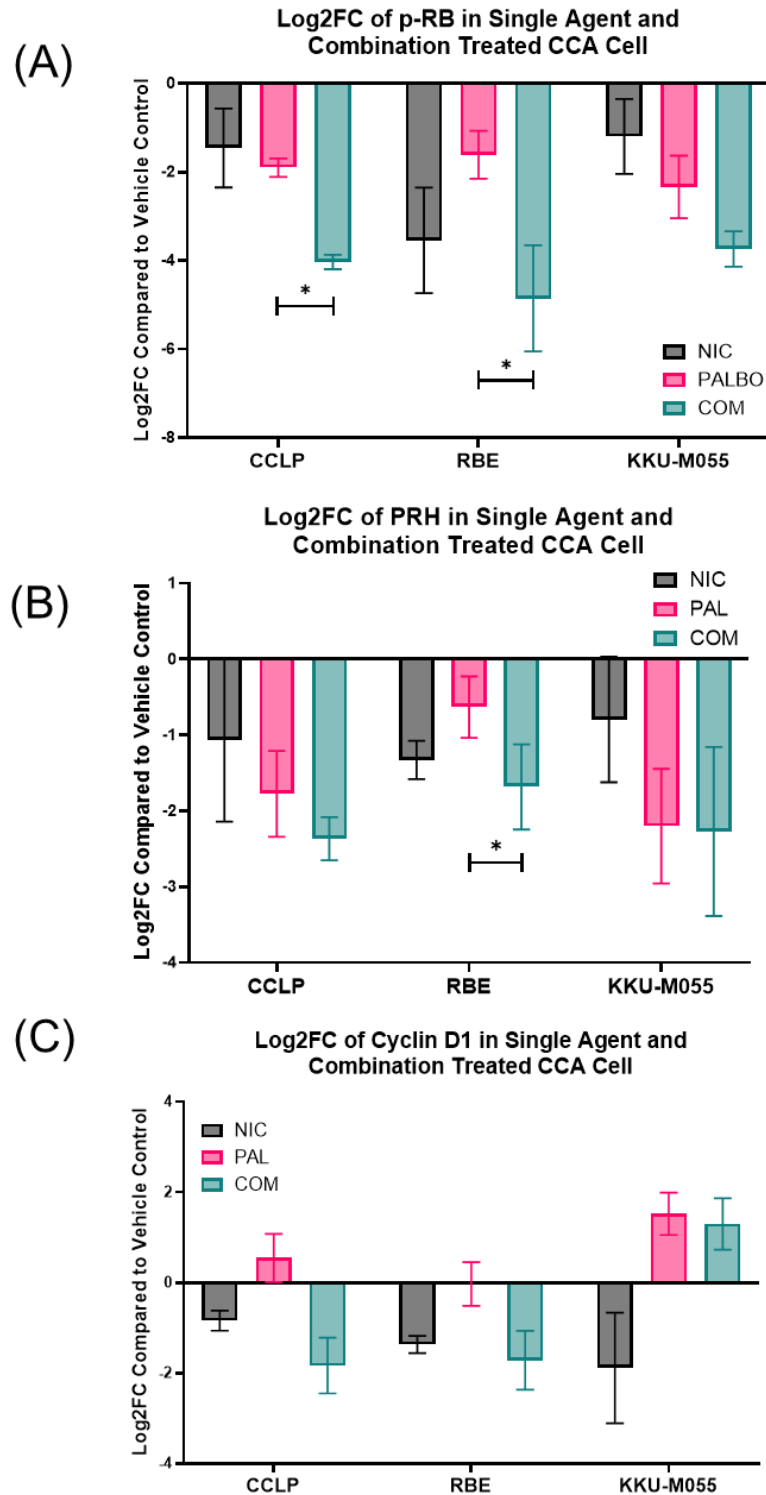


Figure 4.29: Assessing Changes to Protein Expression across the Combination Treated CCA Cell Lines.

A summary of data taken from the western blotting experiments to assess patterns across the CCA cell lines. Graphs show the log2FC in protein expression compared to vehicle control. Error bars present SEM, significance was measured through an unpaired t-test (*= $p < 0.05$) (N=3).

- (A) The decrease in p-RB expression in Niclosamide, Palbociclib or Combination treated CCA cells.
- (B) The decrease in PRH expression in Niclosamide, Palbociclib or Combination treated CCA cells.
- (C) The changes in Cyclin D1 expression in Niclosamide, Palbociclib or Combination treated CCA cells.

Overall, the changes in the levels of p-RB, PRH and Cyclin D1 is similar in all of the drug treated CCA cells. In summary, this analysis shows that the combination of drug has the potential to promote further anti-cell cycle effect compared to use of Niclosamide or Palbociclib alone, by promoting further decrease to cell cycle and cell cycle proteins. In addition, the Niclosamide treatment decreases the upregulation of Cyclin D1 caused by Palbociclib.

4.8 Single and Combination Treatment in 3D

Spheroids

To extend the work performed in 2D cell culture, the effect of the drugs as single agents and in combination were next examined in a 3D CCLP spheroid model. Additionally, to confirm that there is little effect of the drug combination on primary untransformed cells, the primary BECs were also grown using the 3D spheroid protocol to form spheroid like structures and treated as above (Gunit et al., 2021).

Spheroids were generated, as described previously, and left to grow for five days to form a robust 3D structure before adding either vehicle control (VC), the drugs as single agents or in combination (as described in materials and methods section 2.1.8). The concentrations of Niclosamide and Palbociclib added were 0.5 μ M and 1 μ M, respectively, as at these concentrations, the drugs in combination were able to significantly reduce CCLP cell viability further than either drug alone in 2D assays (section 4.3; **Figure 4.4**). The drugs were added to the well and the media was gently mixed before incubating for 72hrs as described previously (section 3.8.2). After the treatment, spheroids were imaged, and the images were used to calculate spheroid volume. In parallel, cell viability was measured through Presto Blue assays. The experiment was performed in triplicate and with three biological repeats. **Figure 4.30** shows representative brightfield images of CCLP spheroids and BEC 3D spheroid like structures after 72hr incubation with either vehicle control, Niclosamide, Palbociclib or both drugs in combination.

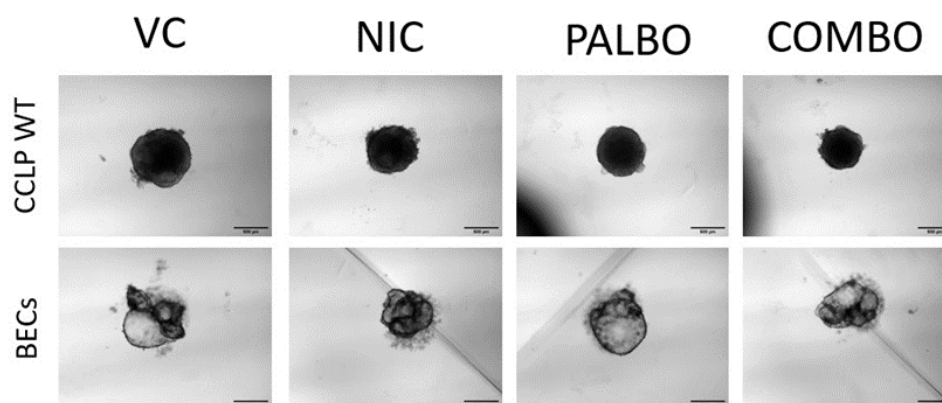


Figure 4.30: Spheroids after 72hrs treatment with Niclosamide, Palbociclib or both in combination.

Spheroids were generated from CCLP WT cell line and primary BECs. The spheroids were left to grow for five days before treating with Niclosamide (0.5 μ M), Palbociclib (1 μ M) or both in combination. The spheroids were treated for a further 72hrs before cell viability was measured through a presto blue assay and images were taken on a Nikon brightfield microscope. Representative images were taken for each cell lines.

Figure 4.31 summarises the effect of the drugs as single agents and in combination on spheroid volume and on spheroid viability. The volume of CCLP WT spheroids was reduced after treatment with either drug, Niclosamide (69%) and Palbociclib (74%) but was further, reduced when the drugs are used in combination (compared to either drug alone (58%) ($p < 0.01$)). The CCLP WT spheroid viability was also significantly reduced after treatment with either drug, Niclosamide to 70% ($p < 0.01$) and Palbociclib to 76% ($p < 0.05$) of the control, and further reduced when the drugs are used in combination to 49% of the control ($p < 0.0001$); this is comparable to the decrease in spheroid volume. This shows the combination treatment is effective in a more complex 3D model. In marked contrast there is a small reduction in spheroid volume and viability when BEC spheroids were treated with the drugs as single agents or in combination but the decreases were not statistically significant (t-test conducted between the viability data of BECs treated with vehicle control and BECs treated with combination; $p = 0.15$). This implies that the drug combination will be able to

have its effects on cancer cells without having comparable effects on normal bile duct epithelial cells.

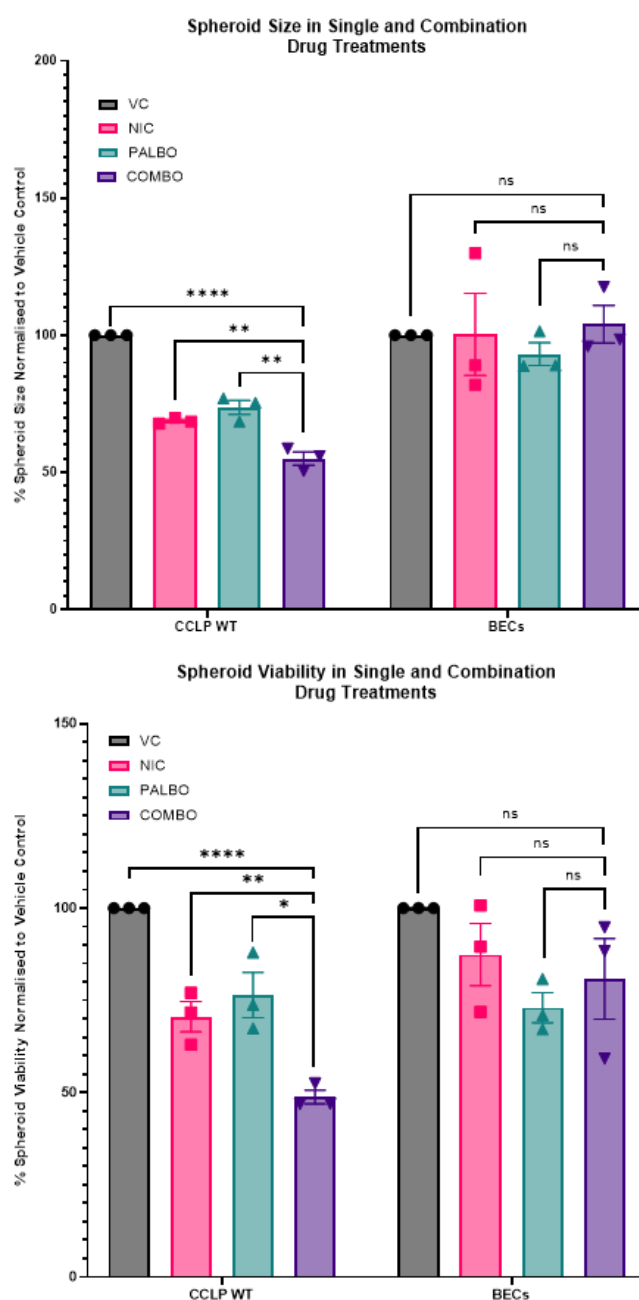


Figure 4.31: Spheroid Viability and Spheroid Size after 72hrs Treatment with Niclosamide, Palbociclib or both in Combination.

Spheroids were generated from CCLP WT cell line and Biliary Epithelial Cells (BECs). The spheroids were left to grow for five days before treating with Niclosamide (0.5uM), Palbociclib (1uM) or both in combination. The spheroids were treated for a further 72hrs before cell viability was measured through a presto blue assay and images were taken on a Nikon brightfield microscope. Spheroid size was calculated through Image J analysis. Spheroid viability and spheroid size was normalised to vehicle control. Three biological repeat were obtained. Error bars represent SEM and significant was calculated through two-tailed paired t-test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$) (N=3).

Since knockdown of PRH resulted in CCLP PRH KD cells showing less sensitivity than controls to Niclosamide treatment at 24 hours (section 3.8) and to Palbociclib at 72 hours of treatment (section 4.2), we next examined whether this change in sensitivity with long term PRH knockdown would also be present in the response to combination therapy in the 3D spheroid model. **Figure 4.32** shows representative images of the drug-treated spheroids and **Figure 4.33** summaries the effect of drug treatments on spheroid area and viability.

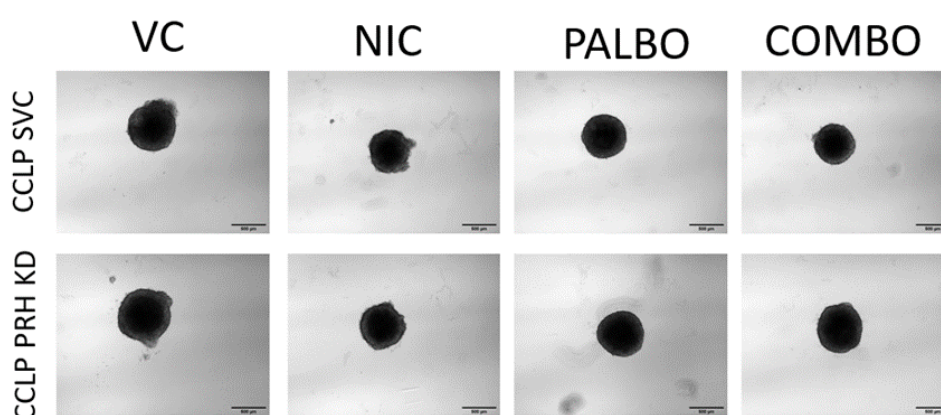


Figure 4.32: CCLP SVC and PRH KD Spheroids after 72hrs treatment with Niclosamide, Palbociclib or both in combination.

Spheroids were generated from CCLP SVC and CCLP PRH KD cell lines. The spheroids were left to grow for five days before treating with Niclosamide (0.5uM), Palbociclib (1uM) or both in combination. The spheroids were treated for a further 72hrs before cell viability was measured through a presto blue assay and images were taken on a Nikon brightfield microscope. Representative images were taken for each cell lines.

Control CCLP SVC spheroids show a significant decrease in volume in response to single drug treatment, and to combination treatments, where both drugs reduce spheroid volume to 55% of the control over 72 hours ($p < 0.0001$). Similarly, there was a reduction in cell viability in CCLP SVC spheroids treated with drugs as single agents and in combination, with combination treatments causing a decrease to 31% of the control viability ($p < 0.0001$). Whilst there was significant decrease in spheroid volume with combination compared to single agents ($p < 0.001$), the combination does not further decrease CCLP SVC

spheroid viability compared to spheroids treated with Palbociclib alone ($p=0.08$). The PRH KD cells showed similar results to the SVC controls in that single agent treatments reduced spheroid volume and cell viability (**Figure 4.33**). However, the combination does not further decrease PRH KD spheroid volume compared to drugs used as single agents, and the viability of combination treated spheroids is not significantly different to the viability of PRH KD spheroids treated with Palbociclib alone ($p=0.10$).

The volume of combination treated CCLP SVC spheroids is smaller than combination treated CCLP PRH KD spheroids ($p<0.05$). Average cell viability in the combination treated CCLP SVC spheroids appears to be decreased compared to combination treated CCLP PRH KD spheroids, but this result is just outside statistical significance ($p=0.08$). Further experiments are required to confirm this observation.

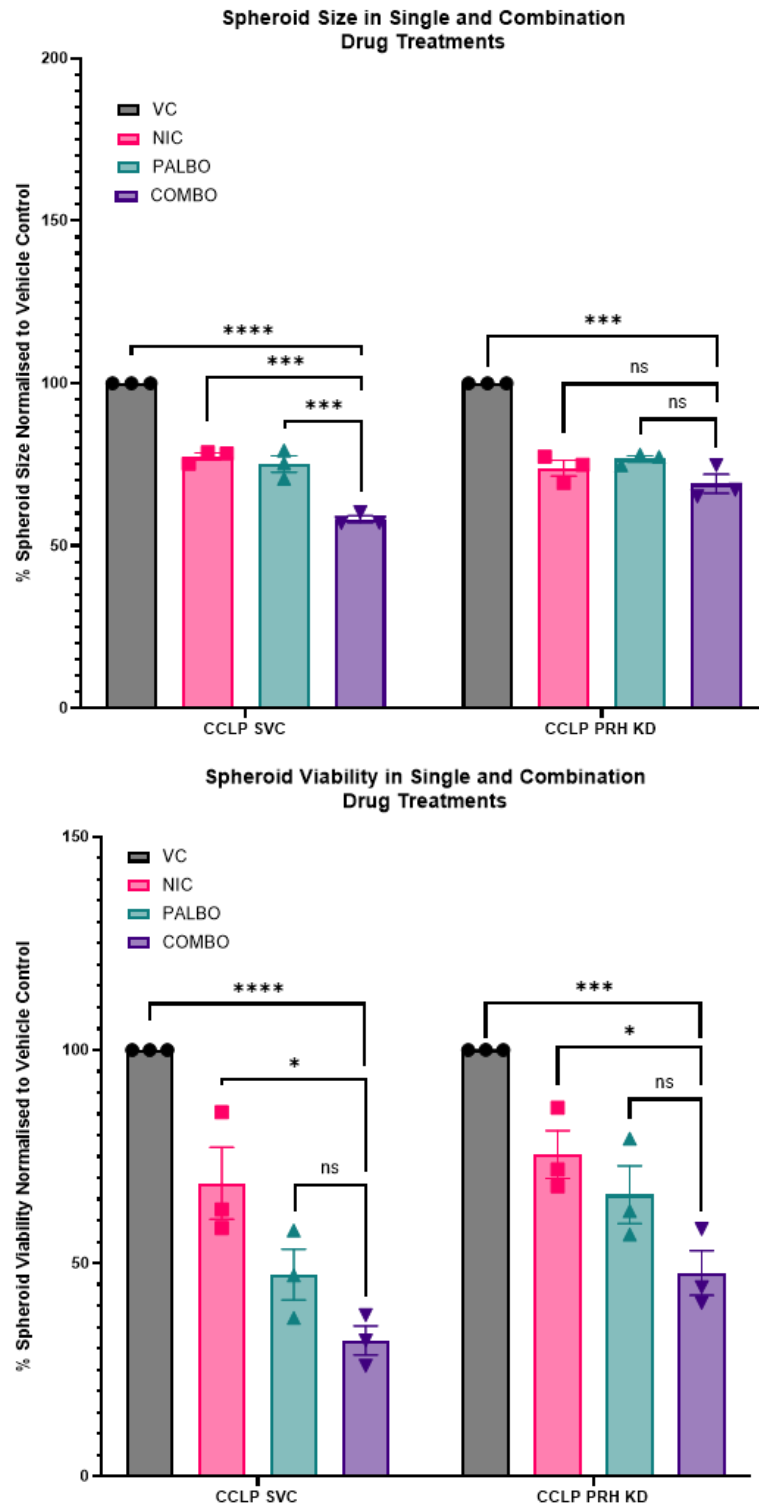


Figure 4.33: CCLP SVC and PRH KD Spheroid Viability and Spheroid Size after 72hrs Treatment with Niclosamide, Palbociclib or both in Combination.

Spheroids were generated from CCLP SVC 2 and CCLP PRH KD 2 cell lines. The spheroids were left to grow for five days before treating with Niclosamide (0.5uM), Palbociclib (1uM) or both in combination. The spheroids were treated for a further 72hrs before cell viability was measured through a presto blue assay and images were taken on a Nikon brightfield microscope. Spheroid size was calculated through Image J analysis. Spheroid viability and spheroid size was normalised to vehicle control. Three biological repeat were obtained. Error bars represent SEM and significant was calculated through two-tailed paired t-test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$) (N=3).

4.9 Discussion

CCA cell line viability is inhibited by Palbociclib with less effect on BECs

Palbociclib sensitivity was investigated in STR-profiled CCA cell lines, alongside BECs. The cell viability assays in all three tumour cell lines confirm that Palbociclib is able to reduce CCA cell viability in a dose-dependent and time-dependent manner. At 24 hours, Palbociclib exerts weak cytotoxic effects on cell viability but few anti-proliferative effects of Palbociclib can be seen at this timepoint. However, at 72 hours, Palbociclib exerts a potent anti-cell cycle effect. The Palbociclib EC₅₀ values obtained after 72 hours incubation in the CCA cell lines ranged from 1-5 μ M. These values are similar to the concentrations reported to achieve 50% growth inhibition using the MTT assay in a range of other cancer cell lines at 72-hours drug incubation (0.032 μ M-7.442 μ M) (Tadesse et al., 2017). Importantly, the data shows that the BECs are not as sensitive to Palbociclib as CCA cells. Indeed, even at 72 hours combination, showing a 6-fold increase to EC₅₀ value compared to the CCA cells. This significant differential response to Palbociclib suggests that the CDK4/6 inhibitor can selectively target CCA cells over the normal cholangiocyte cells. This finding is novel and supports the use of Palbociclib for CCA treatment in the clinic.

Although Palbociclib is able to target the CCA cells preferentially over the BECs cells, the EC₅₀ values do not fall in the range of the maximum serum concentration found in humans, approximately 500nM (Tamura et al., 2016). In an attempt to enhance the anti-CCA cell activity of Palbociclib and thus reduce the concentration needed to reach 50% cell inhibition, Palbociclib was used in combination with Niclosamide.

Niclosamide and Palbociclib act synergistically

Combination therapy is becoming a popular way to enhance and optimise the anti-cancer mechanism of drugs (Mokhtari et al., 2017). Currently, Palbociclib has been shown to synergise with several drugs across different cancers: mTOR inhibitor (Yamamoto et al., 2019), multi-target receptor tyrosine kinase mtRTK inhibitor Sunitinib (Moskovits et al., 2022), Oxaliplatin (Supramote, 2022), BRAF and MEK inhibitors (Martin et al., 2018). Similarly, Niclosamide has been used in synergy experiments with Doxorubicin (Lohiya et al., 2021), EGFR inhibitor Erlotinib (Li et al., 2013) (Shi et al., 2017), Paclitaxel (Chen et al., 2017), BCR-ABL tyrosine kinase inhibitor Dasatinib (Liu et al., 2016). Therefore, Palbociclib and Niclosamide have both been used in multiple synergism experiments, however at the time of this study this is the first time both have been studied in combination.

Niclosamide and Palbociclib combination studies were performed with MTT cell viability assays and synergism was quantified by the Chou-Talalay method through CI values (Chou, 2010). The results demonstrated that the two drugs were synergistic across the CCA cell lines at different drug ratios. To determine whether this synergism can occur in other clinically relevant cell types, experiments were repeated in breast cancer cell line, MCF-7 cells. The drugs showed weak synergism in these cells at higher drug concentrations. The ability of the two drugs to synergise in multiple cell types, with different mutational backgrounds and different dysregulated oncogenic signalling pathways reveals that the drugs are affecting common pathways utilised by the majority of cancer cells. Also, the drugs are synergistic at the lower concentrations which is important as the drugs may not reach the higher concentrations when administered *in vivo*. Further investigation of Niclosamide and Palbociclib synergism could be performed by assessing the drugs at different ratio, as this

has been shown to affect the degree of synergism and may explain the weak synergism in some of the cell lines (Cavanagh et al., 2024).

Many drug combinations do not reach clinical efficacy as although they are efficacious in 2D cell culture they encounter different barriers to efficacy in an *in vivo* setting. The use of 3D spheroids helps bridge this gap. The combination was able to cause a reduction in spheroid volume and viability in CCLP spheroids more than each drug alone, whereas the drugs alone or in combination had little effect on the BECs. This shows that the effect of the combination remains more favourable than using either drug alone in a spheroid model, and the drug combination selectively inhibits CCLP cells at concentrations that do not affect BECs.

To narrow down the mechanisms behind Niclosamide and Palbociclib synergism in CCA, each drug was replaced by an alternative member of their drug class. Niclosamide was used in synergism experiment with alternative CDK4/6 inhibitor Ribociclib, and Palbociclib was observed in synergism experiment with alternative mitochondrial uncoupler, FCCP. Interestingly, both new combinations were synergistic. Of the three combinations, Ribociclib and Niclosamide generated the lowest CI values, hence showed the highest synergism, and FCCP and Palbociclib reported the highest CI values, hence gave the weakest synergism. As the CDK4/6 and Niclosamide synergism experiments generates the lowest CI values, it is likely that cell cycle arrest via CDK4/6 inhibition is required for the synergism. Additionally, as FCCP is not able to produce the same level of synergism as Niclosamide, it is likely that mitochondrial uncoupling is not the only property of Niclosamide that is important for synergism.

Niclosamide and Palbociclib promote potent cell cycle arrest

The canonical mechanism of action of Palbociclib is blocking CDK4/6 complexes and inhibiting the phosphorylation of RB, thus inducing cell cycle arrest (Hydbying et al., 2016). Niclosamide is a well-documented mitochondrial uncoupler, a type of compound that can dissipate the proton gradient and disrupt ATP production in the mitochondria which generally leads to apoptosis (Kumar et al., 2018). Although Niclosamide has also been shown in the literature to induce cell cycle arrest, Niclosamide was only able to significant induce apoptosis, not cell cycle arrest, in the previous chapter. It could be hypothesised that any further decrease in cell viability gained by using the drugs in combination could arise from their ability to promote different mechanisms.

In the combination studies, the promotion of additional cell cycle arrest, and not apoptosis, appears to be a predominant mechanism underlying Niclosamide and Palbociclib synergism across CCA cancer cell lines. Combination treatment is able to induce cell cycle arrest more potently than either drug alone in all of the cell lines tested, and there are significant changes to cell cycle proteins, including loss of p-RB expression, in all the CCA cell lines. Palbociclib is known to decrease p-RB by inhibiting the activity of CDK4/6-Cyclin D complexes (Hydbying et al., 2016), however, the effect of Niclosamide on p-RB has not been published. Here, Niclosamide treatment causes a reduction in p-RB, and, remarkably, in the RBE cell line, causes a more significant reduction of p-RB than Palbociclib. In contrast to the significant increase to cell cycle arrest elicited by the Niclosamide-Palbociclib combination, the induction of apoptosis in the combination studies is poor by both agents alone and in combination.

This new observation of decreased p-RB protein levels with Niclosamide treatment could be related to the ability of Niclosamide to induce protein ubiquitylation and degradation. It has been shown in the literature, and during the previous chapter, that Niclosamide treatment increases total protein

ubiquitylation and can lead to the degradation of specific proteins. It is possible that Niclosamide decreases p-RB, and/or RB, protein levels similarly, through increasing their ubiquitylation and promoting protein degradation at 72 hours. Unfortunately, total RB levels were not possible to quantify because of technical difficulties with two different RB antibodies in Western blotting experiments (see supplemental **Figures 4.1 and 4.2**). If Niclosamide can promote p-RB/RB degradation, and Palbociclib causes a loss of p-RB via CDK4/6 inhibition, this could explain the further decrease to p-RB levels when the drugs are in combination. Moreover it, could explain the potent cell cycle arrest induced by combination and be a mechanism of synergism.

The levels of Cyclin D and p21 may also be linked to the ability of Niclosamide and Palbociclib to synergise. Cyclin D and p21 levels were reduced in the CCA cell lines after 72 hours incubation with 0.5 μ M Niclosamide, potentially through protein degradation. In contrast, Palbociclib caused Cyclin D levels to rise. This is because upon G1 cell cycle arrest, Cyclin D proteins accumulate (Cai et al., 2022). Also, p21 levels have been showed to increase during Palbociclib-induced cell cycle arrest (Wang et al., 2020). The level of Cyclin D and p21 in combination treated CCA cell lines varied, and it is likely that the outcome is dependent on the balance of protein degradation caused by Niclosamide and protein accumulation caused by Palbociclib-induced cell cycle arrest, which will be different in each cell line. In the presence of Palbociclib, the accumulating Cyclin D1 and p21 can still form a complex with CDK4 (Pack et al., 2021), and indeed, Cyclin D overexpressing cancers are associated with Palbociclib resistance (Cai et al., 2022)., Therefore, the decrease in Cyclin D and p21 protein levels elicited by Niclosamide, is likely to reduce any Cyclin D-CDK-p21 signalling that Palbociclib treatment alone fails to inhibit. This theory would explain why combination treated KLU-M055 cells that still show relatively high

levels of Cyclin D, have a much lower degree of synergism compared to combination treated CCLP and RBE cells that have low levels of Cyclin D1. This could be a mechanism by which the synergism between Niclosamide and Palbociclib is influenced.

Cell death may also contribute to the reduction in cell viability seen in combination treated cells, however it is not likely to be influential in combination synergism within the parameters of the experiments performed in this chapter. This is because although there are trends toward increased apoptosis with the combination treatment, they are not consistent amongst cell lines. This may be because the concentrations used for the experiment are not potent enough to elicit apoptosis. For example, 3.2 μ M Niclosamide was shown to significantly increase apoptosis in CCLP cells at 24 hours treatment. However, in combination experiments using 0.5 μ M Niclosamide, there was no significant induction of apoptosis in CCLP at 72 hours. To see any significant effect of the combination on cell death and its signalling, cells could be treated with a range of synergistic combinations of drug and for shorter timepoints, for example 24 hours.

Niclosamide and Palbociclib and PRH

The levels of PRH were substantially reduced by combination treatment in the three CCA cell lines. Previously, it was seen that Niclosamide could reduce PRH expression over 24 hours at concentrations ranging from 1 μ M-8 μ M Niclosamide, in all CCA cell lines and that this could be inhibited by blocking the proteasome. After 72 hours incubation with 0.5 μ M Niclosamide, average PRH expression appears to decrease in all CCA cell lines, but this is only statistically significantly in RBE cells. Palbociclib caused a strong reduction in PRH levels in all CCA cell lines, similar to the reduction in p-RB observed in all cell lines.

There is a much larger reduction in PRH caused by the drug combination than each drug acting as a single agent. As PRH is oncogenic in CCA and drives cell cycle progression, a combination therapy that reduces its expression to low levels is most desirable.

Previously published work had shown that PRH overexpressing CCLP cells were more sensitive to Palbociclib at 24 hours treatment (Kitchen et al., 2020) and we showed in this chapter that PRH shRNA KD cell lines are more resistant to Palbociclib treatment at 72 hours than control. Moreover, they are also more resistant to Niclosamide than controls at 72 hours (chapter 3). In 3D spheroids, PRH KD cells were also more resistant to combination therapy compared to SVC when using spheroid volume as a comparator. However, the difference in cell viability between controls and PRH KD spheroids with combination treatment although apparent was not statistically significant. Different concentrations might be needed to be used to obtain significant results in cell viability experiments in the 3D models. It is possible that there is a drug concentration gradient across the spheroid (Sutherland, 1988). Notably cell viability was not decreased equally in 2D and 3D models with Niclosamide treatment. The same decrease in cell viability occurs at a later time point in 3D models compared to 2D models (see **Figure 3.26** and **Figure 3.2**); thus, CI experiments would need to be performed with 3D spheroids to determine the synergistic concentration range. The resistance of CCLP PRH KD cells to Niclosamide and Palbociclib is not expected, as across all three CCA cell lines CCLP, RBE and KKU-M055, PRH protein depletion is a consequence of drug treatment with both single agents. Therefore, it might be expected that cells with less PRH expression would require less drug treatment and would be more sensitive to the drugs.

CHAPTER 5:

Exploring the Role of PRH in CCA using different model systems

5.1 Introduction

Previous work with a polyclonal shRNA PRH KD cell line showed decreased cell proliferation compared to control cells with little effects on intrinsic apoptosis (Kitchen et al., 2020). The change in PRH expression showed regulation of Notch-3 and Cyclin D2 through RNAseq and western blotting. Here, we extend this work by examining the effects of short-term siPRH knockdown in two CA cell lines, in clonal CRISPR mediated PRH knockout cell lines, and newly generated shRNA PRH KD cell lines through phenotypic experiments and gene expression .

SECTION A:

The effects of PRH KD using
siRNA in CCLP and RBE cells

5.2: Validation of PRH Knockdown via siRNA

PRH knockdown was investigated using silencing RNA (siRNA). SiRNA is duplex RNA synthesised to complement the target mRNA and cause degradation. It enters the cell and assembles into an RNA-Induced Silencing Complex (RISC) with Argonaute, a ribonuclease. The single stranded guide RNA leads the complex to the specific base pair sequence of the target gene where it binds and cleaves the target mRNA, resulting in its degradation. The method uses transient transfection of the oligonucleotide siRNA sequences and does not depend on plasmid integration into the genome. The siRNA knockdown requires no selection via antibiotics and its effects are temporary. Silencing RNA therefore allows us to explore the immediate effect of PRH knockdown on CCA cell phenotype and gene expression.

CCLP and RBE cells were both used in siRNA PRH knockdown experiments. RBE cells were used as they have low expression of Notch-3 protein (see **Figure 3.1**), thus PRH driven mechanisms that may be largely independent of Notch-3 can be observed. Also, the addition of RBE can determine whether previously observed effects of PRH knockdown in CCLP cells are cell line specific.

SiRNA transfection was optimised using RNAiMAX Lipofectamine reagent described at section 2.1.9. and control siRNA or HHEX-targeting siRNA were transfected into CCLP or RBE cells. A 2×10^6 cell suspension was incubated with a transfection mixture, containing siRNA and Lipofectamine, for 8 hours until the cells had adhered, then the media was removed and replaced with fresh media. The cells were left 48 hours post transfection before harvesting cells to

produce RNA samples and protein lysates. RNA was reverse transcribed into cDNA as previously described (section 2.3). Relative gene expression was then measured using *HHEX*/PRH primers and B-actin primers.

Figure 5.1A shows that PRH mRNA significantly decreases in both CCLP and RBE cells following siPRH transfection. The expression of PRH mRNA in siPRH CCLP is reduced to 18% ($p=0.0038$), while the expression in siPRH RBE is decreased to 12% ($p>0.0001$) of the siCtrl. At the same time point whole cell extracts were electrophoresed by SDS-PAGE and transferred to a membrane and probed for PRH and B-actin (details are in section 2.5). PRH protein levels were normalised to the B-actin loading control, then the normalised expression in siPRH cells was made relative to the normalised expression level in siCtrl for each cell line. PRH protein expression was decreased to 3% of the control in CCLP cells ($p>0.0001$) and 2% of the control in RBE ($p>0.0001$) in siPRH treated cells (**Figure 5.1B and C**). In conclusion, successful PRH knockdown was achieved using siRNA in both cell lines.

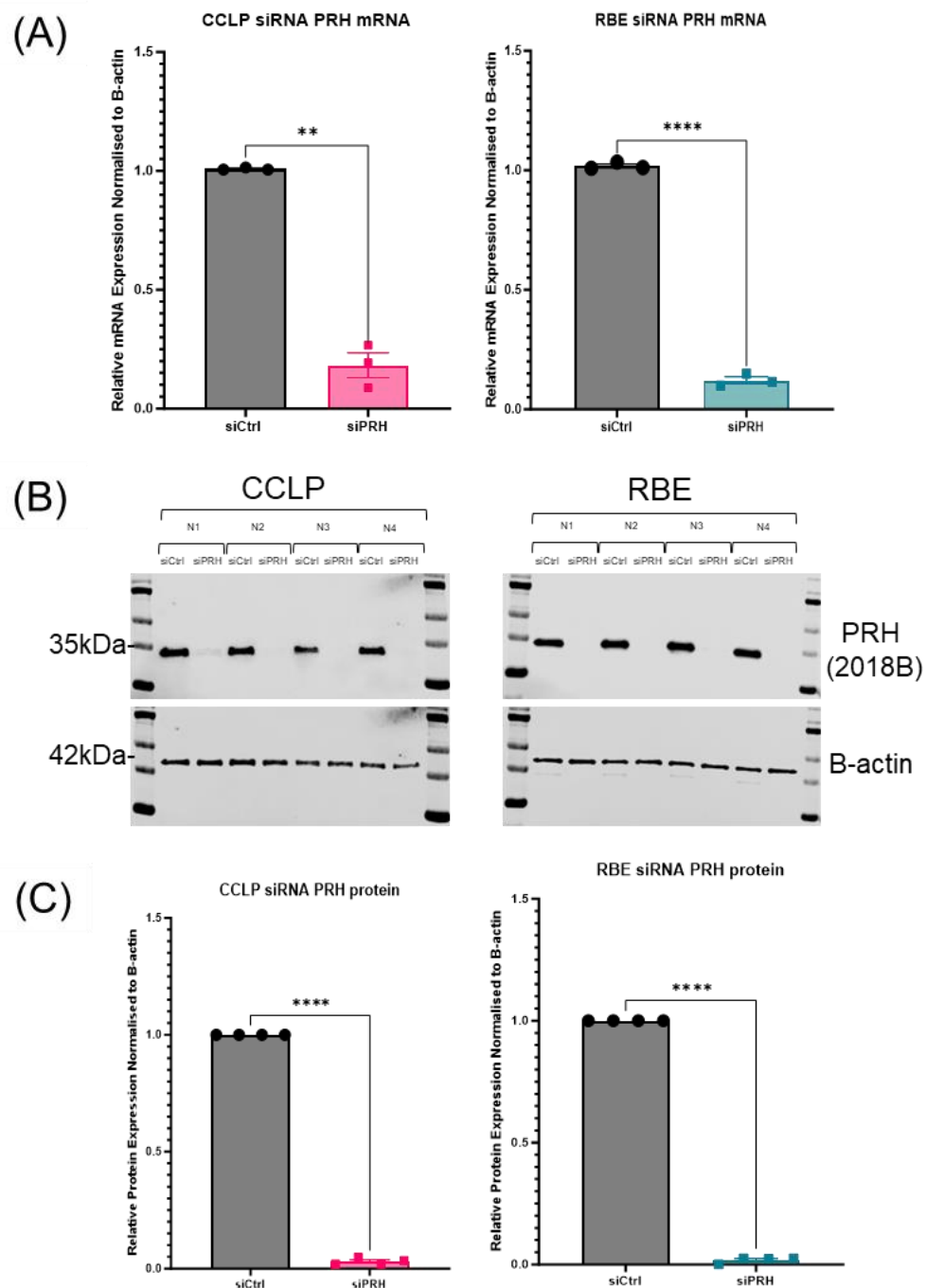


Figure 5.1: PRH mRNA and Protein Expression in siRNA CCLP and RBE.

Cells were transfected and left for 48hrs before harvesting as two sets of pellets for RNA and protein samples.

- (A) PRH mRNA expression in siPRH CCLP or RBE compared to a siCtrl control, measured through qPCR. Ct values were normalised to B-actin mRNA expression, and further normalised to siCtrl. Error bars represent SEM, and statistical significance was measured through two-tailed t-test (N3; **= $p < 0.01$ **** = $p < 0.0001$).
- (B) Western blot showing PRH protein expression in the siRNA CCLP and RBE repeats. Each lane has 30ug protein and was probed with PRH (2018B) and B-actin antibodies.
- (C) PRH protein expression in siPRH CCLP or RBE compared to siCtrl control, measured through western blot. Band densitometry values were normalised to B-actin protein expression, and further normalised to siCtrl. Error bars represent SEM, and statistical significance was measured through two-tailed t-test (N4; **** = $p < 0.0001$).

As the effects of siRNA transfection are transient, the level of PRH in siPRH treated cells was also observed over time in CCLP and RBE cells. After 48hrs of transfection, the cells were harvested and replated in T25 flasks. Pellets were then taken every 24 hours, for 120 hours and protein lysates were prepared for western blotting (**Figure 5.2**). PRH protein expression was lower in the siPRH cells, compared to the siCtrl cells, throughout the five days. However, PRH expression increased with time, with PRH protein expression reaching 64% and 81% of the siCtrl on day five, in CCLP cells and RBE cells, respectively. This is important as it confirms cells maintain low PRH expression through any subsequent experiments exploring the effect of siPRH KD on cell phenotype.

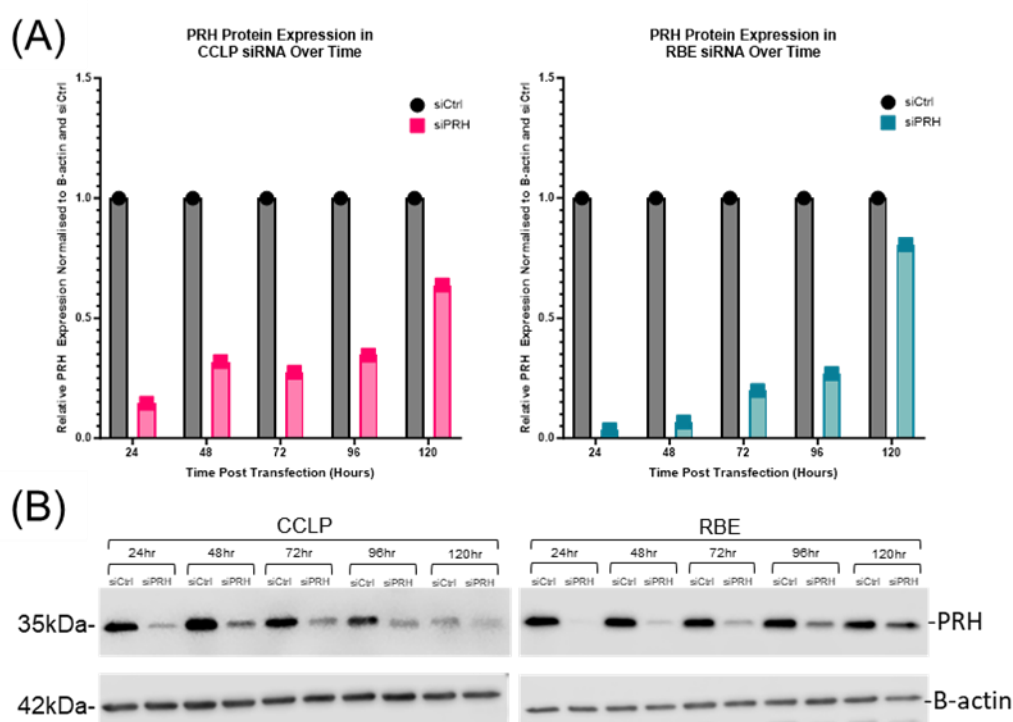


Figure 5.2: PRH knockdown in siRNA CCLP and RBE over 120 hours.

Cells were transfected, as detailed in section 2.1.7, and left for 48hrs before harvesting and seeding into T25 flasks. Pellets were collected 24hr, 48hr, 72hr 96hr and 120hr after transfection to confirm a PRH knockdown is maintained.

(A) PRH protein expression in siPRH CCLP or RBE compared to a siCtrl control over 120 hours. PRH band densitometry was normalised to B-actin, and further normalised to siCtrl (N1)

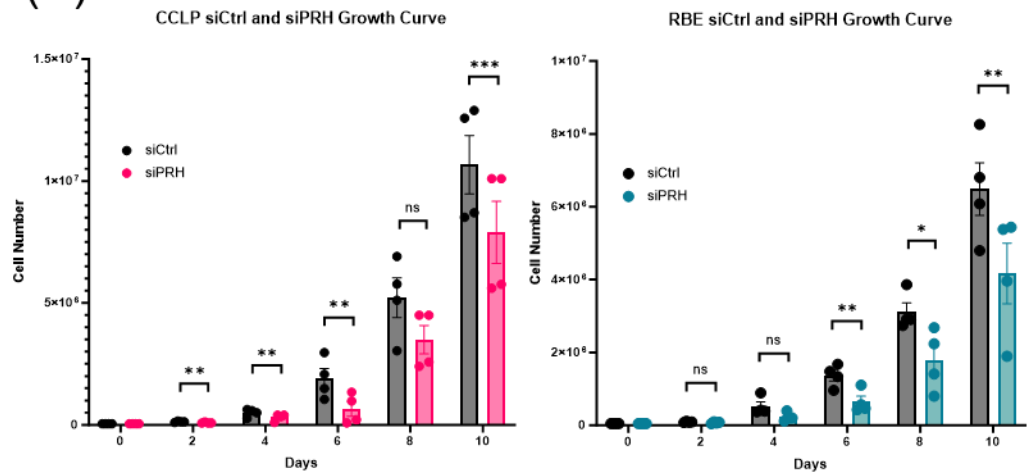
(B) Western blot showing PRH protein expression in the siRNA CCLP and RBE repeats. Each lane has 30ug protein and was probed with PRH (2018B) and B-actin antibodies.

5.3: The Effect of PRH KD via siRNA on Cell Number over Time

To determine the effect of siRNA-induced PRH knockdown on cell number, 5×10^4 cells were plated in duplicate in a 6-well plate 48 hours after transfection and, every two days for ten days, cells were dissociated with trypsin, counted and replated.

Figure 5.3A shows the cumulative cell number of siCtrl and siPRH CCLP or RBE cells over ten days in four biological repeats. Cell number in siPRH treated cells was significantly reduced in both cell lines at multiple timepoints. The reduction in siPRH cell number is similar between the two cell lines, with siPRH CCLP growing to 74% of the siCtrl, and siPRH RBE growing to 64% of the siCtrl, over 10 days. Cell doubling time was calculated from cumulative growth curves (as described in section 2.2.1); the 10-day timepoint was chosen as cell number increases exponentially over time, thus it may be assumed that the greater difference will be seen at the final timepoints. The doubling time calculated from the cell number at day 10 was significantly increased by 2 hours in CCLP cells with PRH KD ($p=0.018$) (**Figure 5.3B**); In RBE cells, although there was a trend of increased doubling time in siPRH treated cells, this was not statistically significant ($p=0.085$) probably because the PRH level increases more quickly in RBE cells with time than it does in CCLP cells.

(A)



(B)

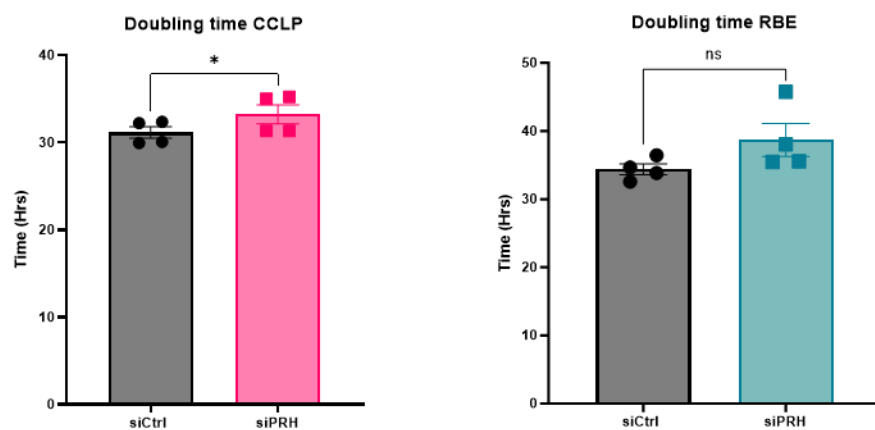


Figure 5.3: Growth Curves of siCtrl and siPRH CCLP and RBE.

Cells were transfected and left for 48hrs before harvesting and seeding for growth curve experiments. In the growth curve experiments, cells were seeded at 5×10^4 cells per well in a 6 well plate and counted via harvesting every two days until 10 days. Cells were re-plated in an appropriately volumed flask to allow for exponential growth. Experiments were set up in duplicate wells and biological replicates of four.

- (A) Cell number counted over every two days in siCtrl and siPRH cells for both CCLP and RBE. Error bars represent SEM, and statistical significance was measured through two-tailed paired t-test (N4; *= $p < 0.05$, **= $p < 0.01$ *** = $p < 0.001$).
- (B) Doubling time as calculated by the cell number over 10 days. Error bars represent SEM, and statistical significance was measured through two-tailed t-test (N4; *= $p < 0.05$).

5.4: The Effect of PRH KD via siRNA on Cell Proliferation and Cell Cycle Progression

To investigate how PRH KD alters cell proliferation, siCtrl and siPRH cells were observed in EdU incorporation assays and cell cycle progression was measured using flow cytometry as previously described.

The EdU incorporation assay measures the ability of cells to synthesise new DNA, via a nucleotide analogue, 5-ethynyl-2'-deoxyuridine or EdU. The EdU is incorporated into DNA over time, and this incorporation is measured through labelling EdU with HRP by a chemical reaction called click technology. The HRP can metabolise a HRP substrate to produce an OD reading at 450nm. Cells that are cycling at a faster rate will incorporate more EdU, causing more HRP labelling and eventually a higher OD reading. The assay works on the basis that the number of cells in each condition are seeded equally; therefore, OD readings are normalised to cell number. Cell number is measured through staining with a calcein-AM dye. This cell-permeable ester becomes a fluorescent green species, calcein, upon cleavage by cytosolic esterases, consequently staining viable cells. Fluorescent is read at 485nm (excit.) and 520nm (emiss.) and correlates with cell number. For full details of EdU incorporation assay, see section 2.2.2.

In EdU experiments, siCtrl and siPRH treated cells were collected after 48 hours of transfection and plated in quadruplicate at 1×10^4 cells per well in 96 well plates. After adhering overnight, the cells were incubated with an optimised concentration of 2 μ M EdU for 3 hours before completing the assay according

to manufacturer's instructions (see section for details) and measuring OD values. Cells that were not incubated with EdU were used to subtract the background from the EdU wells, and values were further normalised to cell number on an identical 96 well plate using calcein-AM staining. The normalised EdU data for the siPRH cells were then made relative to the normalised data for the siCtrl cells.

Figure 5.4A shows that siPRH treated CCLP and RBE cells have significantly lower rates of proliferation compared to siCtrl cells. The CCLP siPRH proliferation rate drops to 58% of siCtrl ($p=0.0058$), and the RBE siPRH proliferation rate drops to 87% of the siCtrl ($p=0.046$). There is a much lesser effect of PRH KD in RBE cells than CCLP cells.

To observe any effect of siPRH treatment on cell cycle progression, PI staining and flow cytometry methods were performed as previously described (see section 2.2.6 for details). **Figure 5.4B**, shows representative images of the raw cell cycle data from CCLP siCtrl and siPRH treated cells or RBE siCtrl and siPRH treated cells, and quantification is shown in **Figure 5.4C**. Two graphs are displayed to illustrate the overall changes and to show the statistical differences. In CCLP cells the siPRH treatment causes little change to the cell cycle profile although there was a small but reproducible decrease in the number of cells in G2 compared to siCtrl ($p=0.049$); (26% reduced to 23% in siPRH cells). Similarly, in RBE cells, there was a decrease in cells in G2 from siCtrl 23% to siPRH 17% ($p=0.029$), however there are no other significant changes to the cell cycle profile in the siPRH RBE samples.

Overall, there is a small reduction in the number of cells in G2, and although there was a major reduction in EdU incorporation in PRH KD using siRNA KD, PI staining showed no change in the number of cells in S phase.

The effect of siPRH on proliferation rate is much lesser in RBE cells than CCLP even though the expression of PRH is much higher in RBE cells than CCLP cells (Figure 3.1). The difference may stem from that fact RBE cells have a slower cycling rate than CCLP cells; this can be seen by the percentage of cells in G2, where CCLP have around 30% of cells in G2 phase, and RBE have around 20% of cells in G2 phase. One reason for the slower cycling rate could be because the Notch-3 ICD is not expressed at high levels in RBE cells. Thus, loss of PRH in RBE cells does not disrupt a PRH-Notch3 ICD feed forward loop and consequently has a lesser effect in RBE than in CCLP cells.

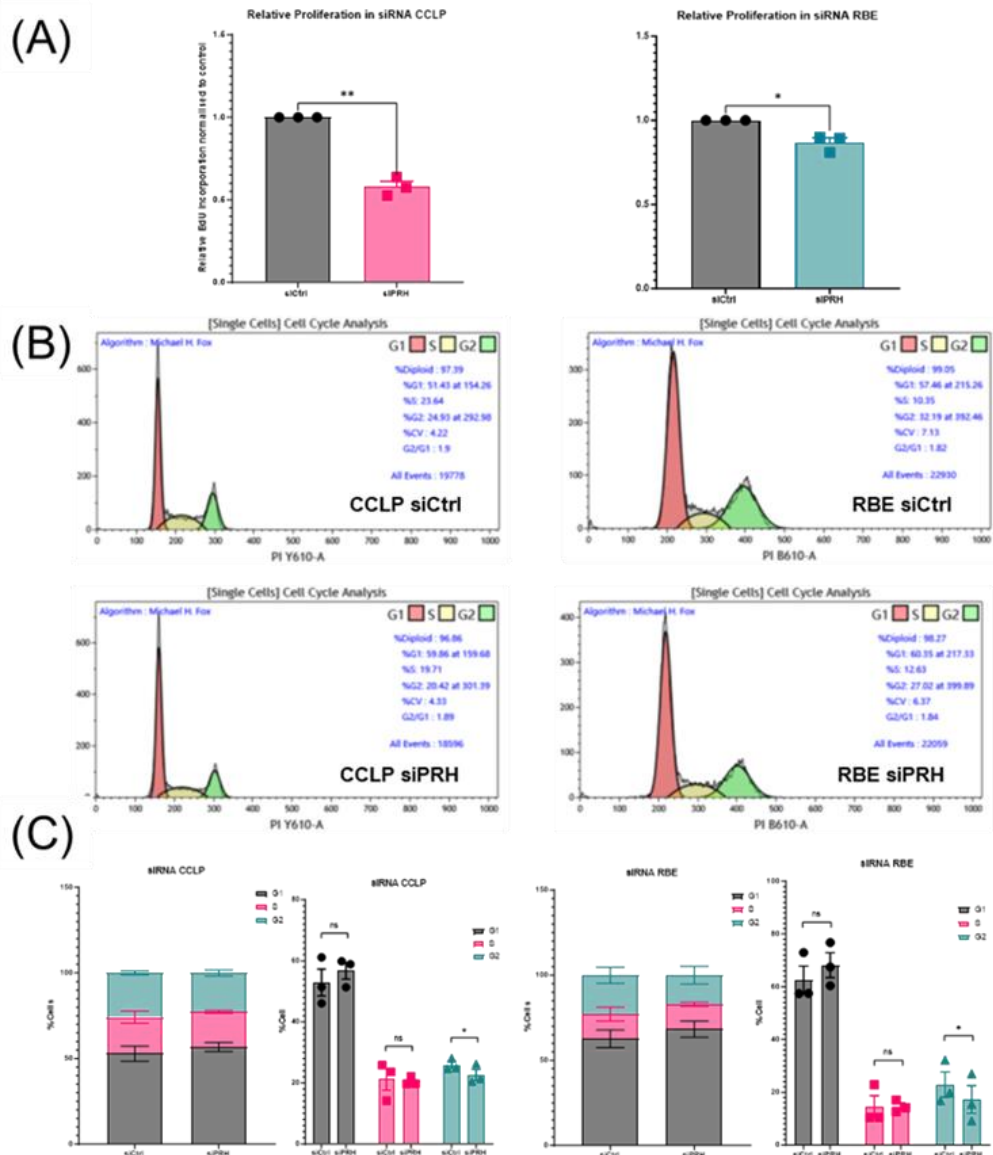


Figure 5.4: The effect of siPRH on CCLP and RBE cell proliferation and cell cycle.

Cells were transfected and left for 48hrs before harvesting and seeding for EdU incorporation proliferation experiments or fixing for Cell Cycle Flow Cytometry. In the EdU incorporation assays, cells were seeded at 1×10^4 cells per well in a 96 well plate in quadruplicate and incubated with 2uM EdU for 3 hours. EdU incorporation was translated into an OD reading, by labelling with HRP and incubating with substrate, which was read at 450nm. Relative proliferation was calculated by normalising siPRH cells to siCtrl cells. In cell cycle flow cytometry, 1×10^6 siCtrl and siPRH cells were washed and fixed in 70% Ethanol. Cells were then washed, subjected to RNase treatment (0.2mg/mL) and PI staining (50ug/mL) before running on a cytometry machine for cell cycle analysis. Analysis gating and cell cycle algorithms were performed through Kaluza software.

- (A) Relative proliferation in siCtrl and siPRH cells for both CCLP and RBE. Error bars represent SEM and statistical significance was measured through two-tailed t-test (N3; **= $p < 0.01$ *** = $p < 0.001$).
- (B) Representative Cell Cycle analysis of siCtrl and siPRH cells in Kaluza.
- (C) Cell Cycle analysis of siCtrl and siPRH for both CCLP and RBE showing the proportion of the cell population in each stage of the cell cycle: G1, S, and G2. Error bars represent SEM and statistical significance was measured through two-tailed paired t-test (N3; *= $p < 0.05$).

5.5 The Effect of PRH KD via siRNA on uninduced and Staurosporine-induced Apoptosis

CCLP shRNA PRH KD cells show decreased apoptosis compared to controls upon Niclosamide treatment. To assess whether acute knockout of PRH plays a role in decreasing intrinsic apoptotic cell death, and whether PRH plays a role in decreasing apoptotic death induced by a canonical inducer of apoptosis, siPRH and siCtrl CCLP or RBE cells were used in apoptosis experiments using flow cytometry as described previously (section 2.2.7). Following transient transfection, the siPRH and siCtrl cells were seeded at 1×10^6 cells in a T25 flask and left to adhere overnight. The siRNA cells were then either treated with a DMSO vehicle control or $1 \mu\text{M}$ Staurosporine for 24 hours. Staurosporine is an anti-microbial and anti-fungal compound that is a non-specific protein kinase inhibitor that can cause rapid caspase-dependent and caspase-independent apoptosis (Belmokhtar et al., 2001). Therefore, in this experimental setup, apoptosis can be investigated in the siCtrl and siPRH cells with, and without, this well characterised apoptotic inducer, to explore their propensity for apoptosis.

After the 24 hours incubation, the cells were assayed for apoptosis. **Figure 5.5** shows representative images of the Kaluza analysis and **Figure 5.6** summarises the impact of siPRH treatment on stimulated and unstimulated apoptosis. CCLP siPRH treatment results in a 5% decrease in live cells ($p=0.0097$), a 1% increase in early apoptotic cells ($p=0.033$) and a 3% increase in late apoptosis cells ($p=0.23$). Similarly, for RBE cells the siPRH treatment

causes a 15% decrease to live cells ($p=0.044$) and a 11% increase in late apoptotic cells ($p=0.046$). Thus, in both cell lines the siPRH treated cells show a decrease in live cells, and a small but significant increase in the percentage of cells entering apoptosis, compared to siCtrl cells.

In 1 μ M Staurosporine treated siCtrl and siPRH CCLP and RBE cells, similar results were observed in both cell lines, with fewer live cells and higher levels of cell death present in the siPRH treated cells (**Figure 5.6C**). Staurosporine treatment in the siPRH treated CCLP cells results in 65% of cells being in late apoptosis. However, this is not significantly different from Staurosporine treated siCtrl CCLP cells as the variation in the late apoptotic fraction in the CCLP siRNA samples is high ($p=0.14$).

Staurosporine treatment of siPRH RBE cells results in a statistically significant decrease of 41% compared to live cells ($p=0.0053$), and a 27% increase in late apoptotic cells ($p=0.04$), compared to siCtrl. Although the trend is similar in siRNA experiments, the CCLP cells are more sensitive than RBE cells to Staurosporine, as shown by the increased amount of cell in late apoptosis compared to RBE cells, and this may explain why the difference between siCtrl and siPRH cells is less apparent in CCLP cells.

To conclude, siPRH treatment increases intrinsic apoptosis in both cell lines. Also, at least for the RBE cell line, it is apparent that the effect of short-term knockdown of PRH is accompanied by increased sensitivity to Staurosporine treatment. Thus, PRH expression is inhibiting Staurosporine induced apoptosis.

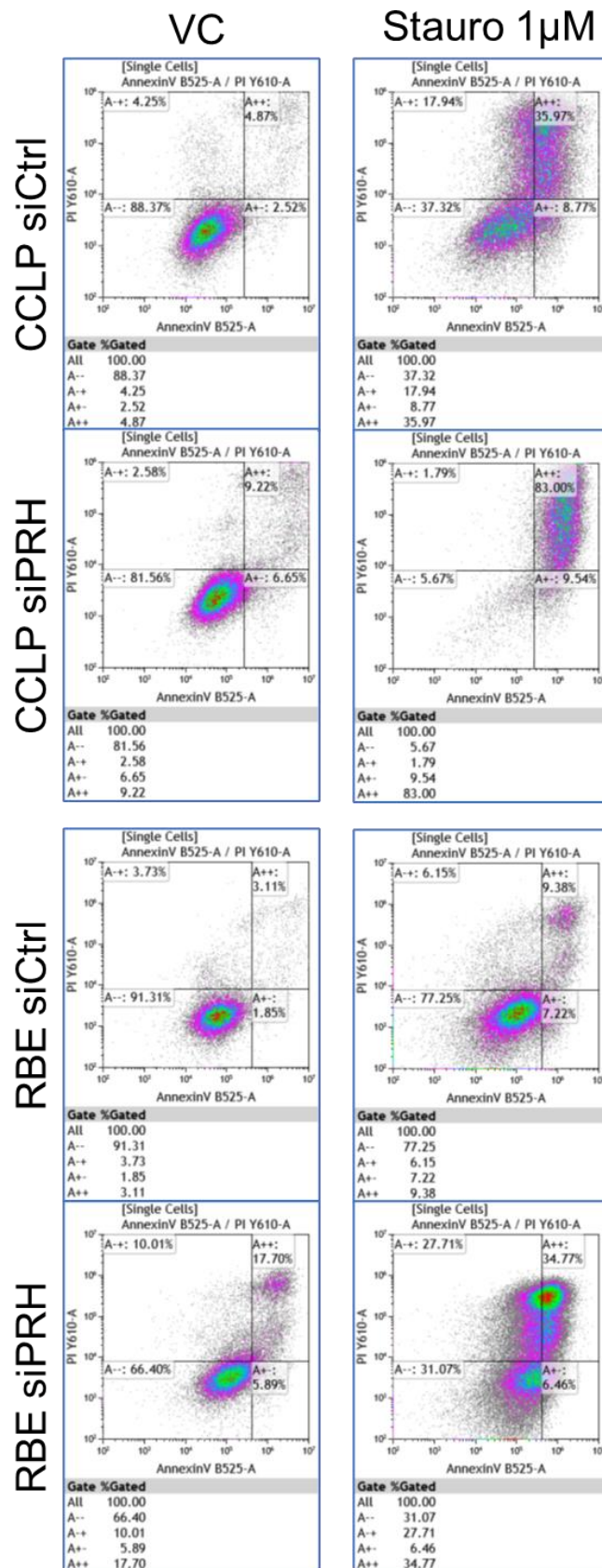


Figure 5.5: Representative images of Kaluza apoptosis analysis in siCtrl and siPRH CCLP and RBE.

CCLP and RBE siCtrl and siPRH were treated with vehicle control (VC) or Staurosporine at 1 μ M (Stauro 1 μ M) for 24hrs before harvesting for apoptosis experiments. Approximately 1x10⁶ cells were washed and stained with PI and Annexin V. The stained cells were run on a flow cytometer and data was analysis in Kaluza software. The analysis shows the proportion of the cell population in each stage of cell death: Live, Early Apoptosis, Necrosis and Late Apoptosis.

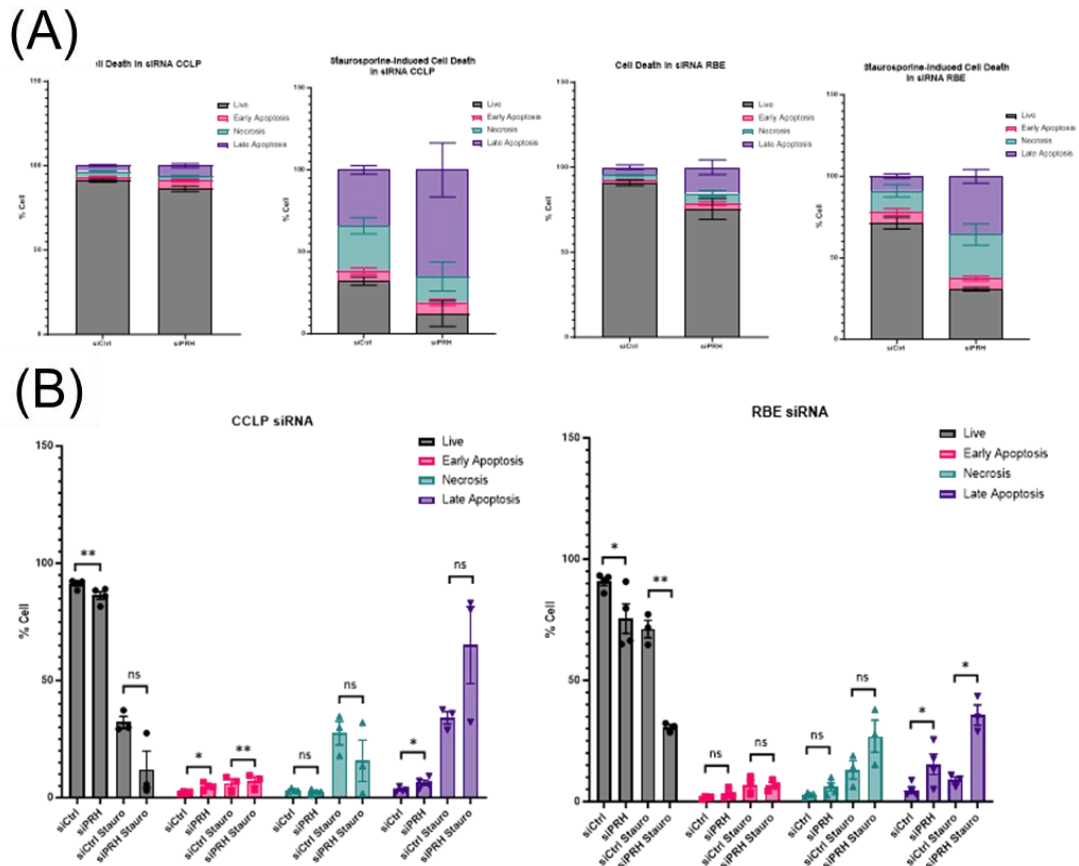


Figure 5.6: Cell death in siCtrl and siPRH CCLP and RBE.

Cells were transfected and left for 48hrs before harvesting and seeding for Flow Cytometry. The siRNA cells were plated at 1×10^6 in a T25, left to adhere for 24hrs, then treated with vehicle control (DMSO) or Staurosporine for 24hrs. siRNA cells were pelleted after treatment and washed before staining with Propidium Iodide and Annexin V. Samples were ran on a cytometry machine. Gating and apoptosis analysis were performed through Kaluza software.

- (A) Cell death in siPRH and siCtrl CCLP or RBE treated with vehicle control or Staurosporine.
- (B) Cell death in siPRH and siCtrl CCLP or RBE treated with vehicle control (N4) or Staurosporine (N3). Statistical analysis was performed with a 2-way ANOVA in Graph Prism (*= $p < 0.05$, **= $p < 0.01$)

5.6 The Effect of Transient PRH

Knockdown on Gene Expression

To understand the phenotype of siPRH KD we investigated the effects of PRH knockdown by siRNA on gene expression. Transcriptomic analysis on RNA samples was performed on three independent repeats of the siCtrl and siPRH treated CCLP cells. For this, RNA samples were extracted from pellets of approximately 2×10^6 cells 48 hours after transient transfection when the protein is known to be strongly down regulated (Figure 5.1) (see section 2.3). To test RNA quality, samples were sent to Deep Seq Next Generation Sequencing facility at the University of Nottingham for a RIN (RNA Integrity Number) test (RIN scores are in the M&M; see section 2.4.2). The test used Agilent TapeStation 4200 kit and quality is inferred by the ratio of 28S rRNA to 18S rRNA. A score above 9 confirms high quality samples. Samples (2000ng RNA in 20uL of RNase free H₂O) were sent to Novogene where RNAseq was performed (section 2.4) as follows: the enriched RNA was reverse transcribed into cDNA, which was fragmented, adapter-ligated and amplified to create a cDNA library. The library underwent quality checks and was then sequenced in the Illumina NovaSeq PE150 platform with 150bp paired end reads. The reads were analysed and aligned to the Hg38 *Homo sapiens* genome for quantification of gene expression. The fragment reads, or counts, for each gene were compared between the three siCtrl samples and three siPRH samples to generate a log fold change (Log2FC), alongside a p-adjusted value. From the resulting DESeq output file, of 27514 genes, 427 genes were significantly differentially expressed genes (DEGs); significance was measured by having a

p-adj value<0.05. Of the significant genes, 157 were up-regulated and 270 were down-regulated in response to the siRNA mediated PRH KD (**Figure 5.7**).

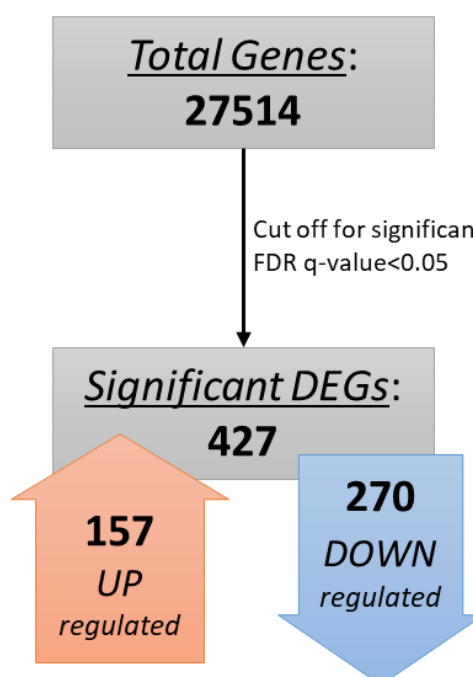


Figure 5.7: Significant Differentially Expressed Genes (DEGs) in CCLP siCtrl and siPRH Bioinformatics.

A diagram to show: the total number of genes accounted for from the RNAseq data, the number of significant DEGs and the proportion of up-regulated and down-regulated genes. A significant DEG is defined by having an FDR q-value<0.05.

The RNAseq data was validated by comparing to qPCR data for *HHEX*, *NOTCH-3* and *CCND2* genes (**Figure 5.8**). **Figure 5.8A** shows the gene expression data from the RNAseq, expressed as log(Feature Counts), and **Figure 5.8B** presents the data from the qPCR experiments, represented as 1/(Ct Value/B-actin Ct Value). It can be seen in **Figure 5.8A and B** that the gene expression data for these genes is similar in RNA sequencing and RT-qPCR experiments. There is a good correlation between RNAseq and qPCR values (Pearson's correlation analysis, $r=0.91$ and $p=0.0116$ **Fig 5.8C**), thus are in agreement. Interestingly, the effect of siPRH did not cause a significant reduction to *NOTCH3* expression in the RNAseq or in qPCR experiments (**Figure 5.9D**) in CCLP cells.

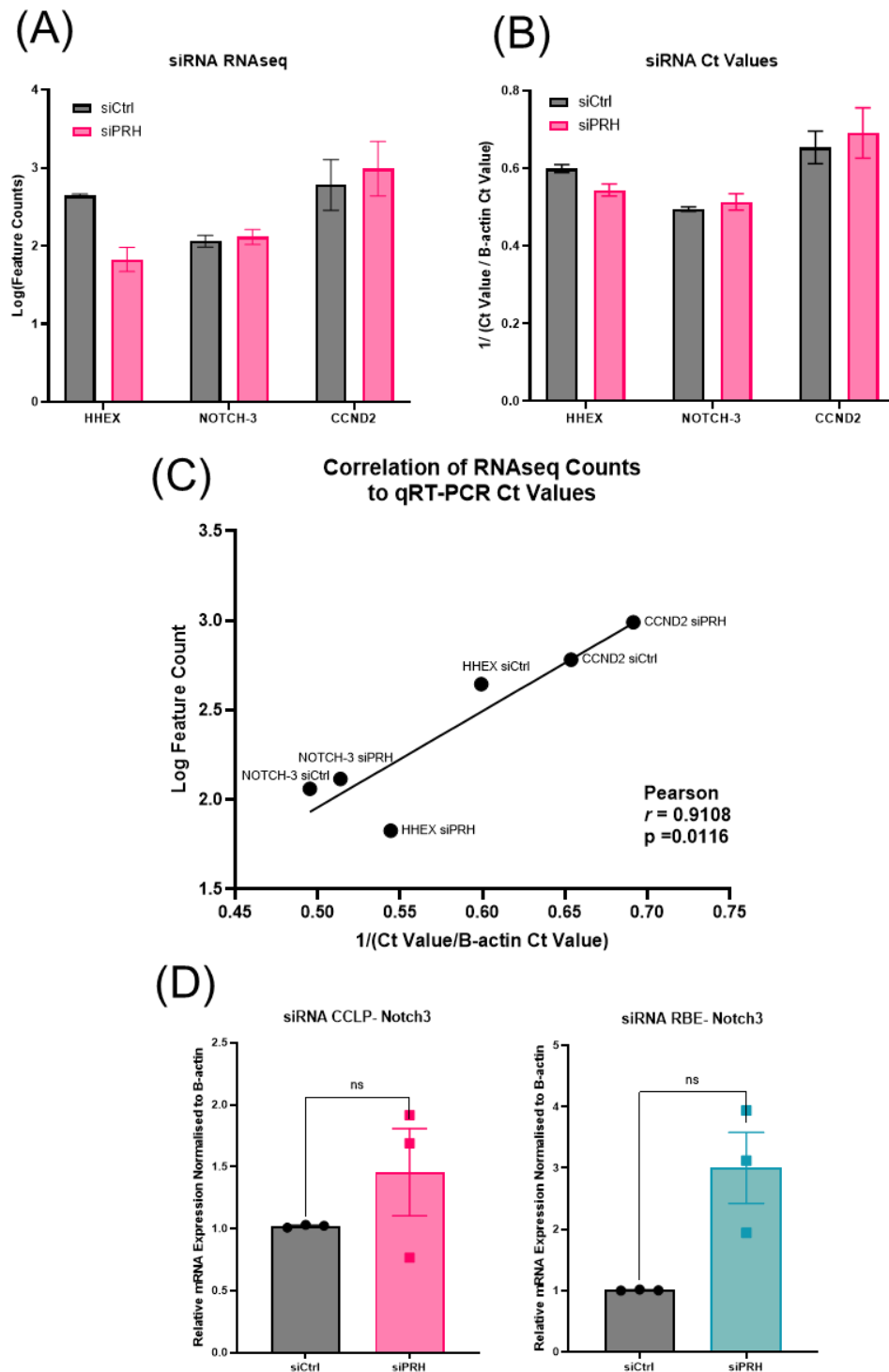


Figure 5.8: Correlation of RNAseq data to qPCR data in siRNA cells.

A summary of mRNA expression data for PRH, Notch-3, and Cyclin in RNAseq and in qRT-PCR.

- The log(Feature Counts) expression of HHEX, NOTCH-3, and CCND2 in CCLP siCtrl and siPRH cells in RNAseq.
- The $1/(\text{Ct Value} / \text{B-actin Ct Value})$ as a measure of mRNA expression of HHEX, NOTCH-3 and CCND2 in CCLP siCtrl and siPRH in qRT-PCR. Experimental were completed to N=3.
- The correlation of the log(Feature Counts) and the $1/(\text{Ct Value} / \text{B-actin Ct Value})$. The data was analysed through correlation analysis in GraphPad Prism. The Pearson correlation regression value and p-value are shown on the graph.
- The mRNA expression of Notch-3 in siCtrl and siPRH CCLP and RBE cells. The qRT-PCR experiment was performed to three biological repeats and significance was measured through an unpaired t-test (N3).

Similarly, Notch-3 gene expression was assessed in the RBE siCtrl and siPRH samples using RT-qPCR (**Figure 5.8D; right panel**) and again there is no difference in *NOTCH3*. Thus, short-term PRH KD using siRNA does not regulate *NOTCH3*.

The RNAseq data was used in an unranked Gene Set Enrichment Analysis (GSEA), to identify PRH-regulated genes and pathways in the CCLP siCtrl and siPRH cells. Gene names and their corresponding normalised counts were uploaded to the GSEA software and were assessed to see whether gene sets were statistically significantly more enriched in either phenotype: the siCtrl or siPRH. To do this, genes were ranked into a list where the top genes are positively enriched in the three siPRH KD samples, and the bottom genes are positively enriched in the three siCtrl samples. The GSEA software calculates an enrichment score (ES). This represents the degree of over-representation a set of genes show in the ranked list. The significance of the ES is calculated through an empirical phenotype-base permutations test, where the ES is recalculated by rearranging the phenotype labels to see if the enrichments can occur by chance. The results are further adjusted by normalising the ES for each gene set, accounting for the number of genes in the sets. For each normalised ES, or NES, a false discovery rate (FDR) is calculated to account for false positives. With the NES and FDR values, the significantly enriched pathways in each phenotype can be observed. Any gene set that is significantly enriched in either phenotype is highlighted.

The ranked list was compared against two gene set databases: Hallmarks MSigDB and KEGG. The Hallmarks gene sets from the Molecular Signatures Database is a comprehensive set of defined biological states or processes gathered by computational methodology and grouping genes that show coordinated expression (Subramaniam et al., 2005). The Kyoto Encyclopaedia

of Genes and Genomes (KEGG) database is a collection of gene sets for various cellular processes, metabolic function and disease mechanisms (Kanehisa et al., 2000). Both databases are widely used in cancer-related research.

Figure 5.9 shows the enrichment of gene sets in the siPRH treated cells and in the siCtrl treated cells when using the (A) Hallmarks MSigDB and (B) KEGG database. The graphs plot the gene set name against the NES, and gene sets that are significantly enriched, where the False Discovery Rate $FDR < 0.25$, are highlighted in magenta. The top ten significant gene sets, with the highest scoring NES, are shown for each phenotype.

In **Figure 5.9A**, Hallmark gene sets that are significantly positively enriched in the siPRH phenotype include gene sets associated with immune response (*TNF- α Signalling via NF κ B, Coagulation and Complement*), metabolic processes (*Oxidative Phosphorylation, Cholesterol Homeostasis and Adipogenesis*) and cellular stress (*Hypoxia, p53 Pathway and KRAS Signalling*). In **Figure 5.9B**, the KEGG gene sets that are significantly enriched in the siPRH phenotype include gene sets that are commonly connected by mitochondrial function or cellular energy metabolism: *Oxidative Phosphorylation, Vibrio Cholerae Infection, Lysosome, Parkinson Disease and Amino Sugar and Nucleotide Sugar Metabolism*. Hallmark gene sets that are significantly positively enriched in the siCtrl cells (which can be alternatively viewed as significantly negatively enriched for siPRH) are cell cycle associated gene sets (*E2F Targets, G2M Checkpoint, Mitotic Spindle and MYC Targets V1*), as well as *Interferon Signalling* and *Hedgehog Signalling* (**Figure 5.9A**). The KEGG pathway gene sets that are positively enriched in the siCtrl cells are grouped under genome instability and cell proliferation: *Cell Cycle, DNA Replication, Homologous Recombination and Mismatch Repair* (**Figure 5.9B**).

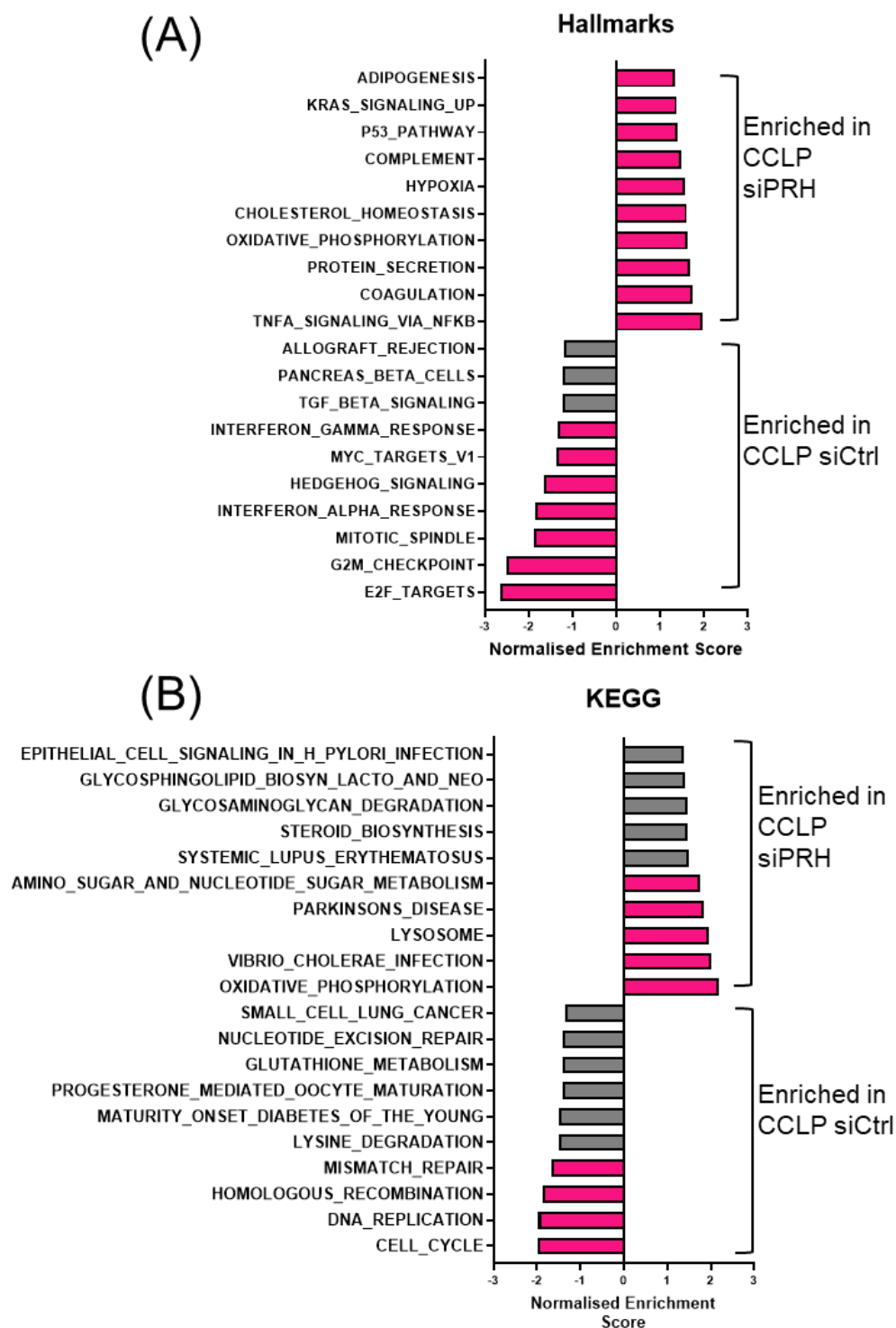


Figure 5.9: Gene Set Enrichment Analysis of CCLP siCtrl and siPRH RNAseq. Three sets of RNA samples from CCLP siCtrl and siPRH cells were used in RNA sequencing experiments. Genes and their respective normalised counts were inputted into Gene Set Enrichment Analysis software. The software ranked the genes into a list of genes starting with genes significantly enriched in the siPRH cells, to genes significantly enriched in the siCtrl cells. Gene sets from different databases were assessed for their enrichment in each phenotype. The top ten gene sets, according to normalised enrichment score (NES), are shown for each phenotype, with significantly enriched gene set in pink; pink shows FDR q -value < 0.25 . The two canonical GSEA databases are used (A) Hallmarks (B) KEGG.

This is consistent with the phenotypic experiments where siCtrl cells have significantly higher cumulative cell number, increased cell proliferation as measured through EdU incorporation and have higher populations of cells that had entered G2 in flow cytometry experiments. To investigate further, a volcano plot was generated for the G2M checkpoint gene set (**Figure 5.10**), where significant negatively regulated DEGs are highlighted in magenta. The figure shows that most G2M genes are down-regulated in response to the siRNA PRH KD, and that 12 genes are significantly down-regulated. These significant DEGs are shown in the figure: *MAD2L1*, *PAFAH1B1*, *FOXN3*, *HOXC10*, *KNL1*, *CDC6*, *STMN1*, *KIF4A*, *CUL5*, *TOP2A*, *CBX1* and *E2F2*. These proteins have functions that span the different stages of mitosis: transcriptional control (*E2F2*, *FOXN3*, *CBX1*, *HOXC10*), regulation of DNA replication (*TOP2A*, *CUL5*, *CDC6*) and microtubule dynamics (*PAFAH1B1*, *KNL1*, *KIF4A*, *STMN1*, *MAD2L1*). The

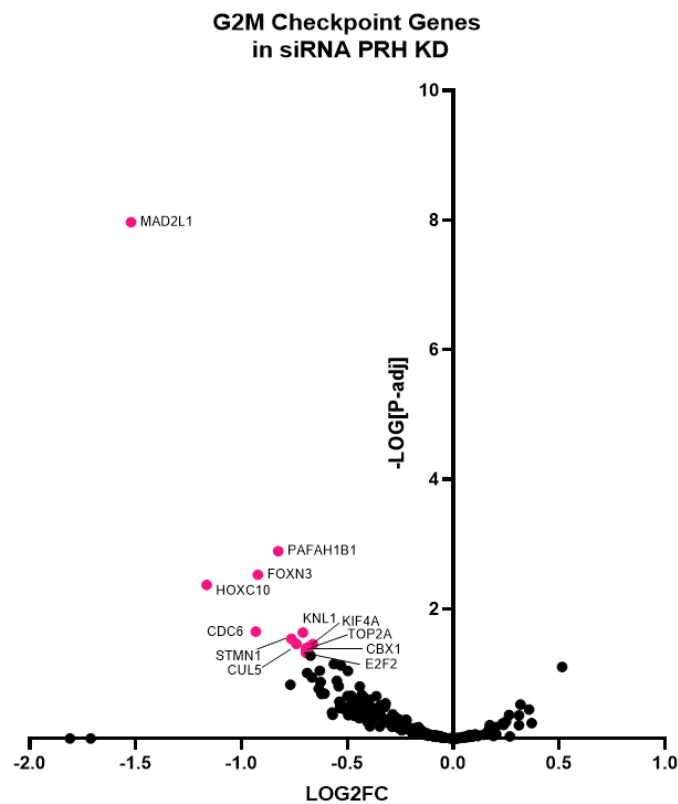


Figure 5.10: A volcano plot of G2M Checkpoint genes in siRNA PRH KD RNAseq
A volcano plot generated from the log2FC and $-\log(P\text{-adj})$ values of G2M Checkpoint genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEGs are highlighted in magenta. The top 12 significantly upregulated DEGs are shown.

decrease in expression of genes involved in mitosis provide a likely explanation for the decrease in cell proliferation observed in EdU incorporation assays and increase in the G2 population observed in flow cytometry experiments.

In apoptosis experiments, siPRH CCLP RNA cells show increased intrinsic apoptosis and siPRH RBE cells show increased intrinsic apoptosis and increased Staurosporine induced apoptosis. The RNAseq shows that siPRH CCLP cells show enrichment in cell stress and apoptosis-related gene sets such as the TNFA signalling pathway and the p53 pathway. To investigate further, a volcano plot was generated for the TNF-A Signalling via NFKB gene set (**Figure 5.11**), where significant DEGs are highlighted in magenta. The figure shows that most genes in this set are upregulated in response to the siRNA PRH KD, and the top ten significant DEG are shown in the figure: *DUSP5*, *MAP2K3*, *PHLDA2*,

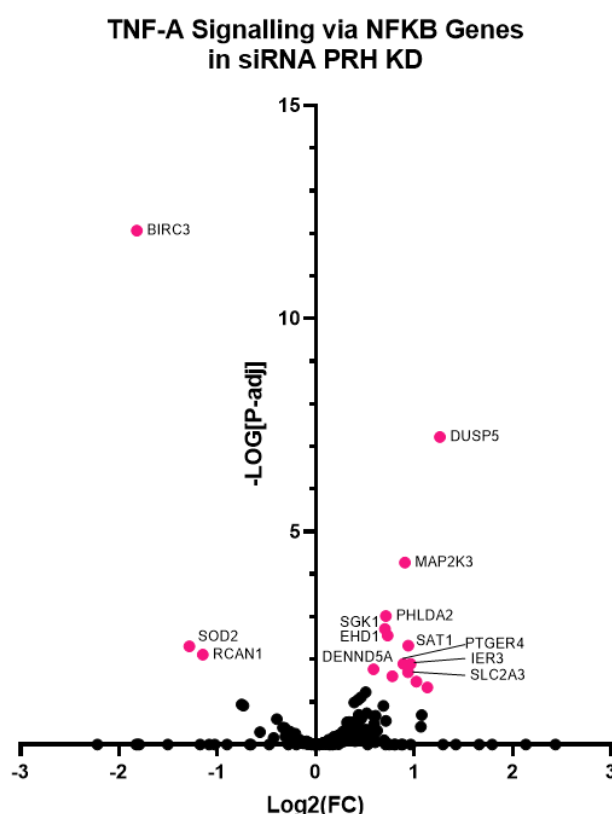


Figure 5.11: A volcano plot of TNF-A Signalling via NFKB genes in siRNA PRH KD RNAseq
A volcano plot generated from the $\log_2FC$ and $-\log(P\text{-adj})$ values of TNF-A Signalling via NFKB genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEG are highlighted in magenta. The top ten significantly upregulated DEGs are shown as well as the three significantly down-regulated DEGs

SGK1, *EHD1*, *SAT1*, *PTGER4*, *IER3*, *DENND5A*, and *SLC2A3*. These genes encode proteins that have a range of functions in cellular stress and survival and may be decreasing the ability of PRH KD cells to survive pro-apoptotic stress, hence increasing intrinsic apoptosis.

The p53 pathway was also shown to be significantly positively enriched in the siPRH phenotype. A volcano plot was generated for the p53 pathway genes (**Figure 5.12**). The figure shows that most genes in this set are upregulated in response to the siRNA PRH KD, and the nine significant DEGs are shown in the figure: *UPP1*, *SAT1*, *HMOX1*, *CDK5R1*, *IER3*, *BLCAP*, *SESN1*, *HBEGF*, and *SFN*. Some of these genes also appear in the TNF- α Signalling via NF κ B gene set (*IER3*, *SAT1*). The p53 pathway is a network of proteins that have roles in multiple cellular processes: cell cycle arrest, DNA repair, cell apoptosis, autophagy, and metabolism, but ultimately the p53 pathway determines whether cells undergo apoptosis or survive under stress conditions (Wang et al., 2023). p53 is activated upon multiple signals, including DNA damage, hypoxia, oncogene, oxidative stress or other cellular stresses. Therefore, the siPRH cells may be in a 'stressed' state, and more likely to respond to apoptotic inducers such as Staurosporine.

In summary, the use of siRNA confirmed previous shRNA PRH KD data showing that PRH KD decreases CCA cell proliferation in phenotypic assays (EdU incorporation and cell cycle progression experiments) as well as GSEA. As there is no change in Notch-3 gene expression, in siRNA CCLP or RBE cells, presumably PRH can alter cell proliferation without altering a Notch-3-PRH feedback loop. The siRNA work also showed that PRH has a role in pro-survival signalling. These effects are the results of short-term transient PRH KD.

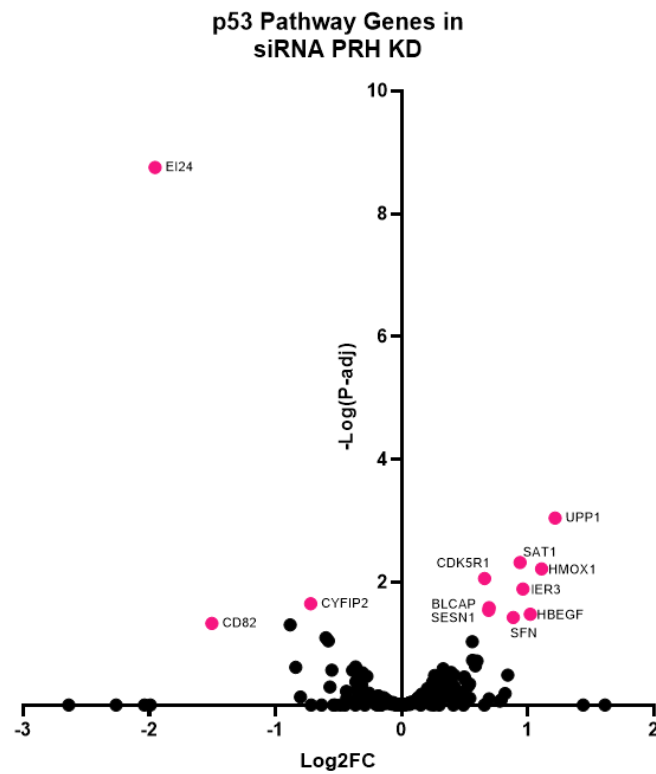


Figure 5.12: A volcano plot of p53 Pathway genes in siRNA PRH KD RNAseq

A volcano plot generated from the log2FC and $-\log(P\text{-adj})$ values of p53 Pathway genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEG are highlighted in magenta. The nine significantly upregulated DEGs are labelled as well as the three significantly down-regulated DEGs

SECTION B:

The effects of PRH KO via CRISPR Technology

5.7 Validation of CRISPR PRH KO Clones

PRH KD using siRNA confirmed that PRH is important for CCA cell proliferation and driving the cell cycle, but also showed new data where PRH loss causes increased intrinsic apoptosis and sensitivity to apoptosis inducers. To further investigate the role of PRH in CCA cells, CRISPR-Cas technology was implemented to produce *HHEX* knockout CCLP cell lines. CRISPR-Cas gene editing was established by Doudna and Charpentier who discovered that the adaptive immune response in bacteria works by integrating foreign DNA sequences into its genome to create complementary complexes and silence foreign material (Jinek et al., 2012). The system has been researched and optimised for gene manipulation and is commonly used to generate ‘knockout’ models, called so as the gene has been rendered inactive. In this system, a guide RNA (gRNA) is designed, which is complementary to a specific sequence in the target gene, and a Cas9 endonuclease enzyme is supplied, to generate double-strand breaks at the target gene. Once the DNA is cut, the cell attempts to repair the break, often via non-homologous end joining, which can create small insertions and deletions at the site. These indels often disrupt the reading of the gene and thus the resulting protein is either non-functional, incomplete or is not translated (reviewed; Ran et al., 2013).

The CRISPR method used to generate the CCLP *HHEX* KO utilised a variant form of Cas9, Cas9 nickase (Cas9n). Due to one of the two nuclease domains being rendered inactive through mutation, Cas9n can only cut a single strand of DNA (Ran et al., 2013). To perform specific gene editing at the *HHEX* loci, a pair of guide RNAs that complement opposite strand of the gene, at close proximity, were generated to produce staggered nicks in the *HHEX* gene, or a “double nicking” strategy. This functions as a double stranded break. Thus, the

use of Cas9n reduces off target effects as both gRNAs are required for the DNA break (Ran et al. 2013).

Generation of HHEX KO was performed in with the help and guidance of Dr. Chris Roberts who designed the guide RNA sequences and helped with genotyping. For the creation of HHEX KO in CCLP cells, cells were plated at 5×10^5 per well in a 6-well plate, left to adhere overnight, then transfected with two pairs of guide plasmids (four plasmids). Each plasmid contained a *HHEX*-targeting single guide RNA, alongside Cas9n and eGFP. One pair of plasmids had guides that targeted exon 1 for editing, the second pair had guides for targeting exon 2. Two pairs of guides were used in the same transfection to increase the chances of KO generation. Alongside the production of KO clones, control clones were also generated by transfecting with a single guide RNA plasmid, as a single-strand break can be repaired without introduction of indels with the Cas9n variant. The plasmids were transfected with plasmid via Lipofectamine and left for 48 hours, as optimised in the shRNA work; details can be found in the materials and methods (section 2.1.11).

Post-transfection, the cells were harvested and sent for fluorescent activated cell sorting (FACS) at the University of Nottingham QMC Flow Cytometry Unit (section 2.1.11.3). Positive cells were isolated by gating for GFP fluorescence and simultaneously plated as single cells in a 96 well plate containing filtered conditioned media (100 μ L/well) taken from a flask of confluent CCLP cells, with the expectation that the conditioned media contains additional growth factors and nutrients that stimulates the growth of single cells into colonies. A 96-well plate of potential KO clones was generated and a plate for the control clones was also generated. Cells were left to grow in the incubator over one month with regular checking for growth. Once a single cell had expanded into a colony that was able to cover 50% of the well, the cells were washed, trypsinised and

replated in a 24-well plate. Once the cells were confluent in the 24-well plate, they were transferred to a 6-well plate. From a confluent well of a 6-well plate, cells were collected and replated in three wells of a 6-well plate. Once confluent, one well was collected for genotyping, one well was collected for preparation of a protein lysate, and the final well was maintained for cell culture. For full details on the generation of CCLP PRH KO cell lines see 2.1.11.

CCLP cells although mesenchymal, are sensitive to growing as single cells. Of the 96-wells, relatively few wells generated colonies. Two control cell lines and four potential HHEX KO cell lines were ultimately screened for editing by genotyping.

For genotyping experiments, genomic DNA was extracted, using a QIAamp DNA kit (Qiagen), from pellets collected from each of the control and potential KO cell lines. The DNA was used in PCR reactions with primers designed to anneal to sequences upstream and downstream of the exon 2 gRNA targeting region (to see diagram to go **section 2.1.11.6**). After PCR amplification, the PCR products generated were electrophoresed on a DNA agarose gel to check for DNA edits. For details on methods see **section 2.1.11.6-8**.

Figure 5.13 shows the PCR products from each control and potential knockout clone, as well as a negative control for the PCR reaction. The negative control showed no PCR products confirming that there was no contamination during the PCR. The control clones have a PCR product that matches the predicted 510bp product from the primers, confirming that *HHEX* is still expressed and has undergone no apparent editing. The products from clones KO 2 and KO 5 are identical to the control clones. It is likely that any editing in these clones has not caused indel mutations in the *HHEX* gene. A product of 700-800bp was present in KO 4 DNA extracts. It is likely that KO 4 has been edited between

the primer sites with an insertion. There was no product formed from KO 1 extracts; this result is unlikely to be due to a failed PCR reaction as PCR products are visible for the controls and the other KO clones. The results could indicate a failure to amplify at this region due to widespread gene editing at the primer sites. The KO4 cloned was taken forward for sequencing to identify the nature of the CRISPR editing.

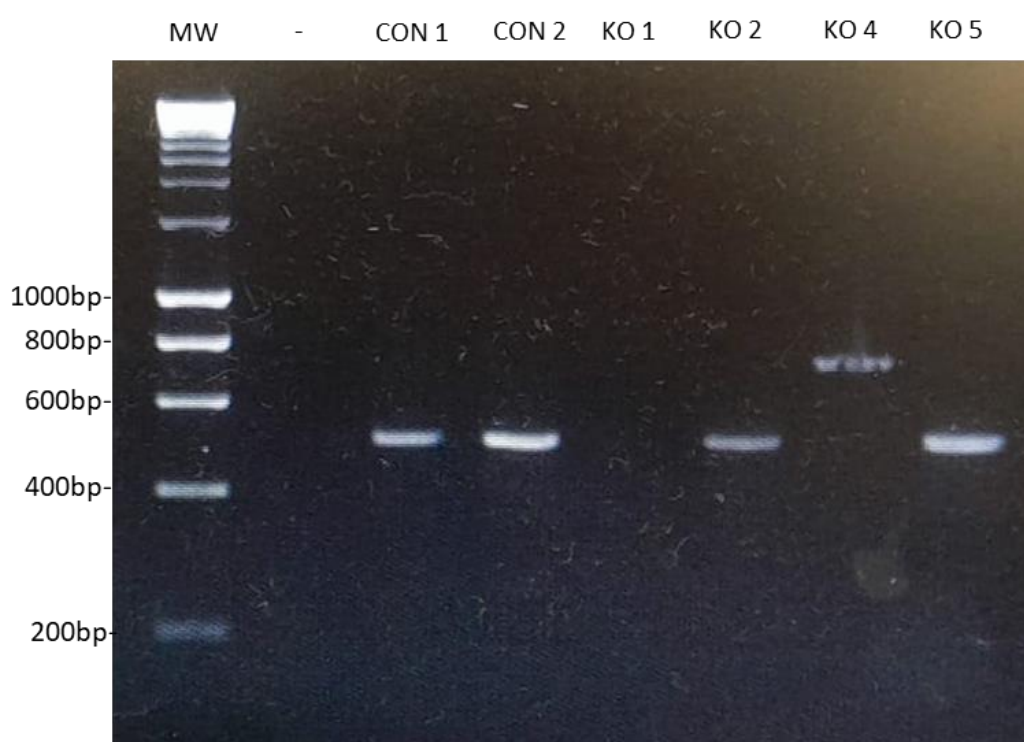


Figure 5.13: HHX PCR Products from a Screen of CCLP control and knockout clones.

CCLP control and knockout cell lines, CON 1 and CON 2, along with KO 1 and KO 4, were harvested and pelleted for obtaining genomic DNA samples. DNA was used in PCR reactions with optimised HHX primers to highlight edits at the CRISPR targeted sequence in Exon 2. PCR products were electrophoresed in an Agarose gel, stained with Ethidium Bromide and imaged on a BioRad GelDoc imager.

From the DNA gel, the PCR product seen in the KO 4 lane was manually excised and sent for sequencing. Sequencing was performed by DeepSeq Lab, QMC, University of Nottingham, and the gel purification was performed by Dr. Christopher Roberts. Sequences were uploaded to SnapGene Viewer software

and annotated to show the edits that had occurred in the *HHEX* gene (**Figure 5.14**). Parts of the sequencing aligned to the first 156bp of *HHEX* exon 2 (yellow feature). This was followed by a 257bp insert from *HHEX* exon 4 (grey feature). This edit has taken place between the two exon 2 gRNA sites which are also labelled. The corresponding amino acid sequence has also been added (purple feature) and shows the sequence terminates with a stop codon (red). It is likely that non-homologous end joining (NHEJ) was performed by the cell in response to a CRISPR-directed double strand breakage. As no PCR product was formed in from the KO 1 clone, no sequencing could be performed.



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In addition to genotyping the pellets were also used for western blotting to determine whether PRH protein is expressed following editing. Pellets were collected from the CCLP CON 1, CON 2, KO 1 and KO 4 cell lines, at different passages to be used in western blot and qPCR experiments. WB was performed as previously described and the blot was probed for PRH and B-tubulin as a loading control. The expression of PRH was divided by B-tubulin expression and made relative to the average expression for PRH in the two CCLP control cell lines. An unpaired t-test between the average control cell line and each knockout cell line was performed to observe statistically significant changes to PRH expression.

Figure 5.15 shows the relative expression of PRH protein in the control and knockout cell lines, accompanied by the blot image. There was no expression of PRH protein detected in either KO 1 or KO 4, resulting in significant differential expression compared to the two controls ($p < 0.0001$). This suggests a successful knockout of the PRH gene in these clones. PRH expression in CON 2 was significantly higher than CON 1 ($p = 0.025$). A possible explanation for this may be that the single cell that the cell line was cultured from had higher PRH expression than the single cell that created the other control line, and the difference in PRH expression observed between clones is the consequence of growing clonal cell lines.

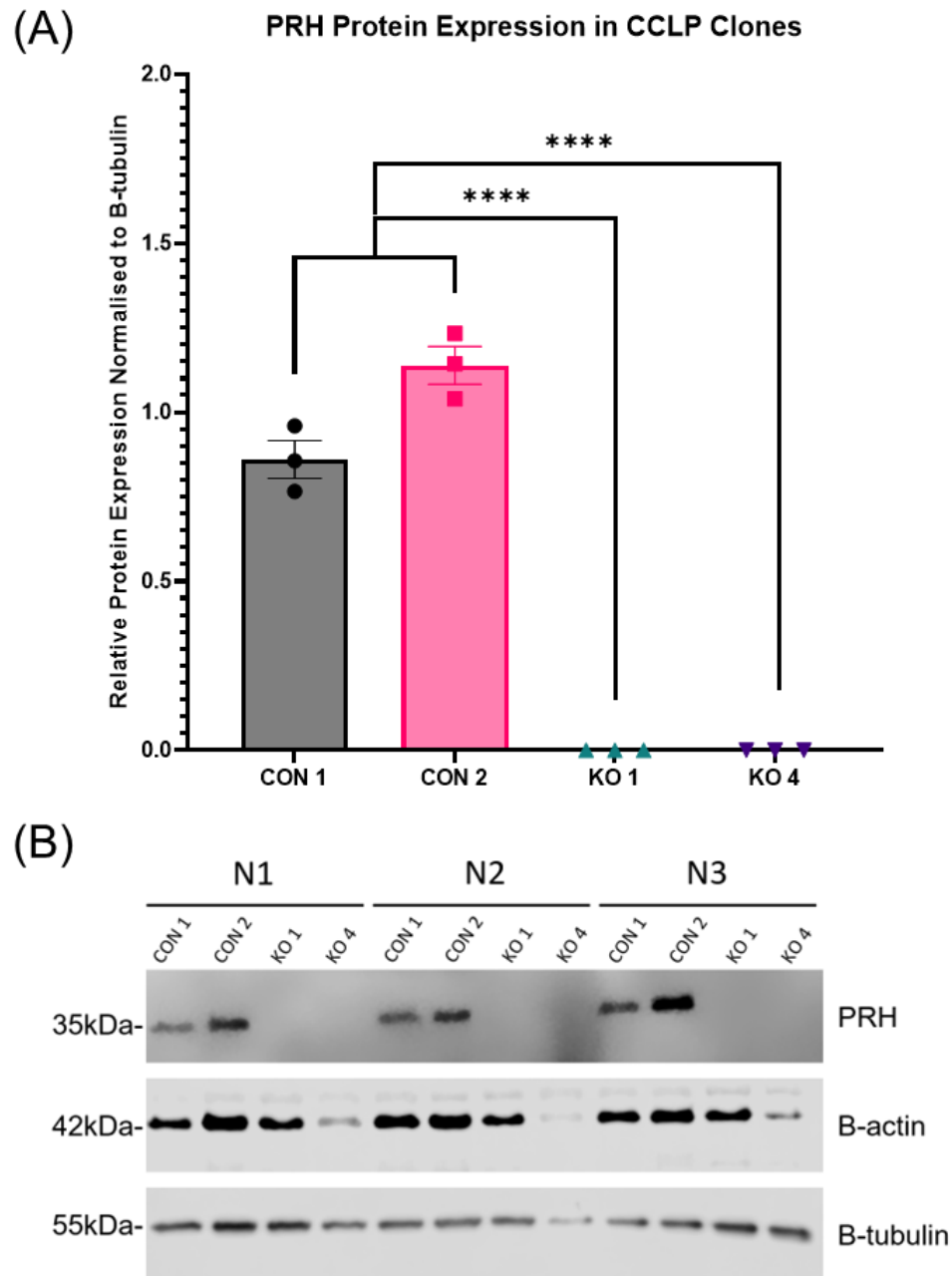


Figure 5.15: PRH Protein Expression in CCLP control and knockout clones. CCLP control cell lines, CON 1 and CON 2, along with CCLP PRH KO cell lines, KO 1 and KO 4, were harvested and pelleted at three different passages for protein samples. Protein samples were used in western blot experiments to assess protein expression.

(A) PRH protein expression across the CCLP clones. Band densitometry values were normalised to B-tubulin, and further normalised to the average of the values of clones CON 1 and CON 2. Error bars represent SEM, and statistical significance was measured through two-tailed t-test (N3; **** = $p < 0.0001$).

(B) Western blot showing PRH protein expression in the CCLP control and knockout clones over three repeats. Each lane has 30ug protein and was probed with PRH (2018B), B-actin and B-tubulin antibodies.

RNA was extracted from duplicate pellets and converted to cDNA to observe PRH mRNA expression in qPCR experiments. *GAPDH* primers were used alongside *HHEX* primers to quantify relative PRH mRNA expression in the CON and KO cell lines. *GAPDH*-normalised *HHEX* Ct values were made relative to the average of the two control cell lines, and significance was measured through an unpaired t-test. PRH mRNA expression is shown in **Figure 5.16**.

PRH mRNA expression in KO 1 was dramatically decreased compared to control cell lines ($p=0.00067$). This indicates that negligible amounts of *HHEX* transcript exist in KO 1. Although there is also a very large decrease in PRH mRNA expression in KO 4, to 15% expression compared to controls ($p=0.0017$), transcripts were still detectable in qPCR experiments for KO 4 samples. This information shows that in KO 4 the *HHEX* gene is still active, but as no PRH protein is detected by western blot, this cell line has no functional full length PRH protein.

In conclusion, two CCLP CON cell lines and two CCLP KO cell lines have been generated and validated. The clones were STR profiled and confirmed as CCLP cells (for STR profile see section 2.1.1) because the use of FACs sometimes introduces unwanted cells to the 96 well plate. The knockout cell lines differ in the type of editing they have endured, where KO 1 produces no detectable PRH protein or mRNA, and KO 4 produces no detectable PRH protein, but some *HHEX* transcripts are present. As PRH protein, not PRH mRNA, acts as a transcription factor, both KO cell lines are useful to examine effects of loss of the PRH protein in CCLP cells.

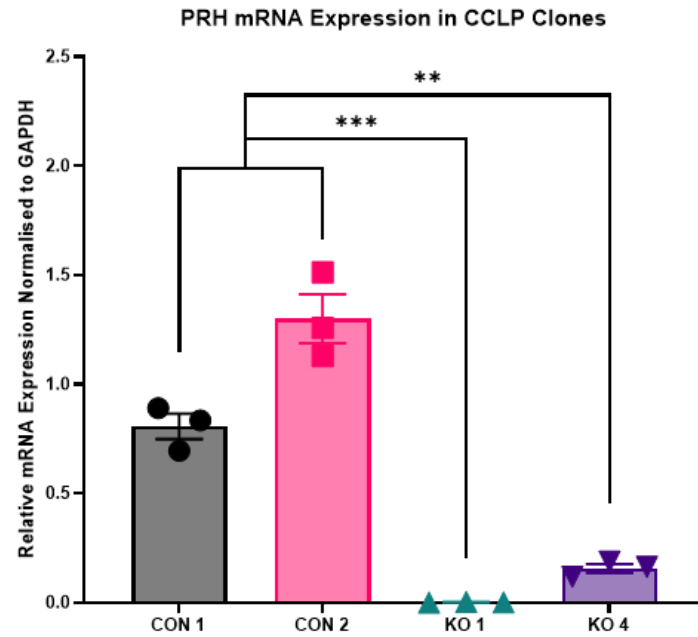


Figure 5.16: PRH mRNA Expression in CCLP control and knockout clones. CCLP control cell lines, CON 1 and CON 2, along with CCLP PRH KO cell lines, KO 1 and KO 4, were harvested and pelleted at three different passages for RNA extraction and cDNA synthesis. Samples were used in qPCR experiments to assess HHEX gene expression. Ct values were normalised to GAPDH, and further normalised to the average of the values of clones CON 1 and CON 2. Error bars represent SEM, and statistical significance was measured through two-tailed t-test (N3; ** = $p < 0.01$ *** = $p < 0.001$).

5.8 The Effect of PRH KO on Cell Growth and Proliferation of CCLP

To investigate the effect of PRH KO on CCLP cumulative cell number and cell proliferation in clones KO 1 and 4, cells were assessed, against CCLP control clones CON 1 and 2, in cumulative growth curves and EdU incorporation assays respectively exactly as described in section 2.2.1 and section 5.3 previously.

Figure 5.17A show that the CCLP KO 1 and KO 4 cell line have decreased rates of growth over time, compared to the control cell lines. Over ten days, KO1 cell count is 14% ($p=0.0008$), and KO4 cell count is 41% ($p=0.006$), of the of the average cell count for the two control cell lines. This is accompanied by a significant increase in the doubling time in both knockout cell lines (**Figure 5.17B**). The doubling time in the KO 1 cell line increases by 16.22 hours ($p=0.0014$), and the doubling time in the KO 4 cell line increases by 5.19 hours ($p<0.0001$), compared to the average value of the two control cell lines (27.95 hours). Overall, PRH knockout results in significantly decreased CCLP cell number.

Interestingly, the decrease in cell number is more pronounced in the KO 1 clone compared to the KO 4 clone. The cell count obtained for KO 4 is significantly higher than KO 1 over eight days ($p=0.029$). Although this could be a biological difference based on the KO deletion it is also very possible that this is occurrence of variation when using cell lines developed by clonal expansion from a single cell.

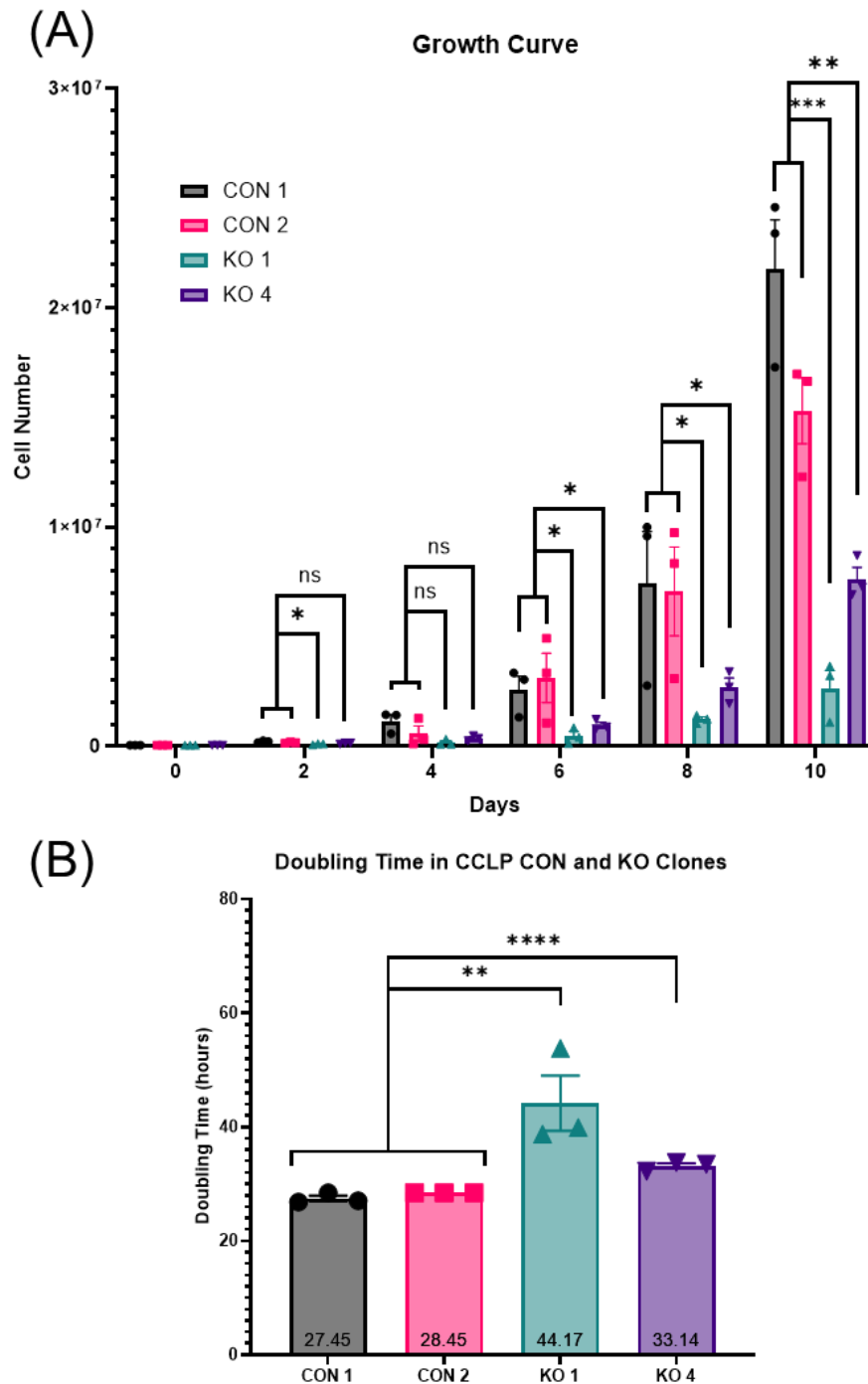


Figure 5.17: The effect of PRH KO on CCLP Cell Growth.

CCLP CON and KO cell lines were harvested and seeded at 5×10^4 cells per well in a 6 well plate for growth curve experiments. Cells were manually counted every two days over 10 days. Cells were re-plated in an appropriately volumed flask to allow for exponential growth. Experiments were repeated three times.

(A) Cell number counted over every two days in CCLP CON 1 and CON 2 control cell lines, alongside CCLP KO 1 and KO 4 knockout cell lines. Error bars represent SEM, and statistical significance was measured between the control cell lines values and either KO 1, or KO 4, through two-tailed paired t-test (N3; $*=p<0.05$, $**=p<0.01$, $***=p<0.001$).

(B) Doubling time as calculated by the cell number over 10 days. Error bars represent SEM, and statistical significance was measured between the control cell lines values and either KO 1, or KO 4, through two-tailed t-test (N3; $**=p<0.01$, $****=p<0.0001$).

Next EdU incorporation assays were performed as previously described to assess cell proliferation. **Figure 5.18** shows that both KO 1 and KO 4 cells have a decreased rate of proliferation compared to the average value for CON 1 and CON 2 cells, with EdU incorporation decreasing to 72% in KO 1 cells ($p=0.0024$), and to 82% in KO 4 cells ($p=0.0042$), compared to the average control value. In conclusion, PRH knockout causes a significant decrease in cell proliferation.

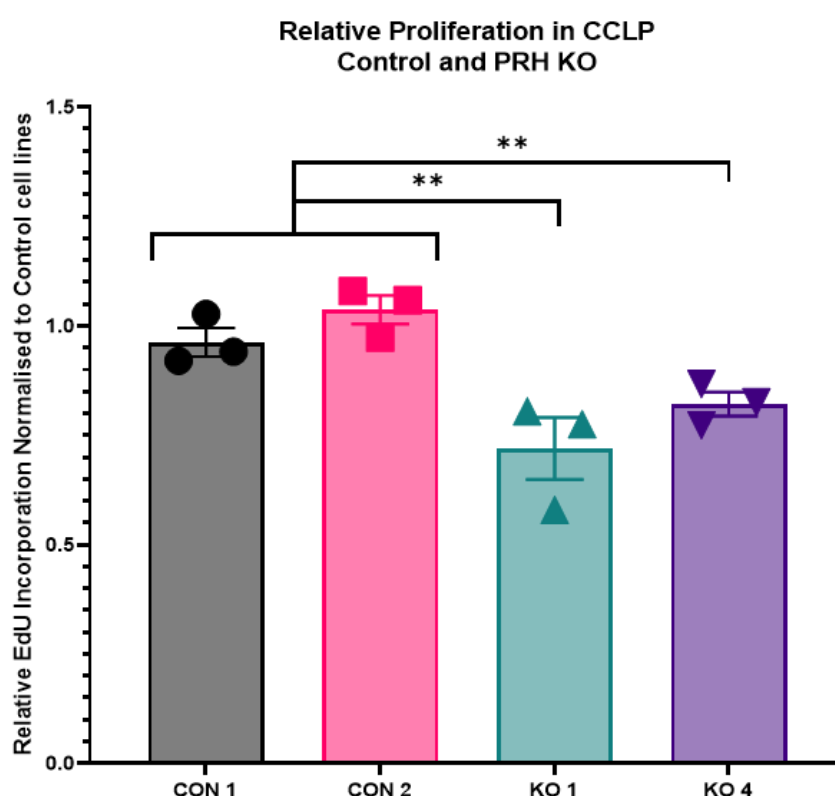


Figure 5.18: The effect of PRH KO on CCLP Cell Proliferation.

CCLP CON and KO cell lines were harvested and seeded at 1×10^4 cells per well in a 96 well plate, in quadruplicate, and incubated with $2 \mu\text{M}$ EdU for three hours. EdU incorporation was translated to an OD reading, by labelling with HRP and incubating with substrate, which was read at 450nm. Relative proliferation was normalised by a calcein loading control and relativised to the average value of the control cell lines for each repeat. Experiments were repeated three times, error bars represent SEM, and statistical significance was measured between the control cell lines values and either KO 1, or KO 4, through two-tailed paired t-test (N3; $*$ = $p<0.05$, $**$ = $p<0.01$ $***$ = $p<0.001$).

5.9 The Effects of PRH KO on Gene Expression

To determine the effect of PRH knockout on gene expression, RNA samples from each of the four cell lines, CCLP CON 1, CON 2, KO 1, and KO 4, were used for RNA sequencing. RNA quality was examined by a RIN test before sequencing. RNAseq was performed via Novogene as previously described and normalised counts for all gene and DEG $p < 0.05$ were identified. Normalised counts were used in Gene Set Enrichment Analysis as described below.

From the PRH KO RNAseq, of 24742 genes, 599 genes were significantly differentially expressed between the two CON cell lines and the two KO cell lines, and 346 of these significant DEGs were up-regulated and 253 were down-regulated (**Figure 5.19**).

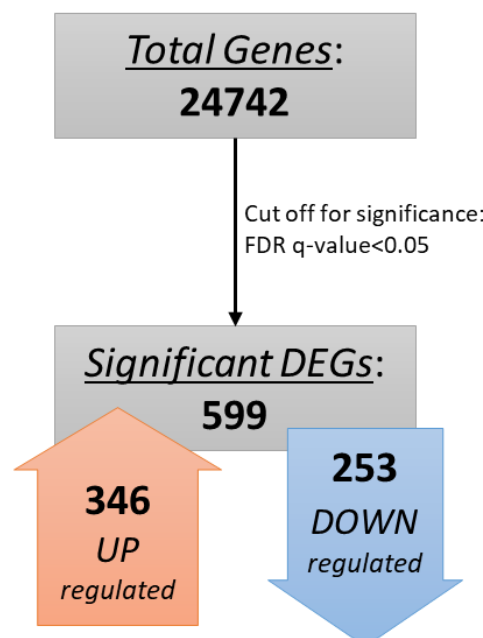


Figure 5.19: Significant Differentially Expressed Genes (DEGs) in CCLP CON and KO Bioinformatics.

A diagram to show: the total number of genes accounted for from the RNAseq data, the number of significant DEGs and the proportion of up-regulated and down-regulated genes. A significant DEG is defined by having an FDR q-value<0.05.

The RNAseq data was compared to qRT-PCR data for *HHEX*, *NOTCH3* and *CCND2* genes as described previously (**Figure 5.20**). The log(Feature Counts) in **Figure 5.20A** and the $1/(\text{Ct Value}/\text{GAPDH Ct Value})$ in **Figure 5.20B** show similar patterns, and in Pearson's correlation analysis, the data showed a regression of $r=0.7997$ and which was statistically significant $p=0.0018$ (**Figure 5.20C**). Thus, gene expression measured by RNAseq counts and RT-qPCR expression is similar for these genes.

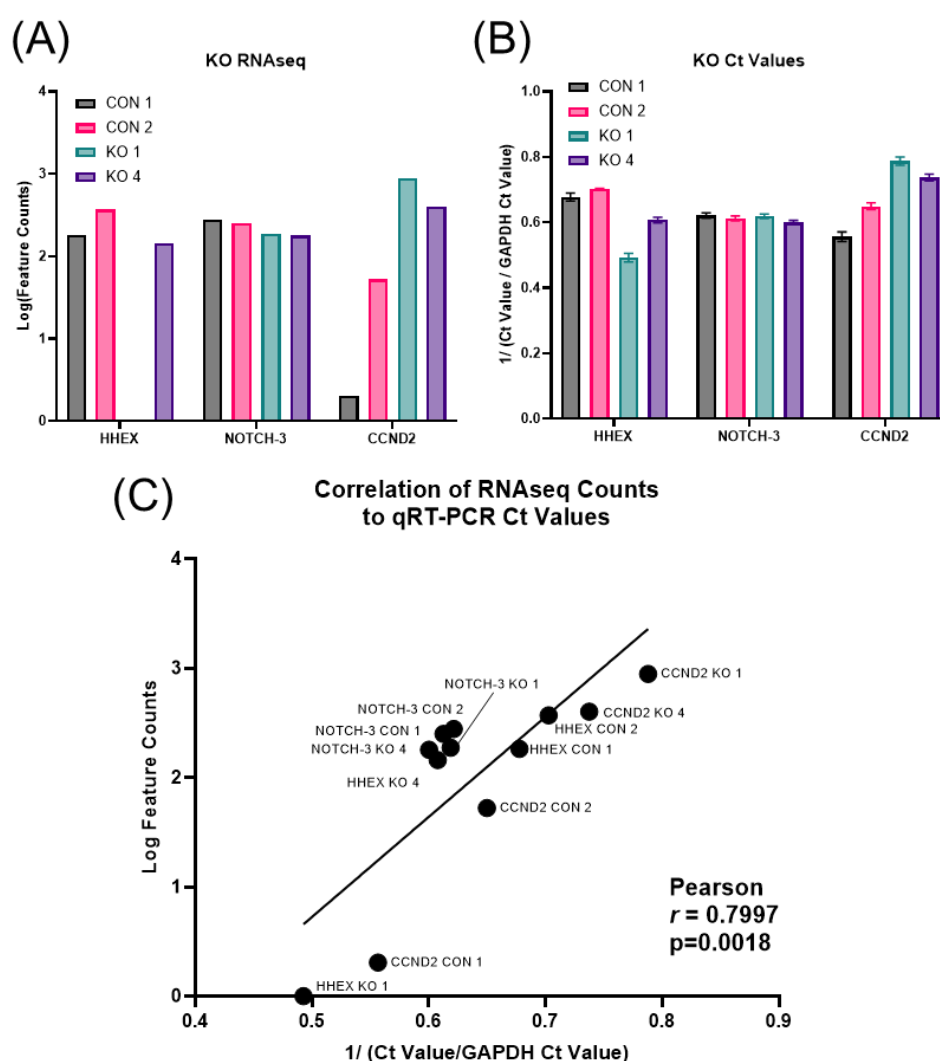


Figure 5.20: Correlation of RNAseq data to qRT-PCR data in CCLP CON and KO cells.
A summary of mRNA expression data for PRH, Notch-3, and Cyclin in RNAseq and in qRT-PCR.
(A) The log(Feature Counts) expression of HHEX, NOTCH-3, and CCND2 in CCLP CON and KO cells in RNAseq.
(B) The $1/(\text{Ct Value}/\text{B-actin Ct Value})$ as a measure of mRNA expression of HHEX, NOTCH-3 and CCND2 in CCLP CON and KO in qRT-PCR. Experimental were completed to N=3.
(C) The correlation of the log(Feature Counts) and the $1/(\text{Ct Value}/\text{B-actin Ct Value})$. The data was analysed through correlation analysis in GraphPad Prism. The Pearson correlation regression value and p-value are shown on the graph.

To identify PRH-regulated genes and pathways in the CCLP knockout cell lines, normalised counts were used to perform unranked Gene Set Enrichment Analysis (GSEA). Data generated from the RNAseq outputs, detailing the gene name along with its normalised counts for each of the phenotypes (CON 1, CON 2, KO 1, KO 4) were uploaded to the GSEA software. The software analysed the data to show enriched gene sets in the KO phenotype, and in the CON phenotype, as previously described (**Figure 5.21**)

In GSEA using the Hallmark MSigDB, the gene sets significantly positively enriched in the PRH KO cell lines were immune response and inflammation (*Interferon Response, Coagulation*), Tissue Dynamics (*Epithelial Mesenchymal Transition, Apical Surface, Angiogenesis*) and signal transduction pathways (*IL2-STAT5 Signalling, KRAS Signalling Up, Estrogen Early Response*). The only Hallmark gene set that was significantly negatively enriched was *Oxidative Phosphorylation*. Although several cell cycle-related gene sets (*G2M Checkpoint, E2F Targets, Mitotic Spindle* and *MYC Targets V1*) were all negatively enriched they were not significantly negatively enriched. This may be because genes have not been changed by the same magnitude in the two KO cell lines, making the overall log2FC not sufficiently large.

In KEGG GSEA analysis, gene sets that were significantly positively enriched in the PRH KO cell lines consisted of gene sets associated with immune response (*Systemic Lupus Erythematosus, Autoimmune Thyroid Disease, Graft vs Host Disease, Allograft Rejection, Type I Diabetes Mellitus*) and metabolism (*Glycosaminoglycan Degradation, Glycine Serine and threonine Metabolism, Drug Metabolism Cytochrome P450, Retinol Metabolism*). There were no significantly negatively enriched KEGG gene sets.

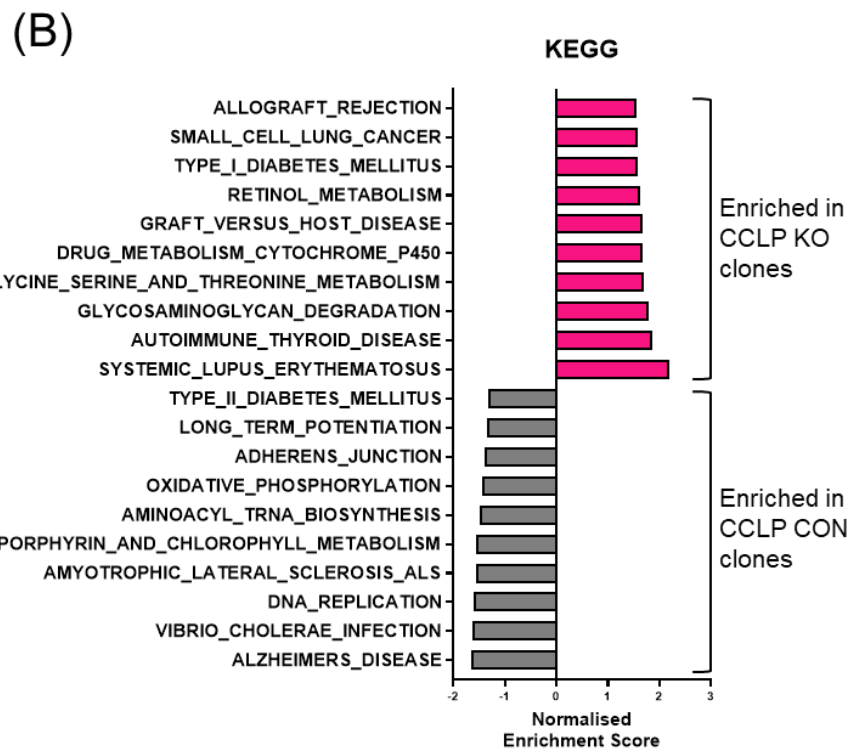
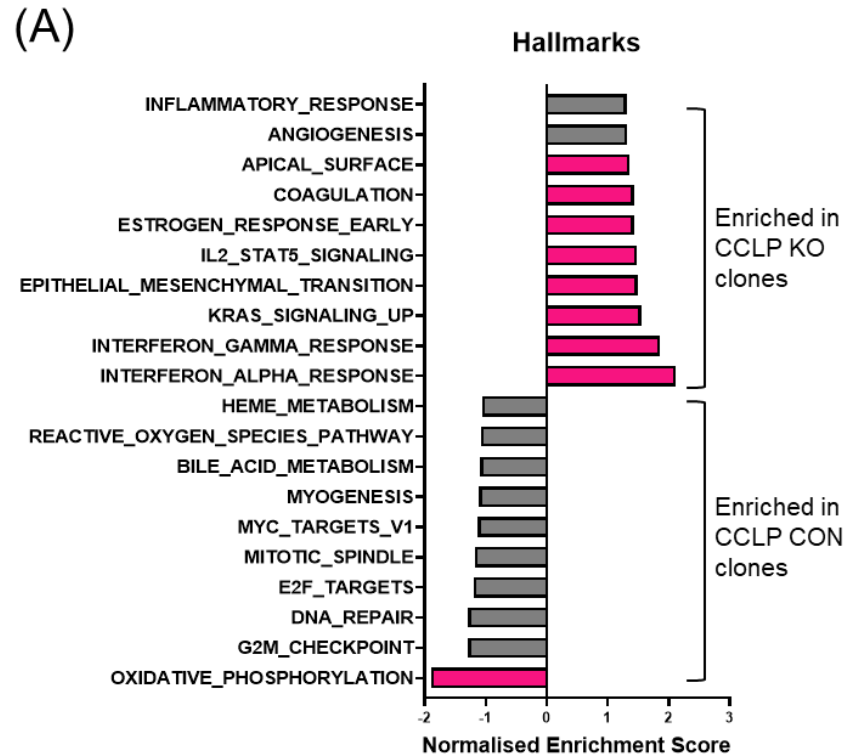


Figure 5.21: Gene Set Enrichment Analysis of between CCLP CON and KO RNAseq.

RNA samples from CCLP CON clones 1 and CON 2, plus CCLP KO clones 1 and 4, were used in RNA sequencing experiments. Genes and their respective normalised counts were inputted into Gene Set Enrichment Analysis software. The software ranked the genes into a list of genes starting with genes significantly enriched in the PRH KO clones, to genes significantly enriched in the CCLP CON clones. Gene sets from different databases were assessed for their enrichment in each phenotype. The top ten gene sets, according to normalised enrichment score (NES), are shown for each phenotype, with significantly enriched gene set in pink; pink shows FDR q -value < 0.25 . The two canonical GSEA databases are used (A) Hallmarks (B) KEGG.

In cumulative growth curve experiments and EdU incorporation proliferation assays, CCLP CON cells had significantly higher growth and proliferation, respectively, compared to CCLP KO cells. To better understand the GSEA a volcano plot was generated for the negatively enriched G2M Checkpoint gene set, even though it did not show statistical significance. **Figure 5.22** shows the volcano plot, where significantly DEGs are highlighted in magenta. As expected, most genes are down-regulated in response to the PRH KO, but the changes in log2FC are not significant because as mentioned above two different clones are grouped which may both have changes in gene expression in the same pathway, but the changes are likely not to the same extent. Interestingly there is one significantly and highly upregulated gene, Cyclin D1, which could indicate that the KO cells proliferate more slowly and are in G1 phase. The first nine negatively enriched genes to show the highest $-\log(P\text{-adj})$ are shown in the plot. Most of these genes are involved in late-stage cell cycle and cytokinesis. The difference in the level of normalised counts for each gene between the two CON clones and between the two KO clones results in low significance in GSEA, and this likely results in the non-significance of sets such as the G2M and E2F gene sets.

The expression of genes in the Oxidative Phosphorylation gene set was also further investigated (**Figure 5.23**). The genes are significantly positively enriched in the CON phenotype (negatively enriched in the KO), which agrees with the GSEA above. Upon further inspection via volcano plot, there were four significant DEGs: *NDUFA4*, *CYCS*, *ATP5FC1* and *SDHA*. Strikingly, these proteins are all part of the electron transport chain, where *NDUFA4* (Complex I) and *SDHA* (Complex II) are involved in transferring electrons to *CYCS* (part of Complex III and IV), which ultimately helps drive *ATP5FC1* (Complex V) to synthesize ATP (Zhao et al., 2019).

In conclusion, this section covers the generation and validation of two PRH KO clones and the effect of PRH KO on CCLP cell phenotype and gene expression. The results further confirm the role of PRH in promoting CCA proliferation. Although the RNAseq GSEA reveals a novel role for PRH in regulating the genes of proteins in the ETC, the analysis does not reach significance for several enriched genes sets including G2M likely due to inter-clonal differences between CCLP KO 1 and KO 4.

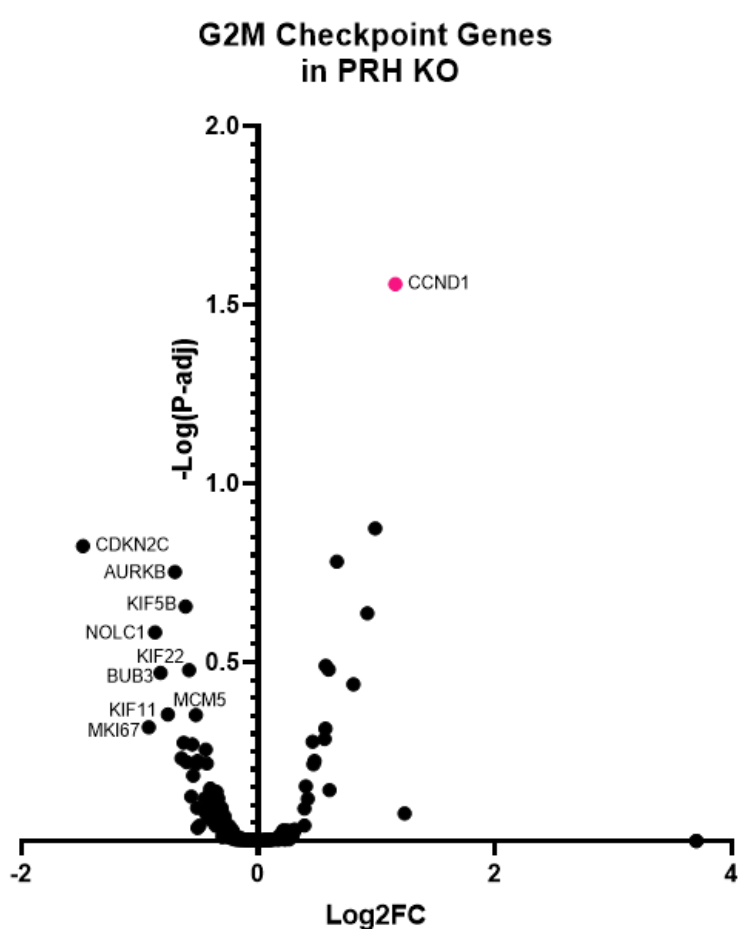


Figure 5.22: A volcano plot of G2M Checkpoint genes in PRH KO RNAseq

A volcano plot generated from the log_2FC and $-\text{log}(P\text{-adj})$ values of G2M Checkpoint genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEG are highlighted in magenta.

Oxidative Phosphorylation Genes in PRH KO

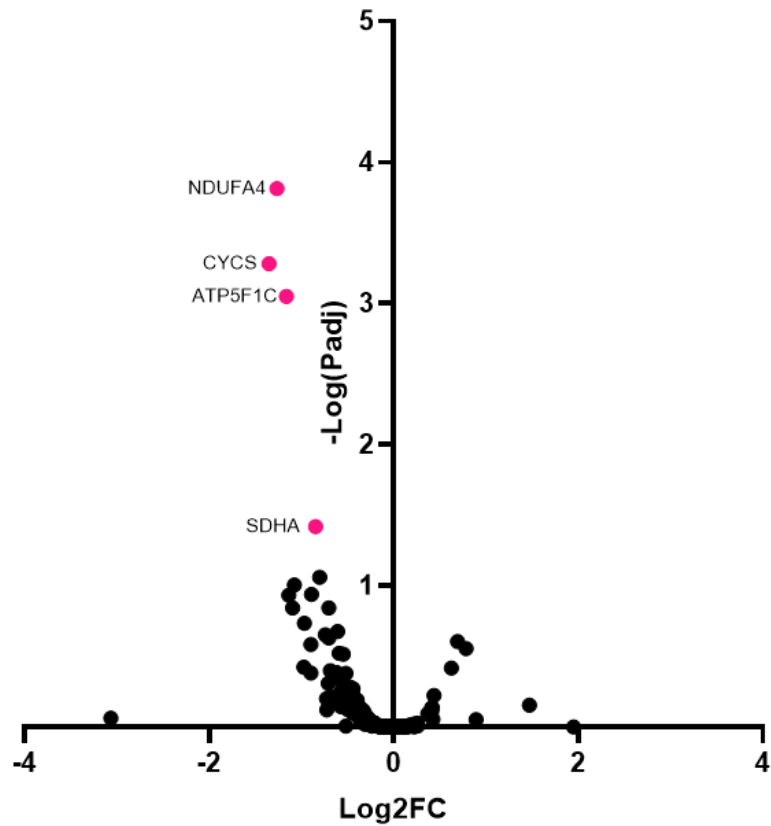


Figure 5.23: A volcano plot of Oxidative Phosphorylation genes in PRH KO RNAseq
A volcano plot generated from the log2FC and $-\log(P\text{-adj})$ values of Oxidative Phosphorylation genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEG are highlighted in magenta.

SECTION C:

The Effects of PRH KD via shRNA

5.10 The effect of PRH KD via shRNA on Cell Growth

To enable a full comparison of PRH knockdown or depletion via siRNA, KO and shRNA, phenotypic and sequencing techniques were employed to examine the effect of PRH KD via shRNA in CCLP cells. The generation and validation of the CCLP SVC and PRH KD cell lines has been described previously (section 3.8).

To determine whether the PRH KD 2 cells show an alteration in cell number with time, CCLP SVC 2 and PRH KD 2 cells were examined in cumulative growth curve experiments. **Figure 5.24** shows the growth of CCLP SVC 2 and PRH KD 2 over ten days. The PRH KD 2 cells have a significantly lower cell number than the SVC 2 cells at multiple timepoints (day 6 ($p=0.016$) and day 8 ($p=0.032$)), although the results for cell count at day 10 fall outside statistical significance ($p=0.09$), which is likely due to variance in the cell counts. Relative doubling time is increased in PRH KD 2 compared to SVC 2, from 33.14 hours to 37.39 hours ($p=0.0083$). These data reproduce the findings reported from previously created shRNA PRH KD clones (Kitchen et al., 2020)

(A) CCLP SVC and PRH KD Growth Curve

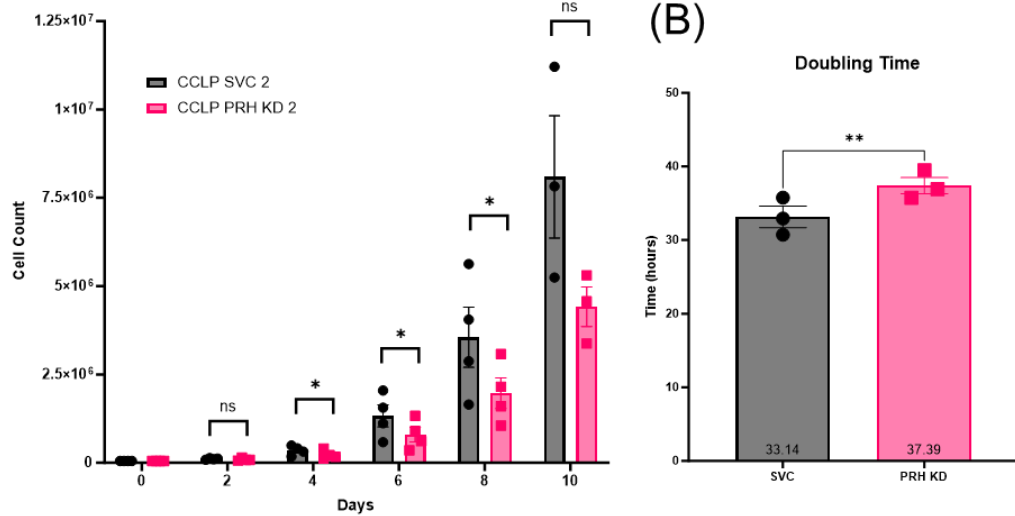


Figure 5.24: Growth Curves of CCLP SVC 2 and PRH KD 2

Cells were seeded at 5×10^4 cells per well in a 6 well plate and counted via harvesting every two days until 10 days. Cells were re-plated in an appropriately volumed flask to allow for exponential growth. Experiments were set up in duplicate wells, counted and re-plated over three biological repeats independently (N=3).

- (A) Cell number counted over every two days in CCLP SVC 2 and CCLP PRH KD 2 cells. Error bars represent SEM and statistical significance was measured through two-tailed paired t-test (N3; *= $p < 0.05$).
- (B) Doubling time as calculated by the cell number over 10 days. Error bars represent SEM and statistical significance was measured through two-tailed paired t-test (N3; **= $p < 0.01$).

5.11 The Effect of shRNA PRH KD on Cell Proliferation and Cell Cycle Progression

To investigate the decrease to growth rate in the CCLP PRH KD cells, CCLP SVC 2 and PRH KD 2 cells were used in EdU incorporation assays and cell cycle progression was analysed via flow cytometry.

Figure 5.25A shows that there is little if any difference in EdU proliferation assays between CCLP SVC 2 and PRH KD 2 cells. The experiment was repeated with increasing times of EdU incorporation to determine whether this would allow small differences in EdU incorporation to be observed, however no difference was seen at 12 hours (N3) or 24 hours (N4) incubation with 2µm EdU (**Figure 5.25B**). This suggests that there is little effect on the rate of DNA synthesis by shPRH KD in these clones.

To determine any role of PRH in other stages of cell cycle, CCLP SVC and PRH KD were examined using flow cytometry. **Figure 5.25C**, shows representative images of the Kaluza analysis and **Figure 5.25D** shows the overall changes to the cell cycle over five biological repeats. PRH KD 2 express no significant difference in cell cycle stages compared to CCLP SVC 2 control. This data is not similar to previous findings with PRH KD cells that showed a decreased proliferation rate following PRH KD using the PRH shRNA in these cells (Kitchen et al., 2020). The discrepancy between the results of the proliferation assays for the current clones and the previous data could occur because the current polyclonal PRH KD cells have less PRH knockdown than the previously characterised cells or because the current cell line population has evolved with time to lose cells with high levels of PRH KD.

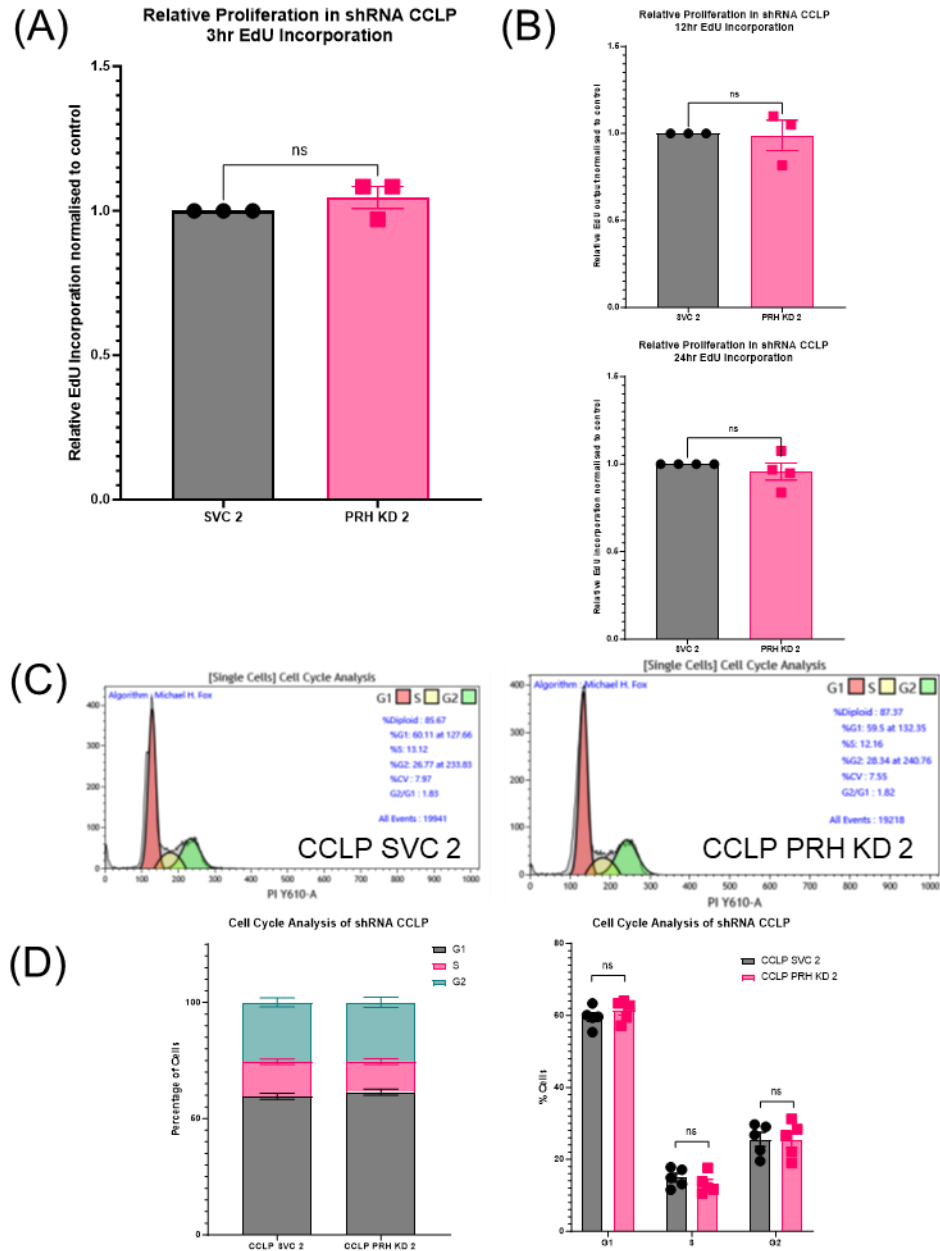


Figure 5.25: EdU Incorporation and Cell Cycle Analysis of PRH KD in shRNA CCLP. CCLP SVC 2 and PRH KD 2 were harvested and seeded at 1×10^4 cells per well in a 96 well plate, in quadruplicate, and incubated with 2uM EdU for 3 hours or longer timepoints. EdU incorporation was translated into an OD reading, by labelling with HRP and incubating with substrate, which was read at 450nm. Relative proliferation was calculated by normalising PRH KD values to SVC values. In cell cycle flow cytometry, 1×10^6 CCLP SVC 2 and PRH KD 2 cells were washed and fixed in 70% Ethanol. Cells were then washed, subjected to RNase treatment (0.2mg/mL) and PI staining (50ug/mL) before running on a cytometry machine for cell cycle analysis. Analysis gating and cell cycle algorithms were performed through Kaluza software.

A) Relative proliferation in CCLP SVC 2 and PRH KD 2 cells. Error bars represent SEM and statistical significance was measured through two-tailed t-test (N3).

B) EdU Incorporation experiments were also performed with longer EdU incorporation time of 12hrs (N3) and 24hrs (N4).

C) Representative cell cycle analysis of CCLP SVC 2 and PRH KD 2 in Kaluza.

D) Quantification of cell cycle analysis of CCLP SVC 2 and PRH KD 2 showing the proportion of the cell population in each stage of the cell cycle: G1, S, and G2 (N=5). Error bars represent SEM and statistical significance was measured through two-tailed paired t-test.

5.12 The Effect of PRH KD via shRNA on Apoptotic Signalling

The CCLP SVC 2 and PRH KD 2 cell lines were used in flow cytometry apoptosis experiments with, and without, the well characterised apoptotic inducer, Staurosporine, to explore their sensitivity to apoptosis. For this, CCLP SVC and PRH KD cells were seeded at 1×10^6 cells in a 6-well plate and left to adhere overnight. The cells were either treated with a DMSO vehicle control or $1 \mu\text{M}$ Staurosporine for 24 hours before Annexin V and PI staining and flow cytometry analysis exactly as described previously for siPRH CCLP cells incubated with Staurosporine.

Figure 5.26 shows the effect of PRH KD on CCLP cell death. **Figure 5.26A** illustrates representative images of the data obtained from Kaluza and **Figures 5.26B and C** show the downstream analysis for the three repeats. CCLP SVC and PRH KD cells that are not stimulated by Staurosporine show minimal cell death, with 95% of cells remaining live in each cell line, and equal rates of cell death. Thus, there appears to be no difference between CCLP SVC and PRH KD cells in unstimulated apoptosis rates.

With Staurosporine treatment, both SVC and PRH KD cells show a significant increase to the number of cells undergoing apoptosis (**Figure 5.26C**). Although there are trends to show PRH KD cells are more sensitive to Staurosporine treatment, (SVC 7% early apoptosis, 25% late apoptosis; PRH KD early apoptosis 6% and late apoptosis 32%) there is no statistical significance across three repeats ($p=0.13$).

In conclusion, the PRH KD cells show a slower increase in cell number with time but surprisingly do not show statistically significant changes in cell division or apoptosis. These results show lack of correspondence between the previously studied shRNA PRH KD in CCLP (Kitchen et al., 2020). There may be differences between the original shRNA clones and the newly generated shRNA clones as the cell lines are polyclonal and the average level of PRH knockdown could be very different, leading to more dramatic effects in the previously reported PRH shRNA KD cell lines.

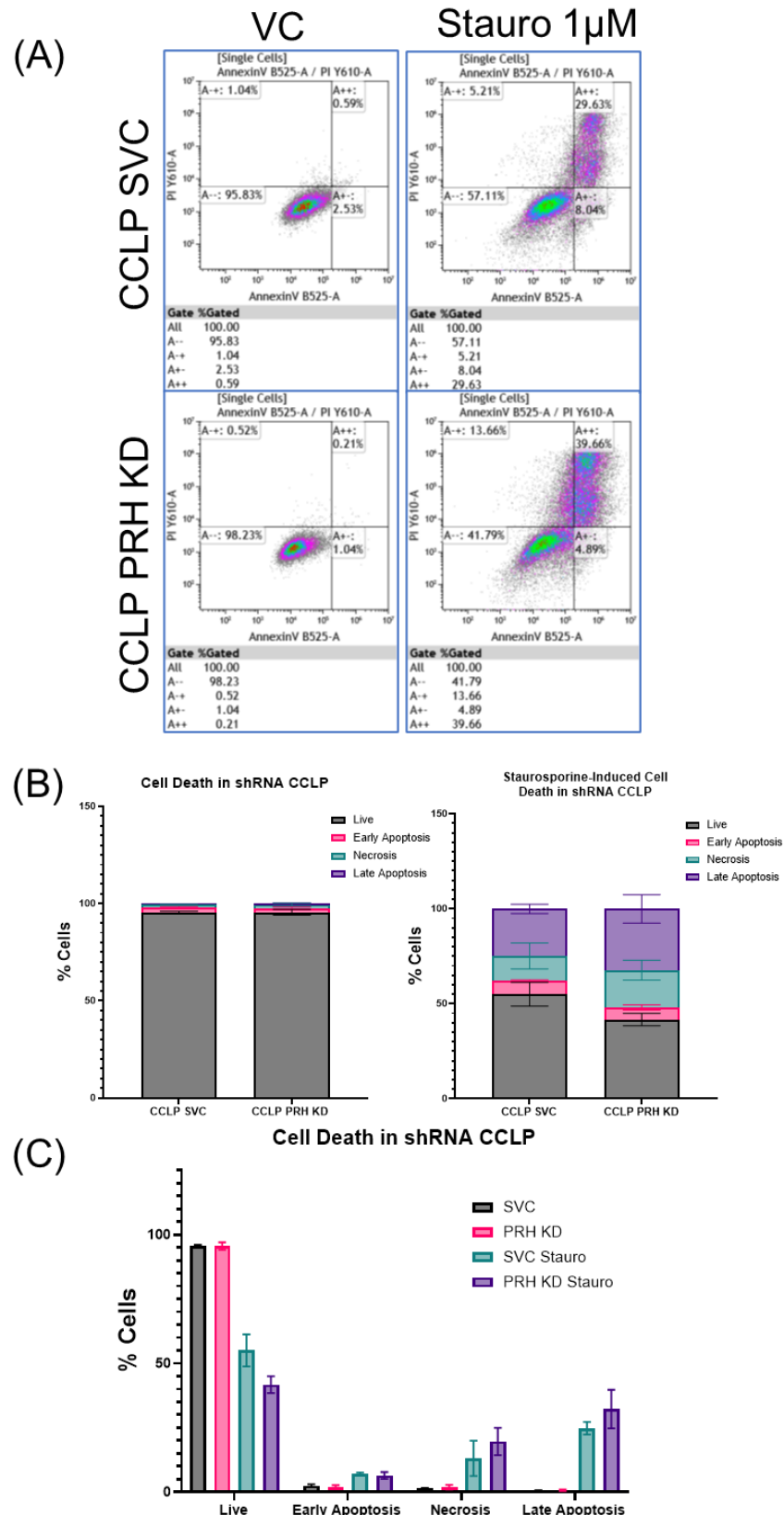


Figure 5.26: Cell death in CCLP SVC and PRH KD cells.

Cells were seeded at 1×10^6 in a T25, left to adhere for 24 hours, then treated with either vehicle control or 1 μ M Staurosporine for 24 hours. After treatment, cells were pelleted and washed before staining with Propidium Iodide and Annexin V. Samples were ran on a cytometry machine. Gating and analysis were performed through Kaluza software.

(A) Representative Kaluza analysis of cell death in CCLP SVC and PRH KD cells treated with vehicle control or Staurosporine.

(B) Apoptosis analysis of CCLP SVC and PRH KD cells treated with vehicle control or Staurosporine.

(C) Overall analysis of Staurosporine-induced, and unstimulated, cell death in CCLP SVC and PRH KD cells, with statistical significance measured through unpaired t-tests (N3).

5.13 The Effect of PRH KD via shRNA on Gene Expression

To investigate the PRH regulated genes and pathways in the shRNA CCLP PRH KD, RNA samples generated from the CCLP SVC 1 and 2 cell lines, as well as the CCLP PRH KD 1 and 2 cell lines, were used in RNAseq analysis. After RIN tests confirmed that samples contained high quality RNA, the four samples were sent to Novogene for sequencing and analysed as described previously. From the DESeq output file, of 26742 genes, 863 genes were significantly differentially expressed (DEGs). Of the significant genes, 546 were up regulated and 317 were downregulated in response to shRNA PRH knockdown (**Figure 5.27**).

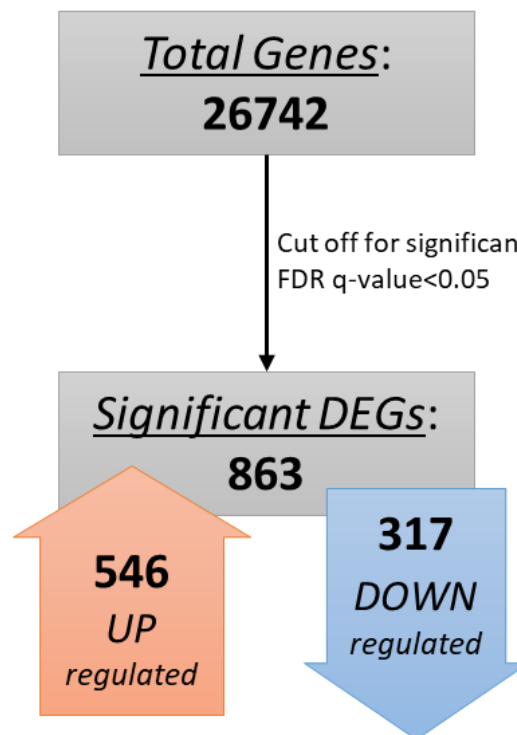


Figure 5.27: Significant Differentially Expressed Genes (DEGs) in CCLP SVC and PRH KD Bioinformatics.

A diagram to show: the total number of genes accounted for from the RNAseq data, the number of significant DEGs and the proportion of up-regulated and down-regulated genes. A significant DEG is defined by having an FDR q-value<0.05.

RNAseq validation was shown through comparing RNAseq data to qPCR data for HHEX, NOTCH-3 and CCND2 genes as described previously (**Figure 5.28**). The log(Feature Counts) in **Figure 5.28A** and the $1/(\text{Ct Value}/\text{GAPDH Ct Value})$ in **Figure 5.28B** are in agreement, although there is less correlation between data sets than for other PRH KD/KO models (Pearsons correlation analysis, $r=0.51$ $p=0.30$) (**Figure 5.28C**), likely due to a low number of data points.

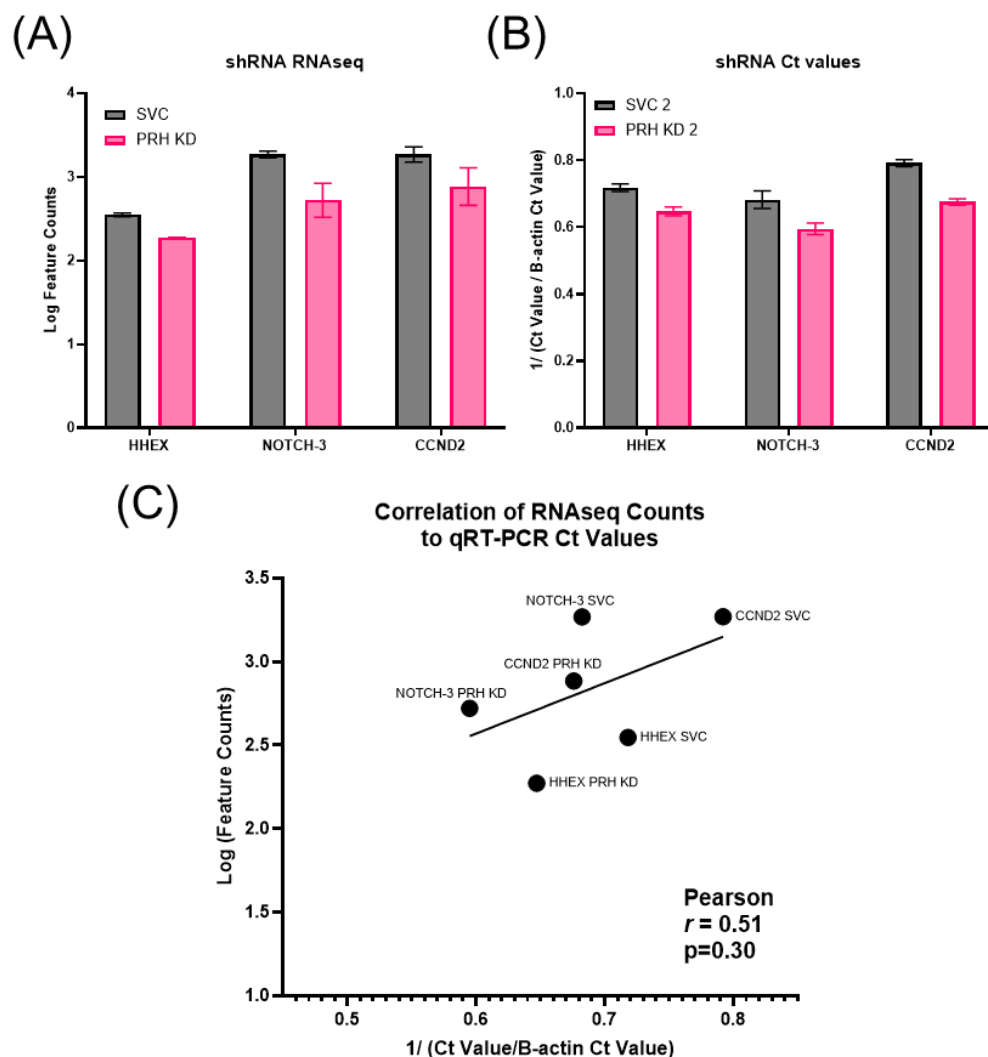


Figure 5.28: Correlation of RNAseq data to qRT-PCR data in CCLP SVC and KD cells. A summary of mRNA expression data for PRH, Notch-3, and Cyclin D2 in RNAseq and in qRT-PCR. (A) The log(Feature Counts) expression of HHEX, NOTCH-3, and CCND2 in CCLP SVC and PRH KD cells in RNAseq. (B) The $1/(\text{Ct Value}/\text{B-actin Ct Value})$ as a measure of mRNA expression of HHEX, NOTCH-3 and CCND2 in CCLP SVC 2 and PRH KD 2 in qRT-PCR. Experimental were completed to N=3. (C) The correlation of the log(Feature Counts) and the $1/(\text{Ct Value}/\text{B-actin Ct Value})$. The data was analysed through correlation analysis in GraphPad Prism. The Pearson correlation regression value and p-value are shown on the graph.

The RNAseq data was used in Gene Set Enrichment Analysis (GSEA), to identify PRH-regulated genes and pathways in the CCLP SVC and PRH KD cells.

Figure 5.29 shows the enrichment of the top ten gene sets in PRH KD cells and in SVC cells when using the (A) Hallmarks MSigDB and (B) KEGG database. In GSEA Hallmarks analysis, PRH KD has significant positive enrichment of late-stage cell cycle associated gene sets including *E2F Targets*, *G2M Checkpoint*, *Mitotic Spindle* and *MYC Targets V1*. Other gene sets, *MTORC1 Signalling*, *Androgen Response* and *Protein Secretion* are also significantly enriched. Gene sets significantly enriched in SVC cells (ie negatively enriched in KD) include sets associated with *KRAS Signalling*, immune response (*Interferon Signalling*, *Allograft Rejection*) and *Inflammation*. *Wnt-Beta Catenin pathways* is significantly enriched in the SVC phenotype, and *Notch Signalling* is enriched in the SVC phenotype but not significantly, showing similarity to previous bioinformatics analysis of genes altered in PRH KD cells studies (Kitchen et al., 2020). However, previous analysis was performed as a pre-ranked GSEA using only significant DEGs with $-0.5 > \log_2FC > 0.5$, as opposed to all genes. This means that the previous published analysis only looked at a subset of regulated genes showing high changes in gene expression. Overall, the current analysis is in broad agreement with the previously published analysis (Kitchen et al., 2020) but in this data set there is an increased emphasis on cell cycle-related gene sets.

In the KEGG analysis, PRH KD shows significant positive enrichment in *Cell Cycle*, *Oocyte Meiosis* and *DNA Replication*. Significantly negatively enriched KEGG gene sets are associated with immune response (*Intestinal Immune Network for IgA Production*, *Autoimmune Thyroid Disease* and *Allograft*

Rejection), diabetes and other signalling pathways. These pathways are similar to those identified in the Hallmark sets.

To identify the genes that show significant and reproducible alteration in the G2M pathways a volcano plot was generated to show the log₂FC and also the significance (-log₁₀Padj) of the G2M checkpoint gene set (**Figure 5.30**). All the genes were plotted from the G2M gene set (200 genes) and the significant DEGs are highlighted in magenta. The figure shows that most genes in this pathway are upregulated in response to the shRNA PRH KD, and that a high proportion of the genes are significantly upregulated. The top ten significantly upregulated DEGs have been shown in the figure: *BIRC5*, *MKI67*, *TOP2A*, *PLK1*, *HMMR*, *AURKB*, *CENPF*, *CENPA*, *KIF4A* and *CDC20*. All these proteins have joint function in the chromosomal passenger complex (CPC); a master controller of cell division (Carmena et al., 2012).

The *p53 Signalling Pathway*, was enriched in the shRNA PRH KD phenotype in the KEGG analysis. Although this may help explain a trend in increased Staurosporine-stimulated apoptosis in shPRH KD cells, cell death gene sets do not seem to be as biologically significant in the RNAseq data as cell cycle gene sets.

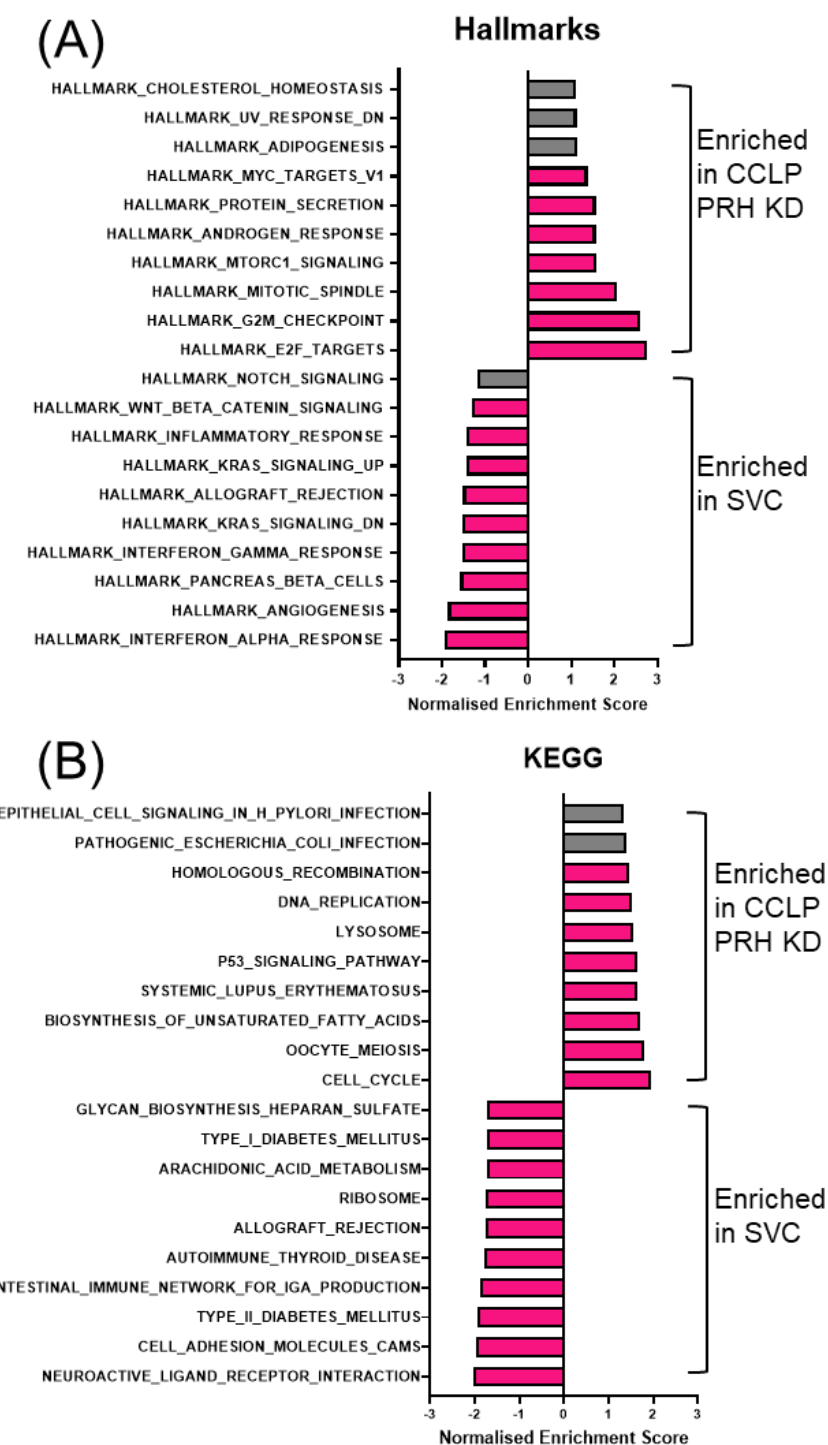


Figure 5.29: Gene Set Enrichment Analysis of CCLP SVC and PRH KD RNAseq. Three sets of RNA samples from CCLP siCtrl and siPRH cells were used in RNA sequencing experiments. Genes and their respective normalised counts were inputted into Gene Set Enrichment Analysis software. The software ranked the genes into a list of genes starting with genes significantly enriched in the siPRH cells, to genes significantly enriched in the siCtrl cells. Gene sets from different databases were assessed for their enrichment in each phenotype. The top ten gene sets, according to normalised enrichment score (NES), are shown for each phenotype, with significantly enriched gene set in pink; pink shows FDR q-value<0.25. The two canonical GSEA databases are used (A) Hallmarks (B) KEGG.

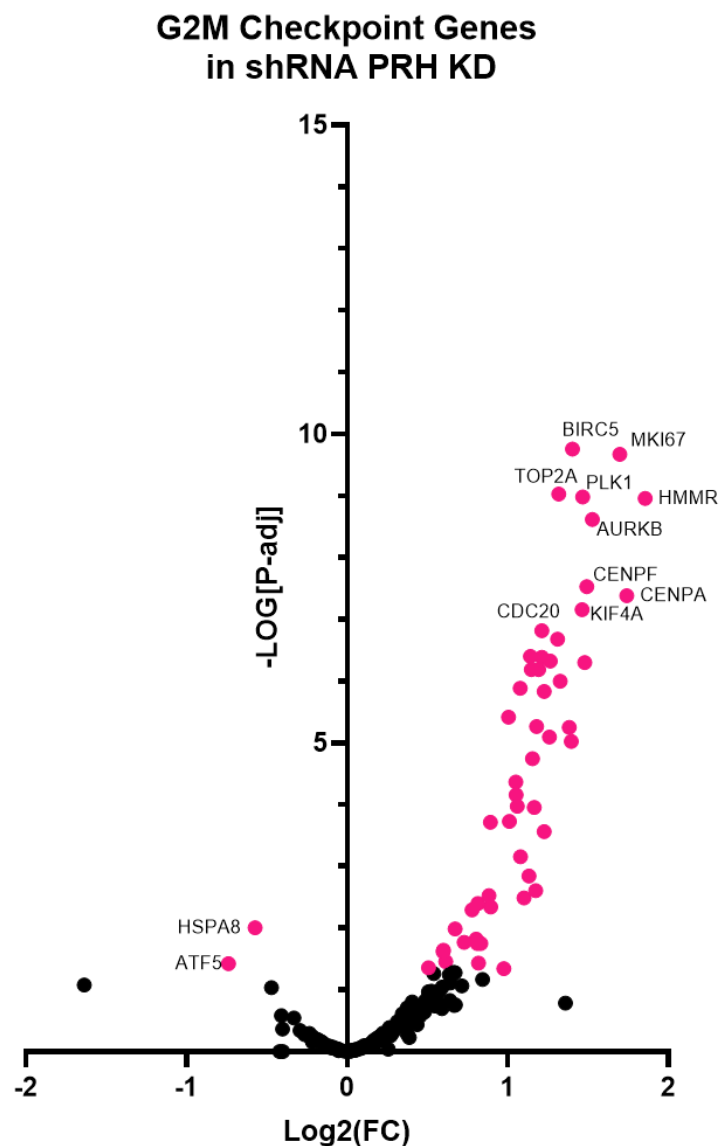


Figure 5.30: A volcano plot of G2M Checkpoint genes in shRNA PRH KD RNAseq
 A volcano plot generated from the log2FC and $-\log(P\text{-adj})$ values of G2M Checkpoint genes. Values were retrieved from the DESeq2 RNAseq file. Significant DEG are highlighted in magenta. The top ten significantly upregulated DEGs are shown as well as the two significantly down-regulated DEGs

SECTION D:

Comparison of PRH KD and PRH KO Models

5.14 Summary of Phenotypic Experiments across PRH KD and KO Models:

The findings in sections A, B and C have summarised below to show the effects of PRH loss across the models on different cell phenotypic experiments (**Figure 5.31**). PRH loss brought about a statistically significant reduction cumulative cell number across the models. Cell proliferation is significantly decreased in the siRNA and KO models, but not in the shRNA model. PRH KD by siRNA increased apoptosis. Although there was a trend toward increased apoptosis in shRNA PRH KD cells, this was not statistically significant. KO cells were not assayed or effects on cell cycle or apoptosis owing to time constraints.

Phenotype	siRNA	KO	shRNA
Growth	↓↓↓	↓↓↓	↓↓↓
Proliferation	↓↓↓	↓↓↓	=
Cell Cycle	↓↓↓		=
Apoptosis	↑↑↑		↑

Figure 5.31: A Summary of the Different PRH KD and PRH KO Models in Phenotypic Experiments.

The effect of PRH KD or KO on multiple phenotypes including cell growth (measured through accumulative growth curve), cell proliferation (measured through EdU incorporation experiments), cell cycle (measured by flow cytometry techniques), cell migration (measured through Transwell migration assays) and cell apoptosis (measured through flow cytometry techniques). Key: ↑↑↑= significantly increased, ↑=non-significant increase, = no change, ↓= non-significant decrease, ↓↓↓= significant decrease.

5.15 Comparison of the Models using RNA Sequencing:

Unranked GSEA considers all genes in an experiment, not just DEGs or those above a significance threshold. In the unranked GSEA analysis, common gene sets are enriched across the models, but they are not enriched in the same direction in all models. These include cell cycle related gene sets such as *G2M Checkpoint*, *E2F Targets*, *Mitotic Spindle* and *MYC Targets V1*.

Figure 5.32 shows the enrichment plots for the cell division related gene sets across the shRNA, siRNA and KO CCLP models. Enrichment plots are a visualisation of how the enrichment score (ES) is calculated. The score is calculated by 'walking down' the ranked gene list; the running-sum is increased when a gene is encountered in the list, and the running-sum is decreased when there is no gene encountered. Thus, if there are genes encountered at the top of the list, where the genes are enriched in the PRH KD/KO phenotype, the running track will be plotted above zero; if the genes are enriched in the control phenotype and have been ranked at the bottom of the list, the plot will reside below zero. The maximum deviation from zero in the random walk is the ES value; the magnitude of the score is dependent upon how enriched the gene set is to the phenotype (Subramanian et al., 2005).

The enrichment is significantly positively correlated to PRH KD in the shRNA model for example, there is upregulation of G2M genes. In contrast, the enrichment is negatively correlated in both the siRNA PRH KD and PRH KO. To investigate further, expression heatmaps for all the cell division related genes were generated to compare their expression between models.

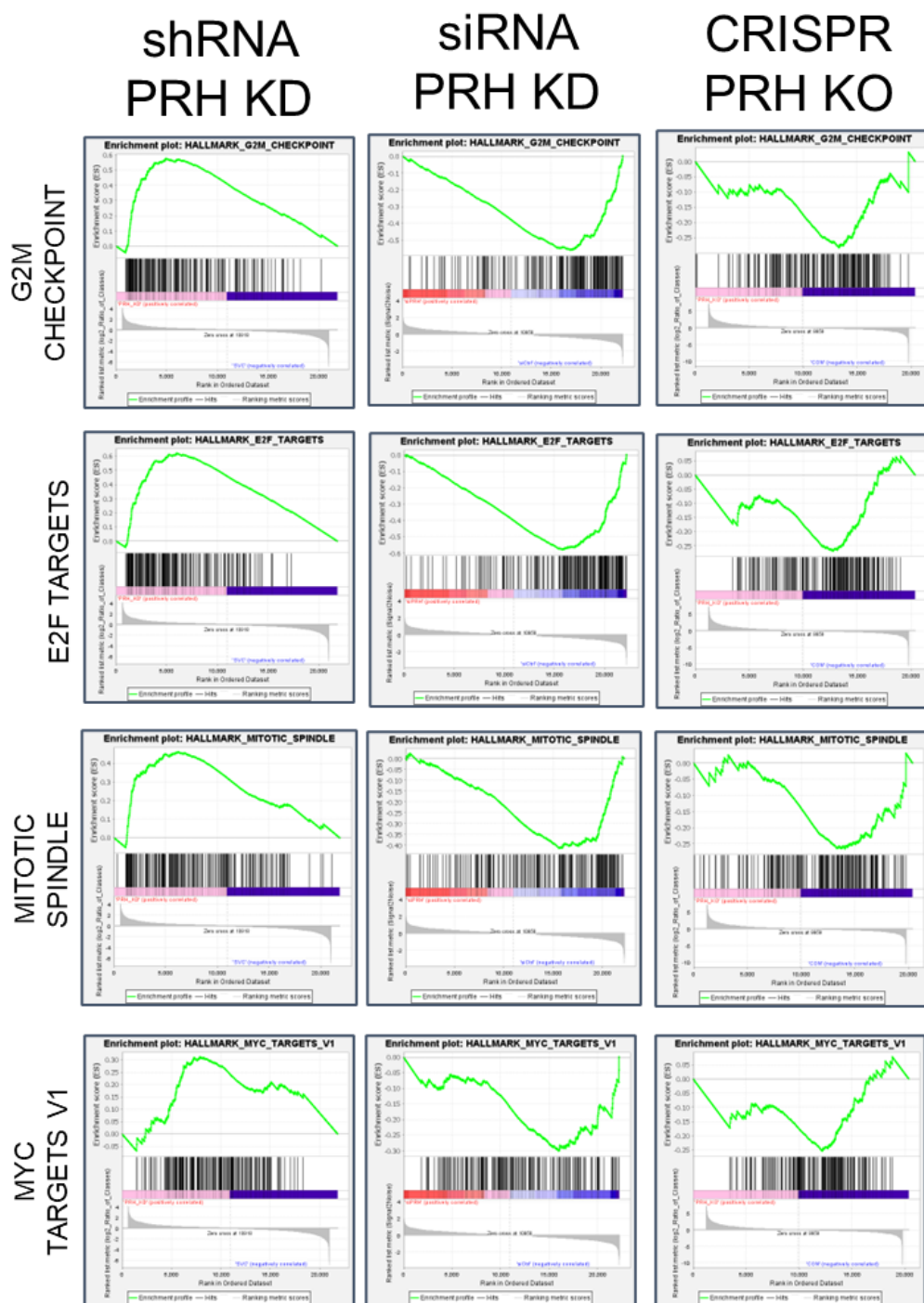


Figure 5.32: Enrichment plots for Cell Cycle Associated Gene Sets across the PRH KD and KO models

Through the GSEA analysis, Enrichment plots were made for significantly enriched gene sets. Across the three models, the shRNA and siRNA PRH KD models, plus the PRH KO model, common gene sets were observed including the following cell cycle associated gene sets: G2M checkpoint, 2F targets, Mitotic Spindle and MYC Targets V1. The green track shows whether the gene set is enriched in the PRH KD/KO model, a positive enrichment score, or its control, a negative enrichment score.

In the KEGG database, the cell division gene set has 125 gene members. The log₂FC was collected for each gene from the three RNAseq datasets. Genes with no counts were removed, and in order to only investigate genes with high levels of differential expression likely to be of biological relevance, genes with log₂FC below 0.5 in all three RNAseq models were removed. This left 60 genes of interest. The genes (rows) and models (columns) were clustered under Euclidean distance method; heatmaps were generated on SRPlot online tool. **Figure 5.33** shows the expression of these genes across models indicated by colour, and this can be directly compared across models.

The figure further illustrates that the siRNA and KO have decreased expression of the highly differentially expressed cell division related genes compared to shRNA cells. This agrees with the ES plots. There is also a minority group of genes that are highly decreased in the siRNA alone, or the KO alone compared to shRNA KD. Interestingly, there are also genes that do not fall into group clusters, such as CCND2.

It can be concluded that the major effect of PRH loss in the siPRH and KO cell lines is the down-regulation of multiple cell division genes including the G2M genes. In marked contrast, there is positive enrichment of these genes in the shRNA PRH KD cell lines. Given the opposite enrichment of these gene sets, it is possible that the shRNA KD cells positively enrich G2M genes to allow cell division in the absence of PRH, that is, as an adaptation to PRH loss rather than as an immediate consequence of PRH loss. This adaptation could have occurred during the antibiotic selection and expansion process needed to generate a shRNA stable cell line.

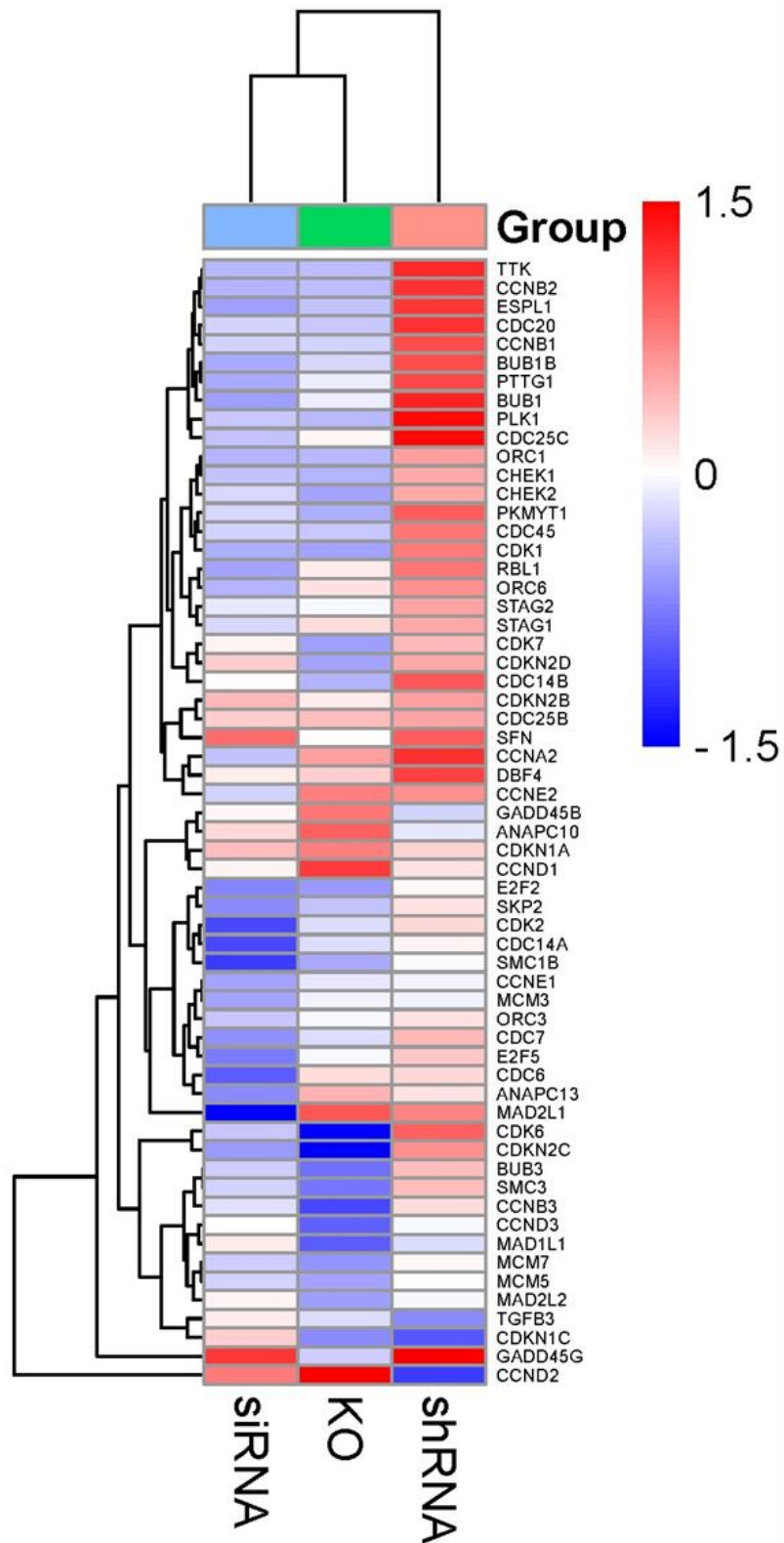


Figure 5.33: A Clustered Heatmap of Cell Cycle across the PRH KD and KO models

The log2FC expression data for gene in the KEGG Cell Cycle gene set were filtered and presented in heatmap format. A colour scale indicates up-regulation (red) and down regulation (blue) of the genes. Genes and column have undergone hierarchical clustering through Euclidean distance method to group genes of similar patterning. The rows of genes have not undergone scaling and therefore expression can be compared between genes.

There are other commonly observed gene sets across GSEA analysis across the RNAseq datasets that are associated with cell viability including the p53 pathway and apoptosis pathways. The enrichment plots for the *p53 Pathway* and *Apoptosis* are shown in **Figure 5.34**.

Apoptosis related genes are not significantly enriched in any of the RNAseq datasets, and this is shown by the lack of directionality in enrichment in all three of the plots. Interestingly whilst the p53 pathway is significantly positively enriched in the siPRH KD phenotype, it is not statistically significant in the shRNA PRH KD or PRH KO models. This could explain the significant differential response to Staurosporine in the siRNA model, and lack of differential response in the shRNA model. Overall, the pattern in *Apoptosis* and *p53 Pathway* enrichment is varied, compared to cell cycle gene set, highlighting that PRH might only have a subtle effect on apoptosis in CCA cells.

Figure 5.35 shows heatmaps of the expression of apoptosis genes amongst the RNAseq datasets. The genes were filtered by $\log_2FC > 0.5$ as described previously. In the apoptosis heatmap, the siRNA and shRNA genes show similar levels of expression whereas the same genes have a very different expression in the PRH KO model. Thus, siPRH RNA and shPRH RNA models cluster more closely for apoptosis. However, again, there were few genes that have similar patterns across all three of the models.

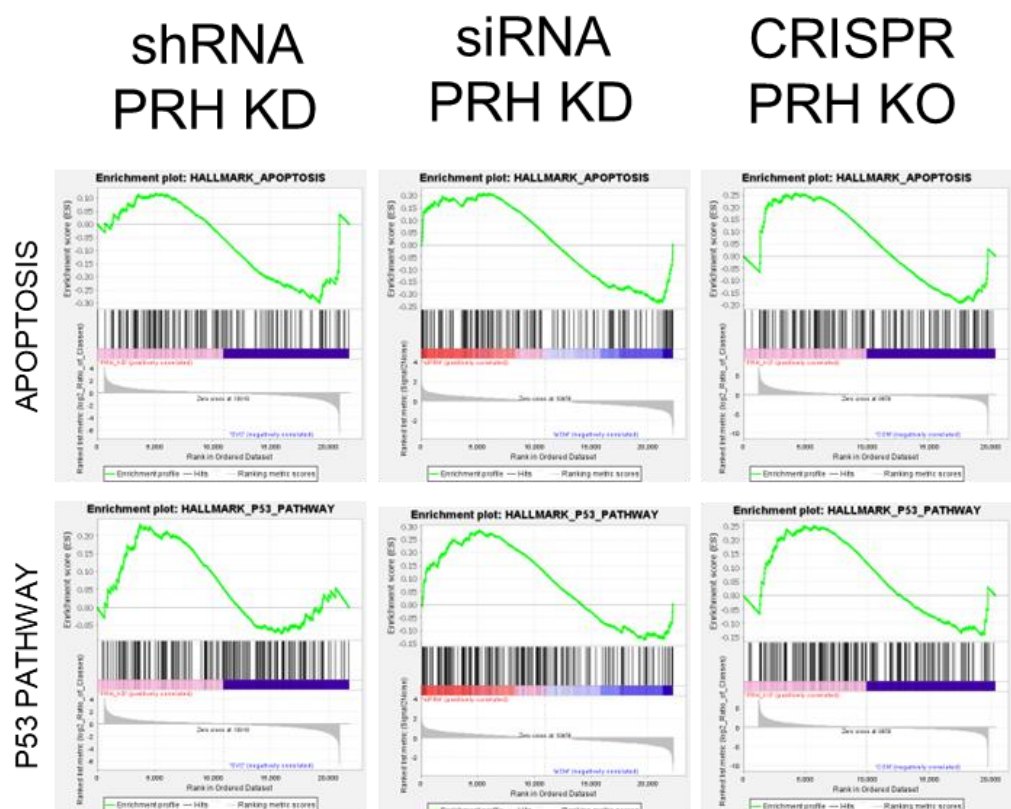


Figure 5.34: Enrichment plots for Cell Death Associated Gene Sets across the PRH KD and KO models

Through the GSEA analysis, Enrichment plots were made for significantly enriched gene sets. Across the three models, the shRNA and siRNA PRH KD models, plus the PRH KO model, common gene sets were observed including the following cell death associated gene sets: Apoptosis and p53 Pathway. The green track shows whether the gene set is enriched in the PRH KD/KO model, a positive enrichment score, or its control, a negative enrichment score.

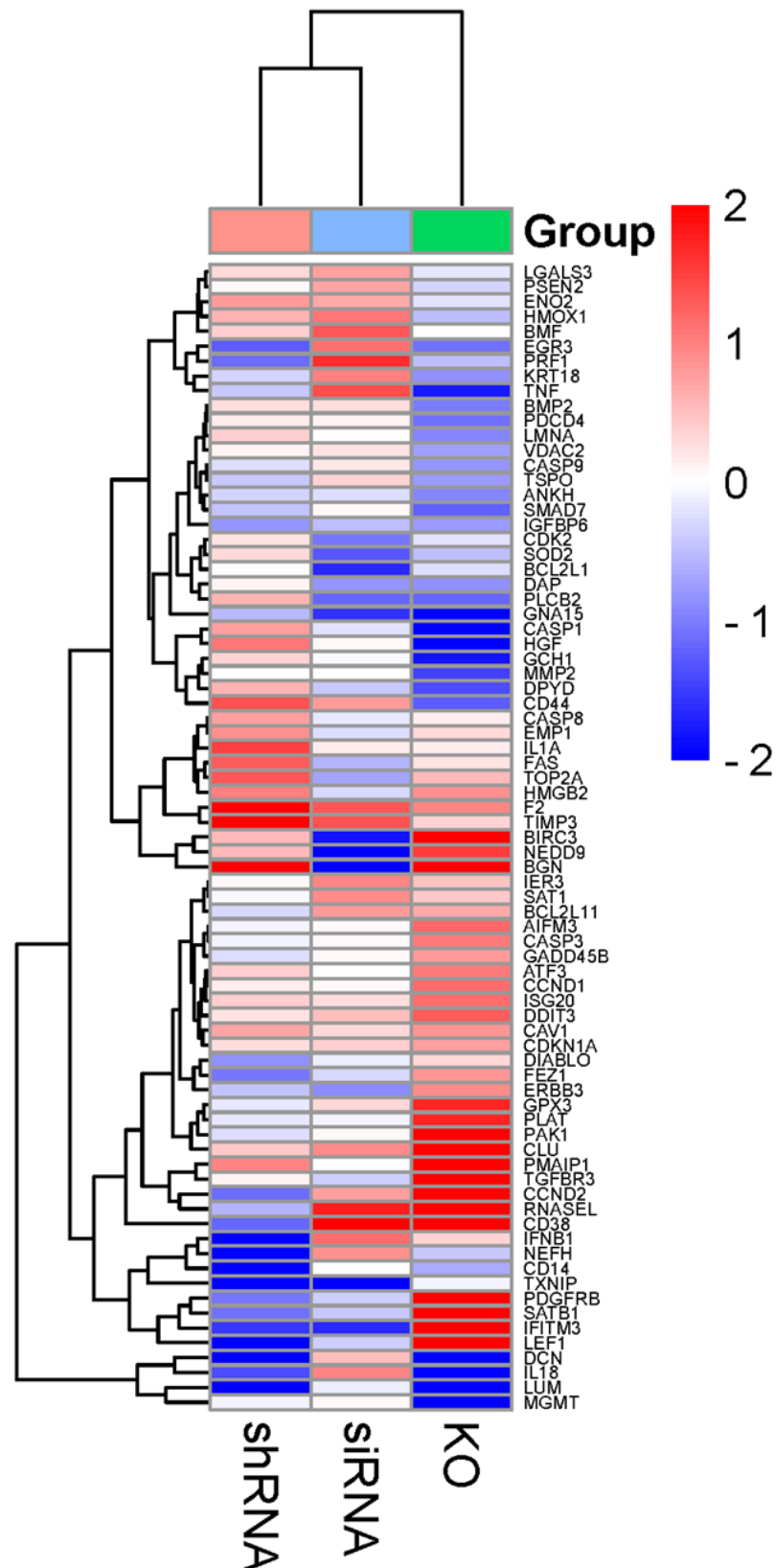


Figure 5.35: A Clustered Heatmap of Apoptosis Genes across the PRH KD and KO models

The log₂FC expression data for gene in the Hallmark Apoptosis gene set were filtered and presented in heatmap format. A colour scale indicates up-regulation (red) and down regulation (blue) of the genes. Genes and column have undergone hierarchical clustering through Euclidean distance method to group genes of similar patterning. The rows of genes have not undergone scaling and therefore expression can be compared between genes.

5.16 Discussion:

The aim of this chapter was to investigate the role of PRH in additional CCA cell line knockdown and knockout models. Three different techniques were used to manipulate the expression of PRH in CCA cells. The siRNA and shRNA methodologies both induce a knockdown through RNA interference, where the sequence of the siRNA/shRNA is complementary to the PRH mRNA target and, upon binding, results in its degradation. They differ in that siRNA is transfected transiently, whilst shRNA is transfected via a DNA plasmid, and can integrate permanently into the genome, forming a stable knockdown. To eliminate PRH expression at a DNA level, CRISPR-Cas knockout technology is required. This is where Cas9 nuclease, directed by guide RNA, introduces double strand breaks at a target gene. Upon DNA repair, mutation in the form of insertion-deletions can occur, disrupting the reading of the gene and leading to a gene knockout. Although this technology is time consuming and requires optimisation, CRISPR editing is permanent and can introduce highly specific effects. Generally, siRNA KD is useful for short-term transient knockdown experiments, shRNA KD is optimal for long-term gene suppression, and CRISPR-Cas generates a model for permanent loss of function. A range of techniques were used to observe PRH through different perspectives.

siPRH model

The use of siRNA generated knockdown models with extremely low expression of PRH at protein level, much lower than levels previously investigated (Kitchen et al., 2020) and were able to maintain PRH KD for at least five days in both the CCLP and RBE cells. The siCtrl and siPRH cells were assessed through phenotypic assays and showed that siPRH KD reduces proliferation, initiates cell cycle arrest, induces apoptosis and enhances cell sensitivity to apoptotic

inducers. These observations confirmed the results seen previously, where PRH drives CCA cell cycle progression (Kitchen et al., 2020) however it also highlights new data, with PRH expression restraining both the level of apoptosis and Staurosporine-induced apoptosis. Previously it was shown that PRH knockdown does not promote apoptosis (Lee, 2021) but is seen clearly in the current siRNA PRH KD.

CCLP cells are known to be non-functional for p53 and harbour *TP53* genetic rearrangement (see table 2.1). Thus, the investigation of PRH function in RBE cells, which are p53 wild type and have a different mutational background (*KRAS* gain of function; *IDH1* switch of function; see table 2.1), show that the role of PRH in promoting cell proliferation, and inhibiting apoptosis occurs independently of these genetic mutations. Loss of p53 function would be expected to influence apoptotic cell death and make the cells more resistant to apoptosis. In this context it is notable that loss of PRH results in increased apoptosis suggesting that PRH might influence pro-apoptotic pathways in the absence of p53.

The siPRH RNAseq GSEA analysis shows positive enrichment for TNF- α and NF κ B signalling pathway and the p53 pathway, which include differentially expressed genes linked to cellular stress and survival signalling. We infer that PRH inhibits cellular stress, and its loss induces cellular stress and apoptosis. The siPRH cells showed reduced cell number with time, decreased proliferation, and alterations of cell cycle. The down-regulated G2M Checkpoint differentially expressed genes, include genes that are involved in mitotic functions such as transcriptional control of cell cycle, regulation of DNA replication and microtubules dynamics. Cell cycle G1-arrest is associated with a decrease in expression of proteins with functions in S phase to G2M phase, and an increase in G1 proteins such as Cyclin D1. This occurs in siPRH cells,

and thus it could be hypothesised that PRH has roles in promoting cell cycle initiation in CCA cells. This would make PRH-driven CCA cells sensitive to CyclinD-CDK4/6/-p21 inhibitors, like Palbociclib in agreement with previous work (Kitchen et al., 2020). As *NOTCH3* gene expression was not changed by short term PRH knockdown with siRNA, in either CCLP or RBE cells, this shows that PRH can promote oncogenic effects without changes in NOTCH3 gene expression. Overall, the siRNA model reveals more clearly the role of PRH as a CCA oncoprotein that promotes cell cycle initiation and cell survival. It will be of interest to investigate the RBE siCtrl and siPRH RNA samples in sequencing to reveal universal PRH-driven signalling pathways.

PRH knock out model

CRISPR knockouts of the *HHEX* gene resulted in PRH KO 1 and KO 4 clones, although both null for PRH protein, the clones had suffered different types of editing, leaving KO 1 with a full gene excision and KO 4 with a truncated *HHEX* transcript. Both KO 1 and KO 4 clones performed similarly in phenotypic assays, showing a significant reduction to cumulative cell number and proliferation although the decrease in cumulative cell number seen for clone KO 1 is more substantial than KO 4 and the effect of KO on CCLP cumulative cell number is much greater than the effect of siRNA KD. The reason for this is likely to be because the effects of the siRNA are transient, whereas KO is permanent and the effect of PRH depletion on cell number is measured over an extended period. The reduction of cell proliferation, which is an assay which is performed over a short period, is very similar to the decreased in cell number observed in siRNA experiments.

In RNAseq experiments, the PRH KO cells were significantly enriched in immune-related, tissue dynamic-related, and signal transduction-related gene

sets. The involvement of tissue dynamic-related gene sets may hint to PRH's role in regulation of genes in tissue development (reviewed: Kershaw et al., 2013), and its ability to regulated important EMT genes (Noy et al., 210) (Cong et al., 2006). Oxidative phosphorylation was the only significantly negatively enriched pathway in the KO cells (positive in cells phenotype), and has revealed a novel potential role of PRH in regulating the expression of electron transport chain proteins. In CCA, mitochondrial oxidative metabolism contributes to cancer stem cell-like phenotype (Raggi et al., 2021), and CCA is commonly associated with mutations of Krebs cycle enzymes IDH (Banales et al, 2020). HHEX polymorphisms are commonly seen in the metabolic disorder diabetes (Alfaifi et al., 2021), and in GSEA, diabetes gene sets are negatively enriched in the siPRH KD and the KO model.

As mentioned above the changes in gene expression for G2M checkpoint pathway genes in the PRH KO cells were compared to G2M checkpoint genes in the siPRH KD cells despite G2M pathway genes not being statistically significant in the GSEA of PRH KO cells. Interestingly genes with decreased expression in the PRH KO had a similar decrease in expression in the siRNA KD experiments. These genes and their corresponding proteins have association with late-stage cell cycle and cytokinesis (*AURKB*, *BUB3*, *MKI67*). As these genes do not appear in ChIP sequencing data for PRH previously obtained for PRH in CCLP cells (Kitchen et al., 2020), it could be postulated that PRH does not directly control the regulation of these late-stage cell cycle genes at a transcriptional level. The expression of Cyclin D1 was significantly increased by PRH KO just as it is in siPRH KD cells, indicating that the KO cells may show G1 arrest. Future cell cycle experiments with the PRH KO cells may be able to confirm this.

It should be noted that due to the clonal difference between the two CON and two KO, a lot of biologically important data is lost through lack of statistical significance. This work could be improved by repeating the RNA seq and GSEA with KO1 data from multiple passages to increase the significance in the gene expression data or by generating a much larger number of KO clones and then using the larger set.

shRNA PRH KD

The shRNA PRH KD 2 cells had significantly lower cumulative cell number than the SVC controls in cumulative growth experiments, however there was no difference between SVC 2 and shPRH KD 2 in proliferation assays or cell cycle progression. This result was unexpected, as previously PRH shRNA KD models, and the other PRH depletion models in this chapter, showed a significant effect of PRH loss on proliferation. In apoptosis experiments, PRH KD 2 cells showed trends in increased sensitivity to Staurosporine, but this was not statistically significant compared to SVC 2.

In RNAseq, the PRH KD 2 cells were found to have significantly positively enriched expression of cell cycle-associated gene sets. Given the negative enrichment of these gene sets in the siPRH KD and the PRH KO cell, this was highly unexpected. The G2M Checkpoint genes were further investigated and showed many significantly up-regulated genes in the PRH KD. The reason for the G2M genes being upregulated in the shPRH KD cells but being down-regulated in the siRNA PRH KD and PRH KO models, could be that the polyclonal population of shPRH KD cells have activated cell division genes to allow cell proliferation despite the down-regulation of PRH. Consistent with this idea, the G1 associated protein Cyclin D2 is significantly downregulated in shPRH KD 2 whilst the G2M genes are enriched.

Interestingly, it is only the shRNA PRH KD model, out of the three models explored, that has a significant decrease in expression of NOTCH-3 mRNA. As no direct regulation of NOTCH-3 by PRH was reported in Chromatin Immunoprecipitation experiments previously (Kitchen et al., 2020), it is possible that the decrease to Notch-3 in the shRNA PRH KD is an indirect effect of the changes that the shPRH cells have undergone to maintain survival. Although the shRNA PRH KD cells behave very differently to the siRNA and PRH KO cells, the model still offers insight into the role of PRH in PRH-CCA biology.

Interestingly, the most up-regulated genes in the cell cycle gene set in the shRNA PRH KD model are mostly down-regulated in the siRNA KD and KO models. This cluster of genes could be downregulated in the siRNA KD and KO due to the siPRH KD/KO-induced cell cycle arrest. However, these same genes could be upregulated in the shRNA PRH KD to compensate for the loss of PRH. Thus, it is this group of proteins that define the role of PRH in early CCA cell cycle. For future work, the expression of these genes in all three models would be useful to examine using qRT-PCR, and expression of their corresponding proteins examined by Western blotting, to refine roles of PRH in cell cycle progression and identify potential PRH-regulated genes.

To carry on from this project, PRH expression at the different stages of cell cycle should be investigated as well as PRH localisation to help better understand its role in the regulation of cell cycle. Furthermore, PRH binding partners should be explored to find potential interactions with the cell cycle proteins. Future work could include determining whether siPRH KD or KO of PRH increases Niclosamide sensitivity and combination therapy and is therefore opposite to the decreased sensitivity to Niclosamide observed in shRNA PRH KD cells.

CHAPTER 6:

Discussion

6.1 Overview

In this project Niclosamide and Palbociclib were investigated as single agents and in combination to better understand the mechanisms contributing to any additive or synergistic effects on CCA cells. Additionally, the role of the PRH protein in regulating the oncogenic phenotype of CCA cells was further investigated with a view to understanding the effect of the drugs on PRH expression and PRH target genes.

Niclosamide inhibits CCA

Niclosamide, an anti-helminth drug, was identified from a drug screen. Through cell viability assays in 2D and 3D models, it was demonstrated that CCA cell lines are significantly more sensitive to Niclosamide than BECs at 72 hours incubation with drug when Niclosamide is able to exert effects on cell proliferation. Although Niclosamide does exert cytotoxic effects at 24 hours on all the tested cell types the lack of further cytostatic effects affords a therapeutic window whereby CCA cells can be treated without further affecting BECs.

Niclosamide is well documented as a mitochondrial uncoupler (Kumar et al., 2018) and is also known to promote global and specific ubiquitylation of proteins (Cheng et al., 2017). It was shown that acute treatment with Niclosamide promoted potent mitochondrial depolarisation in the CCA cell lines. The amount of mitochondrial uncoupling did not correspond with Niclosamide sensitivity, however the levels of Niclosamide-induced total protein ubiquitination did correlate with sensitivity. Niclosamide was able to exert a pro-apoptotic effect in the sensitive CCA cell lines, however the extent of apoptosis was cell type specific, and no change to cell cycle progression was measured in response to Niclosamide treatment. The levels of many different proteins were decreased

by Niclosamide, and they decreased in a dose-dependent manner including B-catenin, Cyclin D proteins, p21 and PRH. B-catenin has already been documented as a target for ubiquitin-direct proteasomal degradation stimulated by Niclosamide (Keng et al., 2023). When the protein stability of PRH was investigated with inhibitors of proteasome activity, it was observed that PRH expression was influenced by proteasome activity. Additional repeats are needed to confirm that PRH is ubiquitylated and degraded by the proteasome in CCA cells. However, as Niclosamide promotes an increase to global protein ubiquitylation, it can be hypothesised that PRH is also a target of this mechanism. Overall, by integrating the results from the experiments performed in Chapter 3, along with evidence from the literature, it is likely that Niclosamide uses mechanisms depicted below (**Figure 6.1A**) to reduce CCA cell viability.

Niclosamide has also been shown to induce mitophagy, a mitochondria-specific autophagy (Huang et al., 2023). To further this work, the effect of Niclosamide in the CCA cell lines, mitophagy could be explored by looking at the levels of PINK1 kinase and Parkin ubiquitin ligase activity across the CCA cells as these proteins are associated with mitophagy and Niclosamide dependent mitochondrial depolarisation (Jeng et al., 2022, Lambourne et al., 2023). It could be that the sensitivity of Niclosamide could depend on factors like PINK1 and Parkin that promote the downstream effects of mitochondrial uncoupling.

Is Niclosamide a suitable candidate for anti-CCA clinical trial?

Niclosamide has been suggested as a potential anti-cancer drug for many types of cancer (review: Jiang et al., 2022), however it is limited by a poor bioavailability profile. Niclosamide has poor water solubility, a relatively low absorption rate, low bioavailability and can induce cytotoxicity. In the CCA cells, only longer incubation with the drug is able to achieve selective inhibition of

cancers cells over BECs. This imposes limitations to using Niclosamide in the clinic. To improve the amount of drug that reaches the tumour, Niclosamide chemistry and administration have been investigated. Niclosamide derivatives have been optimised to improve aqueous solubility whilst maintain the anti-cancer activity of Niclosamide (Chen et al., 2013). Nano-suspension of Niclosamide was also shown to improve the oral bioavailability of the drug (Lin et al., 2016). Also, Niclosamide has been used in early clinical trials against castration-resistant prostate cancer in combination with abiraterone and prednisone, where the solubility and absorption of Niclosamide was increased by supplying the powdered drug in capsules (Parikh et al., 2021). The serum concentration was kept constant between administrations and was maintained in the 0.31 μ M-0.65 μ M range. Also, to reduce the amount of Niclosamide needed to produce of anti-cancer effect, Niclosamide has been studied in combination with multiple chemotherapy regimens, targeted therapy as well as radiotherapy to induce additive or synergistic effects (Ren et al., 2022). Combination therapy is thought to be an easier way of transferring Niclosamide treatment to the clinic for multiple cancer types.

Niclosamide and Palbociclib show synergy through independent effects on critical cell cycle proteins

Palbociclib is a first-line treatment for subtypes of breast cancer (Wang et al., 2024), but it is in clinical trial for a range of cancers (Gopalan et al., 2018). Any drug which can potentiate the actions of Palbociclib is of utmost clinical interest. The rationale for investigating this combination is that both drugs are able to exert selective targeting of CCA cell lines over BECs, thus the combination of both drugs may lead to a potent anti-CCA formulation. Also, as both Niclosamide and Palbociclib have well-documented drug profiles in humans, the aims of pursuing this combination in patients is realistic and translatable.

Similarly, Palbociclib has been previously investigated in CCA patients, but had no obvious effect and thus its activity could be improved by dual therapy (Baghdadi et al., 2019).

The Chou-Talalay method was utilised to quantify synergism through CI values and concluded that the two drugs were synergistic across the CCA cell lines and in a breast cancer cell line. In the clinic, this means that the use of the two drugs together can have a greater effect on the cancer cells. It also means that less drug is required, when the drugs are used as a dual therapy, to have the same effect as drug when used as a single agent; this could reduce side effects or make the treatment more tolerable for patients. When replacing Palbociclib with other CDK4/6 inhibitors and Niclosamide with other mitochondrial uncouplers, synergism was maintained, thus it is apparent that alterations in cell cycle arrest and interference to cellular metabolism are involved in the synergism mechanism.

In flow cytometry experiments, Niclosamide and Palbociclib are seen to further promote cell cycle arrest in combination, however, was little difference to Palbociclib treated cells alone. In addition, the combination of Niclosamide and Palbociclib had little effect on apoptosis at the concentrations studied. At a protein level, both the drugs as single agents were able to cause a reduction in p-RB expression and the drugs in combination had a potent effect on reducing p-RB levels. Palbociclib is well known to suppress RB phosphorylation, however, for Niclosamide, this is a novel finding. As discussed previously, this could be related to a Niclosamide-induced ubiquitin-mediated proteasomal degradation mechanism. Other changes in Cyclin D and p21 protein levels are consistent with Palbociclib causing cell cycle arrest and Niclosamide causing protein degradation. The level of Cyclin D and p21 in combination treated CCA cell lines varies, and it likely depends on the balance of downregulation caused

Niclosamide-induced proteasomal degradation and upregulation caused by Palbociclib-induced cell cycle arrest. Wong et al. report a synergism between Palbociclib and PI3K inhibitors (Wong et al., 2019). They hypothesise that as Palbociclib resistance is associated with Cyclin D up-regulation, they could counteract this increase in expression with PI3K/mTOR inhibition. They showed that a Palbociclib-induced increase of Cyclin D3 was abolished by Omipalisib (PI3K/mTOR inhibitor) (Wong et al., 2019). In this work, we see similar findings with Cyclin D proteins. In combination treated CCLP and RBE cells, the level of Cyclin D1 is low and relatively close to the expression found in Niclosamide treated cells. Whereas Cyclin D1 expression in combination treated KKK-M055 is relatively high and close to the levels found in Palbociclib treated cells. The synergism of Palbociclib and Niclosamide is much higher in CCLP and RBE cells compared to KKK-M055; this may be linked to increased Cyclin D1 levels in combination treated KKK-M055 cells. To observe whether Cyclin D expression is involved in inhibiting Niclosamide and Palbociclib synergism, Cyclin D knockdown models should be generated to determine whether they are less sensitive to the drugs in combination. A potential mechanism to explain Niclosamide and Palbociclib synergism is shown in **Figure 6.1B**.

Niclosamide and Palbociclib: Inhibition of mitochondrial membrane polarity

As well as cell cycle arrest, other routes for synergism should be considered. The cell cycle is closely linked to cell metabolism (Diehl et al., 2024) and Palbociclib has been shown to drive mitochondrial metabolism reprogramming and promotes mitochondrial respiration in a p53-dependent manner in BRAF^{V600E} melanoma cells (Santiappillai et al., 2021). It was also shown that the inhibition of cell cycle by Palbociclib causes the cell to increase its metabolic pathways by increasing cell growth and mitochondrial biogenesis, indicated by

an increase in MitoTracker staining (Uzhachenko et al., 2021). We have observed that Niclosamide acts as a mitochondrial uncoupler in CCA cells. If mitochondrial biogenesis is being up-regulated in the Palbociclib induced cell cycle arrested cells, these cells may be sensitive to Niclosamide treatment. Palbociclib can synergise with another mitochondrial uncoupler, FCCP, therefore this supports the involvement of cell cycle arrest and mitochondrial uncoupling as part of the synergism mechanisms. As mentioned previously, Palbociclib can synergise with other anti-cell metabolism drugs such as mTOR inhibitors. The mTOR pathway is involved in protein synthesis and cell growth, and it is up regulated by Palbociclib treatment (Uzhachenko et al., 2021). In western blot, the mTOR inhibitor and Palbociclib were able to promote decrease to p-RB levels as single agents, and even more so in combination (Yamamoto et al., 2019). The increased effect on p-RB by the combination above is also observed for Niclosamide and Palbociclib in combination. Overall, Niclosamide and Palbociclib promote cell cycle arrest through the additive targeting of Cyclin D-CDK4/6-p21 complex activity, however there may be effects on cell metabolism not investigated in this work.

Niclosamide and Palbociclib: Apoptosis

There is little induction of apoptotic cell death by Niclosamide and Palbociclib in combination. In Chapter 3, we see that CCLP SVC and PRH KD spheroids treated with 1 μ M Niclosamide do not increase in volume, but also do not decrease in volume; only concentrations 2 μ M plus can cause a reduction in viability from day 0. This shows that Niclosamide at lower concentrations has only cytostatic properties. As the maximum serum concentration of Niclosamide is around 0.56 μ M (Schweizer et al., 2018), it may be that the apoptotic capabilities of Niclosamide may not be observed if the drug were to be used in man. Palbociclib is not strongly associated with inducing apoptosis, although it

has been seen in the literature (Bi et al., 2021). Palbociclib is associated with cell senescence (Cayo et al., 2023). Senescent cells are characterised by permanent cell cycle arrest and have increased B-galactosidase (SA-B-gal) activity. Cellular senescence has tumour suppressive and tumour promoting roles. Whilst senescent cells have arrested proliferation, and can activate immune surveillance and inhibit cell transformation, they can also promote EMT, invasion and migration, angiogenesis and immunosuppression (Xiao et al., 2023). In addition, cancerous senescent cells can become resistance to chemotherapy (Demaria et al., 2017). Niclosamide can kill cells at higher concentrations, however as these concentrations may not be achieved at physiologically relevant concentrations, experiments investigating whether the combination can promote cell senescence should be performed, especially as RBE cell do not submit to Niclosamide-induced apoptosis.

As a next step to the *in vitro* work presented in this thesis, the Niclosamide and Palbociclib combination are currently being examined in a mouse model. Mice will be injected with CCLP cells, and when tumours have initiated, the drugs will be administered to mice by intraperitoneal injection as single agent and as a combination. Tumour volume, amongst other outcomes, will be recorded from the experiment. This *in vivo* work will examine whether the drugs, as single agents, are able to display the anti-CCA activity shown in cell culture work and will also interrogate the ability of the drugs so act synergistically in an animal model.

Comparison of the long-term and short-term effect of PRH knockdown and PRH depletion

All three model, siPRH transient knockdown, shRNA integrated knockdown and CRISPR PRH KO, all showed that the loss of PRH was able to reduce cell

number over time. In two of the models, namely the transient siRNA PRH KD and permanent PRH KO models, a decrease in PRH expression caused a reduction in cell proliferation. Upon investigating changes in gene expression, the reduction in proliferation could be linked to a decrease in the expression of genes with proteins in roles in mid-to-late cell cycle. More specifically, the genes were involved in transcriptional control of mitosis, regulation of DNA replication and microtubules dynamics. Simultaneously, there was upregulation of gene found in G1, such as Cyclin D2. Thus, in these two models, siPRH KD and PRH KO result in changes that would be expected to alter CDK4/6 activity.

In the shRNA PRH KD model, proliferation and cell cycle progression was not changed by PRH knockdown. However, upon inspection of the gene expression analysis, G2M checkpoint genes were shown to be up-regulated. We speculate that these cell lines have adapted through compensatory mechanisms on cell division for the loss of PRH. The same genes that are activated by PRH (downregulated in the siRNA and KO models) are strongly upregulated in the shRNA model; presumably in the absence of PRH other regulators increase expression of G2M proteins to allow cell survival and cell proliferation.

Overall, there is a major role of PRH in CCA cell cycle progression. It could be predicted that PRH is involved in the mid-late stages of cell cycle due to the changes in gene expression of genes with proteins that function in S-G2 phase. It would be of interest to investigate whether Palbociclib can cause down-regulation of *HHEX* gene expression, or whether the decrease to PRH in Palbociclib treated cells is at a protein level. To retrieve more information about PRH-driven cell cycle progression, it would be of interest to look at the models as they go through cell cycle and mitosis via confocal imaging and staining of mitotic structures. This would enable the observation of any structural problems with DNA replication or microtubules formation.

This work has also shown a potential role of PRH in regulation of oxidative phosphorylation. As no phenotypic investigation of siPRH KD or PRH KO in cell metabolism was performed, it is not possible to say whether changes to the genes discussed previously have biological relevance. However, this question warrants investigation as this regulation of oxidative phosphorylation will be important for PRH oncogenic activity in cancer and it may also have relevance in other disease such as diabetes, where PRH polymorphisms are common (Alfaifi et al., 2021).

PRH therefore presents itself as a potential target in CCA (Kitchen et al., 2020). Targeting of oncoproteins has been achieved previously. For example, MYC, which, similar to PRH, is an 'undruggable' (lacks known binding pockets for small molecular inhibitors) transcription factor, responsible for promoting cell cycle (Soucek & Evan, 2009), can be regulated by stabilising the upstream G-quadruplex DNA upstream of the MYC gene which prevents transcription (Calabrese et al., 2018). A small molecule, APTO-253, was able to show MYC downregulation, cell cycle arrest and induction of DNA damage in acute myeloid leukaemia cells (Local et al., 2018) and has been assessed in preliminary clinical trials, showing benefit in patients with advanced solid tumours (Cercek et al., 2015). Alternatively, AZD5153 a highly potent BET/BRD4 inhibitor, has been shown to reduce MYC transcription (Rhyasen et al., 2016), however this drug performed modestly at clinical trial, and it was suggested the inhibitor be accompanied in combination with other agents (Hamilton et al., 2023). Inhibitors of mTOR to modulate MYC protein synthesis have been showed to be effective in MYC-driven cancer cells (Pourdehnad et al., 2013), but only at preclinical stages. Thus, Aurora kinase inhibitors (Yang et al., 2010), CDK inhibitors (Horiuchi et al., 2012) PARP inhibitors (Carey et al., 2018) have all been used to successfully to treat MYC-driven tumours. MYC has been studied for

decades as target in cancer (Soucek & Evan, 2009), whereas PRH was found to be oncogenic in CCA in 2020 (Kitchen et al., 2020). As more knowledge is obtained about PRH biology such as *HHEX* transcriptional regulation, regulation of PRH mRNA and protein synthesis, and the discovery of PRH-binding partners, this may expose more mechanism to effective targeting of PRH oncogenic signalling in CCA.

The down-regulation of PRH protein by both Niclosamide and Palbociclib may make this combination particularly beneficial for treating cholangiocarcinoma, where PRH is overexpressed and can be used to selectively target the cancerous cells over the normal tissue. However, this does bring to light that Niclosamide simultaneously targets PRH protein for degradation and shRNA PRH KD cells appear to be more insensitive than control cells. As this seems paradoxical, it is probable that the drug treatment results on shRNA KD are not easily explained as this model is not best representative of PRH loss. Future experiments with drug treatments on the new PRH KO cells are required to address this apparent paradox.

Limitations and Future Directions:

There are still questions and a lack of direct evidence as to how Niclosamide decreases CCA cell viability, as not all the targets of Niclosamide-induced protein ubiquitylation are known and although ubiquitylation-mediated protein degradation correlates with a reduction in cell viability, it was not proven to be causal. A future direction to address this could be conducting proteomics experiments in Niclosamide treated CCA cells and BECs, to identify changes in protein levels. This information would reveal pathways used by Niclosamide to bring about the phenotypic changes and reduce CCA cell viability seen in this work. Additionally, it could confirm whether specific proteins, such as PRH, are

ubiquitylated under Niclosamide treatment, and that this leads to protein degradation, by using specialised techniques to look at protein post-translational modifications, namely 'ubiquitomics' (Sun & Zhang, 2022). This is important as understanding the detailed mechanisms of a drug enables more effective, safer, and personalized treatments.

Niclosamide and Palbociclib dual therapy shows promising results so far, with the ability to synergise and selectively target CCA cells over BECs. The next step would be to assess the combination in robust preclinical evaluation and optimisation to accelerate its progress as an anti-CCA therapeutic. Niclosamide and Palbociclib are currently being explored in mouse models. Alongside observing the tumour growth over time in the presence of the drugs, the tumours should be used in immunohistochemistry staining, to observe the levels of different proteins of interest, like the proteins investigated in this project, p-RB and PRH. These mechanistic studies are essential to assess the ability of the drugs to act on their intended biological pathways *in vivo* and should be considered as future work. As part of the preclinical validation, the Niclosamide-Palbociclib treatment should also undergo pharmacokinetics and toxicology studies, and dose optimisation (Papachristos et al., 2023). A limitation to this study is that synergism was analysed at specific drug ratios and did not consider different dosage schedule (simultaneous vs. sequential). Dose optimisation could identify the most suitable dose, dosage schedule and administration route for the drug combination to show the greatest synergism and efficacy.

In addition, further research into the molecular mechanisms underlying the synergism between Niclosamide and Palbociclib should be conducted, as finding proteins that influence the degree of synergism (e.g. high levels of PRH), would highlight predictive biomarkers where the combination therapy is most likely to be effective and would be useful in treatment stratification.

A limitation to the research interrogating the role of PRH in CCA is that the different knockdown and knockout models displayed different phenotypes and genotypes. To uncover PRH-dependent pathways that are conserved in CCA, it could have been informative to obtain RNA sequencing data from the siRNA PRH knockdown RBE cells. Here, I could have looked at changes to gene expression in response to siPRH that were similar in the CCLP and RBE cells. As the same knockdown techniques were used, the results would be more comparable and may have led to a more detailed and conclusive list of conserved PRH regulated pathways and proteins. This data could be acquired in the future to help reveal a clearer role of PRH in CCA.

With the models that have been investigated, cell cycle was highlighted as a potential PRH-regulated pathway. To follow from this, investigation of PRH expression, localisation, binding partners during cell cycle should be reviewed. The experimental design could employ the use of Palbociclib, a canonical cell cycle inhibitor, which has been shown to influence PRH protein expression within this study. Similarly, new research into the role of PRH in metabolism could be initiated by using the different PRH KD and KO models in applications like the Seahorse Assay that has multiple ways to interrogate cellular metabolism. Finally, the uses of Niclosamide shows that PRH may be a target for ubiquitylation. This warrants investigation as it may identify a novel mechanism of PRH protein turnover that could be exploited as a new strategy for targeting PRH in CCA.

Conclusion

Overall, the results obtained in this project cover the preclinical investigation of two potential anti-CCA drugs that are synergistic when used together. This combination of drugs originated from attempts to target PRH-high CCA cells, however, the combination has also shown potential as a pan-cancer combination due to its effects on cell cycle progression. Thus, the implications of this study not only offer a new combination therapy for investigation in CCA, but a combination therapy that could be extrapolated to other diseases. The project re-confirms the role of PRH in driving the CCA cell cycle and sheds light on new PRH biology to provide grounding for a stronger rationale for targeting PRH-driven cholangiocarcinoma (**Figure 6.1C**).

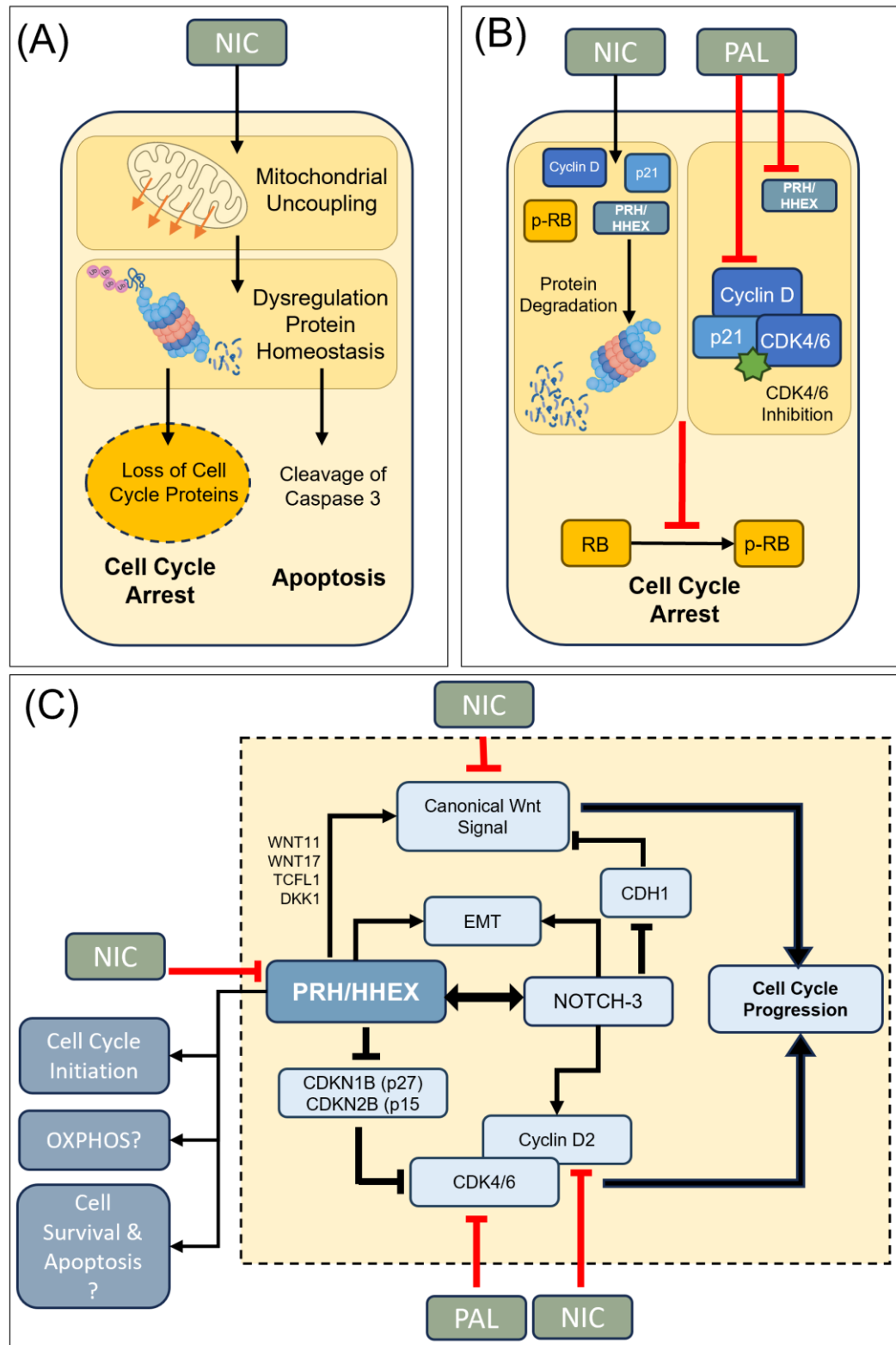


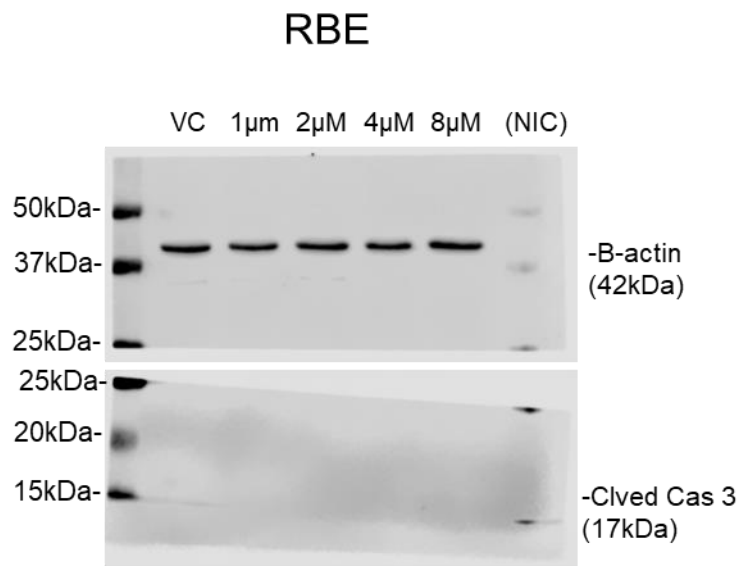
Figure 6.1: Graphical Conclusion.

(A) A summary of the effects Niclosamide induced mechanisms in CCA

(B) Synergism of Niclosamide and Palbociclib

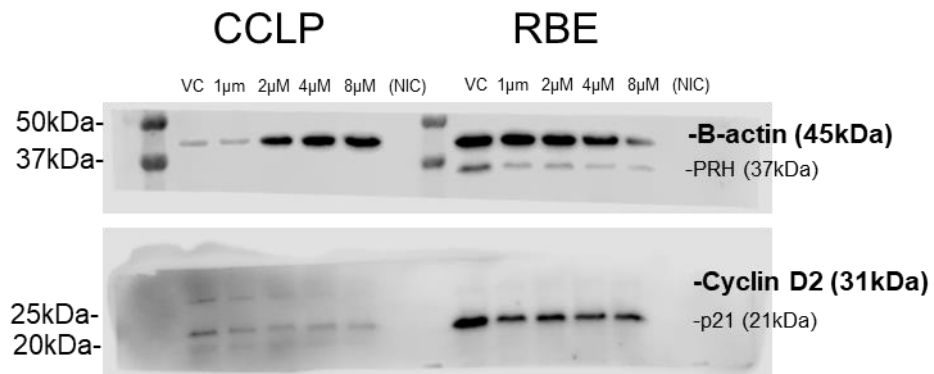
(C) PRH driven CCA and anti-CCA mechanisms of Niclosamide and Palbociclib dual therapy (adapted from Kitchen et al., 2020).

Supplementary Figures:

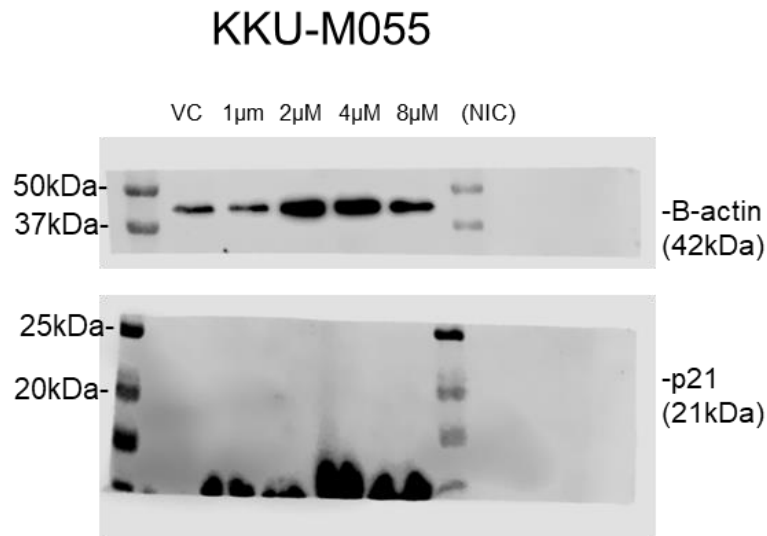


Supplementary Figure 3.1: Cleaved Caspase 3 is undetectable in RBE treated with Niclosamide.

Niclosamide treated RBE cells were used to make protein lysates (section from 3.6) and were examined for multiple protein expression. When probed with anti-cleaved caspase 3 antibody, no protein is detectable.

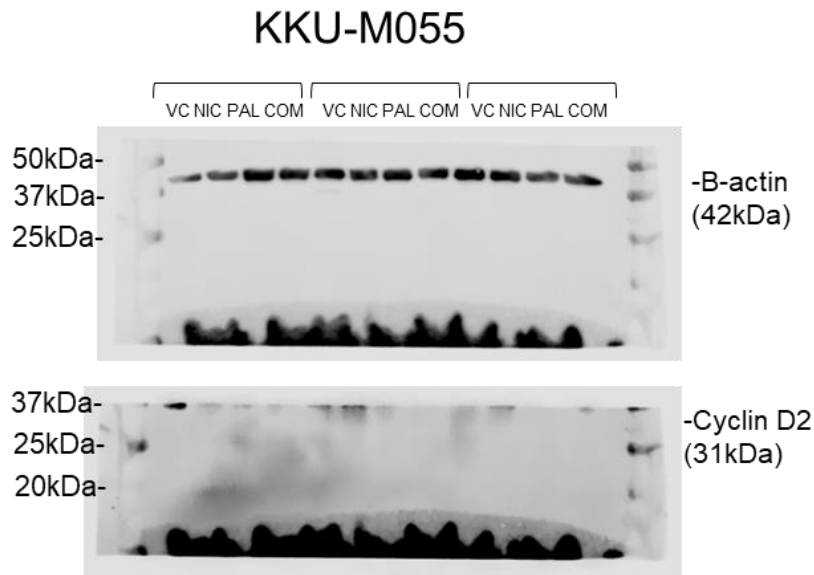


Supplementary Figure 3.2: Cyclin D2 protein is undetectable in RBE treated with Niclosamide; P21 expression is higher in RBE cells compared to CCLP. Niclosamide treated RBE cells were used to make protein lysates (section from 3.6) and were examined for multiple protein expression. When probed with Anti-Cyclin D2, no protein is detectable, whilst bands are present for CCLP cells. This blot also shows that the protein expression of p21 is much higher in RBE cells compared to CCLP cells.



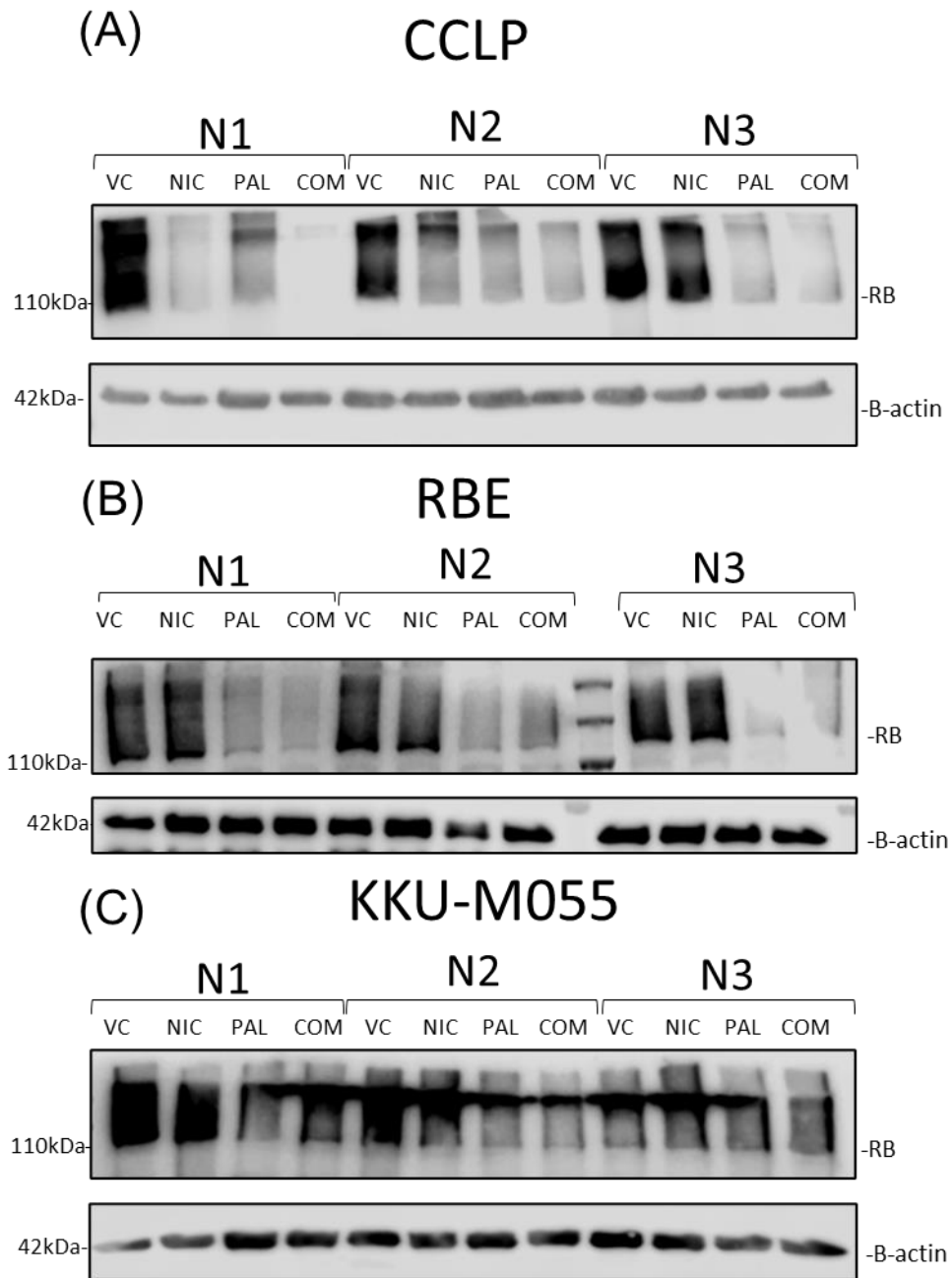
Supplementary Figure 3.3: P21 is undetectable in KKU-M055 cells treated with Niclosamide.

Niclosamide treated KKU-M055 cells were used to make protein lysates (section from 3.6) and were examined for multiple protein expression. When probed with anti-p21 antibody, no protein is detectable.



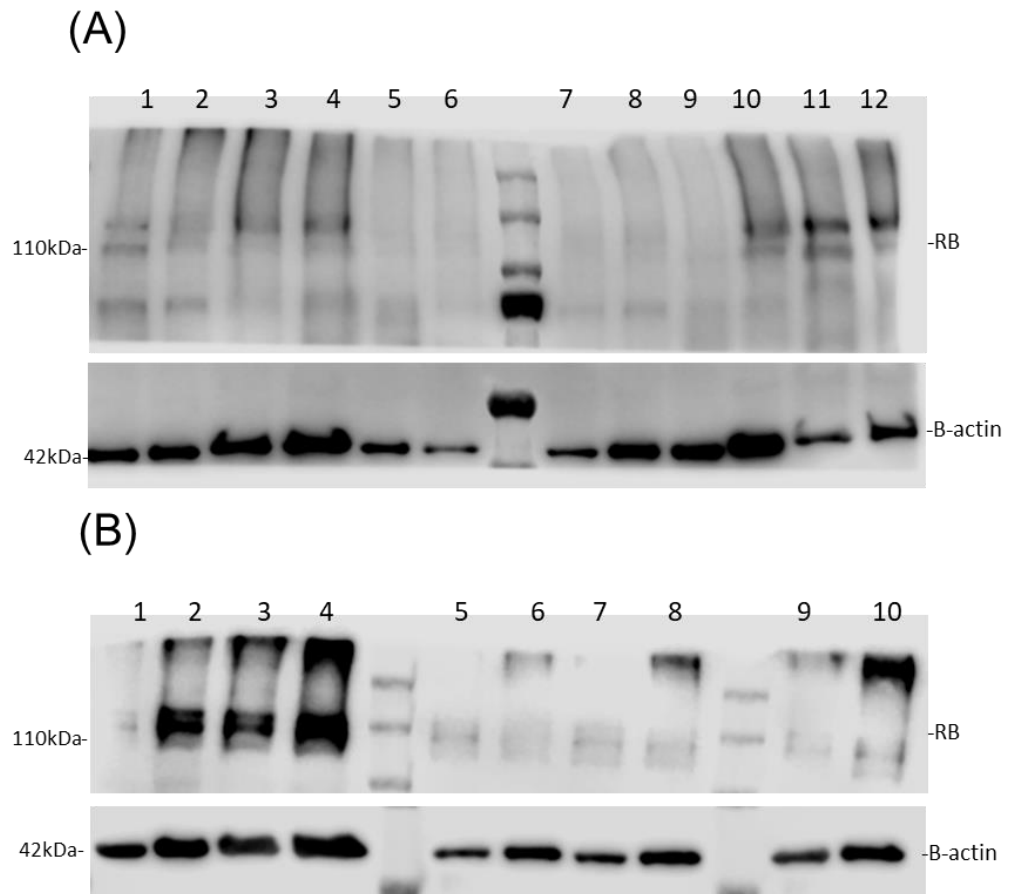
Supplementary Figure 3.4: Cyclin D2 is undetectable in KKU-M055 cells treated with Niclosamide, Palbociclib and Combination.

Niclosamide treated KKU-M055 cells were used to make protein lysates (section from 4.6) and were examined for multiple protein expression. When probed with anti-Cyclin D2 antibody, no protein is detectable.



Supplementary Figure 4.1 : Western Blot of Rb Protein in Combination Treated CCA Cells.

Cells were treated with vehicle control (VC), Niclosamide (NIC), Palbociclib (PAL) or both (COM) for 72hrs before harvesting for western blot. Approximately 2×10^6 cells were washed and lysed, before running on an SDS PAGE gel and transferring to membrane for immunoblotting. The experiment was repeated to three biological replicates. The antibody detected a smear of proteins which comprised Image J densitometry analysis.



Supplementary Figure 4.2: Western Blot of Rb Protein in CCLP SVC and PRH KD cell line lysates in Antibody Optimisation.

CCLP SVC (1, 3, 5, 7, 9, 11) and PRH KD (2, 4, 6, 8, 10, 12) whole cell lysates were electrophoresed on an SDS PAGE gel and transferring to membrane for immunoblotting, to assess different RB antibodies. The detection of RB protein in the lysates is smeared and unquantifiable. The antibodies used were (A) Abcam (AB244373) 1:1000 (B) Cell Signalling (9309S) 1:1000

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