Characterisation of PP2Cβ in regulating tumour suppressor pathways in cancer cell lines

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A feeling of dread hangs over you.
But you stay determined
-Undertale, 2015

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Abstract

Tumour suppressor p53 is a key regulator in preventing neoplastic transformation by inducing cell cycle arrest or death in response to stress-signalling pathways. Consequently, p53 is often non-functional during the early stages of cancer development through either direct mutation or aberrant expression of negative regulators.

PP2Cβ is a protein phosphatase which was recently identified as a negative regulator of p53 and cellular senescence. However, the function of PP2Cβ in cancer development is not fully understood.
The aim of this study was to characterise PP2Cβ and its regulation of p53 pathways in human cancer cell lines. This aim was split into two objectives.

The first objective was to examine the effects of PP2Cβ on p53 pathways and cell proliferation in four cancer cell lines with various genetic backgrounds. A protein analysis using western immunoblotting procedures indicates that p53 pathways are activated in cell lines expressing wildtype p53 and Ras. Consistent with activated p53 pathways, PP2Cβ knockdown significantly reduced proliferation rates, which could be attributed to an increased expression of a p53 target gene, *p21* cell cycle inhibitor.

The second objective was to investigate the mechanisms regulating of PP2Cβ gene expression. Previously, p63 was identified as a potential negative regulator of PP2Cβ gene expression based on a modular relational database that integrated microarray results with a genome-wide search of p53 family member response elements. It was therefore hypothesized that p63 could negatively regulate PP2Cβ gene expression. Mammalian expression vectors carrying either the p63 or p73 expression cassette were constructed and PP2Cβ expression was analysed upon overexpression of p53 family members (p53, p63 and p73) in two human cancer cell lines. A reverse-transcriptase coupled quantitative PCR showed that overexpression of p63 resulted in decreased PP2Cβ expression in p53 wildtype cell line.

Taken together the results presented here suggest that restoration of tumour suppressors such as p53 and Rb activity by PP2Cβ inhibition could be used as a potential therapeutic strategy in cancer treatment

Abbreviations

BSA	Bovine Serum Albumin
CAK	CDK-Activating Kinase
CDK	Cyclin-Dependent protein Kinase
cDNA	Complimentary DNA
CIP	Calf Intestinal alkaline Phosphatase
Con	Control
Cp	Crossing Point
DBD	DNA-Binding Domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxyribosenucleotide Triphosphate
EDTA	Ethylene Diamine Tetra-acetic Acid
FBS	Foetal Bovine Serum
GUSB	β-Glucuronidase
H1299	Human Non-small cell lung carcinoma cell line
HCT116	Human Colorectal Carcinoma cell line
HEK293T	Human Embryonic Kidney 293T cell line
h	Hours
Kb	Kilo bases
kDa	Kilodaltons
KD	Knockdown
M	mol/L
mA	Milliampere
MAPK	Mitogen Activating Protein Kinase
MCF7	Human Breast adenocarcinoma cell line
Min	Minutes
mL	Millilitre
p53	Phosphoprotein-53
p63	Phosphoprotein-63

p73	Phosphoprotein-73
PCR	Polymerase chain reaction
Pen/Strep	Penicillin-Streptomycin
PP2Cβ	Protein Phosphatase 2Cβ
RCF	Relative Centrifugal Force
Rb	Retinoblastoma protein
RNase	Ribonuclease
S	Serine
s	Seconds
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
shRNA	Short-Hairpin RNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBS/T	Tris-Buffered Saline with Tween-20
U2OS	Human Osteosarcoma cell line

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Chapter 1: Introduction

1.1 The project

Protein phosphatase 2Cβ (PP2Cβ) has been linked with cancer development and progression through inhibition of p53, a critical tumour suppressor protein. Knockdown studies of PP2Cβ in human embryonic fibroblasts show that depletion of PP2Cβ increases the activity of p53, resulting in the upregulation of downstream p53 target genes and subsequently induced a permanent arrest in cell cycle progression, termed cellular senescence [1]. This project aims to identify if knockdown of PP2Cβ in human cancer cell lines arrests cell proliferation through activation of p53 and its target genes. Furthermore this project aims to investigate whether PP2Cβ expression is regulated by members of the p53 family through p53-dependent and independent mechanisms.

1.2 Stress-signalling pathways

Stress-signalling pathways prevent the initiation of cancer development and progression in cells exposed to a wide array of physical, chemical and biological stresses from external and internal sources. These include oxidative, genomic, replicative and oncogenic stresses. Core stress-signalling proteins are responsible for the detection of stress and initiating the response (which is highly dependent on the detected insult) through a stress-signalling cascade. These responses range from repair of DNA damage to eliminating damaged cells though apoptosis. These pathways are essential for preventing further damage to the cell and its genomic material. Failure of these mechanisms can result in the rise of cancerous cells through neoplastic transformation. Key proteins in mediating the stress-signalling cascade are therefore essential in preventing neoplastic transformation are commonly dysregulated during early oncogenesis. This usually results from mutation, deletion or epigenetic silencing of the genes encoding these core proteins or through overexpression of their regulators. Epigenetic silencing is a major way through which the expression of genes, including stress-signalling proteins is regulated, which is achieved though chromatin remodelling.

In every eukaryotic cell, genomic DNA is tightly packaged into a higher order structure called chromatin. There are several orders of chromatin, the most basic being a nucleosome where 147 nucleotides of DNA is wrapped around an octamer of histone proteins. The octamer includes two H3 and H4 histones which form a tetramer, and a dimer of histones H2A and H2B [2]. Chromatin remodelling is essential to ensure that DNA is accessible by transcriptional factors and DNA replication machinery. Therefore the tightness in which the DNA is compacted is altered through modifying the chromatin structure through reversible posttranslational modifications of the histones. A variety of enzymes such as kinases, histone acetyl transferases and methyltransferases achieve chromatin remodelling [3]. These enzymes are often recruited to chromatin with transcription factors to facilitate gene expression through modification of the histone tails [4, 5]. Furthermore the DNA itself can be modified through methylation of gene promoters, particularly at C-G rich regions, which is associated with gene silencing [6, 7].

Tumour suppressors p16 ^{INK4A} and p19 ^{ARF} (or p14 ^{ARF} in humans) (hereafter referred to as p16 and p14 respectively), are often silenced through chromatin remodelling in cancer cells through methylation of the gene promoter [8, 9]. As the genes encoding p16 and p14 are located in close proximity to each other in the genome in a region called the INK4A/ARF locus, they are particularly susceptible to deletion and epigenetic silencing through compact chromatin formation [10, 11]. The importance of the epigenetic inhibition of p16 and p14 in cancers is profound as both are essential for modulating the cellular response to stress-signalling and the activation of a critical tumour suppressor, phosphoprotein p53 [12-14]. Tumour suppressor p53 is a prime example of a core mediator of stress-signalling pathways and will be discussed in depth below.

1.3 Phosphoprotein p53

Phosphoprotein 53 (p53) is a potent tumour suppressor protein, acting as a key mediator of the stress-signalling response pathway, by controlling cell proliferation and death in response to stress-signalling. The importance of p53 in coordinating the stress-response to avoid neoplastic transformation cannot be understated, and is clearly demonstrated by how it is directly mutated in half of human cancers [15]. Mutation of p53 resulting in p53 heterozygosity and p53 deficiency is considered a hallmark of early oncogenesis [16]. Individuals with Li-Fraumeni syndrome, a genetic disease resulting from an inherited mutation in the *TP53* allele demonstrate this very clearly through their extreme susceptibility to cancer. Supporting this observation, *TP53*-null mice are severely predisposed to early spontaneous cancer formation [17, 18]. Furthermore, loss of p53 function in cancer cells often results in high genetic instability, increased metastatic potential, increased invasiveness and a lack of cellular differentiation within cancers to such an extent that the status of p53 is commonly used to assess the aggressiveness of the cancer and used to estimate patient prognosis [19-22].

p53 is a transcriptional activator that is activated in response to a vast array of stresses including DNA damage, hyperproliferative signals, oxidative stress and hypoxia (Fig. 1.1) [23-26]. Normally in unstressed cells, the expression of p53 is maintained at very low levels [27-29]. However when stress is detected, the p53 protein is stabilised, accumulates and oligomerizes to form a homotetramer [30]. Once in this conformation, p53 regulates the expression of target genes by binding to conserved sequences, known as p53 response elements, through the DNA-binding domain and transcribes them through interactions with transcription cofactors that coordinate with p53 to direct cell fate [23, 31, 32]. Cell fate decisions are made based on the severity of the stress signals by transcriptionally activating certain target genes which are essential in resolving the stress by coordinating DNA damage repair, cell cycle arrest, cellular senescence or apoptosis [33].

Cell growth inhibition through cell cycle arrest is a defence against cancer development as it prevents the propagation of potentially oncogenic mutations until the stress is resolved. p53 achieves this through the transactivation of cyclin

dependent kinase inhibitor $p21^{Cip1/WAF1}$ (hereafter referred to as p21) [34]. However, if the cell fails to resolve the stress, accumulation of p53 will drive the cell towards a permanent state of cell cycle arrest, termed cellular senescence [35]. How p53 achieves cell cycle arrest and senescence will be discussed in more detail in sections 1.3.1 and 1.3.2.

As well as establishing temporary and permanent cell cycle arrest, p53 can also induce cell death through apoptosis [36]. Apoptosis is programmed cell death that can be induced in response to severe stresses by p53. It achieves this by transcriptionally activating members of the Bcl-2 binding component 3 family, such as *Puma*, *Noxa* and *Bax,* which are major regulators of apoptosis [37]. The transactivation of *Puma* in particular is essential for the pro-apoptotic activity of p53, as deletion of *Puma* abrogates p53-dependent apoptosis in human cell lines [38-40]. Expression of puma results in upregulation of caspase 3, 6 and 7, which is followed shortly after by cell death [41].

Given the extensive control p53 displays in regulating a vast array of tumour suppressor pathways, it is advantageous for tumours to lose the wildtype p53 activity. As a result of this, p53 is commonly mutated or deleted in human cancers or dysregulated through the over expression of p53 regulators [42]. Restoring p53 expression in $p53^{(-/-)}$ mice resulted in a greater average life span and fewer spontaneous tumours than p53 negative mice [43]. Therefore restoring p53 activity in cancers has become a major aim of research over the past decade as it provides a potential mechanism through which to halt cancer progression.

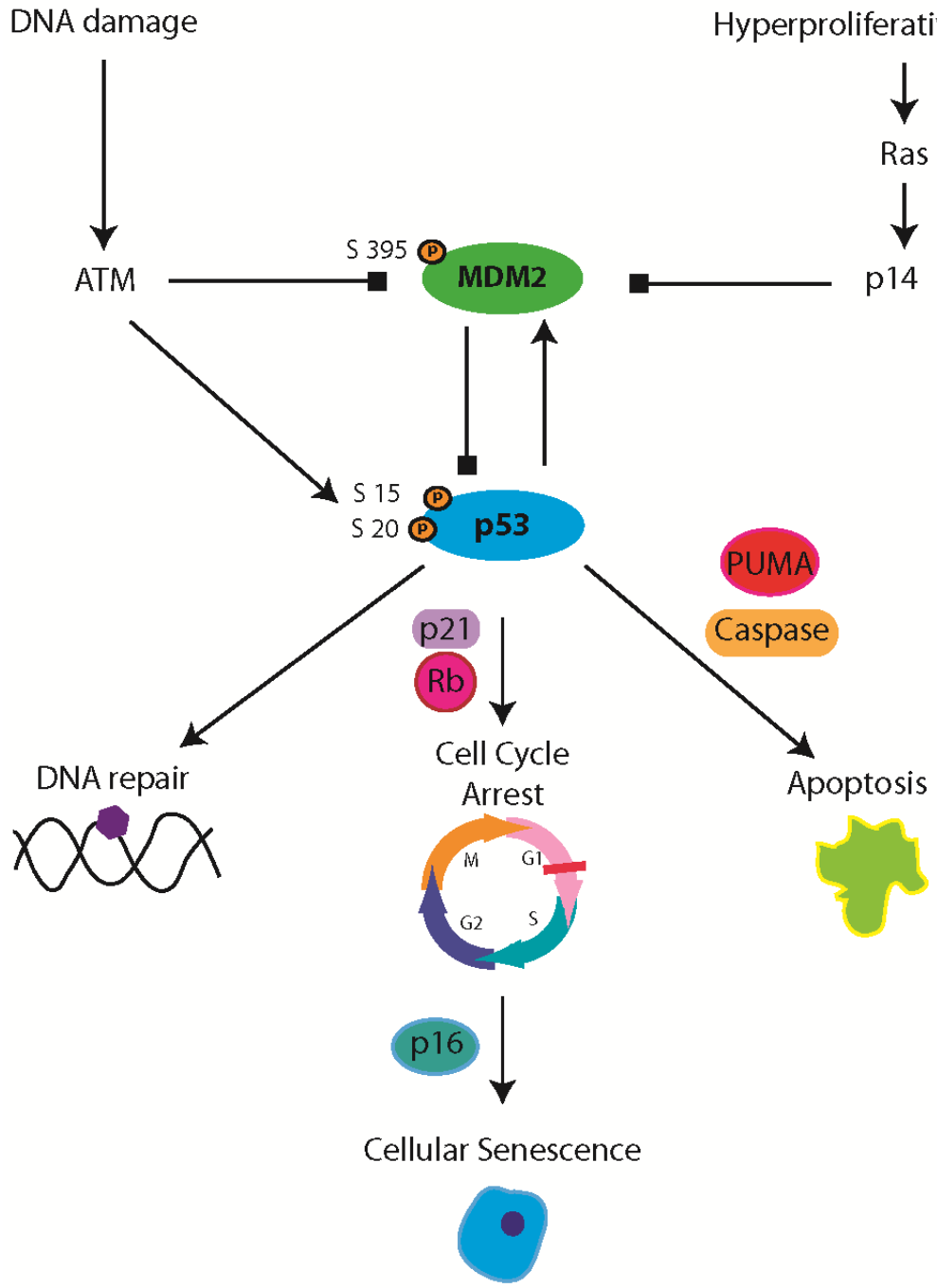


Figure 1.1: A simplified model of p53 regulation, activation and p53-driven cell fate decisions. Tumour suppressor p53 is activated in response to a variety of stress-signalling pathways. p53 is a substrate for ataxia-telangiectasia mutated (ATM), a kinase which is expressed upon acute DNA damage. To drive p53 transcriptional activity ATM indirectly phosphorylates p53 on S15 and S20 through downstream activation of CHK1 and CHK2. The most studied antagonist of p53, mouse double minute 2 (MDM2) is negatively regulated by ATM through phosphorylation of S395, resulting in the increased stabilisation of p53. Hyperproliferative signals activate p53 in a similar manner through Ras. Ras has been shown to promote the stabilisation of p53 through tumour suppressor

p14 which attenuates MDM2 degradation and blocks the nuclear-cytoplasmic export of p53 that is necessary for p53 degradation by MDM2. Upon stabilisation and activation p53 can resolve the stresses through DNA damage, apoptosis, cell cycle arrest or cellular senescence. To induce cell cycle arrest, p53 transcriptionally activates cell cycle inhibitor p21, which in turn drives the dephosphorylation of retinoblastoma (Rb), preventing the progression of the cell cycle from G1 to S phase. This temporary arrest of the cell cycle can become permanent, termed cellular senescence upon heightened expression of tumour suppressor p16. Apoptosis is driven by the increased expression of pro-apoptotic genes such as PUMA which is transcriptionally activated by p53.

1.3.1 Cell cycle arrest

Cell cycle progression is tightly controlled through cell cycle checkpoints. A hallmark of cancer cells is the ability to proliferate in an unrestrained manner, which is achieved mainly through the dysregulation, mutation or deletion of checkpoint proteins. The p53-p21 pathway is essential for the inhibition of cell cycle progression through direct transactivation of p21 by p53 [44]. Mouse models have demonstrated that p21 is a critical inhibitor G1 to S-phase cell cycle progression [45], which it regulates by enhancing the interaction between Retinoblastoma (Rb) and E2F transcription factors. The E2F family are a group of DNA-binding transcription factors that transcriptionally activate genes necessary for cell cycle progression from G1 to S-phase [46]. Rb inhibits this G1-phase transcription by binding directly E2F transcription factors, therefore inhibiting the transcription of target genes necessary for DNA replication and cell cycle progression [47, 48]. The interaction between Rb and E2F is maintained primarily through phosphorylation of Rb. Cell cycle progression occurs when Rb is reversibly hyperphosphorylated by cyclin dependent kinases, dissociating E2F from Rb and allowing its transcriptional activity [49, 50]. p21 prevents the hyperphosphorylation of Rb by indirectly inhibiting cyclin dependent kinase activity, restoring the interaction between Rb and E2F [51, 52].

1.3.2 Cellular senescence

Cell cycle arrest is a temporary state of growth arrest, which can become permanent if the oncogenic stress persists. This permanent state of growth arrest

is termed cellular senescence. Establishment of cellular senescence is dependent on the persistent activity of p53 and Rb. Rb is a critical initiator of senescence and is recruited to E2F target genes with heterochromatin proteins to mediate the formation into specialised domains of heterochromatin, termed senescence-associated heterochromatic foci [53]. Tumour suppressor p16 is highly expressed in senescent cells and is essential in the permanent establishment of senescence through two distinct mechanisms. First, p16, like p21 is a cyclin dependent kinase inhibitor and thus responsible for the inhibition of cell cycle progression through enhancing the interaction between Rb and E2F [54]. Second, p16 is able to form a formidable barrier that prevents the cell from re-entering the cell cycle again by mediating the formation of senescence-associated heterochromatin and orchestrating the silencing of E2F target genes [52, 53]. The reversal of senescence in p53-depleted cells was dependent on the downregulation of p16, where cells expressing low levels of p16 could re-enter the cell cycle while those expressing high levels could not [55]. Furthermore, in senescent cells expressing high levels of p16, knockdown of p16 did not reverse senescence, most likely due to the established heterochromatic foci formation by p16 [55].

Despite being permanently barred from re-entering the cell cycle, senescent cells are metabolically active, morphologically different from cells that have undergone temporary cell cycle arrest and experience wide-spread changes in heterochromatin organisation and subsequently protein expression. Senescent cells also develop a hyper-secretory phenotype where large range of cytokines, growth factors, and chemokines are secreted [35]. This hypersecretory phenotype is intrinsic of all senescent cells, irrespective of what cell type they are derived from [56]. Recently cellular senescence has emerged as a potential therapeutic mechanism to achieve tumour suppression in human cancer cells, in particular in cells resistant to apoptosis. However, this has been debated considerably as a result of the hyper-secretory quality of senescent cells.

Although senescence does prevent aberrant cell cycle progression in cells experiencing extensive stress, senescent cells have been shown to enhance the tumourigenic capacity of the surrounding cells, most likely through the secretion of growth factors. Immunocompromised mice injected with senescent human and

mouse fibroblasts had accelerated initiation and progression of cancer [57-59]. Senescence has also been linked to aging as many of the pro-inflammatory proteins associated with a number of age-related diseases are produced during senescence [35, 52].

On the other hand however, p53-induced senescence in liver tumours in mice was found to have increased expression of inflammatory cytokines, tumour regression and clearance by the innate immune response [60, 61]. A recent study further corroborated this, as the hypersecreted components were identified as critical for the activation and recruitment of Natural Killer and T cells to senescent cells [62]. Moreover, p53 restrains some of the hypersecreted components [56] and preferentially recruits immune cells to senescent cells to clear them out, suggesting that p53 may drive the hypersecretory phenotype towards the protein signals that recruit Natural Killer and T-cells [60].

It is possible that cellular senescence acts as a 'buffer' of sorts, acting both as a barrier to oncogenesis while protecting the organism from extensive cell death that would result in organism death. This is clearly seen in mouse liver cells, where p53 mediates tumour suppression by inducing cellular senescence and recruiting immune system responses [60], whereas loss of p53 resulted in tissue fibrosis and death in mice [63]. This would account for the dual roles cellular senescence and the senescence associated secretory phenotype seem to have in both preventing and stimulating oncogenesis.

1.3.3 Regulation of p53 activity

Although inactivation of p53 results in spontaneous, aggressive tumours, hyper-activation of p53 is just as deleterious to the organism, resulting in a number of age-related degenerative diseases, such as arthritis, possibly due to cellular senescence [64]. Furthermore, enhanced expression of p53 has been reported to cause neuronal cell death and as such is linked to neuropathogenesis [65-69]. Because of this it is essential that the activity of p53 is tightly regulated within the cell.

The activity of p53 is regulated primarily through E3 ubiquitin protein ligase Mouse double minute 2 (MDM2, or HDM2 in humans) [70] which regulates p53 activity in three ways. First MDM2 binds to the N-terminal transactivation domain of p53, preventing p53 from transcriptionally activating target genes [71, 72]. Second, MDM2 facilitates p53 sequestration from the nucleus into the cytoplasm and third, MDM2 ubiquitinates p53, marking it for proteasomal degradation [73]. Normally in unstressed cells, p53 is a short-lived protein that is tightly regulated by MDM2 through the auto-regulatory feedback loop formed by MDM2 which is a transcriptional target of p53 [71]. The interaction between MDM2 and p53 is primarily governed through extensive posttranslational modifications. In the presence of stresses, p53 is displaced from MDM2, allowing p53 to stabilise, accumulate and activate downstream target genes. Ataxia-telangiectasia mutated (ATM) is a well-documented kinase that responds to DNA damage by stimulating p53 stability. Upon DNA damage, ATM is autophosphorylated on S1981 and monomerises, resulting in its activation. Originally, ATM was believed to regulate p53 activity primarily through phosphorylation of S18 and S32 (or S15 and S20 in humans) residues by activating downstream targets CHK1 and CHK2 since phosphorylation correlated with p53 accumulation [74, 75]. However there was a redundancy in these phosphorylation marks as the interaction between MDM2 and p53 S15/-20 mutants was not altered [76]. Furthermore, p53 S15/-20 mutants could still undergo protein stabilisation [77-79]. Instead the interaction between ATM and MDM2 was identified as a possible primary method of negatively regulating the MDM2/p53 interaction in DNA damaged cells through phosphorylation HDM2 S395 residue (S394 in mice) in response to ionizing radiation (IR) [80]. A mouse model study corroborated this and showed that upon whole body irradiation, p53 in mice expressing MDM2 with a serine to alanine substitution (S394A) did not undergo stabilisation upon IR treatment and were comparable to $p53^{-/-}$ mice as both survived IR treatment (radio resistant) and did not experience apoptosis [81]. This demonstrated that the primary role of ATM in regulating p53 activity is due primarily to the phosphorylation of MDM2.

Although post-translational modifications have been shown to displace p53 from MDM2, further studies have suggested that the interaction between p53 and

MDM2 can also be inhibited through localisation. Ras (including H-Ras, K-Ras and N-Ras) is part of an oncogenic family of small GTPases that are responsible for regulating a wide array of cell signalling networks, including cell growth and survival. In contrast to the cell growth promoting function of Ras, overexpression of Ras in primary human cells resulted in p53-dependent cell cycle arrest. This is because Ras promotes the activity of p53 through p14 expression, which negatively regulates the interaction between p53 and MDM2 [82]. p14 has been shown to inhibit MDM2-mediated degradation of p53 through two mechanisms. First, p14 was found to bind directly to MDM2 and promote its degradation [14, 83]. Second, p14 blocks the nuclear-cytoplasmic export of p53 that is essential for p53 degradation by MDM2 [84].

In addition to stabilising p53, posttranslational modifications are used to direct the p53 mediated stress response by modifying p53 conformation, the affinity of p53 for its target genes and the interaction between p53 and its transcriptional cofactors [85, 86]. The severity of the stress directs the response of p53 through the presence of certain acetyltransferases and kinases that are responsible for modifying specific p53 residues. As previously mentioned, although p53 is rapidly phosphorylated on S15 and S20 residues by CHK1 and CHK2 upon DNA damage, they are not essential in stabilising p53. However these phosphorylation marks are required for p53-dependent apoptosis and tumour suppression [77, 87]. Phosphorylation of S15 was found to be necessary for histone acetylation that is essential for p53 to transactivate target genes, such as p21 [88]. It is likely that phosphorylation of S15 recruits coactivators (such as CBP/p300) to p53 [89], resulting in acetylation of K164 which is indispensable for p53-dependent cell cycle arrest through expression of p21 [90].

Specific post-translational modifications to p53 are essential for directing p53-dependent cell fate decisions. p53-dependent apoptosis is mediated solely through acetylation of K120 by the MYST family acetyltransferases (hMOF and Tip60) [91, 92]. Acetylation of K120 resulted in a clear upregulation of *Puma*, a critical target of p53-mediated apoptosis. However what regulates the modification of p53 by TIP60 is currently unknown.

The post-translational modifications of p53 are necessary for p53 activation in response to stress-signalling pathways through both the stabilisation of p53 and modulating p53-dependent cell fate decisions. A common theme in cancers is the inhibition of p53-mediated tumour suppression. This is often achieved through the overexpression of proteins that regulate p53 activity.

1.3.4 Inhibition of p53 and p53 pathways in cancers

Defective p53 signalling pathways occur in more than 80% of tumours [93] through either overexpression of p53-regulators, such as the MDM2 or silencing coactivators necessary for p53 stabilisation and activation such as p16 and p14. The interaction between p53 and MDM2 is often favoured in cancers through overexpression of MDM2 or regulators of the MDM2/p53 interaction. *MDM2* gene amplification occurs in 7% of human cancers and is highly correlated to tumour progression due to its inhibition of p53 stabilisation and accumulation [94-96]. However, as previously discussed (see section 1.3.3) post-translational modification and localisation of MDM2 are equally important for regulating the stability and subsequent accumulation of p53 in response to stress-signalling.

Tumour suppressors p14 and p16 have dual roles in tumour suppression, as they have been shown to play an important part in inhibiting the MDM2/p53 interaction and initiating permanent cell cycle arrest respectively [12-14, 84, 97]. Of particular interest is the critical role p14 plays in p53 accumulation in response to hyperproliferative signals resulting from Ras hyperactivation. Knockout studies conducted on $p19^{-/-}$ mice showed they are particularly vulnerable to spontaneous tumour development compared to wild-type mice. Given the important roles of p14 and p16 in activating p53 and establishing the stress response downstream of p53 respectively, it is not surprising that the INK4A/ARF locus is frequently silenced by epigenetic remodelling (see section 1.2) or deleted in a wide spectrum of tumours [10].

As previously discussed, phosphorylation of MDM2 on S395 upon DNA damage by ATM has been identified as a critical step in the stabilisation and accumulation of

p53 in response to DNA damage [98]. Protein Phosphatase 2C (PP2C) family member, wild-type p53 induced phosphatase (Wip1 or PP2Cδ) is a transcriptional target of p53 [99] and forms an autoregulatory loop with p53, by dephosphorylating MDM2 at S395 [98]. Although this interaction is important for restoring p53 to a low level of expression upon resolution of the DNA-damage, Wip1 is frequently overexpressed in several human cancer types, including breast cancer, neuroblastoma and ovarian cancer [100-103]. Studies conducted in human cancer cell lines identified Wip1 as an essential inhibitor of p53 activity as knockdown of Wip1 restored p53 activity and inhibition of cell growth [104-106], while knockout studies in mouse models resulted in tumour resistant mice [107]. Wip1 also prevents p53 activation by directly binding to and dephosphorylating S15, necessary for the transcriptional activation of p53 target genes [88, 108] (see section 1.3.3). Furthermore, Wip1 also prevents the activation of ATM through dephosphorylation of S1981, preventing ATM monomerization and subsequently preventing the ATM-dependent signalling cascade necessary for p53 activation in response to DNA damage [107]. Through aberrant overexpression of Wip1 in cancers, activation of p53 can be robustly inhibited in both its stability and activity.

Recently another member of the PP2C family, Protein Phosphatase 2C isoform β (PP2Cβ), which is structurally and functionally similar to Wip1 [109] was identified as a regulator of p53 stability in human embryonic fibroblasts.

1.4 The Protein Phosphatase type 2C isoform β

Protein Phosphatase 2Cβ (PP2Cβ) belongs to the Protein Phosphatase type 2C (PP2C) family, a class of serine/threonine protein phosphatases dependent on the presence of Mg^{2+}/Mn^{2+} cofactors for their catalytic activity. There are at least 18 members of the PP2C family, each having a diverse and essential set of functions in cell signalling networks such as cell death/survival signalling, the regulation of stress-signalling pathways, and cell metabolism [109, 110].

There have been six alternatively spliced isoforms variants of PP2Cβ identified so far, β-1, -2, -3, -4, -5 and –X. A mouse model study of PP2Cβ isoform expression revealed that isoforms β-1 and -2 are expressed ubiquitously and are localised exclusively to the cytoplasm [111, 112]. PP2Cβ has been characterised as both a

negative regulator of stress signalling and a negative regulator of cyclin dependent kinase activity (Fig. 1.2).

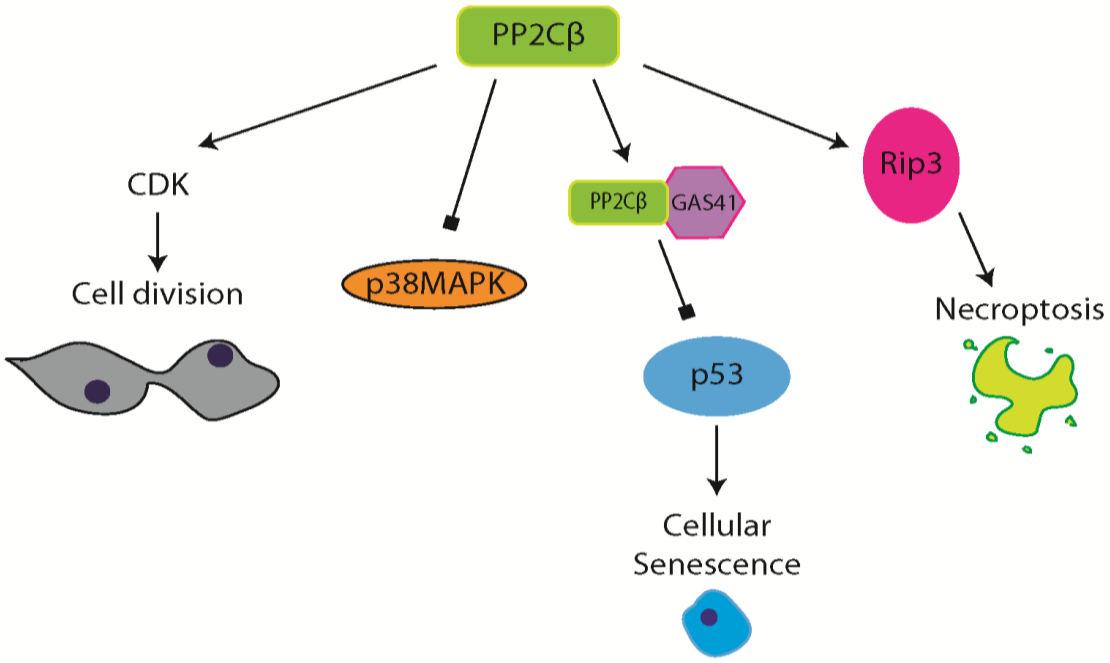


Figure 1.2: Known functions of protein phosphatase 2Cβ. PP2Cβ has been characterised as a negative regulator of necroptosis, stress-signalling pathways and cyclin dependent kinase (CDK) activity. PP2Cβ is able to remove the stabilising T-loop threonine residues on CDKs, resulting in an inhibition of cell proliferation [111, 113]. On the other hand, PP2Cβ was also found to inhibit p38MAPK and -to a larger extent- p53 stress-signalling pathways [1, 114, 115]. In particular, PP2Cβ was found to inhibit p53 stabilisation and consequently p53 pathways after forming a novel complex with glioma amplified sequence 41 (GAS41) [115]. Knockdown of PP2Cβ induced p53-dependent cellular senescence, highlighting PP2Cβ as an important inhibitor of p53. PP2Cβ is also a known inhibitor or necroptosis through the removal of activating phosphate groups from receptor-interacting serine/threonine kinase 3 (Rip3) [116, 117].

1.4.1 Negative regulator of cell cycle progression

The activation of cyclin dependent kinases is necessary for driving eukaryotic cell cycle progression (see section 1.3.1). Cyclin dependent kinase-activating kinases are essential for activation of cyclin dependent kinases through phosphorylation of serine/threonine residues in the T-loop domain. PP2Cβ was found to remove activating T-loop phosphate groups from CDK2, CDK6 *in vitro* [113]. A later

independent study demonstrated that PP2Cβ could directly interact and dephosphorylate T-loop threonine residue in CDK9 in HeLa cells, though the effect was comparatively minor [118]. These results agreed with the original observation that overexpression of PP2Cβ inhibits the cell proliferation of 293T cells [111]. However, further studies showed that PP2Cβ appears to have a dual role in cell proliferation through negative regulation of stress-signalling pathways.

1.4.2 Negative regulation of stress-signalling pathways

Mitogen-activated protein kinase (MAPK) pathways were found to be suppressed by PP2Cβ in mammalian cells [114]. The MAPK pathway regulates a diverse number of cellular functions, including cell proliferation and apoptosis and is regulated through phosphorylation relays. PP2Cβ-1 interrupts this phosphorylation relay by dephosphorylating p38MAPK and upstream activators of a number of MAPK kinases [114]. A later independent study further corroborated these results, where knockdown of PP2Cβ with siRNA increased p38MAPK activity in human embryonic fibroblasts cells [1]. However, in contrast to an earlier study that had identified all PP2Cβ isoforms as inhibitors of cell proliferation in 293T cells [111], Park (2014) found knockdown of PP2Cβ-1 and -2 isoforms in human embryonic fibroblasts induced cellular senescence. Furthermore, p38MAPK was found to be dispensable for this growth arrest. Instead, this growth arrest was contributed to by stabilisation of p53, which was repressed by a novel complex PP2Cβ forms with Glioma Amplified Sequence 41 (GAS41).

1.4.3 The PP2Cβ/Gas41 complex

Gas41, a small nuclear protein which is expressed ubiquitously in mammalian cells is necessary for cell proliferation [119], inhibits p53 activity [120, 121] and is commonly amplified during early stages in human glioblastoma multiforme formation [120-122]. The Gas41/PP2Cβ interaction therefore has important implications in terms of p53 activity and oncogenesis, particularly in glioma formation. The interaction between PP2Cβ and Gas41 was of particular interest as Gas41 was found to reside in a distinct complex with PP2Cβ to regulate p53 stability [115, 120].

Further investigation demonstrated that co-expression of PP2Cβ and Gas41 in UV-treated human cancer cells reduced p53 expression [115]. Furthermore, knockdown of PP2Cβ in the absence of UV-stress, resulted in increased expression of p53. These findings were corroborated by another study that demonstrated knockdown of PP2Cβ in human embryonic fibroblasts induced premature senescence through activation of p53 and p38MAPK pathways [1]. How p53 stability is modified by PP2Cβ is currently still unknown, however the regulation of p53 by Gas41 was comparable to MDM2 [121].

1.4.4 PP2Cβ regulates necroptosis

As well as inhibiting cell proliferation, PP2Cβ was also found to regulate cell death through necroptosis pathways. Necroptosis is a programmed cell death, but is significantly different from apoptosis in that it is induced in a caspase-independent manner and results in cell leakage [123]. Necroptosis is induced through activation of Receptor-interacting serine/threonine kinases (Rip) and downstream targets through a phosphorylation relay. Rip3 is a critical activator of the necroptosis program in response to the expression of tumour necrosis factor-α (TNFα) [123-125]. Rip3 requires phosphorylation of T231 and S232 residues in mice (or S227 in humans) in order to phosphorylate and activate mixed linkage kinase domain-like protein (Mlkl), which is necessary for the induction of necroptosis [117, 126, 127].

PP2Cβ-1 and -2 were found to suppress Rip-3 dependent necroptosis in mouse embryonic fibroblasts by dephosphorylating Rip3 [116] and preventing Mlkl recruitment. Expression of a mutant PP2Cβ lacking phosphatase activity was found to increase the phosphorylation of Rip3, with knockdown inducing TNF-dependent necroptosis pathways [116]. This function of PP2Cβ is dependent on the expression of Rip3 as no cell death occurred in cell lines expressing low levels of Rip3, such as mouse fibroblast cell line (NH3T3) and HeLa cells [116, 128]. This may potentially explain why human embryonic fibroblasts (IMR-90), which express low levels of Rip3, experienced cellular senescence upon PP2Cβ knockdown rather than necroptosis [1, 129].

1.4.5 Regulation of PP2Cβ

Given the role of PP2Cβ in regulating stress-signalling and necroptosis pathways, the activity of PP2Cβ must be modulated. While the catalytic activity of PP2Cβ is dependent on Mn^{2+}/Mg^{2+}, the unvarying physiological levels of divalent cations in living systems suggests that the activity of PP2Cβ may be regulated through its interaction with Gas41, by cellular localisation or expression levels. Immunofluorescence experiments demonstrated that PP2Cβ and Gas41 localised to the nucleus upon UV irradiation in HeLa cell lines [130]. However whether UV irradiation modulates the interaction between Gas41 and PP2Cβ is still unknown as GAS41 was localised to the nucleus and cytoplasm prior to UV irradiation. Furthermore GAS41 is a known component of chromatin remodeler complexes TIP60 and SRCAP and could be involved in DNA damage repair.

A human genome-wide computational search of a modular relational database that integrated microarray results with a genome-wide search of p53 response elements identified *PP2Cβ* as a potential target gene of the p53 family as it contained six p53 family response element motifs [131]. Furthermore PP2Cβ was predicted to be downregulated by a factor of 2-fold by transactivation domain containing p63 isoform-α (TAp63α) [131].

1.5 p53 family members

Given the role of PP2Cβ in inhibiting p53 stabilisation, identifying how PP2Cβ expression is regulated in human cancers may provide means through which to restore p53 activity in cancers. A modular relational database identified PP2Cβ as a potential target of members of the p53 family p63 and p73 [131].

As previously discussed, p53 is a potent tumour suppressor which is commonly deficient in tumour cells through either mutation or overexpression of p53 regulators. However p53 family members, p63 and p73 while structurally and functionally similar to p53 are rarely mutated or deleted in cancers and instead appear to be more important in embryonic development [132-135]. Nonetheless, p63 and p73 have both shown they have roles in tumour suppression through their abilities to regulate cell proliferation, differentiation and death both

dependently and independently of p53. A mouse model study demonstrated that $p63^{(+/-)}$ and $p73^{(+/-)}$ mice were predisposed to spontaneous tumourigenesis [136]. Furthermore, p63 and p73 were found to drive cell fate decisions by coordinating with p53 function [137, 138]. Neural precursor cells (NPCs) in p63 $^{(-/+)}$, p73 $^{(+/+)}$ mice underwent apoptosis while in p63 $^{(-/+)}$, p73 $^{(-/+)}$ mice, the NPCs underwent cellular senescence. This cellular senescence was abrogated upon genetic ablation of p53, demonstrating that p63 and p73 coordinate to determine cell fate through regulation of p53 function in NPCs [138]. These findings were further supported by another study where p63 and p73 were found to be essential in establishing p53-dependent apoptosis in response to DNA damage in both mouse embryonic fibroblasts and mouse brain cells [137].

1.5.1 Tumour Suppressor p63

Discerning the role of p63 in tumour suppression has been a complicated process, owing to the existence of six p63 isoforms, which have a number of diverse and sometimes opposing functions. The *p63* gene contains two promoters allowing the expression of p63 isoforms that contain or lack the C-terminal transactivation domain, referred to as TA and ΔN isoforms respectively [139]. These isoforms have opposing functions in the cell as ΔNp63 isoforms stimulate proliferation and cell survival while TAp63 isoforms inhibit cell cycle progression and stimulate apoptosis [139]. TAp63 is a tumour suppresser which induces cellular senescence independently of p53 in the Ras transformed mouse embryonic fibroblast cell line through transactivation of p21 [140]. Furthermore TAp63 was found to inhibit cell metastasis and invasion; with TAp63 silencing resulting in the migration of epithelium derived human cancer cell line [141]. In addition to the TA and ΔN p63 isoforms, p63 is alternatively spliced to give α, β and γ isoforms.

TAp63α, the longest p63 isoform was identified as a potential inhibitor of PP2Cβ expression and was predicted to bind to 6 possible response elements in a modular relational database, which integrated a genome-wide search of p53 response elements with microarray results produced from overexpressing p53 family members in isogenic cell lines [131]. As p63 is known to coordinate with

p53 to determine cell fate decisions, it is possible that inhibition of PP2Cβ by p63 may be a potential mechanism though which this is achieved [138].

1.5.2 Tumour suppressor p73

Like p63, *p73* encodes isoforms from two promoters that allow isoforms that either contain or lack a transactivation domain (TAp73 and ΔN763 isoforms respectively) and are alternatively spliced to give the α, β and γ isoforms. Like the p63 isoforms, the TAp73 and ΔNp73 isoforms have opposing functions in the cell acting as a tumour suppressor or oncogene respectively [142, 143]. The TAp73 isoforms can transcriptionally activate p53 target genes associated with cell cycle arrest and apoptosis independently of p53 [144-146]. Furthermore, like p63, p73 is capable of coordinating with p53 to determine cell fate. This was shown in neural progenitor cells where p73 haplodeficiency resulted in a shift from p53-dependent apoptosis to senescence [138]. As p63 is predicted to inhibit PP2Cβ expression through response elements, and the DNA binding domain (DBD) of p63 and p73 share a ~85% amino acid identity, it would be possible that p73 may also regulate PP2Cβ expression.

1.6 Hypothesis

The hypothesis for this project will be split into two separate sections which will be addressed by two different aims.

1. Knockdown of PP2Cβ restores p53 pathways in human cancer cell lines resulting in a slower cell proliferation.
2. Constitutive expression of TAp63α reduces PP2Cβ expression.

1.6.1 Aim 1

The first aim of the project will be to examine if stable PP2Cβ knockdown in human cancer cell lines restores the p53 stress-signalling pathway and halts cancer cell proliferation.

1.6.1.1 Objectives

1. **Achieve knockdown in three human cancer cell lines: U2OS, MCF7 and H1299.**

   Cultured human cancer cell lines will be infected with lentiviral particles containing either non-targeted shRNA or PP2Cβ shRNA. Knockdown will be confirmed using western blot analysis of the whole cell lysate.

2. **Determine if PP2Cβ knockdown arrests the proliferation of human cancer cell lines.**

   A CyQuant® cell proliferation assay will be used to compare the proliferation of cancer cell lines expressing PP2Cβ shRNA and non-targeted shRNA over seven days.

3. **Investigate whether p53 pathway activation occurs in response to PP2Cβ knockdown.**

   To test if PP2Cβ knockdown can activate p53 and its downstream targets, p21 and Rb phosphorylation, both a western blot and RNA expression analysis will be utilised.

1.6.2 Aim 2

The second aim of my project was to determine if p53 family members could alter PP2Cβ expression.

1.6.2.1 Objectives

1. **Clone *p63* and *p73* cDNA into a transient mammalian expression vector.**

   To investigate whether p53 family members can alter the expression of PP2Cβ, full length of *p63* (TAp63α) and *p73* (TAp73α) will be cloned into a CβF transient mammalian expression vector.

2. **Overexpress p53 family members in human cancer cell lines.**

   p53 family members will be overexpressed using a mammalian expression vector in human cancer cell lines H1299 and U2OS to study PP2Cβ protein expression in p53 negative and p53 wt conditions, as p53 family members coordinate to direct cell fate decisions.

3. **Examine PP2Cβ expression in a H1299 and U2OS cell lines**

   Two approaches will be utilised to address this objective. Firstly protein expression of PP2Cβ isoforms 1 and 2 will be examined in response to p53 family member overexpression. Secondly, a quantitative RNA analysis of PP2Cβ expression through use of a RT-qPCR in response to p53 family member overexpression will be undertaken to determine if p53 family members can alter the RNA expression of PP2Cβ.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General

Materials	Manufacturer/Supplier
Agarose	Bioline, England
Molecular Imager ® Gel Doc™ XR	BioRad, USA
Absolute Ethanol, Boric Acid, Methanol, Isoproponal β-mercaptoethanol, Bromophenol blue Ethidium bromide, Ethylenediaminetetraacetic acid (EDTA), DirectLoad™ DNA marker, Glycerol, Glycine, Polytheylene glycol (PEG), Potassium Chloride, Tween-20	Merck, Germany
Microfuge tubes, PCR Tubes, Pipette tips	Axygen, USA
5 mL polystyrene round-bottom tube	BD Falcon, USA
Plastic Tubes, 60 mm Tissue culture plates	Grenier Bio-One, Germany
Nanodrop spectrophotometer ND-1000	Thermoscientific, USA

2.1.2 Cloning

Materials	Manufacturer/Supplier
Ampicillin, Luria Broth base	Sigma chemical company, USA
T4 Ligase Buffer, T4 Ligase, T4 Polymerase, *EcoRV*, *HindIII*, Calf intestinal phosphatase (CIP)	New England Biolabs, USA
dNTP	Clontech, USA
Plasmid purification (Minikit)	Epigenetics Research Technologies, USA
Plasmid purification (Midikit), Bovine	Thermoscientific, USA

Serum Albumin (BSA), CyQUANT® cell proliferation assay kit	
Petridish	Axygen, USA
UV light block	Alpha Innotech Corporation, USA
Kapa HiFi Hotstart ReadyMix PCR kit	KAPA Biosystems, South Africa

2.1.3 Cell Culture

Materials	**Manufacturer/Supplier**
Dulbecco's Modified Eagle Medium (DMEM), OptiMEM, Fetal Bovine Serum (FBS), Penicillin/Streptomycin, Tyrpsin,	Gibco
Puromycin	Sigma-Aldrich, USA
FuGENE HD	Promega
XtremeGENE 9	Roche
60 mm Tissue culture plates, 100 mm Tissue culture plates, 5 mL pipette, 10 mL pipette, 25 mL pipette, 96 well Tissue culture plate, Cryo.S tube	Greiner Bio-One, Germany
POLARstar Omega plate reader	BMG Labtech, Germany
HEK 293T, HCT116, H1299, MCF7, U2OS cell lines	ATCC, USA

2.1.4 RNA extraction

Materials	**Manufacturer/Supplier**
Trizol ® LS reagent	Thermo Fisher Scientific, USA
RNA miniprep kit	Epigenetics Research Technologies, USA
DNase, DNase buffer	Roche, NZ

2.1.5 Protein extraction and western blotting

Materials	Manufacturer/Supplier
40% Acrylamide-bis solution, p21-WAF1-Cip 1(05-345) monoclonal antibody	Merck, Germany
Goat anti-rabbit HRP conjugated antibody (SC2054), Goat anti-mouse conjugated antibody (SC2055), Bovine anti-sheep HRP conjugated antibody (2918) PP2Cβ monoclonal antibody (small isoform only), p53 polyclonal antibody	Santa Cruz Biotechnology, USA
FLAG M2 monoclonal antibody (F1365), β-actin antibody	Sigma chemical company, USA
Retinoblastoma total monoclonal antibody (D20), Phospho- Retinoblastoma (S807/811) monoclonal antibody	Cell signalling technology, USA
PP2Cβ polycolonal antibody (both isoforms)	R&D Systems, USA
Ammonium persulphate (APS) Bradford protein assay	BioRad, USA
Pierce ECL western Blotting Substrate Halt Protease Inhibitor Cocktail, Super Signal West Femto maximum sensitivity substrate	Thermoscientific, USA
N,N,N',N'-Tetramethylethylenediamine (TEMED), Phenylmethanesulfonyl fluride (PMSF), Sodium dodecyl sulphate (SDS)	Sigma chemical company, USA
Nitrocellulose blotting membrane PVDF blotting membrane	GE Healthcare life sciences, UK
Ultrospec 3000	Pharmacia Biotech, Sweden

2.1.6 Reverse transcriptase quantitative real-time PCR

Materials	Manufacturer/Supplier
SensiFAST SYBR No-Rox one step kit	Bioline, UK
LightCycler® 480 instrument	Roche, NZ
Frame Star 480 Q-PCR plate (96 well)	4titude, UK
q-PCR lentivirus titration kit	Applied Biological Materials, Canada

Primer pairs	
Caspase-7 GUSB p27 PP2Cβ PUMA p21	Qiagen, Germany
WPRE	Integrated DNA technologies, USA

2.2 Methods

2.2.1 Mammalian Cell Culture

Cell culture work was carried out in a class II ESCO biological safety cabinet (Model LA241A). UV irradiation and spraying surfaces with 70% ethanol ensured sterile conditions were maintained. All equipment and solutions utilised for cell culture were sterilised using an autoclave and sprayed with 70% ethanol.

2.2.1.1 Cell lines

Four human cancer cell lines were used in this study including U2OS osteosarcoma, MCF7 non-invasive adenocarcinoma, H1299 non-small cell lung carcinoma and HCT116 colorectal carcinoma.

Table 2.1: Summary of p53, Ras and p14/p16 expression in human cell lines used in this study

	p53	Ras	p14/p16
HCT116	+	++	-
H1299	-	++	+
MCF7	+	+	-
U2OS	+	+	-

+	Wild-type
-	Not Expressed
++	Hyperactive mutant

2.2.1.2 Cell Culturing

All cell lines (U2OS, HEK293T, H1299, HCT116 and MCF7) were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 0.5% penicillin and streptomycin (Pen/Strep) in sterile conditions. All cells were incubated in humidified incubators at 37 °C with an atmosphere of 5% CO_2.

2.2.1.3 Thawing cell lines

Cell lines were kept frozen at -80°C with 10% DMSO (see section 2.2.1.5). For U2OS, H1299, HCT116 and HEK293T cell lines were thawed briefly in a 37°C water bath and transferred to a 10 cm plate and supplemented with 10 mL of pre-warmed growth medium. After 24 h the growth medium was replaced to remove the DMSO. For the MCF7 cell line the cells were thawed briefly in 37°C water bath and transferred to a sterile 15 mL plastic tube containing 5 mL of pre-warmed media. The cell suspension was then centrifuged at 200 rcf for 5 min to obtain a cell pellet. The growth medium was replaced with 10 mL of fresh growth medium and the pellet was resuspended which was then added to a 10 cm plate.

2.2.1.4 Subculturing cell lines

After reaching a confluence of ~80%, the cell line was subcultured. After the growth media was removed, the cells were washed with 7 mL of 1x PBS. HEK293T cell line was trypsinised with 1 mL of pre-warmed 0.5x Trypsin/EDTA by swirling and followed by immediate removal. U2OS, H1299, HCT116 and MCF7 cells were trypsinised with 1 mL of pre-warmed 1x trypsin/EDTA by swirling and left to stand for 3 min before removal of trypsin. The cells were then dislodged after 1 min with palm tapping and resuspended in 10 mL of growth medium. One mL of the resuspended cells was then added to 9 mL of growth medium in a fresh plate.

2.2.1.5 Freezing cells

Cell lines were frozen when they reached a high confluency (~90%) to ensure survival when they are thawed. Removal of growth medium was followed by washing the cells in 1x PBS. The cells were then trypsinised according to their needs (see section 2.2.1.4) and dislodged by palm tapping. They were resuspended in 5 mL of growth medium per 10 cm plate and transferred to a sterile 15 mL plastic tube which was centrifuged at 300 rcf for 5 min. The growth medium was then removed and the cell pellet was resuspended in 2 mL of freezing media (FBS and 10% DMSO). The cell suspension was then transferred to a Cryo.S tube (Gernier Bio-one) which was frozen at -80°C in a cell freezing container (Biocision). The cell lines were stored at -80°C for short term storage. For long term storage (>1 year) cell lines were stored in liquid nitrogen.

2.2.2 Transfection and Lentivirus Preparation

2.2.2.1 Transfection of human cancer cell lines

Human cell lines were transfected with the CβF-p53 family constructs (see section 2.2.8.1). After transfection, the cell lines were incubated for 48 to 72 h.

FuGENE® HD transfection method: Prewarmed OptiMEM was dispensed into sterile microfuge tubes (500 µL/10 cm plate). The DNA to the desired transfection reagent ratio was then added in respective order, followed by immediate tapping of the microfuge tube. The microfuge tube was centrifuged briefly at 3,000 rcf to spin-down the solution and was then incubated at room temperature for 15 min before being added dropwise to the human cell lines.

Calcium phosphate transfection method: Prewarmed 2x HEPES was dispensed into sterile 5 mL polystyrene round-bottom tubes (500 µL/ 10 cm plate). Four µg of DNA was then added to the HEPES followed by 500 µL 250 mM $CaCl_2$ which is added dropwise with constant tapping. The tubes were left to incubate at room temperature for 15 min before being added dropwise to the human cell lines.

2.2.2.2 Lentivirus packaging

Lentivirus PP2Cβ and non-targeted shRNA plasmid constructs used for PP2Cβ knockdown was purchased from Sigma.

Preparation: HEK293T cells were seeded onto 10 cm plates at a density of ~5.3 x 10^6 cells per plate with 10 mL growth medium 19 h prior to transfection. These plates were incubated as in section 2.2.1.2

Transfection: Prepared cell lines were co-transfected with a viral packaging-plasmid mix (5 µg) and a lentivirus vector (3 µg) containing either shRNA for PP2Cβ or non-targeting shRNA as a negative control. The transfection reagent used was FuGENE HD (Promega). Pre-warmed OptiMEM was dispensed to sterile microfuge tubes (500 µL/10 cm plate) followed by the required volume of DNA and packaging plasmid mix. Lastly 32 µL of transfection reagent was added, followed by immediate tapping of the microfuge tube. The microfuge tube was centrifuged briefly at 3,000 rcf to spin-down the solution. The transfection mix was

incubated at room temperature for 15 min before being added to the HEK 293T cells dropwise with gentle swirling. The HEK 293T cells were then incubated for 24 h.

Replacing media: The medium on the HEK 293T cells was replaced with medium containing 1 mM/µL of caffeine 24 h after transfection, as previous studies have shown caffeine increases the viral titre [147]. The HEK 293T cells were then incubated for a further 24 h.

Viral Harvest: Medium containing viral particles was collected 48 h after cell transfection in a 50 mL plastic tube and stored at 4°C. The medium on the HEK293T cells was replaced (containing 1mM of Caffeine) and harvested again after another 24 h.

2.2.2.3 Concentrating lentivirus

The medium containing harvested viral particles was centrifuged immediately after the final viral harvest at 3,000 rcf for 5 min. The supernatant was then transferred to a fresh 50 mL plastic tube and was mixed thoroughly with 6 mL of 50% PEG 6000. Then 3 mL of 4 M $NaCl_2$ was added and the solution was mixed thoroughly. The solution was then stored at 4°C for 24 h to precipitate the viral particles. The mixture was centrifuged at 7,000 rcf for 10 min at 4°C, followed by 3,000 rcf for 5 min in a bucket rotor to pellet the precipitate in the bottom of the tube. The supernatant was removed and 1 mL of DMEM per 10 cm plate the virus was collected from was added to the pellet and incubated overnight at 4°C. The pellet was gently resuspended in the DMEM to avoid bubble formation and was dispensed 500 µL aliquots into cryovials which were stored at -80°C. Excess virus was collected for viral quantification and stored at 4°C.

2.2.2.4 Viral Quantification using RT-q PCR

Concentrated viral particles (see section 2.2.2.3) were lysed using viral lysis buffer (Applied Biological Materials) at a dilution factor of 10. RT-q PCR was then carried out on the lysed viral solution at the conditions listed in Table 2.1 using a commercially purchased q-PCR Lentivirus Titration Kit (Applied Biological

Materials). The primers for RT-q PCR were provided with the titration kit and the sequences were not disclosed.

Table 2.2: RT-q PCR program for viral titration

Step	Temperature	Time	Cycles
Reverse Transcription	42◦C	20 min	
Enzyme Activation	95◦C	10 min	
Denaturation	95◦C	15 s	40 Cycles
Annealing/Extension	60◦C	1 min	

2.2.2.5 Infection and Selection

Preparation: Mammalian cell lines was trypsinised and seeded onto 10 cm plates in 10 mL of growth medium at a density of ~5.3 x 10^6 cells per plate 24 h prior to infection.

Infection: The medium containing viral particles was made in a 15 mL plastic tube with 9 mL of growth medium, 1 mL of concentrated virus and 10 µL of Polybrene (8 mg/mL), which increases infection efficiency [148]. The tube was immediately inverted to mix the solution thoroughly. The spent medium on the cells was removed and replaced with the viral medium solution.

Selection: The viral medium was replaced after 24 h with 10 mL of fresh growth medium containing 2 µg/mL of puromycin for selection of infected cells and over a period of 48 h.

2.2.3 Infection of human cancer cell lines

To achieve a broad understanding of the mode through which PP2Cβ knockdown may affect stress-signalling pathways in human cancers it is essential that PP2Cβ knockdown be studied in several different human cancer cell lines. In particular, as PP2Cβ has been identified as an inhibitor of p53, it is essential that PP2Cβ knockdown is studied in human cancer cell lines that express functional wild-type p53 and cell lines in which p53 function is lost by deleted or mutation.

2.2.3.1 U2OS Osteosarcoma

U2OS human osteosarcoma cell line expresses all tumour suppressor proteins properly, with the exception of INK4A/ARF locus, which is silenced by heterochromatin formation. Because of this genetic background it was likely that U2OS would be the most comparable human cancer cell line to the human fibroblast IMR-90 cell line, which entered cellular senescence upon knockdown of PP2Cβ [1].

2.2.3.2 H1299 non-small cell lung carcinoma

Human non-small cell lung carcinoma cell line H1299 has a partial homozygous deletion of the p53 gene. The H1299 cell line has been used to study the effect of stress-signalling pathways in a p53 deficient environment. As the effect of PP2Cβ depletion needs to be investigated in a p53 independent manner, knockdown of PP2Cβ in H1299 cells would provide a suitable experimental system.

2.2.3.3 HCT116 colorectal carcinoma

Human colorectal carcinoma cell line HCT116 expresses wild type p53 tumour suppressor and a hyperactive mutant K-Ras. The effect of PP2Cβ knockdown in HCT116 cell line in conjunction with H1299 and U2OS would provide some insight into whether any observable effect of PP2Cβ depletion was dependent on p53 and whether Ras hyperactivity affects p53 activation upon PP2Cβ knockdown. Furthermore HCT116 is comparable to U2OS as it has a frameshift on the INK4A/ARF locus, and as a result, does not express p16 and p14.

2.2.3.4 MCF7 breast adenocarcinoma

Breast adenocarcinoma cell line MCF7 expresses p53 tumour suppressor and wild-type Ras. Like U2OS and HCT116, MCF7 does not express p14 or p16, owing to a deletion of the INK4A/ARF locus. Determining the effect of PP2Cβ depletion in the MCF7 cell line would allow insight into the role that PP2Cβ may play in the presence of p53.

2.2.4 Protein Analysis

2.2.4.1 Harvesting mammalian cells

Mammalian cells were harvested by removing the growth media and adding 1 mL of chilled (4 °C) 1xPBS to the plates. The cells were then scraped using a silicon

scraper and collected in microfuge tubes, which were centrifuged at 3,000 rcf for 5 min at 4 °C. The pelleted cells were then resuspended in 300 µL of chilled 1xPBS. For RNA purification 100 µL of the cell suspension was dispensed into TRIzol® (see section 2.2.4.1) while the remaining suspension was used for used for protein extraction and analysis (see section 2.2.3.2)

2.2.4.2 Protein extraction

The remaining suspension from section 2.2.3.1 was centrifuged at 3,000 rcf at 4 °C for a further 5 min. The supernatant was removed and the pellet was resuspended in a 100 µL mixture of F-buffer (25 mM Tris (pH 7), 50 mM NaCl, 30 mM tetrasodium pyrophosphate, 50 mM NaF, 10% glycerol, 0.5% Triton X-100), 10% Protease Inhibitor (Roche) and 1% PMSF (Biochemika). The cell suspension was kept on ice for 15 min and then vortexed at maximum speed for 1 min. The solution was centrifuged at 16,000 rcf for 10 min at 4 °C to separate the cell lysate from cell debris. The supernatant was then added to a fresh microfuge tube and was stored at -20 °C until use.

2.2.4.3 Bradford protein quantification

Bradford reagent was used to quantify cell lysate solution from section 2.2.3.2. Bovine Serum Albumin (BSA) was diluted to concentrations between 0-0.8 mg/mL at 0.2 mg/mL intervals with 1xPBS. 10 µL of each BSA concentration solutions or 1 µL of extracted protein was added to 1 mL of diluted Bradford dye reagent (200 µL) in triplicate in microfuge tubes. The samples were left to stand for 5 min to allow colour development. Solution absorbance was measured by spectrophotometer (Ultraspec 3000) in a 0.5 mL cuvette at a wave length of 595 nm. A standard curve from the BSA absorbance spectrum was generated in Microsoft Excel (2010) and used to determine the concentration of extracted protein. These concentrations were then normalised to 25 µg/mL to be loaded into a SDS PAGE gel.

2.2.4.4 SDS-PAGE gel electrophoresis

Analysis of PP2Cβ knockdown: The range of target protein sizes analysed for the knockdown study varied between 140 kDa and 20 kDa. A gradient gel from 15% to 4% acrylamide was therefore used to analyse the protein expression. (See table 2.2)

Analysis of p53/p63/p73 transient expression: The range of protein sizes analysed for transient expression of p53, p63 and p73 expression analysis varied between 100 kDa and 20 kDa. Therefore a gel of 10% acrylamide was used. (See table 2.2)

A 1.5 mm-spaced gel casting system (Mini PROTEAN®) was filled with 10 mL of running gel and left to polymerize for 20 min under a layer of 70% v/v ethanol. After the running gel had polymerized ~3 mL of 4% stacking gel was poured on top of the running gel with either a 10 or 15 well comb and left to polymerize for at least 30 min.

Samples of protein were added to 6x SDS loading dye and heated at 92°C for 5 min before being loaded into the SDS-PAGE gel. The 20 µL of 25 µg protein samples were then added to the gel wells along with 6 µL of protein marker (ThermoScientific) and electrophoresed at a constant current of 40 mA for 1 hr in SDS running buffer.

Table 2.3: Ingredients for polyacrylamide resolving gel

		Gradient SDS-PAGE gel	
Percent gel	**10% (10 mL)**	**4% (5 mL)**	**15% (5 mL)**
40% Acrylamide	2.5 mL	0.5 mL	1.5 mL
4x Tris buffer (pH 8.8)	2.5 mL	1.25 mL	1 mL
50% Glycerol	-	0.125 mL	1 mL
Distilled Water	5 mL	3.125 mL	1.5 mL

10% APS: 1:200 v/v of gel solution

TEMED: 1:5 v/v of 10% APS

2.2.4.5 Western Blotting

Transfer: The SDS-PAGE gel was soaked in chilled western transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for ~5 min before being electro-blotted to a membrane at a constant current of 150 mA for 2 h at 4°C. Either nitrocellulose or charged PVDF (pre-soaked in methanol) membranes were used for western blotting. After electro-blotting the membrane was incubated on an orbital shaker

(Labnet) in 5 mL of blocking buffer (5% skim milk powder, 0.02% NaN_3) for at least 30 min at room temperature. After blocking, the membrane was rinsed for 5 min with TBS/T (10 mM Tris, 150 mM NaCl, pH adjusted 7.6 with HCl and 0.1% Tween-20) three times.

Probing: The blocked membrane was incubated overnight at 4°C on a shaker in 5 mL of diluted primary antibody (see Table 2.3) to detect target proteins. The membrane was washed in TBS/T for 10 min intervals three times on an orbital shaker. After washing, the membrane was incubated on orbital shaker at room temperature for 30 min in 10 mL of TBS/T, 1 µL HRP-conjugate secondary antibody and 100 µL blocking buffer. The membrane was then washed again for 15 min intervals three times. The target proteins were visualised using a chemiluminescent reagent (Thermoscientific) and exposed to medical X-ray film (Kodak).

Table 2.4: Primary antibody dilution factors

Primary Antibody	Secondary Antibody	Dilution Factor
β-Actin	Mouse	1:5,000
α-FLAG	Mouse	1:500
Phosphoprotein p53	Rabbit	1:50
Phosphoprotein p21	Mouse	1:500
PP2Cβ	Sheep	1:500
Retinoblastoma (pRb) (PhosS807/S811)	Rabbit	1:1,000
Retinoblastoma Total	Rabbit	1:200

Diluted in TBS/T

20% blocking buffer (5% skim milk powder, 0.02% NaN_3)

2.2.4.6 Membrane stripping

For further detection of other target proteins, membrane stripping was sometimes necessary to remove primary and secondary antibody which may occlude the results of future probing (such as detection of phosphorylated (PhosS807/811 and total retinoblastoma (Rb) protein). After protein detection, membranes were

washed for 10 min in TBS/T to remove remaining detection reagent. The TBS/T was then removed and the membrane was incubated in a water bath at 60 °C in stripping buffer (9 mL 4x Tris SDS (pH 6.8), 1 mL of 10% v/v SDS and 70 µL β-mercaptoethanol (0.1 M)) for 30 min with shaking every 10 min. The membrane was then washed in TBS/T three times for 10 min intervals to remove any remaining stripping buffer. Finally the membrane was incubated in blocking buffer for at least 30 min before reprobing with primary antibody (as in section 2.2.3.5).

2.2.5 RNA analysis

2.2.5.1 RNA isolation and purification

After harvesting the mammalian cells as in section 2.2.3.1, 100 µL of the cold cell suspension in PBS was transferred to a microfuge tube containing 300 µL of TRIzol® LS reagent which was stored at -80 °C until RNA purification. Samples stored in TRIzol® were thawed and 400 µL of 98% Ethanol (v/v) was added, followed by vortexing to mix the solution. The supernatant was collected after centrifugation at 12,000 rcf for 1 min. Thereafter the specified protocol for Zymo Direct-Zol™ RNA purification kit was used to purify the RNA.

2.2.5.2 RNA normalisation

RNA concentrations were estimated by absorbance using a Nanodrop® ND-1000 spectrophotometer at 280 nm.

2.2.5.3 Reverse-Transcriptase Quantitative Polymerase Chain Reaction (RT-q PCR)

The normalised RNA samples were loaded in triplicate into a 96-well plate (4titude). Gene expression was quantified in real-time using a LightCycler® 480 (Roche). RNA was reverse transcribed using SensiFAST SYBR no-ROX one step kit (Bioline) according to the manufacturer's instructions using the conditions in table 2.4. Amplification of Caspase-7, GUSB, PP2Cβ, PUMA and p27 cDNA was carried out using specific primers pairs. Primer specificity was confirmed with a melt curve and analysis of amplified cDNA.

Table 2.5: RT-q PCR cycling conditions for amplification of target genes

Stage	Temperature	Acquisition Mode	Time	Cycles
Reverse Transcription	45◦C	None	10 min	1 cycle
Enzyme Activation	95◦C	None	2 min	
Denaturation	95◦C	None	5 s	40 cycles
Annealing	60◦C	Single	20 s	
Melting Curve	95◦C	None	1 s	1 cycle
	65◦C	Continuous	1 s	
	97◦C	None		
Cooling	40◦C			

2.2.5.4 Relative quantification and statistical analysis

Crossing point (Cp) values for the RT-qPCR analysis were put into Microsoft Excel program and a ratio of the test sample to control sample was calculated using Efficiency (calculated by LightCycler 480 software) (Efficency $^{Cp\ (control)-\ Cp\ (test\ sample)}$). The ratio was then normalised by multiplying it by the ratio for a reference gene. The difference between the ratio of control and treated cells was calculated to find the fold difference change. The standard error was found for all the ratios. A student T-test was used to determine whether gene expression differences were statistically significant.

2.2.6 Cell Growth assay

2.2.6.1 Cell Counting & Harvesting

Five days post infection; infected cell lines were trypsinised and resuspended in 4 mL of growth medium. 10 μL of cell suspension was loaded via capillary action onto a hemocytometer and counted in triplicate. The concentration of cells per mL was determined and ~2,000 cells were loaded in triplicate into a 96 well cell culture microplate. One 96 well plate was removed every 2nd day over a period of 7 days, residual growth medium was washed off with PBS and stored at -80°C for a maximum of 4 weeks.

2.2.6.2 CyQUANT® Cell Proliferation assay

A CyQUANT® Cell Proliferation Assay was used to measure the proliferation rate of the infected cell lines. The assay kit contains a dye which fluoresces when bound to nucleic acids enabling the measurement of DNA content. This translates into the quantification of the cell density and thus can be used to determine a difference in cell proliferation within a given cell line.

Harvested and frozen 96 well plates (section 2.2.5.1) were thawed at room temperature and the reagents from the kit were added as per the manufacturer's instruction. After 5 min incubation in the dark, the fluorescence of the samples was read using a POLARstar Omega plate reader (BMG Labtech).

2.2.6.3 Data analysis

Fluorescence intensity from the Cyquant assay was analysed on Microsoft excel and the average for each well was found. The fluorescence intensities for the control and treated cells for each day were plotted on a bar graph. The standard error was calculated from the fluorescence readings. A student t-test was used to determine whether the results were statistically significant.

2.2.7 DNA

Table 2.6: Primers used in this study

Name	Sequence (5'-3')
Tp63 (Forward)	GTT GGA TAT CAT GAA TTT TGA AAC TTC ACG GTG T
Tp63 (Reverse)	ATG GAA GCT TTC ACT CCC CCT CCT CTT TGA T
Tp73 (Forward)	CGT GGA TAT CAT GGC CCA GTC CAC CGC CAC C
Tp73 (Reverse)	GGC GAA GCT TTC AGT GGA TCT CGG CCT CCG T
WPRE (Forward)	CCT TTC CGG GAC TTT CGC TTT
WPRE (Reverse)	GCA GAA TCC AGG TGG CAA CA

2.2.7.1 Standard PCR amplification

Standard PCR amplification of Tp63 and Tp73 was performed using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems). The reaction mixture contained 5 µL of 5x Fidelity buffer, 5 µL of 5x GC Buffer, 10 mM dNTPs, 2.5 mM $MgCl_2$, 1 U/µL

of KAPA HiFi HotStart DNA Polymerase, 1 µL *p63*/*p73* full length cDNA template (10 ng/µL) and 1.5 µL of Forward/Reverse Primer (at a concentration of 5 µM). The PCR reaction was run at the conditions as in table 2.6.

Table 2.7: PCR conditions used in this study

Stage	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1 cycle
Denaturation	98°C	20 s	30 cycles
Annealing	68°C	15 s	
Extension	72°C	2 min	
Final Extension	72°C	5 min	1 cycle

2.2.7.2 PCR product purification

Excess nucleotides and primers were removed from the PCR product using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions.

2.2.7.3 Gel electrophoresis

To separate DNA fragments, gel electrophoresis was carried out on 1% agarose (w/v) in 0.5x TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 10 mM EDTA). Samples were loaded into wells with the addition of 6x (v/v) DNA loading dye (30% glycerol, 0.25% bromophenol blue). Directload™ DNA marker was loaded to analyse the fragment sizes. Gel electrophoresis was carried out at either 120 V or 150 V in a small or large tank respectively for 1 hr. To visualise the DNA, agarose gels were stained for 15 min in ethidium bromide (0.5 µg/µL) in 0.5x TBE solution followed by a 5 min destain in water. Visualisation of DNA was done using a Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software #170-8195 (Bio-Rad).

2.2.7.4 DNA Quantification

The concentration of DNA was estimated by absorbance at 260 nm using a Nanodrop® ND-1000.

2.2.8 Cloning

2.2.8.1 Cloning strategy

To investigate whether PP2Cβ expression is regulated by p53 family members, two constructs containing p63 and p73 cDNA were prepared using the CβF expression vector. The CβF expression vector has both a constitutively active CMV promoter and a FLAG epitope sequence upstream of the multiple cloning site (see appendix I). PCR was carried out on human full-length *p63* and *p73* cDNA (purchased from Thermoscientific) (see appendix V) using the primers listed in Table 2.5. These primers were designed to include restriction enzyme *EcoRV* and *HindIII* cut sites in the 5' and 3' ends respectively of both p63 and p73 cDNAs. PCR amplified *p63* and *p73* cDNAs were then cloned into a CβF vector at the *EcoRV* and *HindIII* multiple cloning site. Fig. 2.1 illustrates the cloning strategy utilised for the construction of *CβF-p63* and *CβF-p73*.

After double digestion with *EcoRV* and *HindIII*, *p63*, *p73* and linearized CβF vector were subjected to agarose gel purification (described in section 2.2.8.4). Ligation reactions were then set up between gel purified CβF and *p63*/*p73* DNA using T4 DNA ligase enzyme as described in section 2.2.8.5.

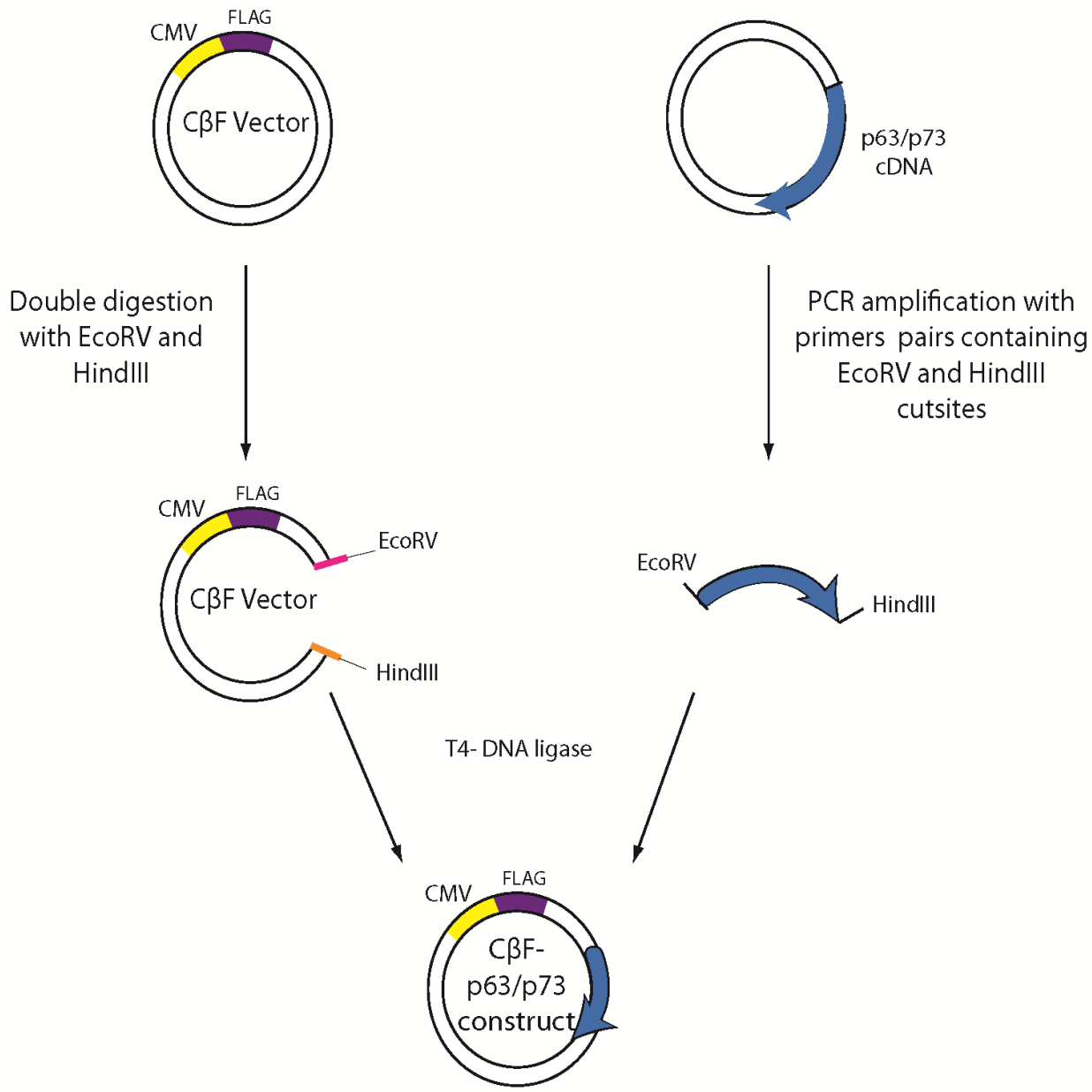


Figure 2.1: Cloning strategy used to construct CβF-p63 and CβF-p73 constructs. Mammalian transient expression vector CβF was linearized using *EcoRV* and *HindIII* (left). The commercially purchased *p63* and *p73* cDNA was PCR amplified with primer pairs containing cutsites for *EcoRV* and *HindIII* (right). The PCR amplified cDNAs were then inserted separately into the CβF plasmid by ligating at the *EcoRV* and *HindIII* sites.

2.2.8.2 Restriction Enzyme digestion

Reactions containing 1 µg vector DNA and PCR amplified insert were double digested with 2 µL of *Eco*RV *HF* (40 U), 2 µL of *Hind*III (40 U), 4 µL of NEB 10x

reaction buffer 2.1 and water to make a final volume of 40 μL. The reactions were incubated in a water bath for 2 h at 37°C.

2.2.8.3 Calf Intestinal alkaline phosphatase (CIP) treatment

To remove phosphate groups of 5' ends of digested vector DNA to prevent relegation, digested vector DNA was treated with 1 μL of calf intestinal alkaline phosphatase (CIP) (10 U) and incubated in a 37°C water bath for 1 hr.

2.2.8.4 Agarose Gel Purification

To isolate, extract and purify linearised vector or PCR products fragments, ethidium bromide stained agarose gels were visualised with a UV light block (Alpha Innotech Corperation) and the correctly sized DNA bands were excised out of the gel with a sterile scalpel blade. The gel slice was transferred to a microfuge tube and the DNA purified using E.Z.N.A® Gel Extraction Kit (Omega Bio-tek) according to the manufacturer's instructions.

2.2.8.5 Ligation

For ligation, 150 ng of gel-purified vector was combined with gel-purified insert at a 3:1 ratio of insert to vector in a 10 μL reaction mix containing 1 μL T4 ligase (40 U) (NEB) and 2 μL of 5x T4 ligase NEB reaction buffer. The reactions were incubated overnight at room temperature (~22°C).

2.2.8.6 *E. coli* Transformation

Chemically competent DH5α *E. Coli* cells were transformed with plasmids or ligation reactions by heat-shock method. 3 μL of the ligation product (containing ~50 ng of vector DNA) was added to 100 μL of competent cells, mixed by gentle tapping and incubated on ice for 30 min. The cells were heat-shocked at 42°C for 1 min and transferred to ice immediately afterwards. 800 μL of sterile Luria Broth was added to the cells, which were then incubated on a shaker (at 225 rpm) at 37°C for 2 h. The cell suspension was then pelleted by centrifugation at 3,000 rcf for 10 min. 800 μL of the supernatant was removed and the pellet was resuspended in the remaining 100 μL, which was spread onto LB agar plates containing ampicillin (100 μg/mL). The plate was dried and incubated overnight at 37°C.

2.2.8.7 Colony Screening

Colonies were selected from the LB amp plate, picked using sterile p200 pipette tips and incubated in Luria Broth containing 100 μg/mL ampicillin on a shaker (at 225 rpm) for 16 h at 37°C.

2.2.8.8 Plasmid purification

Small Scale Plasmid purification: 1.5 mL of inoculated Luria Broth was added to a microfuge tube and pelleted by centrifugation at 3,000 rcf for 10 min. The supernatant was removed and the plasmids were extracted and purified using the High Pure Plasmid Isolation kit (Roche) according to the manufacturer's instructions.

Medium Scale Plasmid purification: 50 mL of inoculated Luria Broth was added to a 50 mL plastic tube and centrifuged at 5,000 rcf for 10 min. The supernatant was removed and the plasmids extracted and purified using GeneJET Plasmid Midiprep Kit (Thermoscientific) according to the manufacturer's instructions.

2.2.8.9 DNA Concentration

DNA was concentrated by adding 1/10th volume of 3 M sodium acetate and 1 volume of 98% ethanol. DNA was pelleted by centrifugation and washed with 70% (v/v) ethanol. After ethanol removal the pellet was left to dry out in a sterile fume hood. The pellet was resuspended by vortexing in 200 μL of sterile TE buffer.

Chapter 3: Knockdown of PP2Cβ in Human Cancer Cell Lines

3.1 Introduction

Protein phosphatase type 2Cβ (PP2Cβ) has been identified as an inhibitor of p53 stress signalling pathways by directly dephosphorylating and destabilising p53 in human osteosarcoma cell line (U2OS) [115]. By inhibiting p53 activity, PP2Cβ prevents the transcriptional activation of p21 and inhibits p53-dependent induction of the cellular senescence program in human foetal lung fibroblasts [1]. Therefore, PP2Cβ was identified as a potential target through which to restore p53 activity in human cancers. To investigate whether PP2Cβ inhibition restores p53 activity and induces cellular senescence in human cancers, PP2Cβ was knocked down in four established human cancer cell lines (see section 2.2.3 for more details). This knockdown was achieved through stable infection of human cancer cell lines with lentiviral particles expressing PP2Cβ short hairpin RNA (shRNA). shRNAs are targeted to degrade a specific mRNA and thus prevent protein expression. This allows an opportunity to examine and identify pathways and functions which are regulated directly or indirectly by PP2Cβ *in vivo*.

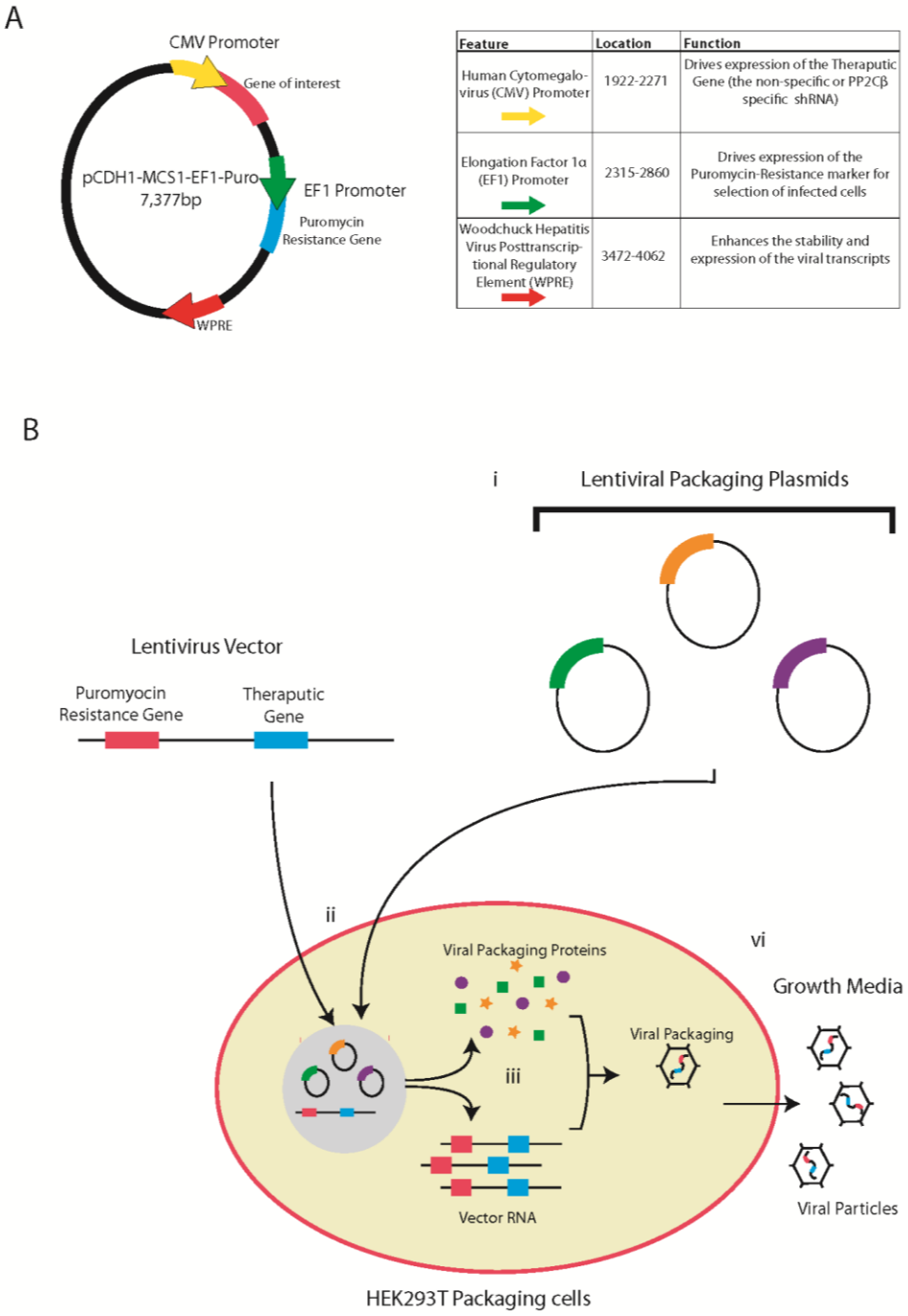
After infection with lentivirus encoding PP2Cβ shRNA, activation of stress-signalling pathways was examined. This included an analysis of cell proliferation and an examination of p53 signalling pathways through western blot analysis of p53 and its downstream targets p21 and the phosphorylation status of retinoblastoma (Rb) protein.

3.2 Lentiviral production

The lentiviral gene delivery system allows stable, long term expression of the inserted gene (>two weeks). Production of lentivirus particles requires two major components; a lentiviral vector containing the gene of interest (Fig 3.1 A) and packaging plasmids, which encode genes necessary for the production of structural proteins necessary to generate viral particles (Fig 3.1 B i) [149, 150]. The lentiviral plasmids for PP2Cβ

knockdown were commercially purchased and contained either PP2Cβ shRNA (knockdown) or non-targeted shRNA (control). To produce lentiviral particles, one of the lentiviral vectors and a mix of packaging plasmids are co-transfected into human embryonic kidney (HEK) 293T cells, which are commonly used as a packaging cell line (Fig 3.1 B ii). Once introduced into the cell, the packaging plasmids are transcribed and translated into viral structural proteins while the lentiviral vector is transcribed into single stranded RNA, which are assembled into lentiviral particles or virons (Fig 3.1 B iii). These virons are then released by the cells into the growth media (Fig 3.1 B iv). To maximise the knockdown efficiency, a puromycin resistance gene was incorporated into the lentiviral plasmid to enable selection of infected cells using puromycin containing media. Two different promoters were used to drive expression of the gene of interest and the antibiotic resistance gene; the cytomegalovirus promoter (CMV) and the elongation factor-1 alpha promoter (EF-1) respectively (Fig 3.1 A).

As viral preparation, infection and selection are lengthy processes, it was essential that all stages be optimised to obtain a consistently high lentiviral efficiency and knockdown efficiency of PP2Cβ. The method of transfection and the ratio of lentivirus plasmid DNA to packaging plasmid mix was optimised to achieve this (see section 2.2.2.2) and tested through quantification of lentiviral particles through reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR). Three lentiviral plasmids containing PP2Cβ shRNA were commercially sourced (Sigma) and named #3, #4 and #5.



Feature	Location	Function
Human Cytomegalo-virus (CMV) Promoter	1922-2271	Drives expression of the Theraputic Gene (the non-specific or PP2Cβ specific shRNA)
Elongation Factor 1α (EF1) Promoter	2315-2860	Drives expression of the Puromycin-Resistance marker for selection of infected cells
Woodchuck Hepatitis Virus Posttranscrip-tional Regulatory Element (WPRE)	3472-4062	Enhances the stability and expression of the viral transcripts

Figure 3.1: Map of lentivirus and diagram of lentiviral particle production in human embryonic kidney (HEK) 293T cell line. Lentivirus was utilised for stable expression of PP2Cβ (knockdown) and non-targeted (control) shRNA in human cancer cell lines. **(A)** A vector map of commercially purchased lentivirus vector (pCDH1-MCS1-EF1-Puro). Expression of the shRNA was driven by the cytomegalovirus (CMV) promoter and the puromycin resistance gene was driven by the elongation factor 1α (EF1) promoter. **(B)** Lentiviral packaging system. Human embryonic kidney (HEK) 293T cells were co-transfected with the lentiviral constructs and a mix of three helper plasmids encoding packaging-proteins necessary for the production of viral

particles. After the virus was produced it was released into the growth media and harvested over a period of 2-3 days.

3.2.1 Transfection method optimisation

To optimise the transfection method for lentiviral production, lentiviral particles produced from the PP2Cβ shRNA (#3) lentivirus were quantified using a RT-qPCR lentivirus titration kit (see section 2.2.2.4) and compared across two transfection methods; calcium phosphate and commercially purchased FuGENE® (Fig. 3.2) (see section 2.2.2.1). The viral particle titration (infectious units (IU)/ mL) was calculated from the equation provided by the manufacturers (see Fig 3.2). The viral titre obtained from calcium phosphate transfection method was 7.97 x 10^6 IU/ mL while the titre from FuGENE® method was 2.14 x 10^8 IU/ mL. As FuGENE® resulted in a notably higher viral titre, all transfections for lentivirus production were done using FuGENE®.

Despite optimising the transfection method, difficulties were encountered in producing consistently high titres of lentivirus particles from HEK293T cells. However, replacing the passaged HEK293T cells with freshly thawed HEK293T cells seemed to resolve this problem. In hindsight, this difficulty was likely the result of using highly passaged HEK293T cells.

Sample	Average Cp value	Viral Titre (IU/mL)
STD1	13.24	$5x10^7$
STD2	16.05	$6.25x10^6$
Negative	30.51	
Calcium Phosphate	15.72	7.97 x 10^6
FuGENE®	11.275	2.14 x 10^8

Viral Titre Formula: Dilution Factor (10) x (5 x 10^7)/2E(3x(A-B)/(C-B)

A= Average Cp Value for unknown sample
B= Average Cp Value for STD1
C= Average Cp Value for STD2

Table 3.1: Quantification of lentiviral particles from non-targeted shRNA lentiviral vector using RT-q PCR. Lentiviral particles were lysed with viral lysis buffer and quantified by real-time RT-q PCR using a q-PCR lentivirus titration kit. The DNA was amplified using a primer

targeted to the CMV promoter site and quantified using SYBR green dye. Standard 1 (STD1) contained $5x10^7$ IU/mL and Standard 2 (STD2) contained $6.25x10^6$ IU/ mL.

3.3 Lentiviral infection optimization

To achieve a robust knockdown of PP2Cβ in U2OS cells, infection with PP2Cβ shRNA lentivirus (see section 2.2.2.5) was initially done by combining the viral particles expressing PP2Cβ shRNA (#3, #4 and #5). However, although U2OS cells were successfully infected with PP2Cβ knockdown lentivirus, as demonstrated by the cells surviving puromycin selection (Fig. 3.2A), a protein analysis of U2OS cell lysate 8 days after infection showed that PP2Cβ knockdown had not been achieved (Fig. 3.2B). It was speculated that the lack of knockdown in the U2OS cell line was due to a non-functional shRNA from one of the three lentiviral constructs. Preliminary results generated by Dr Jeong Park indicated that PP2Cβ knockdown in U2OS cells reduced the cell proliferation rate (Jeong Park, *unpublished data*). It was therefore possible that U2OS cells with PP2Cβ knockdown were being outgrown by those U2OS cells infected with the faulty lentivirus construct, resulting in inefficient depletion of PP2Cβ in the whole cell population. Therefore, PP2Cβ shRNA expressing lentiviral vectors #3, #4 and #5 were prepared separately and quantified using the q-PCR lentiviral titration kit to ensure the viral particles had been generated (Table 3.3).

U2OS cells were then infected, selected with puromycin resistance and subjected to a protein analysis of PP2Cβ eight days after infection. Although the protein was not evenly loaded in the western, as seen by the difference in β-actin detection (Fig. 3.3), it is clear that PP2Cβ knockdown construct #5 (Fig. 3.3 Lane 4) was capable of PP2Cβ knockdown whereas constructs #3 and #4 (Fig. 3.3, Lanes 2 and 3) did not achieve knockdown of either the small (43 kDa) or large isoform (58 kDa) of PP2Cβ. As PP2Cβ shRNA lentivirus #5 successfully depleted PP2Cβ expression, this construct was utilised for all future PP2Cβ knockdown experiments.

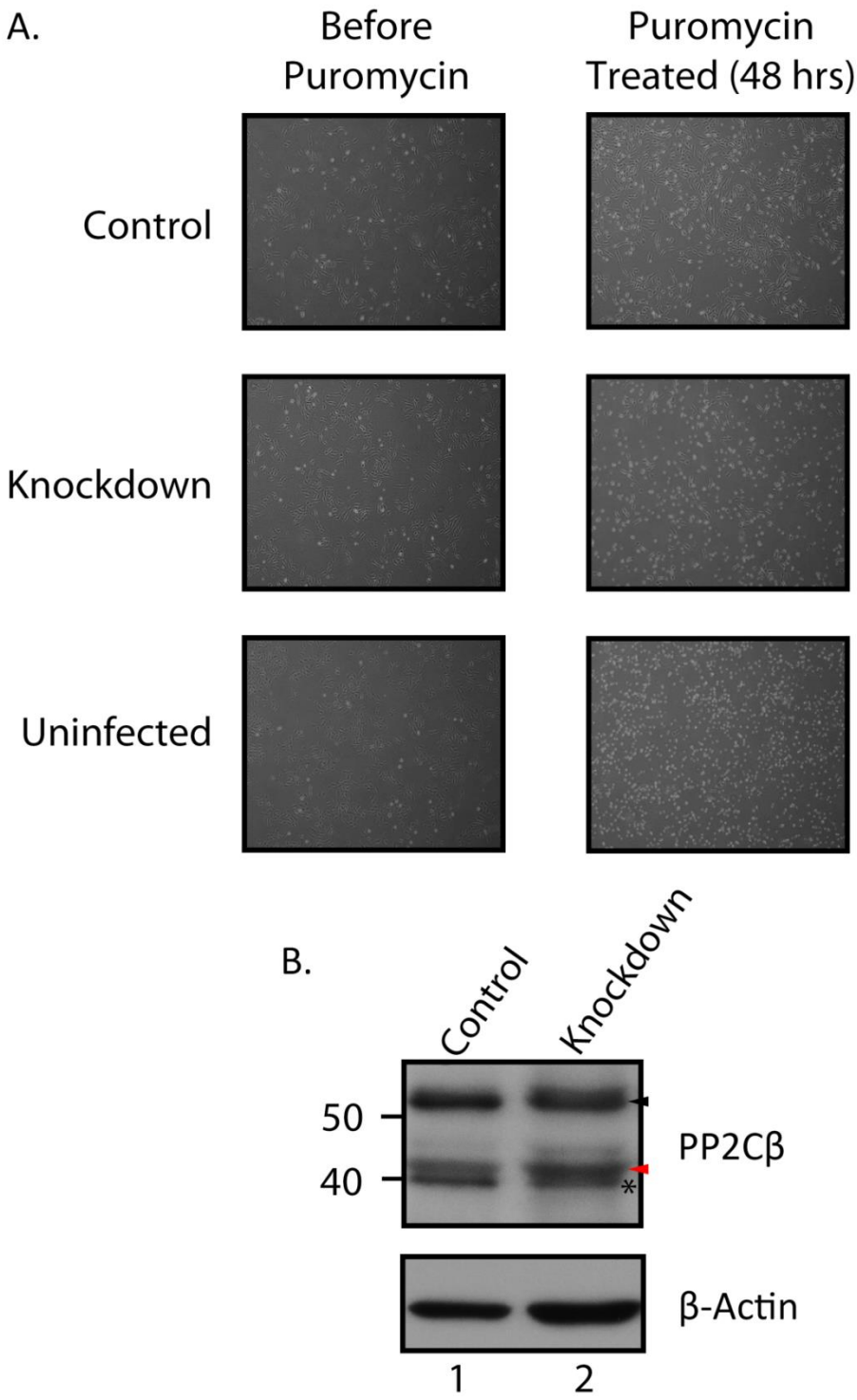


Figure 3.2: Combining the PP2Cβ shRNA lentivirus does not achieve knockdown of PP2Cβ. **(A)** U2OS cells infected with non-targeted shRNA (control), PP2Cβ shRNA (knockdown) or uninfected at x40 magnification undergoing puromycin selection over 3 days. **(B)** 25 µg of whole cell lysate from U2OS cells 8 days after infection were run on a 15% to 4% gradient polyacrylamide gel. Cells were infected with lentivirus particles expressing (lane 1) non-targeted shRNA and (lane 2) PP2Cβ shRNA, prepared from all three lentivirus constructs (#3,

#4 and #5). Both the small (43kDa) and large (58kDa) PP2Cβ isoforms were detected and are indicated by the red and black arrow respectively. Non-specific binding is indicated by the asterisk.

Sample	Average Cp	Viral Titre (IU/mL)
STD1	12.625	$5x10^7$
STD2	16.11	$6.25x10^6$
Negative	30.46	
Control	10.735	$1.54x10^8$
#3	11.75	$8.42x10^7$
#4	10.565	$1.70x10^8$
#5	10.43	$1.85x10^8$

Viral Titre Formula: Dilution Factor (10) x (5×10^7)/2E(3x(A-B)/(C-B)

A= Average Cp Value for unknown sample
B= Average Cp Value for STD1
C= Average Cp Value for STD2

Table 3.2: Quantification of lentiviral particles from PP2Cβ shRNA lentiviral constructs #3, #4 and #5 using RT-q PCR. Lentiviral particles were lysed with viral lysis buffer and quantified by real-time RT-q PCR using a q-PCR lentivirus titration kit. The DNA was amplified using a primer targeted to the CMV promoter site and quantified using SYBR green dye. Standard 1 (STD1) contain $5x10^7$ IU/ mL and Standard 2 (STD2) contain $6.25x10^6$ IU/ mL.

Although PP2Cβ shRNA lentivirus #5 was determined to be the best of the commercially purchased PP2Cβ shRNA constructs for knockdown of PP2Cβ (Fig. 3.3), this was not always achieved after puromycin selection for cells successfully infected with the lentivirus. The prepared lentivirus seemed to be functional as it enabled cell survival in puromycin containing medium. Furthermore, previous optimisation of the knockdown efficiency (Fig. 3.3) demonstrated that 8 days after infection was ample time for depletion of PP2Cβ protein expression.

It was speculated that the CMV promoter driving PP2Cβ shRNA expression was being silenced upon integration of the expression cassette into the host genome. As the

promoter that drives PP2Cβ shRNA expression (CMV) is different from the promoter driving puromycin resistance (EF1) (Fig. 3.1), it was possible that the CMV promoter was being silenced after infection while the EF1 promoter was not. This is a common phenomenon with CMV promoter-driven genes in embryonic stem cells [151] and would account for the active transcription of the puromycin resistance gene while the PP2Cβ shRNA was silenced. To overcome this, the volume of concentrated lentivirus used to infect the U2OS cells was doubled to 2 mL (Fig. 3.4). This restored the knockdown efficiency of the knockdown lentivirus (Fig. 3.4 Lane 2 compared to Lane 3) and so 2 mL of prepared lentivirus was used for all future infections.

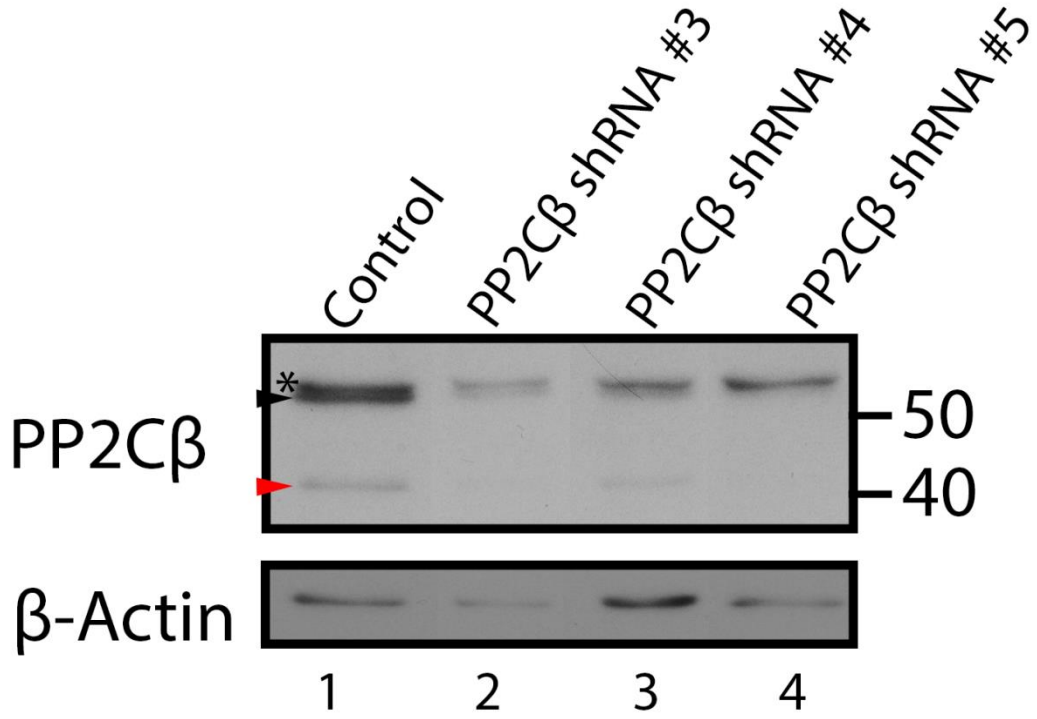


Figure 3.3: Protein analysis of U2OS cells infected separately with PP2Cβ shRNA lentivirus particles prepared from constructs #3, #4 and #5. 30 µg of whole cell lysate from U2OS cells harvested 8 days after infection were run on a 15% to 4% gradient polyacrylamide gel. Cells were infected separately with lentivirus particles expressing (lane 1) non-targeted shRNA as a control and (lane 2) PP2Cβ shRNA #3; (lane 3) #4; and (lane 4) #5. Both the small (43kDa) and large (58kDa) PP2Cβ isoforms were detected and are indicated by the red and black arrow respectively. Non-specific binding is indicated by *.

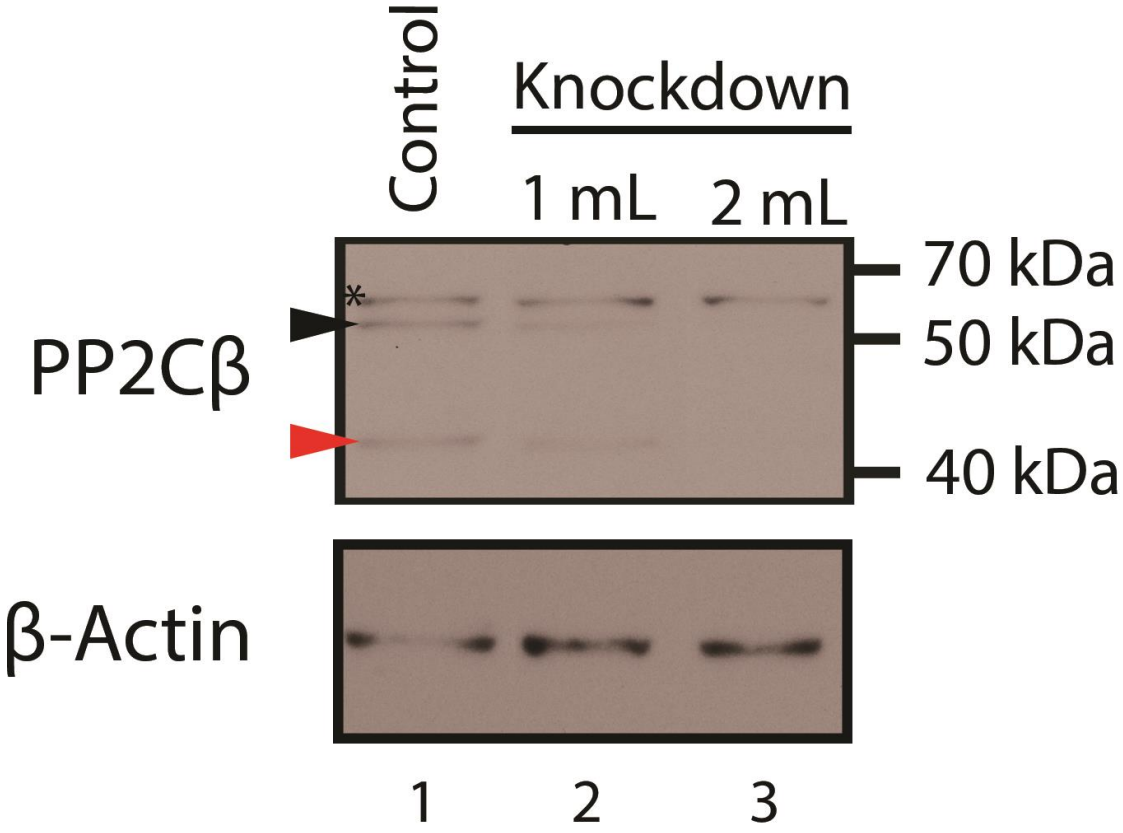


Figure 3.4: Knockdown of PP2Cβ in U2OS cells occurs only when the virus quantity is doubled. 30 µg of whole cell lysate from U2OS cells harvested 8 days after infection were run on a 15% to 4% gradient polyacrylamide gel. U2OS cells were infected with (lane 1) non-targeted shRNA expressing lentivirus particles and either; (lane 2) 1 mL; or (lane 3) 2 mL of lentivirus particles expressing #5 PP2Cβ shRNA. Both the small (43kDa) and large (58kDa) PP2Cβ isoforms were detected and are indicated by the red and black arrow respectively. Non-specific binding is indicated by *.

3.4 Cell proliferation and p53 pathway examination in human cancer cell lines upon PP2Cβ knockdown

3.4.1 Cell proliferation in MCF7 and U2OS cells decreases upon PP2Cβ knockdown

In human embryonic fibroblasts, PP2Cβ knockdown was shown to permanently arrest cell proliferation to induce cellular senescence [1]. Therefore, PP2Cβ knockdown in human cancer cell lines was hypothesised to result in an arrest of cell proliferation. A CyQUANT® cell proliferation assay (Invitrogen) was used to quantify the cell density over a period of 7 days immediately after puromycin selection for 48 h (Fig. 3.5) (see section 2.2.6.2). The cell proliferation of MCF7 (Fig. 3.5 A), U2OS (Fig. 3.5 B) and H1299

(Fig. 3.5 C) cell lines was tested three times, while the proliferation of the HCT116 cell line (Fig 3.5 D), was tested by Dr Jeong Park at least twice using #5 PP2Cβ shRNA lentivirus.

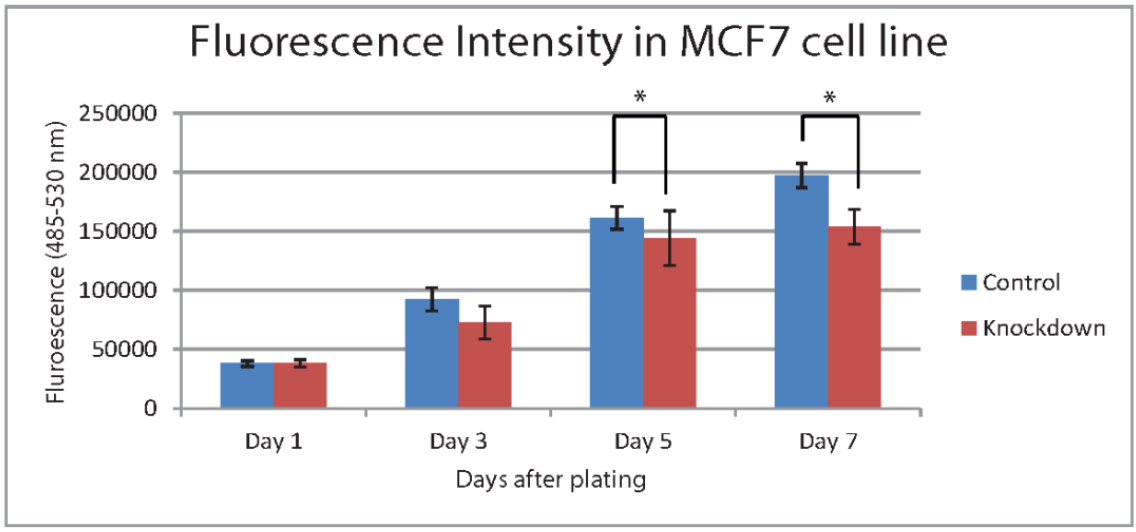
An equal number of cells (~2,000 cells) expressing either non-targeted (control) or PP2Cβ shRNA (knockdown) were loaded into four 96-well plates in triplicate and each plate was grown for either one, three, five or seven days (see section 2.2.6.1). The cell density in the sample was determined by quantifying the DNA content using fluorescence measurements from a DNA intercalating dye at 485-530 nm (see section 2.2.6.3). A western blot analysis of total protein extract (see section 2.2.4) was also carried out in tandem with the proliferation assay to confirm PP2Cβ had been knocked down (see section 3.4.2).

Both U2OS and MCF7 cells showed a decreasing trend in the cell proliferation upon knockdown of PP2Cβ (Fig. 3.5 A and B respectively), which was most evident at day 7 in MCF7 cells. Although there was a decrease in cell proliferation upon PP2Cβ knockdown in MCF7 and U2OS cell lines, it should be noted that this was not as severe as the growth arrest reported in the IMR-90 cell line [1].

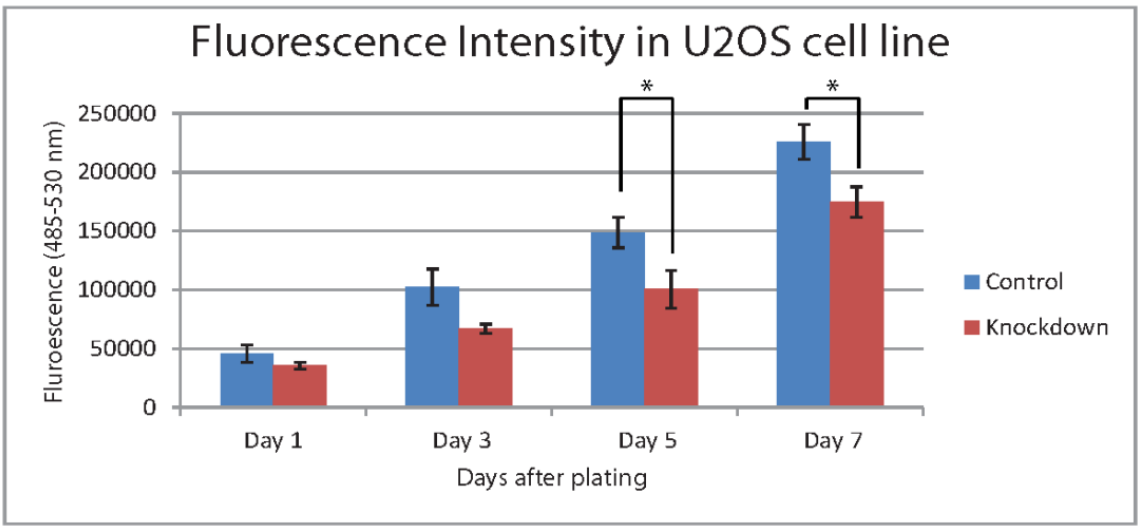
There was no discernible difference in cell proliferation between the control and PP2Cβ knockdown cell populations in the H1299 cell line (Fig. 3.5 C). Previous literature identified p53 stabilisation as the most likely mechanism through which cellular senescence was induced upon PP2Cβ knockdown [1]. This result was therefore expected as H1299 cells do not express p53. However, there was also little difference in cell proliferation between the control and PP2Cβ knockdown in the HCT116 cell line (Fig. 3.5 D). This was unexpected as HCT116 cells express wildtype p53. Therefore, this result suggests that the pathways activated by depletion of PP2Cβ in IMR-90, U2OS and MCF7 cells may be compromised in the HCT116 cell line.

Although there was an inhibitory trend in the cell proliferation, it is still unclear whether there is a true reduction in cell proliferation in MCF7 and U2OS cell lines. It is important to note that the results of these experiments were difficult to replicate (see section 5.2.1) and so the data presented here should be regarded with some caution and preliminary at best.

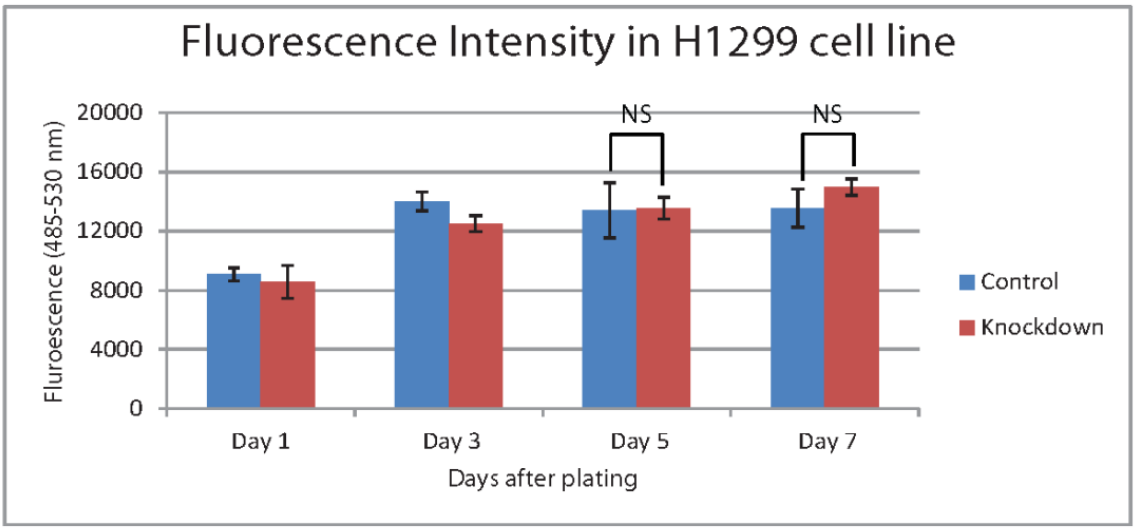
A.


Fluorescence Intensity in MCF7 cell line
Fluroescence (485-530 nm)
250000
200000
150000
100000
50000
0
*
*
Day 1
Day 3
Day 5
Day 7
Days after plating
Control
Knockdown

B.


Fluorescence Intensity in U2OS cell line
Fluroescence (485-530 nm)
250000
200000
150000
100000
50000
0
*
*
Day 1
Day 3
Day 5
Day 7
Days after plating
Control
Knockdown

C.


Fluorescence Intensity in H1299 cell line
Fluroescence (485-530 nm)
20000
16000
12000
8000
4000
0
NS
NS
Day 1
Day 3
Day 5
Day 7
Days after plating
Control
Knockdown

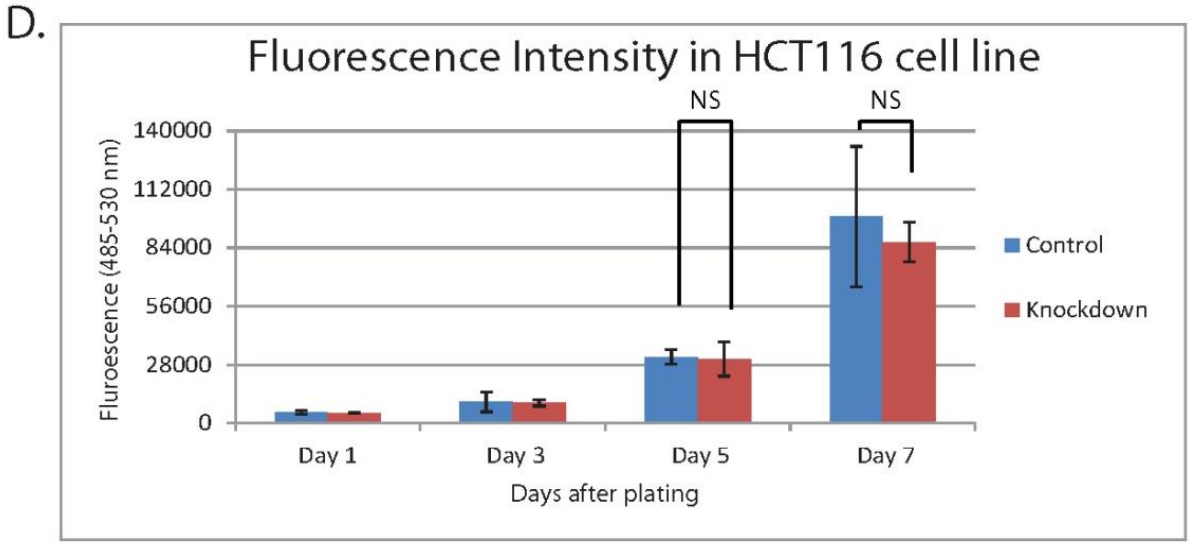


Figure 3.5: Cell proliferation analysis in human cancer cell lines expressing PP2Cβ knockdown. The proliferation rate of cells expressing PP2Cβ shRNA and non-targeted shRNA was examined using a cyQUANT® cell proliferation assay kit every 2 days over a period of 7 days. Four days after infection cells were counted using a haemocytometer and ~2,000 cells were plated into each well of transparent 96 well plates in triplicate. The cell proliferation was determined by comparing the fluorescence between cells infected with PP2Cβ shRNA (knockdown) and non-targeted shRNA (control). Examination of cell proliferation in **(A)** U2OS **(B)** MCF7 **(C)** H1299 and **(D)** HCT116 cell lines. The HCT116 cell line CyQUANT® analysis was carried out by Dr Jeong Park. Error bars are representative of two standard errors of the mean. The statistical significance of differences was calculated using the student t-test: *, $p < 0.01$; NS, not significant)

3.4.2 PP2Cβ knockdown increases the expression of p21 in MCF7 and U2OS cell lines

Although there is a decreasing trend in cell proliferation upon knockdown of PP2Cβ in U2OS and MCF7 human cancer cell lines (Fig. 3.5 A and B), the pathways activated by PP2Cβ depletion are unknown. In human fibroblasts, PP2Cβ knockdown resulted in increased expression of p21, a direct transcriptional target of p53 [1]. Therefore, alteration of p21 expression was investigated upon PP2Cβ knockdown eight days after infection in the human cancer cell lines. Furthermore a downstream target of p21; dephosphorylation of S807/811 of retinoblastoma (Rb) protein was also investigated. The protein expression analysis was performed on total cell lysate from each cell line (see section 2.2.4).

In all cell lines, with the exception of the HCT116, the small (43 kDa) and large (58 kDa) PP2Cβ isoforms were depleted compared to the cells infected with lentivirus expressing the non-targeted shRNA control. Depletion of the large PP2Cβ isoform in the HCT116 cell line however could not be confirmed as PP2Cβ expression, which was conducted by Dr Jeong Park, was analysed using an antibody for PP2Cβ that could only detect the small isoform of PP2Cβ (purchased from Santa Cruz).

Upon knockdown of PP2Cβ, there was an increase in the expression level of p53 in the U2OS cell line; however this increase in p53 was not observed in any of the other examined cell line. There was an increase in p21 expression seen in both U2OS and MCF7 cell lines while there was no increase in p21 expression in either the H1299 or HCT116 cell lines. These results correlate with the proliferation assay results as p21 is a known inhibitor of cell proliferation through inhibition of cyclin dependent kinases. There was also a notable decrease in total Rb and subsequently in Rb phosphorylated at S807/811 in the MCF7 cell line. However there was no clear change in the total or phosphorylated Rb levels in U2OS cells when comparing the protein levels of Rb and phospho-Rb with the unequal level of β-actin. This was unusual as increased p21 expression usually correlates with Rb dephosphorylation at S807/811 or a decrease in total RB levels.

As there was no increase in expression of p21 in H1299 cells it is tempting to speculate that PP2Cβ depletion sensitivity in MCF7 and U2OS cell lines was- at least in part- a result of p53-dependent stress-response activation. However despite expression of wildtype p53 in the HCT116 cell line, there was no increase in p21 expression, correlating with the proliferation assay. It is possible that p53 activation is being blocked upstream of PP2Cβ in the HCT116 cell line.

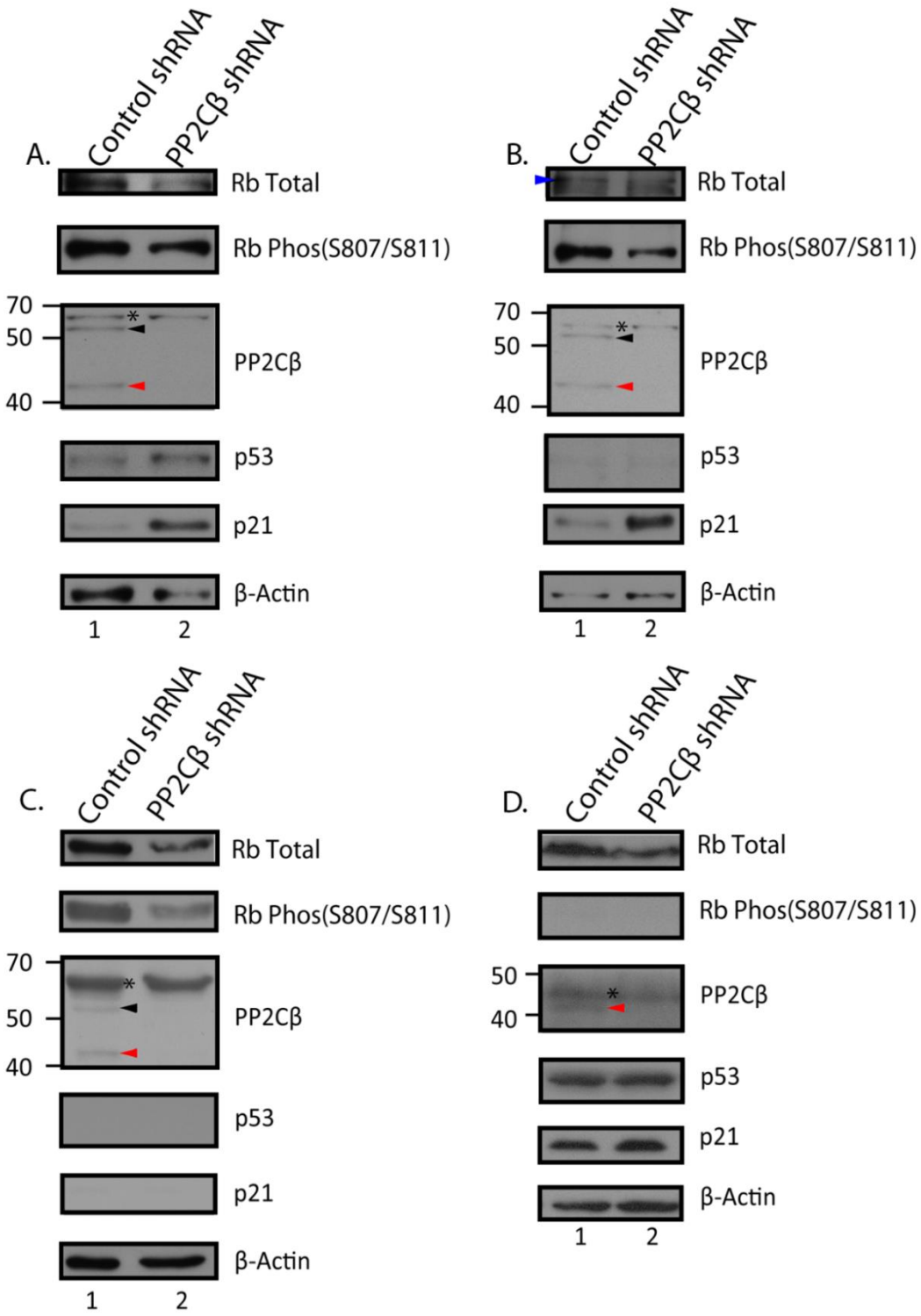


Figure 3.6: PP2Cβ knockdown protein analysis in human cancer cell lines. Whole cell lysate from human cancer cells lines infected with lentivirus expressing non-targeted shRNA (control) or PP2Cβ shRNA (knockdown) was prepared 8 days after infection. 25 μg of cell extract was separated on 15% to 4% gradient polyacrylamide gel. Membranes were examined for p21 and p53 proteins which were detected using antibodies with β-actin antibody as a loading control. The phosphorylation of retinoblastoma (Rb) was examined using Rb-P

(S807/811) antibody and total Rb was used as a secondary loading control. **(A)** U2OS cell lysate from cells expressing (lane 1) non-targeted shRNA; or (lane 2) PP2Cβ shRNA. Rb total is indicated with the arrow. **(B)** MCF7 cell lysate from cells expressing (lane 1) non-targeted shRNA; or (lane 2) PP2Cβ shRNA. Rb total is indicated with the blue arrow. **(C)** H1299 cell lysate from cells expressing (lane 1) non-targeted shRNA; or (lane 2) PP2Cβ shRNA. For A-C the membranes were examined using α-PP2Cβ which detected the small (43 kDa) and large (58 kDa) PP2Cβ isoforms, which are indicated by the black and red arrows, respectively. Non-specific binding is indicated by the asterisk (*). **(D)** HCT116 western blot was performed by Dr Jeong Park. Lysate from HCT116 cells expressing (lane 1) non-targeted shRNA; or (lane 2) PP2Cβ shRNA. Antibody for PP2Cβ used here could only detect the small PP2Cβ isoform (indicated by black arrow).

3.5 MCF7 and U2OS cells do not undergo permanent growth arrest

PP2Cβ knockdown in IMR-90 cells showed clear stress-signalling activation through induction of cellular senescence and increased p53 and p38MAPK pathway activation [1]. While it is clear that p53 pathway activation is occurring in U2OS and MCF7 cell lines, the growth arrest is not as severe as that seen in the IMR-90 cell line under the same conditions. The milder stress-response seen in U2OS and MCF7 cell lines may have resulted from the inactivation of the INK4a/ARF locus (Fig. 3.7 B). Both $p16^{INK4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in humans) (hereafter referred to as p16 and p14 respectively) have demonstrated critical roles in the establishment and maintenance of cellular senescence [52, 55, 152, 153] (see sections 1.3.2 and 1.3.3). The importance of p16 and p14 in establishing cellular senescence appears to be dependent on cell type. Homozygous deletion of p14, but not p16 caused murine fibroblasts to become immortal [154] while knockdown of p16, but not p14 immortalised mammalian epithelial cells [155]. Given the role of p14 in establishing cellular senescence is primarily due to its role as an inhibitor of p53 degradation, and that p53 pathway activation is clearly occurring in MCF7 and U2OS cells, as evidenced by the increase in p21 expression, it is likely that the loss of p16 is essential for the senescent response seen in IMR-90 cells. Given the importance of p16 in establishing cellular senescence, is it possible the loss of the INK4A/ARF locus may provide an explanation as to why cellular senescence was not seen in U2OS and MCF7 cell lines.

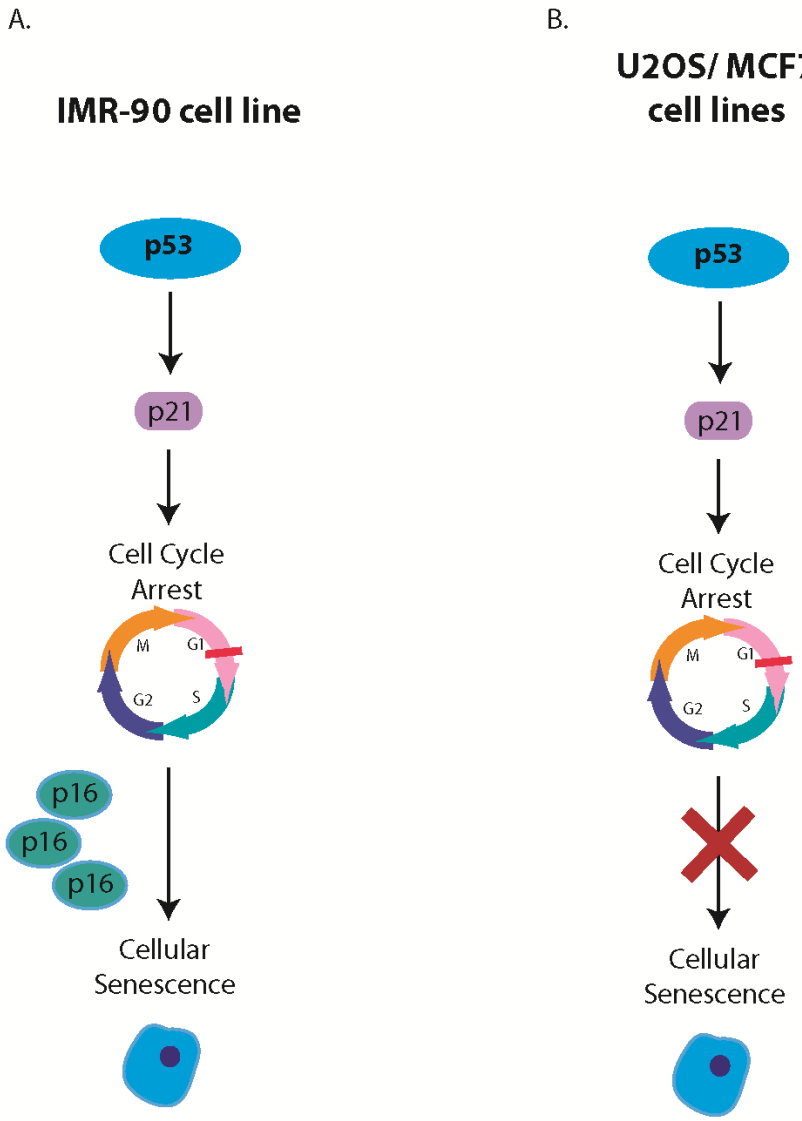


Figure 3.7: A model to account for the lack of senescence seen in U2OS and MCF7 cell lines. **(A)** Upon knockdown of PP2Cβ in IMR-90 cell line, cellular senescence was established due to the expression of p16. **(B)** However in MCF7 and U2OS cells which do not express p16, knockdown of PP2Cβ did not establish cellular senescence and instead resulted in a milder response through cell cycle arrest. This response could potentially be due to a lack of p16 expression.

3.6 PP2Cβ depleted HCT166 cell line did not experience p53 activation

Knockdown of PP2Cβ in IMR-90, MCF7 and U2OS cell lines resulted in increased expression of p21 and an arrest in cell proliferation, whereas no such response was seen in wildtype p53 expressing HCT116 cell line. It was hypothesised that the lack of

response to PP2Cβ knockdown in HCT116 was the result of expression of a hyperactive K-Ras mutant (Fig. 3.8).

Ras is a proto-oncogene which is essential in many signalling pathways within the cell, including cell cycle progression. Ras forms a feedback loop with p53, increasing p53 expression through p14, which is essential for p53 activation in response to Ras [82]. A mouse study demonstrated that in the absence of p14, constitutive expression of a downstream target of Ras, Raf, increased the expression of MDM2, inhibiting p53 accumulation. However when a constitutively active form of Raf was induced in the presence of p14, p53 accumulated in the cell [156]. The INK4A/ARF locus is lost in both U2OS and MCF7 cell lines suggesting that p16 and p14 are not required for p53 stabilisation by PP2Cβ knockdown in these particular cell lines. However as Ras-driven p53 expression is dependent on p14 it is possible that loss of the INK4A/ARF locus, coupled with the hyperactivity of Ras in HCT116 cells prevented the activation of p53 and subsequently the arrest cell proliferation despite PP2Cβ knockdown (Fig 3.8 B).

Unfortunately because both HCT116 and H1299 cell lines express hyperactive Ras mutants, no conclusions can be drawn in terms of the p53 dependent nature of PP2Cβ depletion driven cell proliferation arrest seen in IMR-90 [1], U2OS or MCF7 cell lines. This would need to be addressed in future experiments (see section 5.3.3)

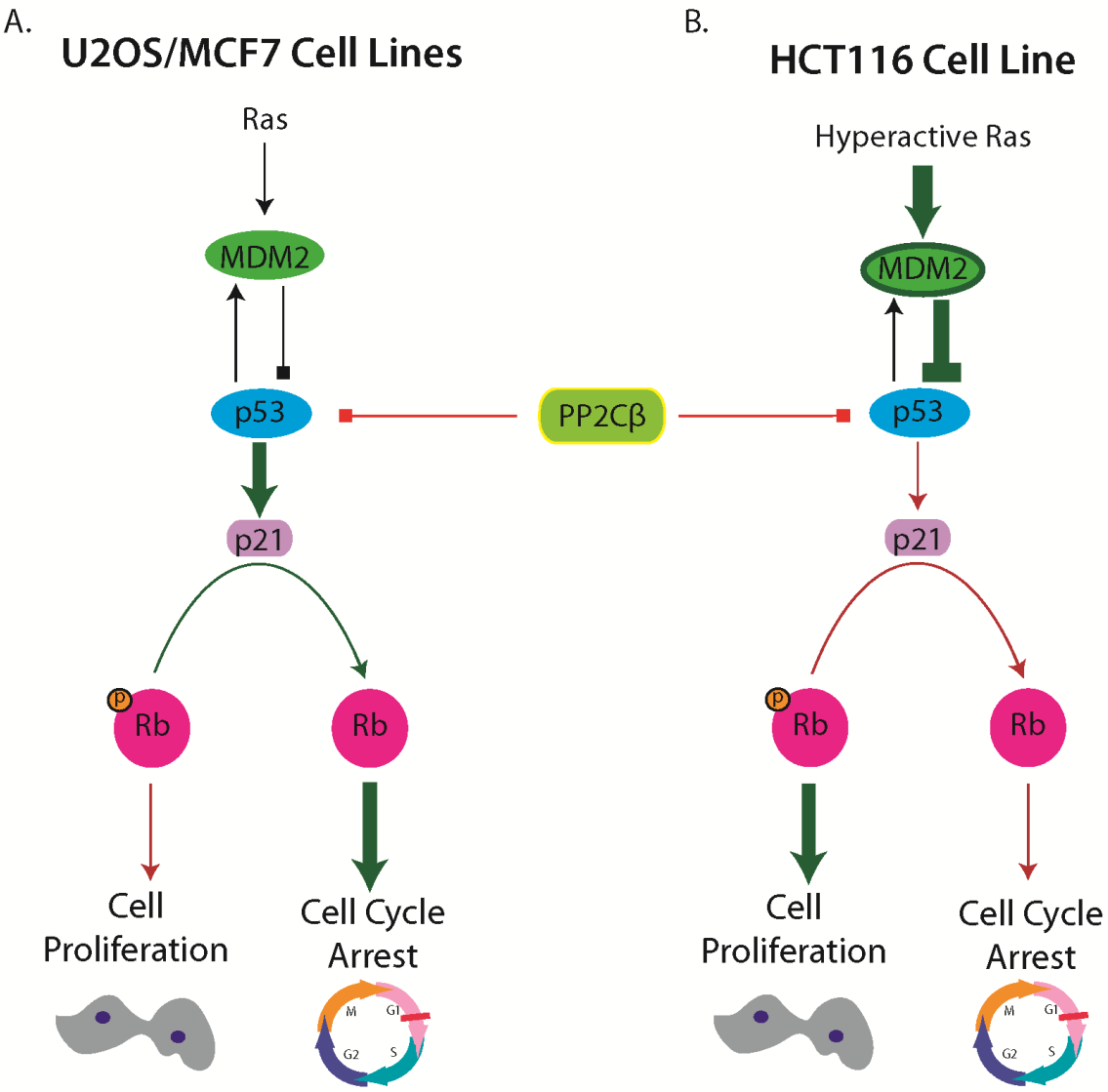


Figure 3.8: Model to compare the cellular response seen in p53 expressing HCT116 and U2OS/MCF7 cell lines. **(A)** Knockdown of PP2Cβ in U2OS and MCF7 cell lines resulted in an increase in p53 expression, driving the cell towards cell cycle arrest. **(B)** HCT116 cell line expresses a hyperactive form of Ras, which drives the expression of MDM2, resulting in upstream inhibition of p53. This upstream inhibition overrides the effect PP2Cβ knockdown, resulting in cell proliferation instead of cell cycle arrest.

3.7 Knockdown of PP2Cβ in U2OS cell line resulted in floating cells

Several days after selection, U2OS cells infected with lentiviral particles expressing PP2Cβ shRNA often had a large proportion of floating cells compared to cells infected with the lentivirus expressing non-targeted shRNA. As previously discussed (see section 1.4.4), PP2Cβ knockdown in mouse embryonic fibroblasts caused induction of Rip3

dependent necroptosis [116]. However this was very unlikely to account for the floating cells as U2OS cells are Rip3 deficient [157]. Another possible explanation was PP2Cβ knockdown was inducing apoptosis, possibly through p53 activation. As p53 pathways were active in U2OS cells as evidenced by the protein analysis (section 3.4.2), the transcriptional activation of p53-activated apoptotic genes were investigated using an RT-q PCR.

3.7.1 Reverse Transcriptase-coupled quantitative real-time PCR

To investigate if apoptosis had occurred in U2OS cells upon knockdown of PP2Cβ, an RNA analysis of three p53-activated pro-apoptotic genes, PUMA, Caspase-7 and p27 were investigated in PP2Cβ knockdown and control U2OS cells. This was done in three biological replicates using reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR) of infected cells in triplicate. The RNA was detected and quantified using commercially purchased primers for p27, Caspase-7 and PUMA and housekeeping gene glucuronidase-β (GUSB). The expression fold difference of the pro-apoptotic genes in PP2Cβ knockdown cells was compared to U2OS cells infected with lentivirus expressing non-targeted shRNA to determine a gene expression base-line and the RNA expression was normalised against GUSB expression.

Caspase-7 is the only pro-apoptotic gene analysed that was statistically significantly up regulated (Fig 3.9). This increase in caspase-7 expression could be explained by the increased stability of p53 expression seen in the protein analysis of U2OS cells (Fig. 3.6 A). However whether this increased level of expression is enough to induce apoptosis in U2OS cells is unclear as caspase-7 activity must reach a threshold before the apoptosis programme is induced [158]. The increase in caspase-7 expression seen here is so marginal that no firm conclusions can be reached. There are many possible reasons besides apoptosis that could account for the cells floating in the medium. Therefore, it is still unclear if knockdown of PP2Cβ induces apoptosis in U2OS cells, and so must be investigated further, possibly through use of flow cytometry fluorescence activated cell sorting assay to identify whether cells are becoming apoptotic (see section 5.3.2).

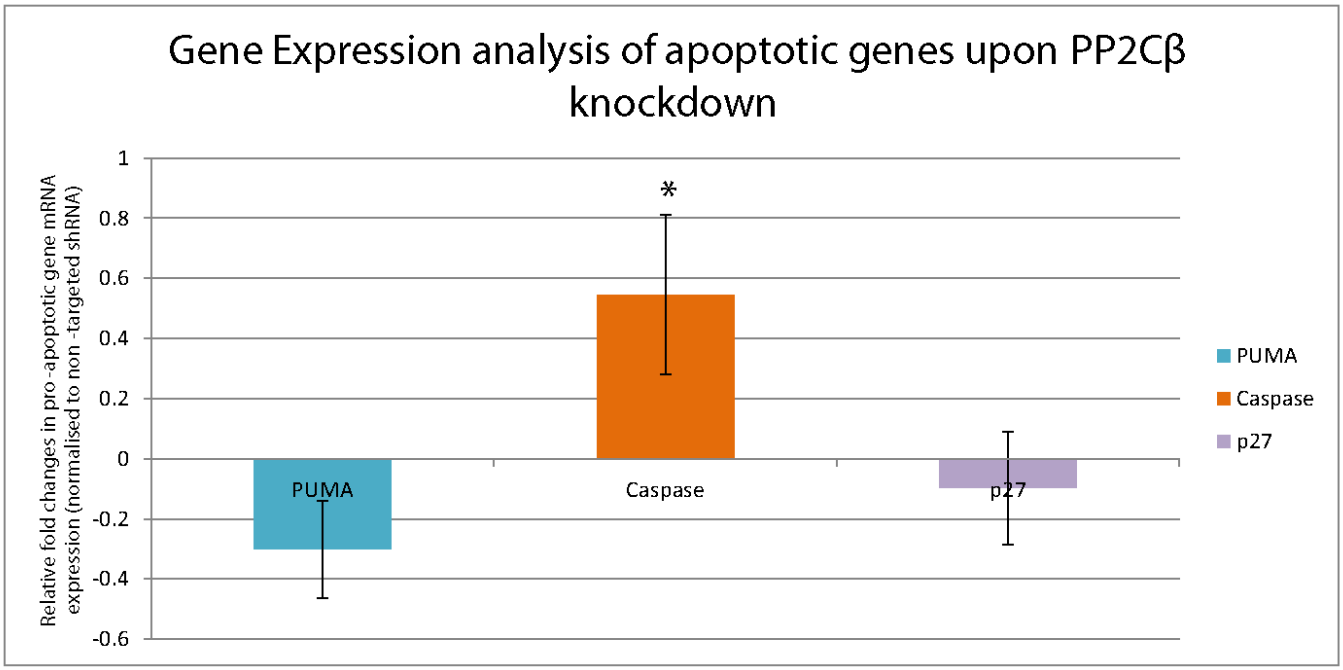


Figure 3.9: Expression analysis of pro-apoptotic genes in U2OS cell line. RNA was harvested 8 days after infection. Relative quantification in gene expression of apoptotic genes PUMA, Caspase-7 and p27 was determined by RT-qPCR in response to PP2Cβ knockdown in three biological replicated. Each sample was tested in duplicate and normalised against GUSB expression. Error bars are representative of two standard errors of the mean. The statistical significance of differences was calculated using the student t-test: *, $p < 0.05$.

Chapter 4: Roles of p53 Family Members in the Expression of PP2Cβ

4.1 Introduction

In the previous chapter PP2Cβ was identified as a novel target through which p53 activity could be restored in two human cancer cell lines. However, how PP2Cβ activity is modulated is not yet fully understood. Identifying and understanding how PP2Cβ expression is regulated could provide further insight into how p53 signalling pathways are regulated by PP2Cβ.

Wip1, a member of the PP2C family, and forms a negative feedback-loop with p53, where *Wip1* is transcriptionally activated by p53 and Wip1 enhances the interaction between p53 and MDM2 to inhibit p53 activity [98, 159, 160]. As Wip1 is a member of the PP2C family and is similar to PP2Cβ in terms of its structure, function and regulation it is possible that PP2Cβ expression could be regulated in a similar manner [160]. p53 was therefore investigated as a potential regulator of PP2Cβ expression. As well as p53, two other member of the p53 family, p63 and p73 were investigated as potential regulators of PP2Cβ expression. This was because full length p63 isoform, TAp63α was identified as a potential negative regulator of PP2Cβ gene expression through use of a modular relational database that integrated microarray results with a genome-wide search of p53 family member response elements [131]. Although no p73 isoforms were identified as potential regulators of PP2Cβ in this database, p63 and p73 share a ~85% homology between their DNA binding domains [161] and often share response elements and target genes [162]. Furthermore, full length p63 and p73 isoforms are capable of inducing cell cycle arrest, apoptosis and cellular senescence both dependently and independently of p53 [137, 163, 164]. Taken together these observations prompted an investigation into PP2Cβ expression in response to overexpressed p53 family members.

Transient mammalian expression vectors were constructed to constitutively express the members of the p53 family. A transient expression vector for p53 (pCDNA-FLAG-p53) was previously constructed and was available in the laboratory in which p53 was constitutively expressed by the cytomegalovirus (CMV) promoter and tagged with FLAG for easy protein detection. To ensure observations were comparable between each of the p53 family members, p63 or p73 were constructed into a CβF transient expression vector containing a CMV promoter and FLAG epitope sequence.

In order to determine whether p53 family members could alter PP2Cβ expression, the investigation was carried out in a p53 wildtype (wt) (U2OS) and a p53-null (H1299) human cancer cell line. This was because p53 family members are known to interact both directly and indirectly with each other to drive cell fate decisions [137, 138]. Western blot analyses of total cell lysate and gene expression analyses were performed to determine if p53, p63 or p73 could alter the expression of PP2Cβ in either a p53 dependent or independent manner.

4.2 Construction of transient mammalian expression vectors for p63 and p73

4.2.1 Screening CβF constructs

Escherichia coli strain DH5α was transformed (see section 2.2.8.6) with the ligation reactions between full length p63 or p73 and the CβF vector (see section 2.2.8.1) and plated onto LB/agar plates containing ampicillin (100 μg/mL). The β-Lactamase gene (Amp^r), encoded by the CβF vector ensured that only successfully transformed *E. coli* would grow into a colony. Candidate clones were screened (see section 2.2.8.7) by two-step sequential digestion with *EcoRV*, followed by *HindIII* (see section 2.2.8.2). The digested DNA fragments were analysed by agarose gel electrophoresis and stained with ethidium bromide (Fig. 4.1).

The expected bands for the CβF-p63 and CβF-p73 constructs double digested with *EcoRV* and *HindIII* were 2.1 kb/ 4.7 kb and 1.9 kb/ 4.7 kb respectively. This matched the fragment sizes shown in Fig. 4.2 (Lane 3 and 8). Size comparisons were also made between the digested construct (Fig. 4.1, Lane 3 and 8), linearised

CβF (Lane 5) and gel-purified *p63* and *p73* cDNA (Fig. 4.2 Lane 4 and 9). The size of the expected vector portion from the *EcoRV*/*HindIII* digested construct is identical to the linearized CβF (Lane 5). There is a slight difference in the migration of the gel-purified *p63* and *p73* cDNA compared to the lower bands of the digested constructs (Lane 3 and 4 & Lane 8 and 9 Fig. 4.1). However this difference could be explained by a higher concentration of PCR amplified cDNA, causing a migration shift on the agarose gel. To further confirm that p63 and p73 were correctly cloned into the CβF vector, the p63 and p73 cDNA was sequenced by Marcogen® (Appendices VI and VII) using the same primers that were used for the PCR amplification of *p63* and *p73* cDNA (Table 2.5). By comparing the reference sequences of p63 and p73 (obtained from NCBI) to the sequencing data, it was confirmed that PCR of p63 and p73 cDNA did not result in any undesired mutations (see appendices VI and VII).

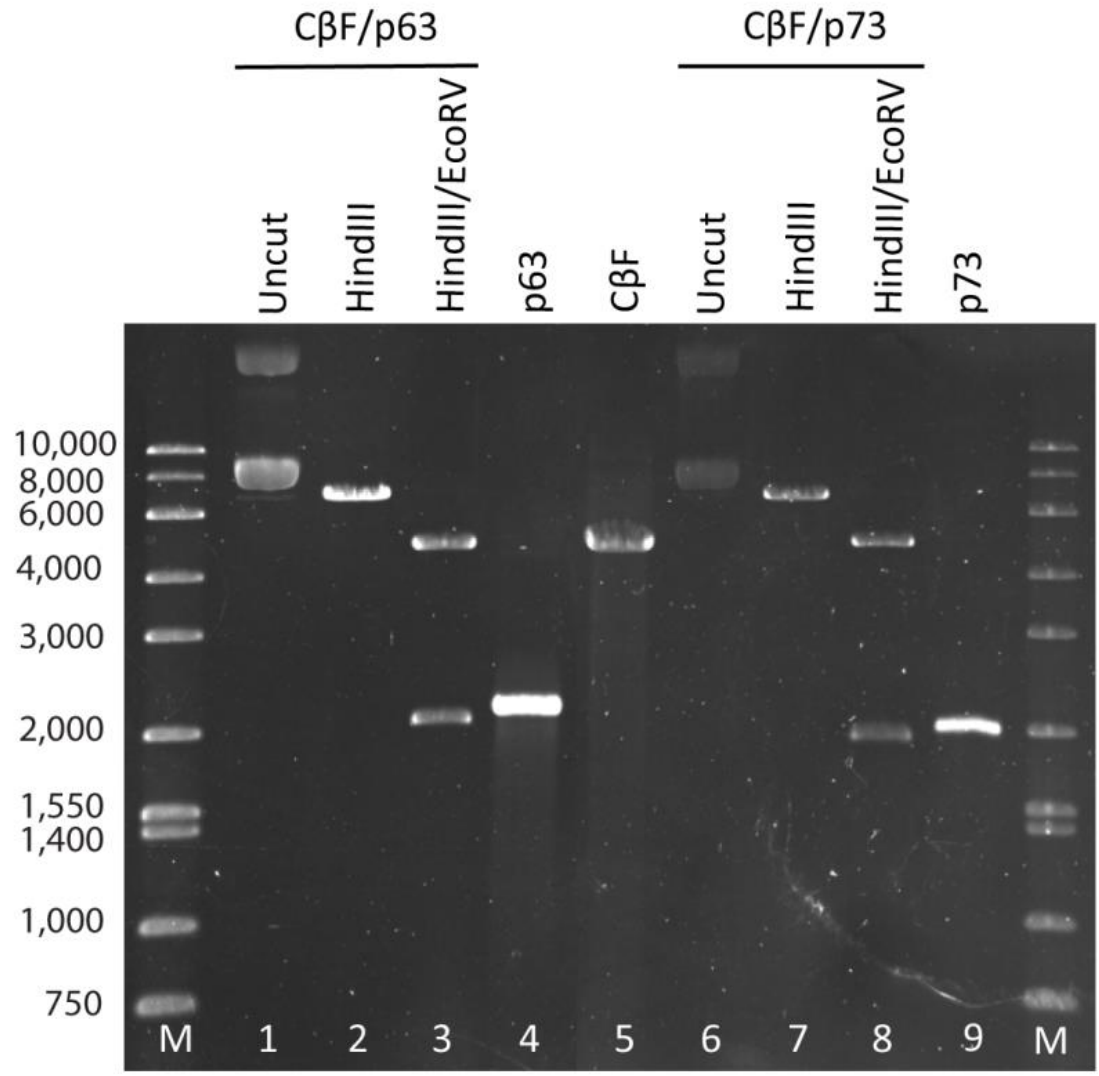


Figure 4.1: Screening digests of CβF-p63 and CβF-p73 constructs with *EcoRV* and *HindIII*. 100 ng of digested CβF-p63 and CβF-p73 construct DNA was separated by agarose gel electrophoresis on a 1% agarose gel alongside linearized empty CβF vector and gel purified p63 and p73 cDNA. M: DNA molecular weight marker. Lane 1: CβF-p63, uncut. Lane 2: CβF-p63 *HindIII* digested. Lane 3: CβF-p63 *HindIII*/*EcoRV* digested. Lane 4: Gel purified PCR amplified *p63*. Lane 5: Linearized CβF digested with *HindIII*/*EcoRV*. Lane 6: CβF-p73 uncut. Lane 7: CβF-p73 *HindIII* digested. Lane 8: CβF-p73 *HindIII*/*EcoRV* digested. Lane 9: Gel purified PCR amplified *p73*.

4.3 Confirmation of ectopic p63 and p73 expression in HEK 293T cells

Correct expression of the p53 family member constructs was confirmed in the human embryonic kidney (HEK) 293T cell line. Human embryonic kidney (HEK)

293T cell line is a fast-growing cell line that is easily and inexpensively transfected using calcium phosphate. For these reasons, HEK293T cells were used to confirm the correct expression of p53 family members constructs.

4.3.1 Confirming correct expression for p53 family members

HEK 293T cells were grown in 10 cm plates at ~60% confluence and were transfected using calcium phosphate method with either the empty CβF vector (as a negative control), *CβF-p63* or *CβF-p73* (see section 2.2.2.1). The cell lysate was harvested 48 h after transfection and was subjected to SDS-PAGE followed by western blot. α-FLAG antibody was utilised to detect p63 and p73 expression (see section 2.2.4).

Expression of FLAG-tagged p63 and p73 proteins were detected at their predicted molecular weights of 78 kDa and 69 kDa respectively (Fig. 4.2, Lane 1&2 respectively). Furthermore, no α-FLAG tagged proteins were detected in the empty vector control (Fig. 4.2, Lane 3), indicating that the α-FLAG antibody exclusively binds to transiently expressed FLAG-p63 and FLAG-p73. This confirms the gene delivery system successfully introduced the expression constructs into HEK 293T cells and the ectopically expressed proteins were at the expected molecular weights.

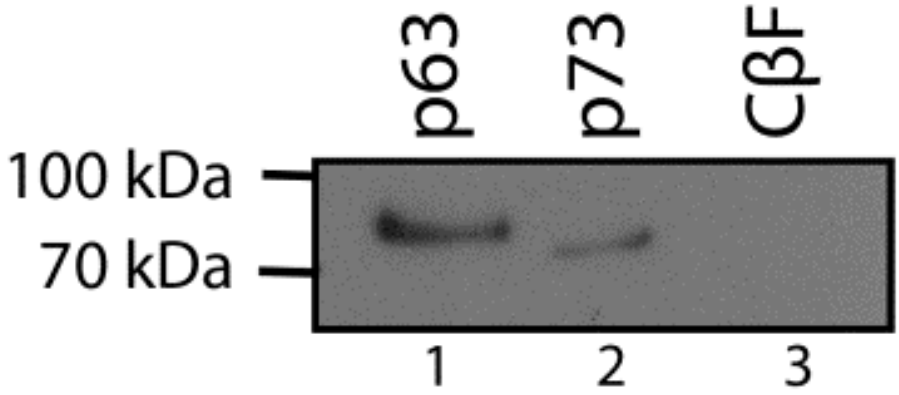


Figure 4.2: Confirming expression of p63 and p73 constructs in HEK293T cells. Whole cell lysate prepared from HEK293T cells transfected with empty CβF, CβF-p63 or CβF-p73 expression vectors were separated by SDS-PAGE gel at 40 mA for 1 hr and then transferred onto a charged PVDF membrane at 150 mA for 2 h. Detection of Flag tagged p63 and p73 was done using α-FLAG antibody. Lane 1: CβF-p63. Lane 2: CβF-p73. Lane 3: Empty CβF

4.4 PP2Cβ expression in response to p53 family members in U2OS and H1299 cell lines

The HEK293T cell line is not an appropriate cell line to characterise signalling pathways, as p53 signalling is defective in HEK293T cells, which stably express the SV40 large T antigen. As SV40 is a known inhibitor of p53 and Rb-family of tumour suppressors, HEK293T cells should not be used in a study where p53 expression and signalling pathways are to be observed [165]. As p53 family members have been shown to interact together to drive cell fate decisions, it was important to investigate PP2Cβ expression in response to p53 family members in a $p53^{wt}$ and p53-null human cancer cell line. To this order, two cancer cell lines were investigated: $p53^{wt}$ U2OS osteosarcoma and p53-null H1299 non-small cell lung carcinoma.

4.4.1 Transfection strategy and optimisation

Optimising the transfection strategy was necessary for examining the effect of PP2Cβ expression in response to p53 family members. DNA:FuGENE ratios were tested by examining the expression of p53 family members 48 h after transfection by western blot analysis with β-actin as a loading control. The predicted molecular weight of each FLAG-tagged p53 family member is as follows:

FLAG-p53: 45 kDa

FLAG-p63: 78 kDa

FLAG-p73: 69 kDa

The ratios of DNA:FuGENE tested were 1:3 and 1:5 (see section 2.2.2.1). U2OS cells transfected with a 1:3 ratio (Fig. 4.3 Lanes 2, 4 and 6) all displayed low to undetectable levels of FLAG-tagged protein, compared to the 1:5 ratio (Fig. 4.3 Lanes 3, 5 and 7). Although transfection of H1299 cells was not optimised, the western blot analyses of H1299 show that there is a relatively high level of protein expression for p53, p63 and p73 48 h after transfection using the 1:5 ratio (Fig. 4.5).

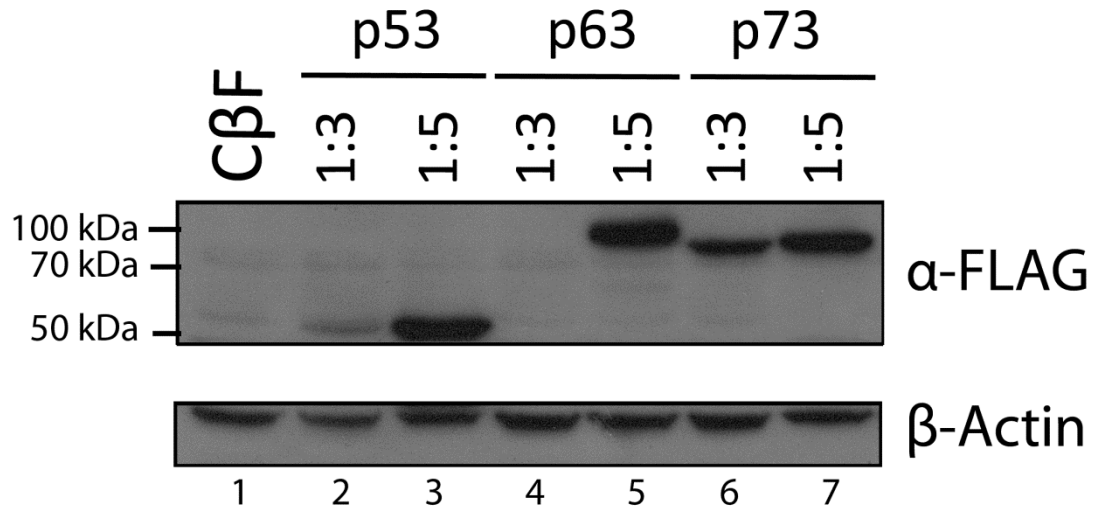


Figure 4.3: Western Blot analysis of U2OS cells to optimise transfection strategy. U2OS cells were transfected with p53 family member constructs using ratio of DNA: transfection reagent (FuGENE®) at either 1:3 or 1:5. 50 µg of whole cell lysate was extracted from U2OS cells 48 h after transfection and was run on a 10% polyacrylamide gel. Lane 1: Transfection with empty CβF at a ratio of 1:2.5. Lane 2&3: Transfection with pCDNA-p53 construct at a ratio of either 1:3 (lane 2) or 1:5 (lane 3). Lane 4&5: Transfection with CβF-p63 construct at a ratio of either 1:3 (lane 4) or 1:5 (lane 5). Lane 6&7: Transfection with CβF-p73 construct at a ratio of either 1:3 (lane 6) or 1:5 (lane 7).

4.4.2 PP2Cβ protein expression is not altered significantly by p53 family members

H1299 and U2OS cell lines were transiently transfected with the p53 family constructs using FuGENE® HD transfection reagent using the optimal DNA:FuGENE ratio which was found in section 4.4.1. To analyse the protein expression levels of PP2Cβ, the whole cell lysate of U2OS and H1299 cells was collected at 48 h and 72 h after transfection. To ensure equal protein loading for the western blot, the total protein concentration was determined using bradford protein assay (see section 2.2.3.3) and 25 µg of the total cell lysate was separated by SDS-PAGE and then blotted onto a PVDF membrane. The membranes were incubated with antibodies specific for FLAG (to detect FLAG-tagged p53 family members), PP2Cβ and β-actin overnight (see section 2.2.4.5).

Although ectopic expression of the p53 family members was detected 48 h after transfection in both U2OS and H1299, they were undetectable after 72 h in U2OS with p53 undetectable after 72 h in H1299 (Fig. 4.4 and 4.5). As a result of this, analysis of PP2Cβ protein expression in response to ectopic expression of p53 family members could only be accurately determined 48 h after transfection.

The protein levels of PP2Cβ after 48 h in both U2OS (Fig. 4.4) and H1299 cell lines (Fig. 4.5) were not significantly different upon overexpression of p53 family members (Fig. 4.4 and 4.5, Lane 2-4) compared to the control cells transfected with empty CβF vector (Fig. 4.4 and 4.5, Lane 1). However, a previous knockdown study of PP2Cβ (Jeong Park, *unpublished data*), identified PP2Cβ as a highly stable protein. This was because while a reserve transcriptase-quantitative coupled real-time PCR (RT-q PCR) analysis of PP2Cβ at >72 h after infection with constitutively expressed PP2Cβ shRNA confirmed a significant decrease in the expression of PP2Cβ mRNA, there was no significant reduction in level of PP2Cβ protein detected. This discrepancy suggests that PP2Cβ has a high degree of protein stability.

Therefore, if PP2Cβ protein expression was inhibited by p53 family members, any reduced expression would not be detectable until >72 h after transfection. As constitutive expression of p53 family members could not be detected at 72 h after transfection, any significant decrease in PP2Cβ protein expression would not be detectable.

In light of this, a protein analysis of PP2Cβ expression while using a transient expression construct is not a viable means for analysing PP2Cβ protein expression. As the regulation of PP2Cβ by p53 family members is speculated to be at the level of transcription, a gene expression analysis of PP2Cβ by RT-q PCR was instead employed to measure PP2Cβ expression.

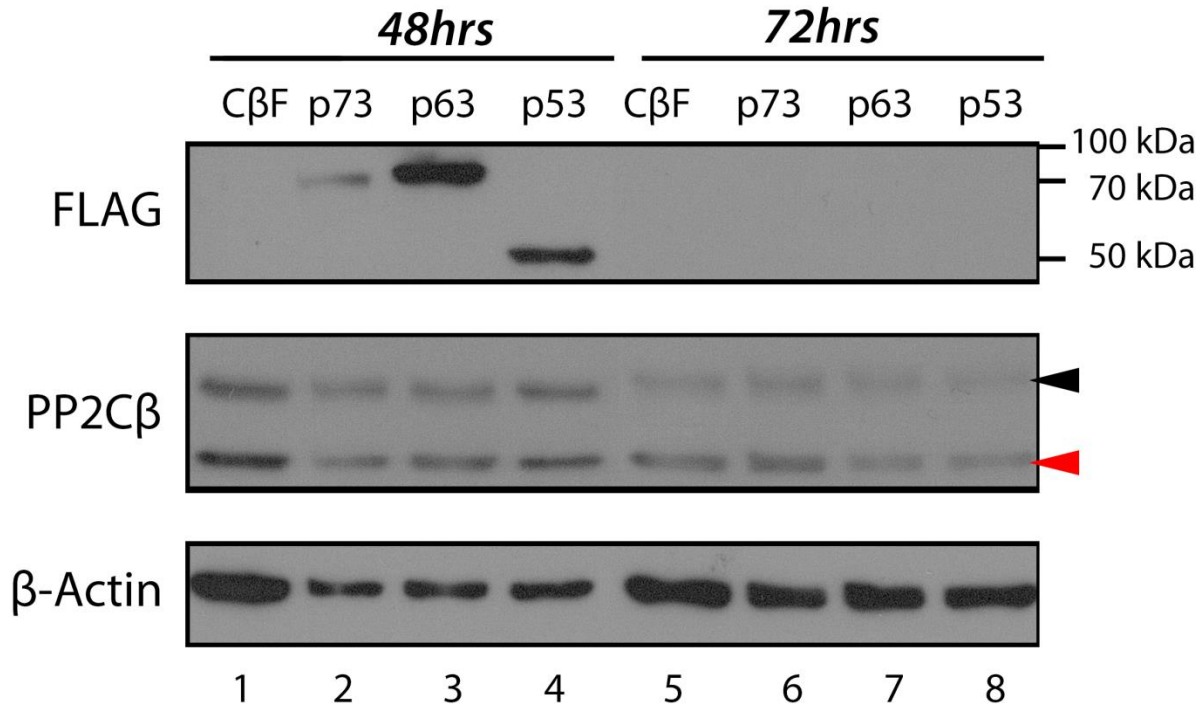


Figure 4.4: Western blot analysis of U2OS cell line transiently expressing p53 family members. 25 µg of whole cell lysate from U2OS cells constitutively expressing FLAG-tagged p53, p63 and p73 at 48 and 72 h post transfection was run on a 10% polyacrylamide gel. Lane 1 and 5: Transfection with Empty CβF vector over 48 and 72 h respectively as a negative control; Lane 2 and 6: Transfection with CβF-p73 expression vector over 48 and 72 h respectively; Lane 3 and 7: Transfection with CβF-p63 expression vector over 48 and 72 h respectively; Lane 4 and 8: Transfection with pCDNA-p53 expression vector over 48 and 72 h respectively. The α-PP2Cβ antibody used to examine expression of PP2Cβ could detect both the large (58 kDa) and small (43 kDa) PP2Cβ isoforms and are indicated by the black and red arrows respectively.

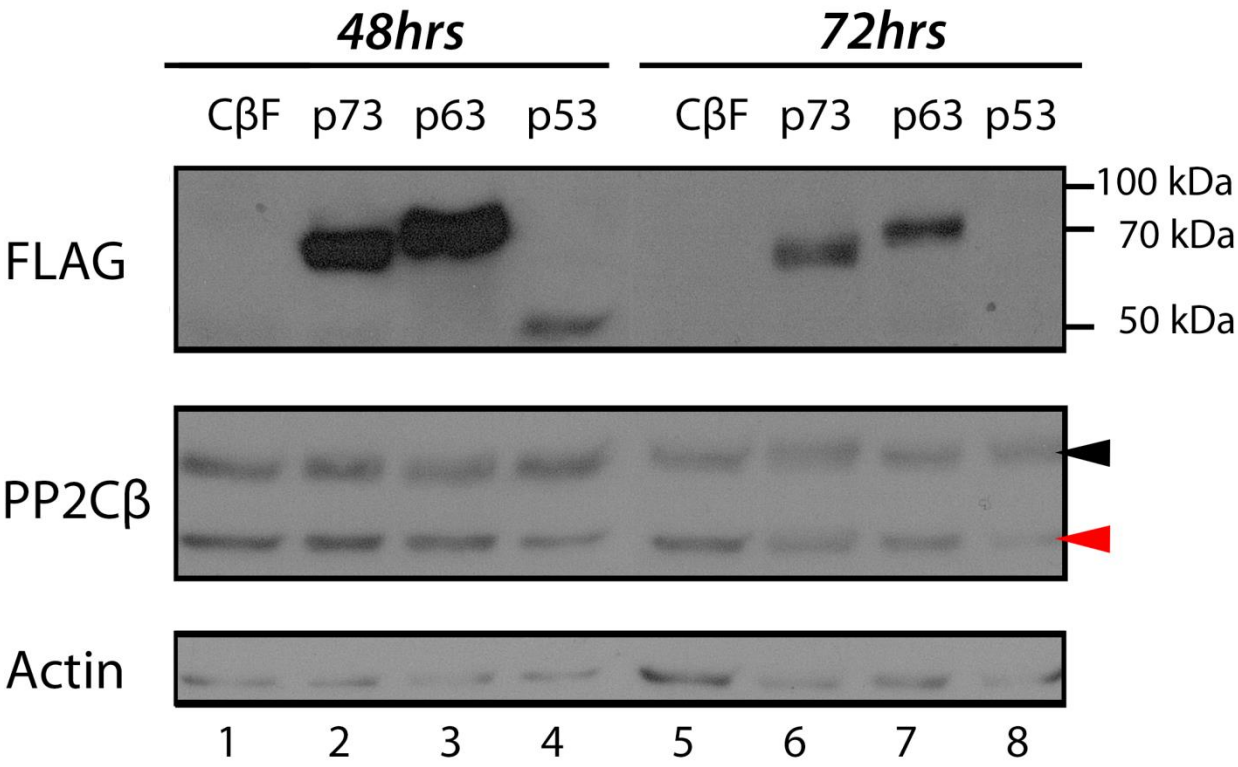


Figure 4.5: Western blot analysis of H1299 cell line transiently expressing p53 family members. 30 µg of whole cell lysate from H1299 cells constitutively expressing FLAG-tagged p53, p63 and p73 at 48 and 72 h post transfection was run on a 10% polyacrylamide gel. Lane 1 and 5: Transfection with Empty CβF vector over 48 and 72 h respectively as a negative control; Lane 2 and 6: Transfection with CβF/p73 expression vector over 48 and 72 h respectively; Lane 3 and 7: Transfection with CβF/p63 expression vector over 48 and 72 h respectively; Lane 4 and 8: Transfection with pCDNA/p53 expression vector over 48 and 72 h respectively. The α-PP2Cβ antibody used to examine expression of PP2Cβ could detect both the large (58 kDa) and small (43 kDa) PP2Cβ isoforms and are indicated by the black and red arrows respectively.

4.4.3 Reverse Transcriptase-coupled quantitative real time PCR analysis of PP2Cβ mRNA expression

A reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR), an extremely sensitive method for the detection and quantification of mRNA, was utilised to measure the expression of PP2Cβ mRNA in response to ectopic expression of p53 family members. RNA was extracted and purified from H1299 and U2OS cell lines 48 h after transfection with empty CβF vector and its derivatives expressing p53 family members (described in section 2.2.5.1).

Commercially purchased primers (Qiagen) were used to detect and quantify the fold change in PP2Cβ and house-keeping gene, glucuronidase-β (GUSB) expression. Cells transfected with the empty CβF expression vector were used to determine a gene expression base-line and the RNA expression was normalised against the house keeping gene, GUSB (see section 2.2.5.4). A protein analysis was carried out in tandem with the RT-qPCR to confirm expression of p53 family members (Fig. 4.4 and 4.5).

In the $p53^{wt}$ U2OS cell line (Fig. 4.6), there was a significant inhibition of PP2Cβ expression which decreased by a factor of 2-fold in cells transfected with p63. These data are consistent with the microarray study that identified a 2-fold decrease in PP2Cβ expression upon expression of full length p63 (TAp63α) [131]. However in the p53 negative cell line, H1299 (Fig. 4.7), PP2Cβ mRNA expression did not appear to be significantly altered by p63. These results suggest that transcriptional control of PP2Cβ by p63 may be dependent on native expression of wildtype p53. Furthermore PP2Cβ mRNA expression appeared to be inhibited in response to p53 overexpression in U2OS cells, though this was to a lesser extent than inhibition by p63. A student t-test conducted on PP2Cβ expression indicates that inhibition of PP2Cβ by p53 in H1299 cells and p73 in both cell lines was not statically significant. This is likely to be a result of discrepancies between biological replicates and therefore PP2Cβ expression is unlikely to be altered by p73.

The results presented here suggest a strong inhibition of PP2Cβ mRNA expression by p63 in $p53^{wt}$ U2OS cell line. Whether p53 can significantly inhibit PP2Cβ expression is debatable as inhibition of PP2Cβ RNA showed a less than 1 fold difference. Unfortunately this inhibition cannot be verified by western blot and thus cannot be confirmed while using a transient expression vector. Furthermore, RT-q PCR data presented here was only repeated twice for each treatment of individual cell lines and hence, the reproducibility remains to be verified. Additionally, gene expression was normalised against GUSB, which appears, upon further investigation to have been a bad choice of house-keeping gene to normalise the data against as regulation of GUSB is often unstable in cancer cell lines [166].

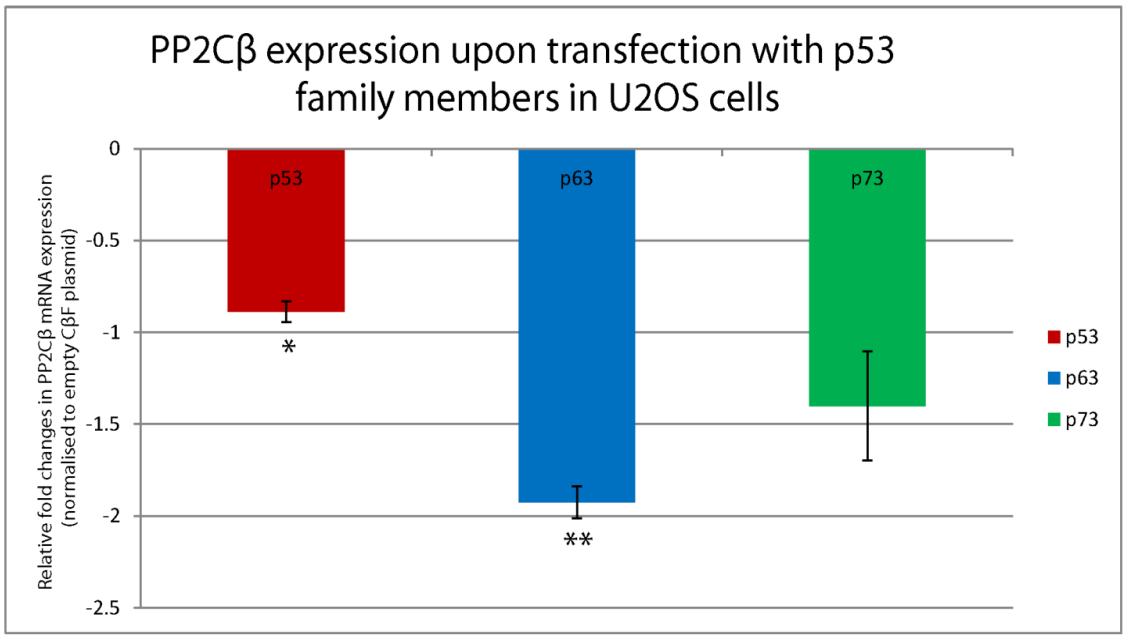


Figure 4.6: Relative fold change in PP2Cβ mRNA expression in response to p53 family members in U2OS cell line using RT-q PCR. RNA was collected 48 h after transfection of U2OS cells. Relative quantification of gene expression changes in PP2Cβ mRNA was measured in response to constitutive expression of p53, p63 or p73 and U2OS cells transfected with empty CβF plasmid were used to determine the gene expression base-line. Two biological replicates were carried out for each sample and each sample was tested in triplicate and normalised against GUSB house-keeping gene. Error bars are representative of two standard errors of the mean. The statistical significance of differences was calculated using the student's t-test: *p<0.05; **p<0.01)

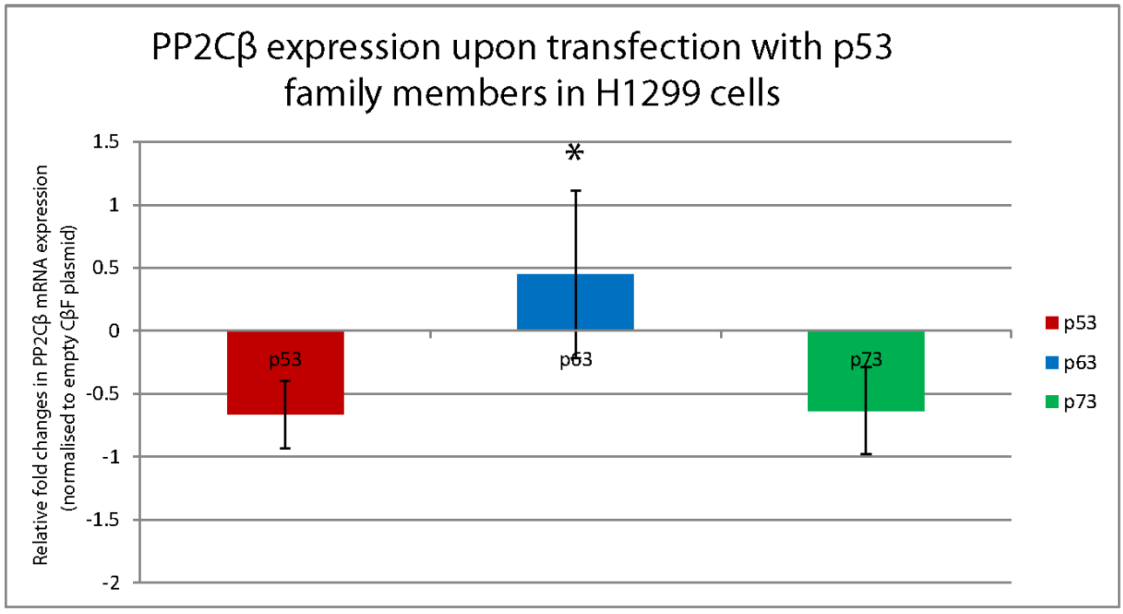


Figure 4.7: Relative fold change in PP2Cβ mRNA expression in response to p53 family members in H1299 cell line using RT-q PCR. RNA was collected 48 h after transfection of H1299 cells. Relative quantification of gene expression changes in PP2Cβ mRNA was measured in response to constitutive expression of p53, p63 or p73 and H1299 cells transfected with empty CβF plasmid were used to determine a gene expression base line. Two biological replicates were carried out for each sample and each sample was tested in triplicate and normalised to GUSB house-keeping gene. Error bars are representative of two standard errors of the mean. The statistical significance of differences was calculated using a student's t-test: *p<0.05.

4.4.4 PP2Cβ is a stable protein

Analysis of PP2Cβ mRNA expression through RT-q PCR identified p63 as an inhibitor of PP2Cβ mRNA expression in U2OS cells (Fig. 4.6). However protein analysis of PP2Cβ expression (Fig. 4.4) did not reflect this inhibition of PP2Cβ by p63. It was speculated that this was the result of high protein stability. As previously discussed, PP2Cβ was identified as being a relatively stable protein, with constitutive expression of PP2Cβ shRNA in the short term having no discernible effect on PP2Cβ protein expression until at least >72 h post infection (Jeong Park, *unpublished data*). Unfortunately, a major limitation of the experimental design implemented here was the transient expression of p53 family members. Transient expression resulted in non-sustainable expression of p53 family members up to 48 h. Therefore any decrease in PP2Cβ protein expression as a result of the p53 family members would have been undetectable. Therefore,

future experiments to test whether p53 family members negatively regulate PP2Cβ expression need to be investigated in a system capable of sustaining their expression over a long period of time (see section 5.3.4).

4.4.5 Functional interactions between p53 and p63 may drive PP2Cβ inhibition

There was a significant decrease in PP2Cβ mRNA expression in response to p63 overexpression in the U2OS cell line (Fig. 4.6). Although this finding supported the microarray data integrated into the modular relational database which identified TAp63α as an inhibitor of PP2Cβ expression [131], it does not explain why p63 did not alter PP2Cβ mRNA expression in H1299 cells. There are a number of possible reasons that may account for this observation; the first and foremost reason being that inhibition of PP2Cβ expression by p63 is p53-dependent. Although p63 is capable of controlling cell fate independently of p53 [167, 168], p53 and p63 have been shown to determine cell fate through functional interactions [137, 138].

A finding that p53-dependent apoptosis requires p63 expression in order to transcriptionally activate target genes in the developing brain of mice [137] is of particular interest as inhibition of p53 by PP2Cβ is implicated in glioma formation [115]. As p53 requires p63 in order to induce apoptosis in brain cells, it is possible that p63 may be required in part due to its ability to inhibit PP2Cβ mRNA expression.

How p63 inhibits PP2Cβ is still up to conjecture, however it is tempting to speculate that p53 and p63 may coordinate to alter PP2Cβ expression through RNA interference. Recently p63 was identified as suppressor of metastasis through transcriptional activation of *Dicer*, an enzyme which is necessary in the production of siRNA and miRNA [169]. Wild-type p53 has also been shown to promote miRNA maturation through its interaction with a miRNA processing complex, to achieve cell growth arrest [170]. Therefore it is possible that p53 and p63 may regulate PP2Cβ through their shared interactions with miRNA processing complexes to achieve tumour suppression.

Another possible reason that may account for inhibition of PP2Cβ by p63 in the U2OS but not the H1299 cell line could be due to the tissue specific tumour suppressor functions of p63. Carcinomas derived from p63-null mice were found to be more aggressive than carcinomas from p63 haploinsufficient mice and showed high levels of genomic instability while on the other hand, sarcomas derived from p63-null mice were less aggressive and displayed high levels of senescence [171]. As H1299 cells are derived from carcinoma and U2OS from sarcoma, it is possible inhibition of PP2Cβ in U2OS cells is cell-line dependent rather than p53 dependent.

Chapter 5: Summary, Limitations and Future Directions

5.1 Summary

Restoring p53 activity in human cancer cells through inhibition of p53 regulators has been a major aim of many research studies hoping to halt the progression of cancer. This study focuses on the inhibition of p53 by PP2Cβ and aims to investigate: 1) the potential of utilising PP2Cβ expression to activate p53 pathways in human cancer cell lines and 2) whether members of the p53 family can regulate PP2Cβ expression.

A previous study identified PP2Cβ as an inhibitor of p53 through its interaction with Gas41 [115]. Knockdown of PP2Cβ in human fibroblasts further demonstrated that PP2Cβ is an important inhibitor of cellular senescence through regulating the activity of p53 and to a smaller extent, p38MAPK [1]. This study sought to investigate whether PP2Cβ knockdown could induce cell proliferation arrest and p53 pathway activation in a variety of human cancer cell lines. Therefore PP2Cβ was knocked down in four established human cancer cell lines with various genetic backgrounds and the proliferation and protein expression of downstream targets of p53 were examined.

Two human cancer cell lines, U2OS and MCF7 both showed a decreasing trend in cell proliferation upon knockdown of PP2Cβ. This was supported by an increase in p21 expression upon PP2Cβ knockdown. However, the cells did not enter a senescent state upon PP2Cβ knockdown as reported for human fibroblasts [1]. In contrast H1299 and HCT116 cells expressing PP2Cβ shRNA did not show any significant difference in cell proliferation or any change in p21 expression, suggesting that p21 expression was being blocked by upstream effectors. The data presented here demonstrates that PP2Cβ knockdown alone is not a viable method for establishing cellular senescence in these human cancer cell lines.

The transient mammalian expression vectors CβF-p63 and CβF-73 were both successfully constructed to express full length p63 and p73 at the predicted molecular weights in HEK293T, U2OS and H1299 cell lines over a period of 48 h.

Full length p63 isoform, TAp63α was identified as a potential inhibitor of PP2Cβ expression through a modular relational database that integrated microarray results with a genome-wide search of p53-response elements. As p53 is known to alter the expression of WIP1, a member of the PP2C family with a similar structure and function to PP2Cβ, this study investigated whether PP2Cβ expression was altered by the full length p53 family isoforms. While this study could find no evidence that PP2Cβ protein expression was altered by p53 family members after 48 h, an RNA analysis suggested that PP2Cβ mRNA expression was negatively regulated by TAp63α in U2OS cells, but not H1299 cells. As H1299 and U2OS cells are derived from epithelial and non-epithelial cells respectively and p63 is known to behave differently in epithelial cells, this study cannot conclude that negative regulation of PP2Cβ mRNA by TAp63α is p53 dependent. The change in PP2Cβ mRNA expression has not previously been reported *in vivo* and warrants further investigation.

5.2 Limitations

This study was extremely limited by the design of many experiments. First and foremost there were a number of discrepancies between results, which made them difficult to replicate. The age and the passaging of the all of the cell lines used in this study are unknown and likely varied considerably. As the age and passaging of cells often accounts for random differences between replicates, it is possible this played a part in the difficultly of replicating the experiments. It could also account for the difficulties experienced when producing lentiviral particles.

5.2.1 Cell proliferation assay

As previously addressed in Chapter 3 (see section 3.4.1) the CyQUANT® cell proliferation assay was difficult to replicate. The difficulty of reproducing the experiments was believed to be due in part to discrepancies that cannot be accounted for such as the movement of plates that occurs every week for general

maintenance of the incubators. This environmental control is further exacerbated when the cells are loaded into 4 plates for the cyQUANT® analysis over 7 days. This exposes the cells to different environments that may account for the variability in the experiments. Ideally an assay to measure cell proliferation would need to be done on the same population of cells within one plate over a period of days during which the plate is left undisturbed (see section 5.3.2).

There were also a number of discrepancies between the fluorescence measurements of triplicates which often varied considerably to each other, suggesting that there had not been an equal number of cells loaded into each well. However, another reason that might account for the differences in fluorescence could be due the use of transparent 96-well plates instead of the recommended black 96-well plates [172]. This could mean that the fluorescence was 'leaky', and may account for the variability in the fluorescence readings between replicates. Furthermore, a CyQUANT® assay does not take into account floating cells as they get washed off with PBS before freezing the plate at -80°C. As PP2Cβ knockdown seemed to increase the number of floating cells in the U2OS cell line, a CyQUANT® assay may not be the best method of analysing the proliferation of cells expressing PP2Cβ shRNA.

5.2.2 PP2Cβ protein expression

Knockdown of PP2Cβ was found to increase p53 stability and the activity of p38MAPK [1]. This study does not address whether p38MAPK activation occurs in those cells that experienced a decrease in cell proliferation. Nor does it address whether PP2Cβ induces cell cycle arrest or apoptosis. Although an attempt was made to determine whether PP2Cβ knockdown resulted in an increase in gene expression of pro-apoptotic genes through an RNA analysis, the analysis could not determine if apoptosis was being induced upon PP2Cβ knockdown.

Whether PP2Cβ protein expression changed in response to the overexpression of p53 family members could also not be confirmed through western blot as the nature of transient expression meant the effect of p53 family members on PP2Cβ expression could only be examined over a period of 48 h.

Additionally, originally the experiment was designed to investigate whether p53 family members could alter PP2Cβ expression in a $p53^{wt}$ and p53-negative cell lines, U2OS and H1299 respectively. However as U2OS and H1299 cells are not derived from the same cell type, no conclusions can be drawn as to the p53 dependent nature of the regulation of PP2Cβ by p53 family members.

5.3 Future Directions

5.3.1 PP2Cβ depletion using an inducible promoter

This study utilised constitutively expressed PP2Cβ shRNA to investigate the effects of PP2Cβ knockdown. Unfortunately there were a number of problems with PP2Cβ shRNA expression as discussed in section 3.3 where PP2Cβ knockdown failed despite successful lentiviral infection. Although increasing the volume of virus for infections did resolve this, over-infection of cell lines is an undesirable strategy to overcome this problem. It was speculated that the reason for this may be due to epigenetic silencing of the CMV promoter driving production of PP2Cβ shRNA, a common occurrence in embryonic stem cells [151].

Another limitation of the experimental design is the nature of constitutive expression of PP2Cβ shRNA. PP2Cβ knockdown in U2OS cells appeared to result in floating cells and an RT-q PCR of the adhering cells identified an increase in the mRNA of pro-apoptotic gene Caspase-7. As puromycin selection and PP2Cβ knockdown occurs simultaneously due to the constitutive expression of the PP2Cβ shRNA by the CMV promoter, it is difficult to identify if any cell death was due to knockdown of PP2Cβ. Therefore, for future experiments it would be advantageous to use an inducible promoter to drive production of PP2Cβ shRNA in the lentiviral vector.

5.3.2 Cell proliferation, cell cycle and death

As previously mentioned in the limitations section (see section 5.2.1), a CyQUANT® assay may not be the best method of assessing cell proliferation upon PP2Cβ knockdown as there are too many environmental variables to account for. Instead, future proliferation assays should instead be done using xCELLigence real-time cell analysis systems (ACEA Biosciences). This would provide a real-time method of monitoring the proliferation, morphology and anchorage of cells.

Furthermore this assay does not require the removal of the plate from the incubator, providing and a stable environment and enabling multiple analyses of the same population of cells over a period of several days. This would eliminate discrepancies between replicate samples and any differences between the numerous plates usually required for a CyQUANT® assay.

Furthermore, a flow cytometry fluorescence associated cell sorting (FACS) assay should be used to examine the cellular state in response to PP2Cβ knockdown. A FACS analysis would determine if knockdown of PP2Cβ results in a cell cycle arrest (and elucidate what phase the cell cycle is arrested in) or apoptosis. As discussed in section 3.7, a number of floating cells were seen in U2OS cells expressing PP2Cβ shRNA. Although an RNA analysis showed that Caspase-7 expression increased upon PP2Cβ knockdown, the increase was considered to be negligible. A flow cytometry FACS assay would be able to determine whether knockdown of PP2Cβ results in apoptosis.

5.3.3 Characterize the role of hyperactive mutant Ras and p53 in PP2Cβ depletion

Neither HCT116 nor H1299 cell lines experienced a decrease in cell proliferation upon knockdown of PP2Cβ. As discussed in section 3.6, the hyperactive Ras mutant present in both HCT116 and H1299 was suspected to play some role in the cell response, or lack of. Therefore no conclusions could be drawn about whether the cell proliferation arrest upon PP2Cβ knockdown was dependent on p53 activity. Therefore to further study this, U2OS and MCF7 cell lines should be co-infected with PP2Cβ shRNA and p53 shRNA to determine if the cell response is dependent on p53 expression. Furthermore, PP2Cβ knockdown U2OS and MCF7 cell lines should be infected with a hyperactive N-Ras or K-Ras mutant to analyse cell proliferation and activation p53 pathways.

5.3.4 Clone p53 family members into a stable mammalian expression lentiviral vector

A major weakness of the p53 overexpression study presented here is the short period of time they were overexpressed, due to the transient nature of their expression. As PP2Cβ is a stable protein, inhibition of PP2Cβ by TAp63α could not be confirmed with a protein analysis. Therefore to investigate if overexpression of

p53 family members can alter PP2Cβ expression, p53 family members need to be stably expressed. This could be achieved by cloning p53 family members into a lentiviral vector.

5.3.5 Inhibition of PP2CB by p63: is it a cell type or p53 dependent mechanism?

Originally p53 family members were expressed in both p53-negative and $p53^{wt}$ human cancer cell lines. However, although TAp63α did appear to inhibit PP2Cβ mRNA expression in U2OS cells, it could not be concluded that this effect was the result of p53 expression or the cell-type dependent manner in which p63 is known to behave. To determine of TAp63α inhibits PP2Cβ expression in a p53 dependent manner, p53 should be knocked down in the U2OS cell line allowing an examination of regulation of PP2Cβ by TAp63α in a p53 dependent and independent manner.

5.3.6 Study PP2Cβ knockdown in Brain Cancer cell lines

The interaction between p53 and PP2Cβ is dependent on the interaction between PP2Cβ and Gas41. As Gas41 is commonly overexpressed in glioma multiforme, it is likely that PP2Cβ is a potent inhibitor of p53 in gliomas. Therefore, the effect of PP2Cβ knockdown should be investigated in human derived glioma.

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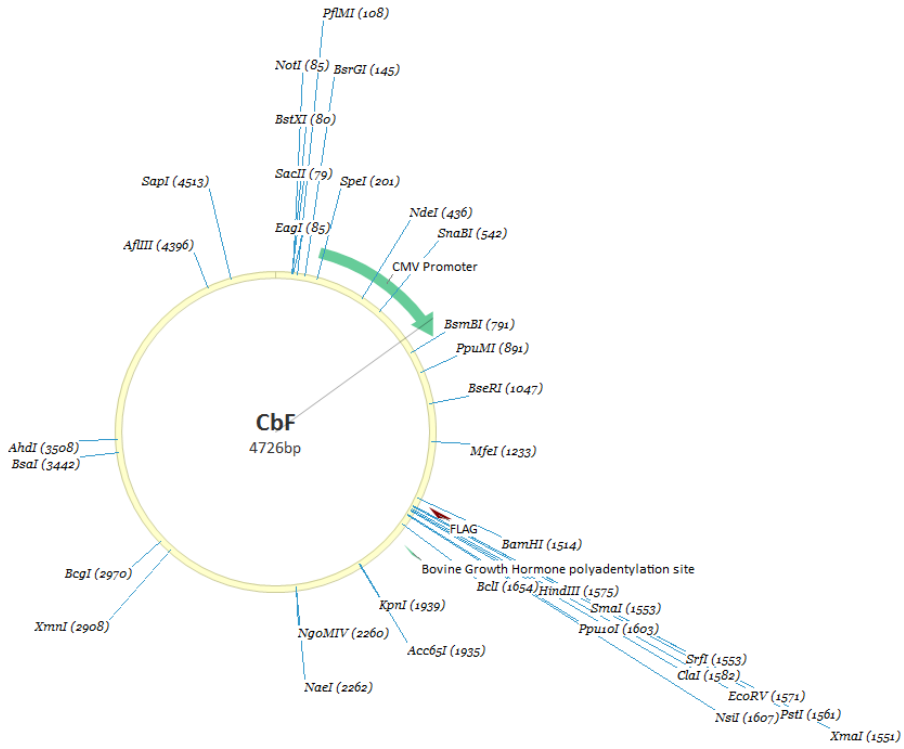
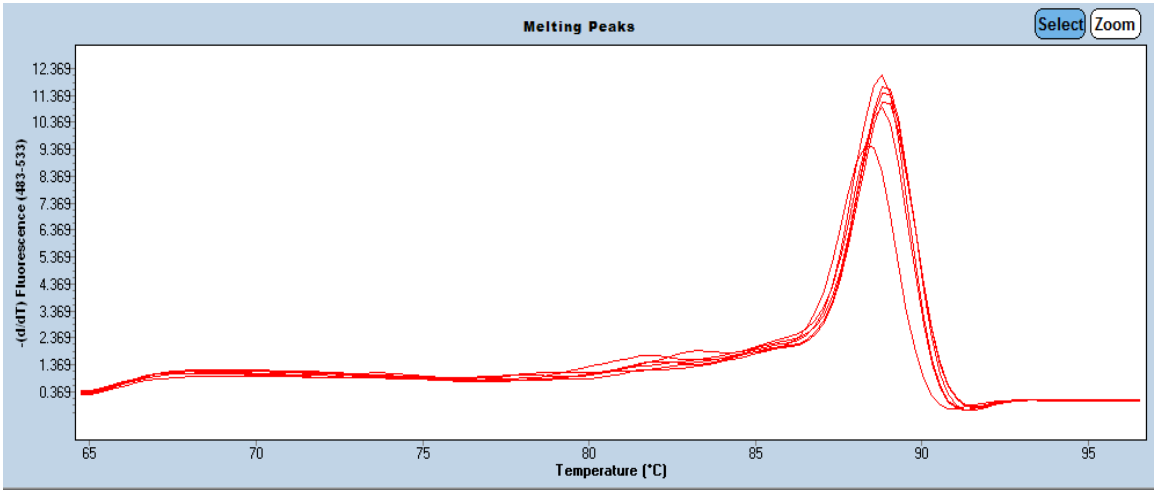
Appendices

Appendix I: A vector map of the CβF transient mammalian expression vector

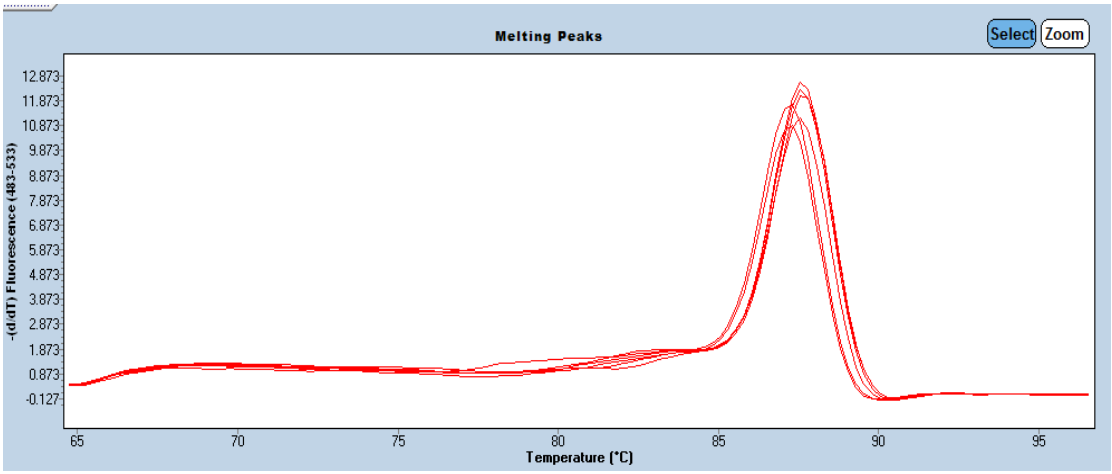
Vector map determined from Invitrogen Vector NTI software



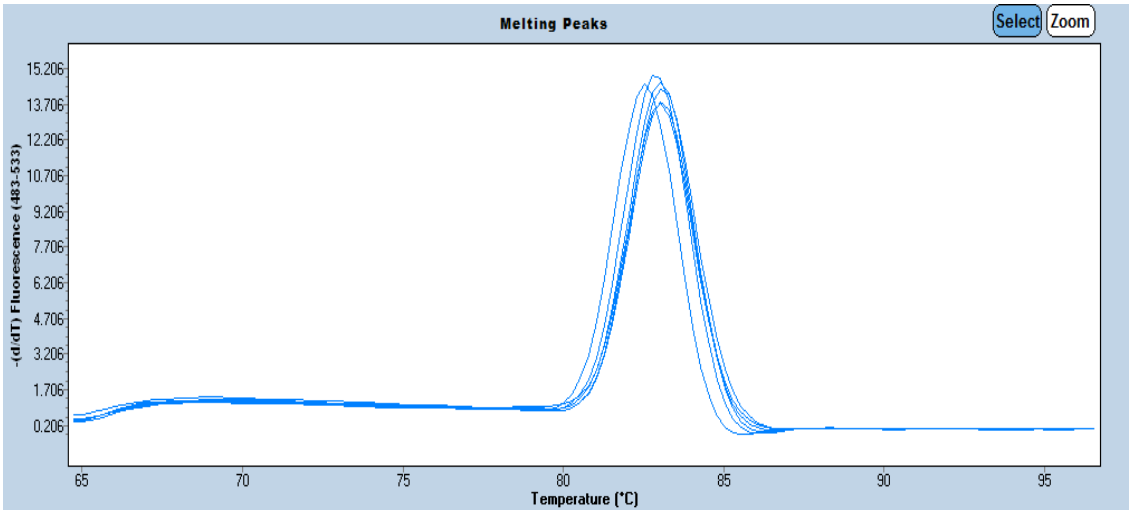
Appendix II: Melting peaks for Caspase-7



Appendix III: Melting peaks for PUMA

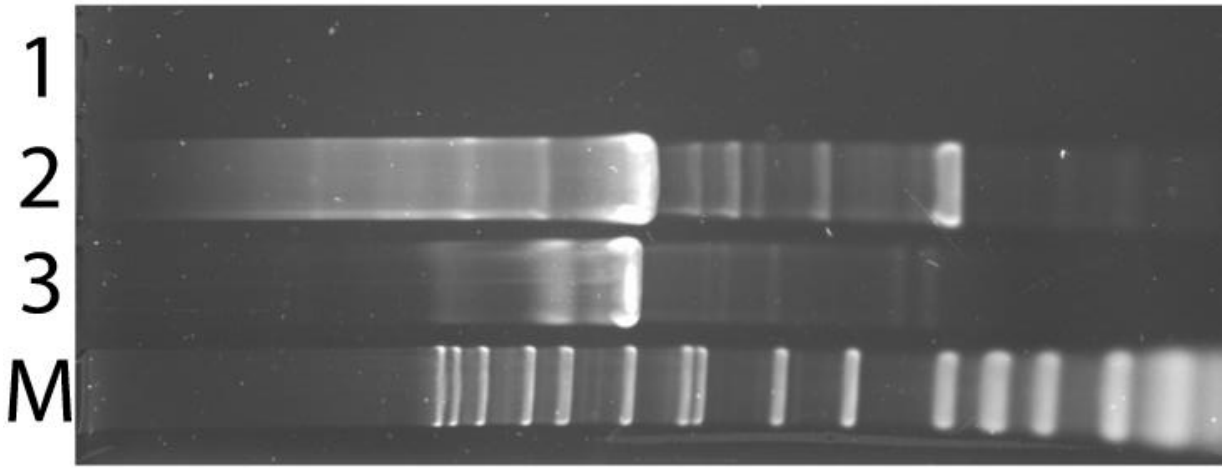


Appendix IV: Melting peaks for p27



Appendix V: PCR amplification of full length human p63 and p73 cDNA.

Commercially purchased constructs containing the cDNA for *p63* and *p73* were PCR amplified using primers carrying restriction enzyme cutsites on the flanking regions (see Table 2.2.1). A PCR amplification of *p63* (Lane 3) and *p73* (Lane 2) was carried out with a negative control containing only water (Lane 1). The PCR products were PCR purified and run on a 1% agarose gel at 120V for 1 hour. The full length size of *p63* cDNA is 2040bp and 1946bp for *p73* cDNA.



Appendix VI: Sequence alignment for *p63* cDNA

The reference sequence is indicated in black while the sequence obtained from Macrogen for the cDNA of *p63* is indicated in blue.

```
1       atgaattttgaaacttcacggtgtgccaccctacagtactgccctgaccc
        ||||||||||||||||||||||||||||||||||||||||||||||||||
1       atgaattttgaaacttcacggtgtgccaccctacagtactgccctgaccc


51      ttacatccagcgtttcgtagaaaccccagctcatttctcttggaaagaaa
        ||||||||||||||||||||||||||||||||||||||||||||||||||
51      ttacatccagcgtttcgtagaaaccccagctcatttctcttggaaagaaa


101     gttattaccgatccaccatgtcccagagcacacagacaaatgaattcctc
        ||||||||||||||||||||||||||||||||||||||||||||||||||
101     gttattaccgatccaccatgtcccagagcacacagacaaatgaattcctc


151     agtccagaggttttccagcatatctgggattttctggaacagcctatatg
        ||||||||||||||||||||||||||||||||||||||||||||||||||
151     agtccagaggttttccagcatatctgggattttctggaacagcctatatg


201     ttcagttcagcccattgacttgaactttgtggatgaaccatcagaagatg
        ||||||||||||||||||||||||||||||||||||||||||||||||||
201     ttcagttcagcccattgacttgaactttgtggatgaaccatcagaagatg


251     gtgcgacaaacaagattgagattagcatggactgtatccgcatgcaggac
        ||||||||||||||||||||||||||||||||||||||||||||||||||
251     gtgcgacaaacaagattgagattagcatggactgtatccgcatgcaggac


301     tcggacctgagtgaccccatgtggccacagtacacgaacctggggctcct
        ||||||||||||||||||||||||||||||||||||||||||||||||||
301     tcggacctgagtgaccccatgtggccacagtacacgaacctggggctcct


351     gaacagcatggaccagcagattcagaacggctcctcgtccaccagtccct
        ||||||||||||||||||||||||||||||||||||||||||||||||||
351     gaacagcatggaccagcagattcagaacggctcctcgtccaccagtccct


401     ataacacagaccacgcgcagaacagcgtcacggcgccctcgccctacgca
        ||||||||||||||||||||||||||||||||||||||||||||||||||
401     ataacacagaccacgcgcagaacagcgtcacggcgccctcgccctacgca


451     cagcccagctccaccttcgatgctctctctccatcacccgccatcccctc
        ||||||||||||||||||||||||||||||||||||||||||||||||||
451     cagcccagctccaccttcgatgctctctctccatcacccgccatcccctc


501     caacaccgactacccaggcccgcacagtttcgacgtgtccttccagcagt
        ||||||||||||||||||||||||||||||||||||||||||||||||||
501     caacaccgactacccaggcccgcacagtttcgacgtgtccttccagcagt


551     cgagcaccgccaagtcggccacctggacgtattccactgaactgaagaaa
        ||||||||||||||||||||||||||||||||||||||||||||||||||
551     cgagcaccgccaagtcggccacctggacgtattccactgaactgaagaaa
```

```
601    ctctactgccaaattgcaaagacatgccccatccagatcaaggtgatgac
       ||||||||||||||||||||||||||||||||||||||||||||||||||
601    ctctactgccaaattgcaaagacatgccccatccagatcaaggtgatgac


651    cccacctcctcagggagctgttatccgcgccatgcctgtctacaaaaaag
       ||||||||||||||||||||||||||||||||||||||||||||||||||
651    cccacctcctcagggagctgttatccgcgccatgcctgtctacaaaaaag


701    ctgagcacgtcacggaggtggtgaagcggtgccccaaccatgagctgagc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
701    ctgagcacgtcacggaggtggtgaagcggtgccccaaccatgagctgagc


751    cgtgaattcaacgagggacagattgcccctcctagtcatttgattcgagt
       ||||||||||||||||||||||||||||||||||||||||||||||||||
751    cgtgaattcaacgagggacagattgcccctcctagtcatttgattcgagt


801    agaggggaacagccatgcccagtatgtagaagatcccatcacaggaagac
       ||||||||||||||||||||||||||||||||||||||||||||||||||
801    agaggggaacagccatgcccagtatgtagaagatcccatcacaggaagac


851    agagtgtgctggtaccttatgagccaccccaggttggcactgaattcacg
       ||||||||||||||||||||||||||||||||||||||||||||||||||
851    agagtgtgctggtaccttatgagccaccccaggttggcactgaattcacg


901    acagtcttgtacaatttcatgtgtaacagcagttgtgttggagggatgaa
       ||||||||||||||||||||||||||||||||||||||||||||||||||
901    acagtcttgtacaatttcatgtgtaacagcagttgtgttggagggatgaa


951    ccgccgtccaattttaatcattgttactctggaaaccagagatgggcaag
       ||||||||||||||||||||||||||||||||||||||||||||||||||
951    ccgccgtccaattttaatcattgttactctggaaaccagagatgggcaag


1001   tcctgggccgacgctgctttgaggcccggatctgtgcttgcccaggaaga
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1001   tcctgggccgacgctgctttgaggcccggatctgtgcttgcccaggaaga


1051   gacaggaaggcggatgaagatagcatcagaaagcagcaagtttcggacag
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1051   gacaggaaggcggatgaagatagcatcagaaagcagcaagtttcggacag


1101   tacaaagaacggtgatggtacgaagcgcccgtttcgtcagaacacacatg
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1101   tacaaagaacggtgatggtacgaagcgcccgtttcgtcagaacacacatg


1151   gtatccagatgacatccatcaagaaacgaagatccccagatgatgaactg
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1151   gtatccagatgacatccatcaagaaacgaagatccccagatgatgaactg


1201   ttatacttaccagtgaggggccgtgagacttatgaaatgctgttgaagat
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1201   ttatacttaccagtgaggggccgtgagacttatgaaatgctgttgaagat


1251   caaagagtccctggaactcatgcagtaccttcctcagcacacaattgaaa
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1251   caaagagtccctggaactcatgcagtaccttcctcagcacacaattgaaa
```

```
1301   cgtacaggcaacagcaacagcagcagcaccagcacttacttcagaaacag
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1301   cgtacaggcaacagcaacagcagcagcaccagcacttacttcagaaacag


1351   acctcaatacagtctccatcttcatatggtaacagctccccacctctgaa
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1351   acctcaatacagtctccatcttcatatggtaacagctccccacctctgaa


1401   caaaatgaacagcatgaacaagctgccttctgtgagccagcttatcaacc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1401   caaaatgaacagcatgaacaagctgccttctgtgagccagcttatcaacc


1451   ctcagcagcgcaacgccctcactcctacaaccattcctgatggcatggga
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1451   ctcagcagcgcaacgccctcactcctacaaccattcctgatggcatggga


1501   gccaacattcccatgatgggcacccacatgccaatggctggagacatgaa
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1501   gccaacattcccatgatgggcacccacatgccaatggctggagacatgaa


1551   tggactcagccccacccaggcactccctcccccactctccatgccatcca
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1551   tggactcagccccacccaggcactccctcccccactctccatgccatcca


1601   cctcccactgcacacccccacctccgtatcccacagattgcagcattgtc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1601   cctcccactgcacacccccacctccgtatcccacagattgcagcattgtc


1651   agtttcttagcgaggttgggctgttcatcatgtctggactatttcacgac
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1651   agtttcttagcgaggttgggctgttcatcatgtctggactatttcacgac


1701   ccaggggctgaccaccatctatcagattgagcattactccatggatgatc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1701   ccaggggctgaccaccatctatcagattgagcattactccatggatgatc



1751   tggcaagtctgaaaatccctgagcaatttcgacatgcgatctggaagggc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1751   tggcaagtctgaaaatccctgagcaatttcgacatgcgatctggaagggc


1801   atcctggaccaccggcagctccacgaattctcctccccttctcatctcct
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1801   atcctggaccaccggcagctccacgaattctcctccccttctcatctcct


1851   gcggaccccaagcagtgcctctacagtcagtgtgggctccagtgagaccc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1851   gcggaccccaagcagtgcctctacagtcagtgtgggctccagtgagaccc


1901   ggggtgagcgtgttattgatgctgtgcgattcaccctccgccagaccatc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1901   ggggtgagcgtgttattgatgctgtgcgattcaccctccgccagaccatc
```

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1951   tctttcccaccccgagatgagtggaatgacttcaactttgacatggatgc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1951   tctttcccaccccgagatgagtggaatgacttcaactttgacatggatgc


2001   tcgccgcaataagcaacagcgcatcaaagaggaggggggagtga   2043
       |||||||||||||||||||||||||||||||||||||||
2001   tcgccgcaataagcaacagcgcatcaaagaggaggggggagtga   2043
```

Appendix VII: Sequence alignment for *p73* cDNA

The reference sequence is indicated in black while the sequence obtained from Macrogen for the cDNA of *p73* is indicated in blue.

```
1       atggcccagtccaccgccacctcccctgatgggggcaccacgtttgagca
        ||||||||||||||||||||||||||||||||||||||||||||||||||
1       atggcccagtccaccgccacctcccctgatgggggcaccacgtttgagca

51      cctctggagctctctggaaccagacagcacctacttcgaccttccccagt
        ||||||||||||||||||||||||||||||||||||||||||||||||||
51      cctctggagctctctggaaccagacagcacctacttcgaccttccccagt

101     caagccgggggaataatgaggtggtgggcggaacggattccagcatggac
        ||||||||||||||||||||||||||||||||||||||||||||||||||
101     caagccgggggaataatgaggtggtgggcggaacggattccagcatggac

151     gtcttccacctggagggcatgactacatctgtcatggcccagttcaatct
        ||||||||||||||||||||||||||||||||||||||||||||||||||
151     gtcttccacctggagggcatgactacatctgtcatggcccagttcaatct

201     gctgagcagcaccatggaccagatgagcagccgcgcggcctcggccagcc
        ||||||||||||||||||||||||||||||||||||||||||||||||||
201     gctgagcagcaccatggaccagatgagcagccgcgcggcctcggccagcc

251     cctacaccccagagcacgccgccagcgtgcccacccactcgccctacgca
        ||||||||||||||||||||||||||||||||||||||||||||||||||
251     cctacaccccagagcacgccgccagcgtgcccacccactcgccctacgca

301     caacccagctccaccttcgacaccatgtcgccggcgcctgtcatccccctc
        ||||||||||||||||||||||||||||||||||||||||||||||||||
301     caacccagctccaccttcgacaccatgtcgccggcgcctgtcatccccctc

351     caacaccgactaccccggaccccaccactttgaggtcactttccagcagt
        ||||||||||||||||||||||||||||||||||||||||||||||||||
351     caacaccgactaccccggaccccaccactttgaggtcactttccagcagt

401     ccagcacggccaagtcagccacctggacgtactccccgctcttgaagaaa
        ||||||||||||||||||||||||||||||||||||||||||||||||||
401     ccagcacggccaagtcagccacctggacgtactccccgctcttgaagaaa

451     ctctactgccagatcgccaagacatgccccatccagatcaaggtgtccac
        ||||||||||||||||||||||||||||||||||||||||||||||||||
451     ctctactgccagatcgccaagacatgccccatccagatcaaggtgtccac

501     cccgccacccccaggcactgccatccgggccatgcctgtttacaagaaag
        ||||||||||||||||||||||||||||||||||||||||||||||||||
501     cccgccacccccaggcactgccatccgggccatgcctgtttacaagaaag

551     cggagcacgtgaccgacgtcgtgaaacgctgccccaaccacgagctcggg
        ||||||||||||||||||||||||||||||||||||||||||||||||||
551     cggagcacgtgaccgacgtcgtgaaacgctgccccaaccacgagctcggg
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601    agggacttcaacgaaggacagtctgctccagccagccacctcatccgcgt
       ||||||||||||||||||||||||||||||||||||||||||||||||||
601    agggacttcaacgaaggacagtctgctccagccagccacctcatccgcgt

651    ggaaggcaataatctctcgcagtatgtggatgaccctgtcaccggcaggc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
651    ggaaggcaataatctctcgcagtatgtggatgaccctgtcaccggcaggc

701    agagcgtcgtggtgccctatgagccaccacaggtggggacggaattcacc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
701    agagcgtcgtggtgccctatgagccaccacaggtggggacggaattcacc

751    accatcctgtacaacttcatgtgtaacagcagctgtgtaggggcatgaa
       ||||||||||||||||||||||||||||||||||||||||||||||||||
751    accatcctgtacaacttcatgtgtaacagcagctgtgtaggggcatgaa

801    ccggcggcccatcctcatcatcatcaccctggagatgcgggatgggcagg
       ||||||||||||||||||||||||||||||||||||||||||||||||||
801    ccggcggcccatcctcatcatcatcaccctggagatgcgggatgggcagg

851    tgctgggccgccggtcctttgagggccgcatctgcgcctgtcctggccgc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
851    tgctgggccgccggtcctttgagggccgcatctgcgcctgtcctggccgc

901    gaccgaaaagctgatgaggaccactaccgggagcagcaggccctgaacga
       ||||||||||||||||||||||||||||||||||||||||||||||||||
901    gaccgaaaagctgatgaggaccactaccgggagcagcaggccctgaacga

951    gagctccgccaagaacggggccgccagcaagcgtgccttcaagcagagcc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
951    gagctccgccaagaacggggccgccagcaagcgtgccttcaagcagagcc

1001   cccctgccgtccccgcccttggtgccggtgtgaagaagcggcggcatgga
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1001   cccctgccgtccccgcccttggtgccggtgtgaagaagcggcggcatgga

1051   gacgaggacacgtactaccttcaggtgcgaggccgggagaactttgagat
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1051   gacgaggacacgtactaccttcaggtgcgaggccgggagaactttgagat

1101   cctgatgaagctgaaagagagcctggagctgatggagttggtgccgcagc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1101   cctgatgaagctgaaagagagcctggagctgatggagttggtgccgcagc

1151   cactggtggactcctatcggcagcagcagcagctcctacagaggccgagt
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1151   cactggtggactcctatcggcagcagcagcagctcctacagaggccgagt

1201   cacctacagcccccgtcctacgggccggtcctctcgcccatgaacaaggt
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1201   cacctacagcccccgtcctacgggccggtcctctcgcccatgaacaaggt

1251   gcacgggggcatgaacaagctgccctccgtcaaccagctggtgggccagc
       ||||||||||||||||||||||||||||||||||||||||||||||||||
1251   gcacgggggcatgaacaagctgccctccgtcaaccagctggtgggccagc
```

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1301   ctccccccgcacagttcggcagctacacccaacctggggcccgtgggcccc
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1301   ctccccccgcacagttcggcagctacacccaacctggggcccgtgggcccc


1351   gggatgctcaacaaccatggccacgcagtgccagccaacggcgagatgag
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1351   gggatgctcaacaaccatggccacgcagtgccagccaacggcgagatgag


1401   cagcagccacagcgcccagtccatggtctcggggtcccactgcactccgc
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1401   cagcagccacagcgcccagtccatggtctcggggtcccactgcactccgc


1451   cacccccctaccacgccgaccccagcctcgtcagtttttttaacaggattg
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1451   cacccccctaccacgccgaccccagcctcgtcagtttttttaacaggattg


1501   gggtgtccaaactgcatcgagtatttcacctcccaagggttacagagcat
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1501   gggtgtccaaactgcatcgagtatttcacctcccaagggttacagagcat


1551   ttaccacctgcagaacctgaccattgaggacctgggggccctgaagatcc
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1551   ttaccacctgcagaacctgaccattgaggacctgggggccctgaagatcc


1601   ccgagcagtaccgcatgaccatctggcggggcctgcaggacctgaagcag
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1601   ccgagcagtaccgcatgaccatctggcggggcctgcaggacctgaagcag


1651   ggccacgactacagcaccgcgcagcagctgctccgctctagcaacgcggc
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1701   caccatctccatcggcggctcaggggaactgcagcgccagcgggtcatgg
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1701   caccatctccatcggcggctcaggggaactgcagcgccagcgggtcatgg


1751   aggccgtgcacttccgcgtgcgccacaccatcaccatccccaaccgcggc
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1751   aggccgtgcacttccgcgtgcgccacaccatcaccatccccaaccgcggc


1801   ggcccaggcggcggccctgacgagtgggcggacttcggcttcgacctgcc
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1801   ggcccaggcggcggccctgacgagtgggcggacttcggcttcgacctgcc


1851   cgactgcaaggcccgcaagcagcccatcaaggaggagttcacggaggccg
       ||||||||||||||||||||||||||||||||||||||||||||||||||||
1851   cgactgcaaggcccgcaagcagcccatcaaggaggagttcacggaggccg


1901   agatccactga
       ||||||||||||
1901   agatccactga
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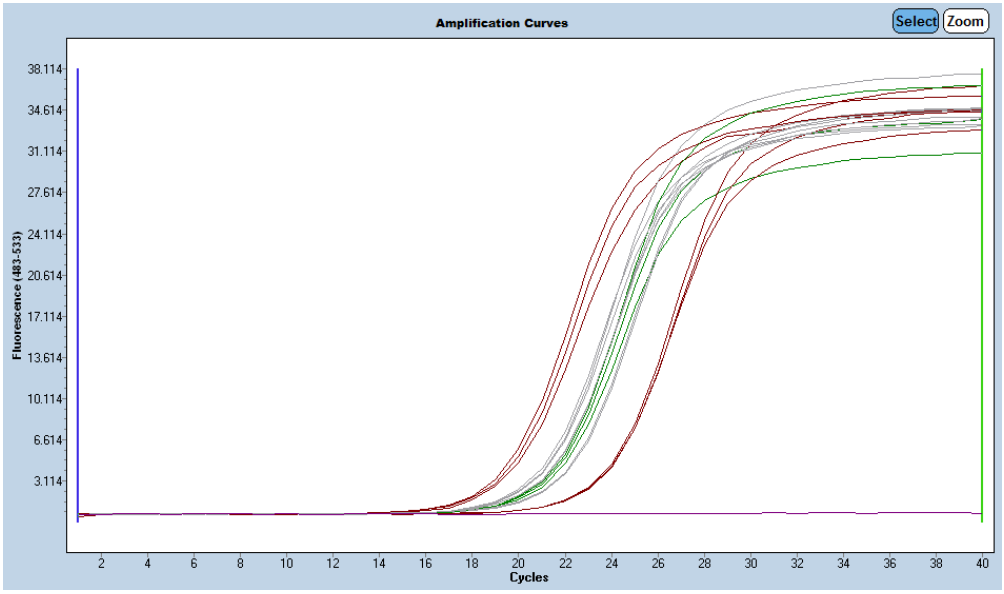
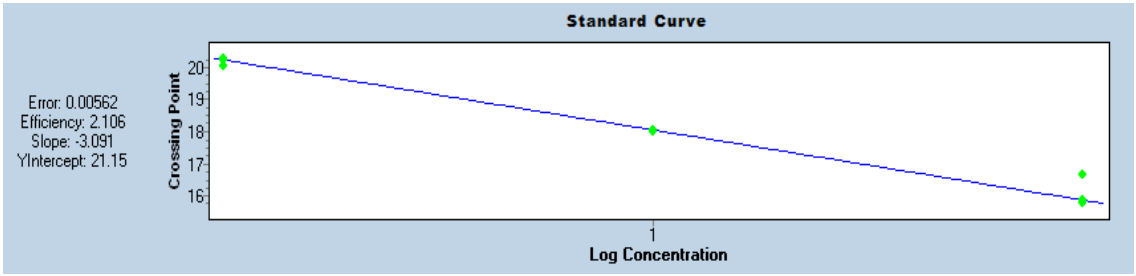
Appendix VIII: Amplification curves, standard curve and melting peaks for PP2Cβ Efficiency in U2OS cells

A.



B.



C.

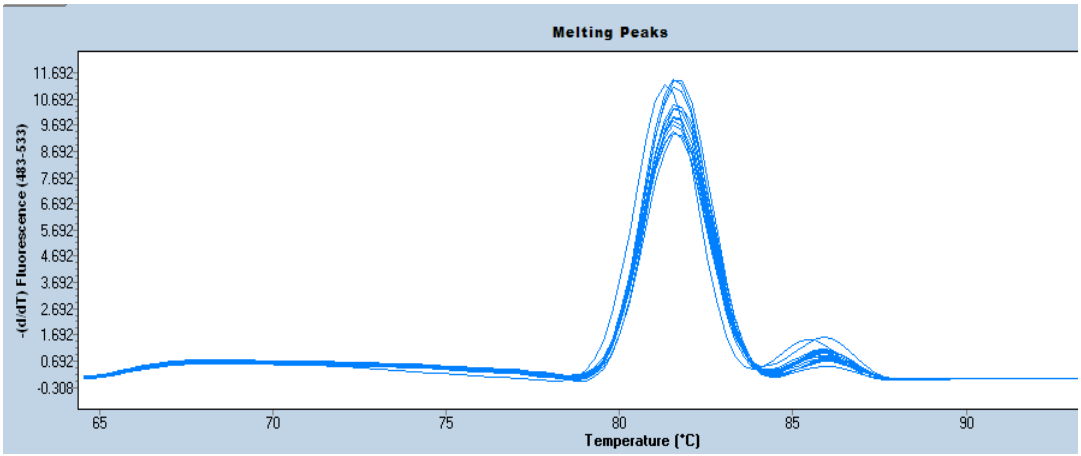
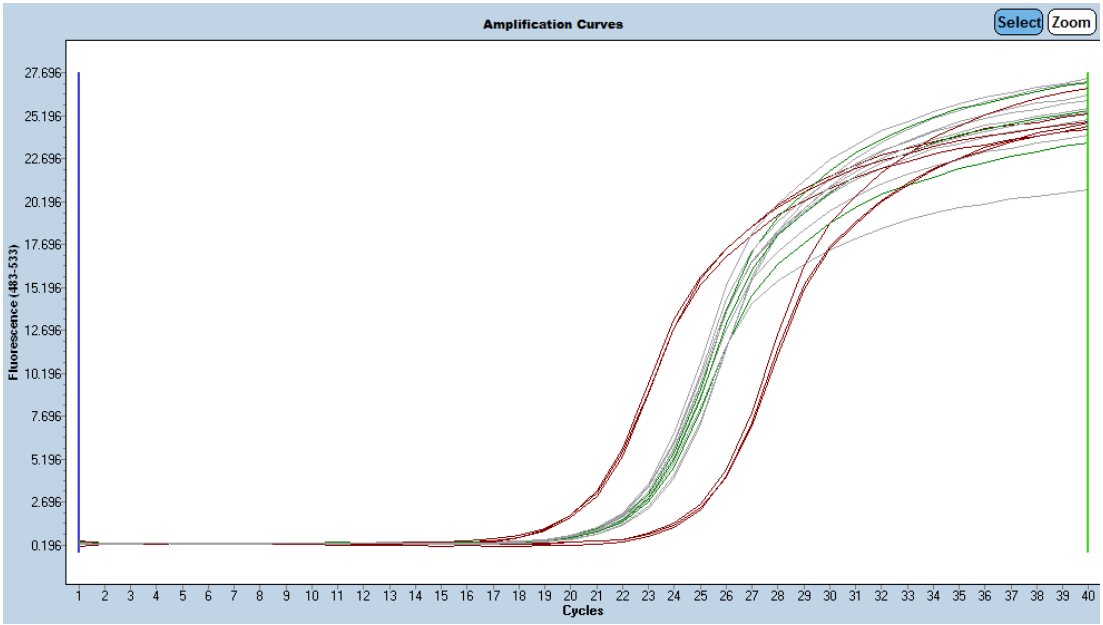


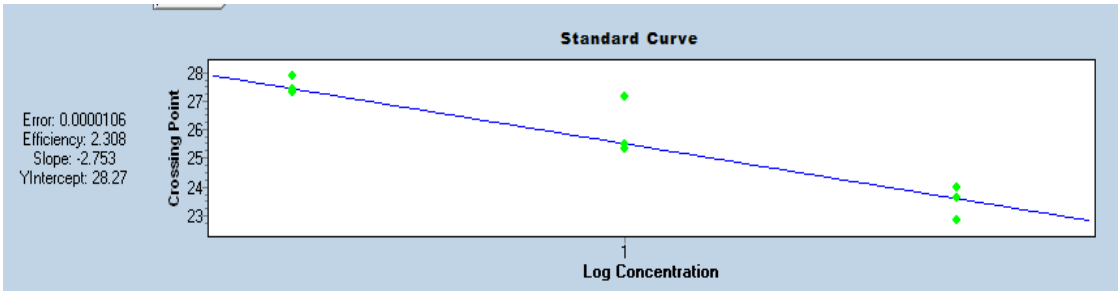
Figure VIII: Standard curve optimisation of PP2Cβ amplicon in U2OS cells using RT-q PCR. **(A)** Amplification curves of 10 fold serial dilutions were used to construct a standard curve. **(B)** Standard curve with efficiency 2.106. **(C)** Melting peaks indicating the specificity of PCR.

Appendix IX: Amplification curves, standard curve and melting peaks for PP2Cβ Efficiency in H1299 cells

A.



B.



C.

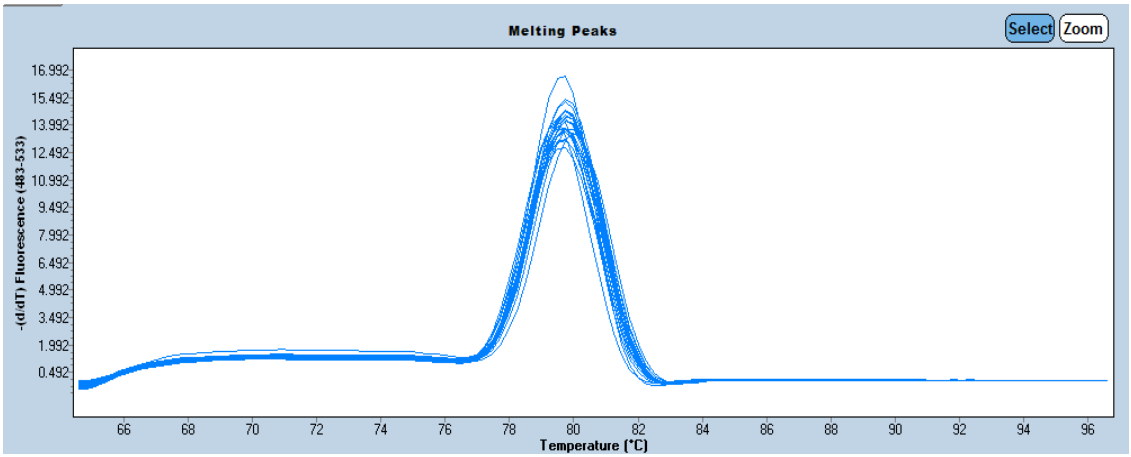


Figure IX: Standard curve optimisation of PP2Cβ amplicon in H1299 cells using RT-q PCR. **(A)** Amplification curves of 10 fold serial dilutions were used to construct a standard curve. **(B)** Standard curve with efficiency 2.308. **(C)** Melting peaks indicating the specificity of PCR.