An investigation into the role of protein phosphatase 4 in breast cancer

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Abstract

Breast cancer is the most common malignant tumour among females world-wide. The complexity of the pathogenesis of the disease and its heterogeneous clinical presentation, with an increasing incidence of the development of chemotherapeutic resistant cases, make its treatment challenging.

This report describes the analysis of the role of the catalytic subunit of PP4 (PP4c) in controlling survival and death of breast cancer cells. These investigations suggested that PP4c regulates both apoptosis and proliferation in human breast cancer cells and have shown that the level of PP4c has a strong influence on the cell cycle and on the anchorage-dependent growth of these cells. This wide variety in the functions of PP4 enzyme is likely to be related to its interacting proteins which determine the location and the functions of the PP4 holoenzyme. This work has identified the effects of modulating the expression level of some of the PP4c-regulatory subunits on the function of PP4c in breast cancer cells. This study has also highlighted a novel PP4c-PEA15 signalling axis in the control of breast cancer cell survival. This study has showed that modulation in the endogenous expression level of PP4c changes the phosphorylation status of important proteins in the Akt-mTOR signalling pathway. Most of these proteins are implicated in the regulation of a variety of cellular functions including cell survival, proliferation, apoptosis and protein translation. Furthermore, this study has revealed important differences between hormone positive and triple negative breast cancer cells in the term of changes in phosphorylation status of some of downstream proteins in the Akt-mTOR pathway as a result of modulating endogenous expression of PP4c. These differences may partly explain the different behaviour of these cell lines and require further

investigation in the future which may open up new opportunities for developing therapeutic approaches to breast cancer.

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Abbreviations

4E-BP1	4E-Binding Protein1
5-FU	5-Fluorouracil
7-AAD	7-Amino-Actinomycin D
AFs	Activation Functions
AGC	Protein Kinase A, G, and C
Akt	Protein Kinase B
AMPK	5' Adenosine Monophosphate-Activated Protein Kinase
Apaf-1	Apoptotic protease activating factor –1
ATCC	American Type Culture Collection
ATF2	Activating Transcription Factor 2
ATM	Ataxia- Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR (Rad3-Related)	Ataxia Telangiectasia and Rad3-Related Protein
Bad	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BCRP	Breast Cancer Resistant Protein
BRCA1	Breast Cancer Associated Gene 1
BRCA2	Breast Cancer Associated Gene 2

BSA	Bovine Serum Albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic Adenosine Monophosphate
Caspase	Cysteine-aspartic proteases
CCT	Chaperonin Containing TCP-1
CDK2	Cyclin Dependent Kinase2
cDNA	complementary DNA
CHK1	Checkpoint Kinase 1
CHK2	Checkpoint Kinase 2
CRC	Colorectal Cancer
CRTC2	CREB Regulated Transcription Coactivator 2
CS	Cowden Syndrome
C_t	Cycle Threshold
CTCL	Cutaneous T Cell Lymphoma
CTLs	Anti-Tumour Cytotoxic T Lymphocytes
DBC1	Deleted in Breast Cancer-1
DCIS	Ductal Carcinoma In Situ
DDR	DNA Damage Response
DED	Death Effector Domain

DEPTOR	DEP Domain-Containing mTOR-Interacting Protein
DISC	Death Inducing Signalling Complex
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA Dependant Protein Kinase
DNA-PKcs	DNA-Dependent Protein Kinase, catalytic subunit
dNTP	Deoxyribonucleotide Triphosphate
DSBs	Double-Strand Breaks
DUSPs	Dual-Specificity Phosphatases
E2	17-β Estradiol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
eIF4E	Eukaryotic Translation Initiation Factor 4E
eNOS	endothelial Nitric Oxide Synthetase
ER	Endotoxin Removal
ER	Oestrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
FACS	Fluorescence Activated Cell Sorting

FADD	Fas-Associated Death Domain Protein
Fas	First Apoptosis Signal
Fasl	Fas Ligand
FBS	Foetal Bovine Serum
FKBP	FK506 Binding Protein
FKHLR	Forkhead in Rhabdomyosarcoma
FRAP	FKBP12-Rapamycin-Associated Protein
GFP	Green Fluorescent Protein
GnRH	Gonadotropin-Releasing Hormone
GSK3-A	Glycogen Synthase Kinase 3-A
H2AX	H2a Histone Family Member X
HCC	Hepatocellular Carcinoma
HDAC3	Histone Deacetylase 3
HEK 293T	Human Embryonic Kidney 293 cells
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HER2/EGFR	Human Epidermal Growth Factor Receptor
HIF-1α	Hypoxia-Inducible Factor 1 alpha
HM	Hydrophobic Motif
HMEC	Human Primary Mammary Epithelial Cell

HPK1	Haematopoietic Progenitor Kinase 1
Hprt	hypoxanthine phosphoribosyl transferase
HR	Homologous Recombination
Hs578T	Homo sapiens mammary gland/breast Carcinoma
Hsp25	Heat shock 25 kDa protein
Hsp27	Heat shock 27 kDa protein beta 1
IGBP1	Immunoglobulin (CD79A) Binding Protein 1
IGF-1R	Insulin-Like Growth Factor Receptor
IkBs	Inhibitory kB Proteins
IKK	Inhibitor of Nuclear Factor Kappa-B Kinase
INPP4B	Inositol Polyphosphate 4-Phosphatase Type II
IRS4	Insulin Receptor Substrate 4
JAK	Janus Kinase
JNK	JUN-N-terminal Protein Kinase
KAP-1	KRAB-Domain-Associated Protein 1
kDa	kilodalton
LB	Lysogeny Broth
LCIS	Lobular Carcinoma In Situ
LKB1	Liver Kinase B1

LNCaP	Androgen-Sensitive Human Prostate Adenocarcinoma Cells
MAC	Mitochondrial Apoptosis-Induced Channel
MAPKs	Mitogen-Activated Protein Kinases
MCF7	Michigan Cancer Foundation 7 Breast Cancer Cell Line
MCT	Multiple Comparison Test
MDA-MB-231	Breast cancer cell line derived from metastatic site
MDR	Multidrug Resistance
MDSCs	Myeloid-Derived Suppressor Cells
MEG1	Protein Phosphatase 4 Regulatory Subunit 1
mLST8	mammalian LST8
MMP-2/9	Matrix Metallopeptidase 2/9
mRNA	Messenger Ribonucleic Acid
MT	Microtubule-Destabilizing Protein
mTOR	Mammalian Target of Rapamycin
MTS1	Multiple Tumour Suppressor 1
MTX	Mitoxantrone
Ndel1	nudE-like1
NF-κB	Nuclear Factor kappa-Light-Chain-Enhancer of Activated B Cells

NHEJ	Non-Homologous End Joining
NK	Natural Killer
NPC	Neural Progenitor Cell
NSCLC	Non-Small Cell Lung Cancer
OVCAR-3	Ovarian Cancer Cells
p16INK4a	Cyclin-Dependent Kinase Inhibitor 2A,
PAM	Phosphoinositide 3 Kinase (PI3K)/Akt/Mammalian Target of Rapamycin (mTOR)
PARP	Poly ADP Ribose Polymerase
PATZ1	POZ-, AT hook-, and Zinc Finger-Containing Protein 1
PBS	Phosphate Buffered Saline
PC-3 and LNCaP	Prostate Cancer Cell Lines
PCD	Programmed Physiological Cell Death
PDAC	Pancreatic Ductal Adenocarcinoma
PDK1	3-Phosphoinositide-Dependent Protein Kinase-1
PEA15	Phosphoprotein Enriched in Astrocytes
PED	Phosphoprotein Enriched In Diabetes
PES	Phenazine Ethosulfate
PH	Pleckstrin Homology

PI	Propidium Iodide
PI3K	Phosphoinositide 3-Kinase
PICT-1	Protein Interacting with C-terminus
PIF	PDK1-Interacting Fragment
PIKKs	PI3K Related Kinases
PIP2	Phosphatidylinositol 4,5 Biphosphate
PKC	Protein Kinase C
PKR	Double-Stranded RNA-Dependent Protein-Serine Kinase
PMSF	Phenylmethanesulfonyl Fluoride
PP2A	Protein Phosphatase 2A
PP2Ac	PP2A catalytic subunit
PP4	Protein Phosphatase 4
PP4c	Protein Phosphatase 4 catalytic subunit
PP4cs	Protein Phosphatase 4, cisplatin-sensitive complex
PP4R1	PP4 Regulatory Subunit 1
PP4R2	PP4 Regulatory Subunit 2
PP4R3 α	PP4 Regulatory Subunit 3 alpha
PP4R3 β	PP4 Regulatory Subunit 3 beta
PP4R4	PP4 Regulatory Subunit 4

PPMs	Metal Dependent Protein Phosphatases
PPPs	Phosphoprotein Phosphatases
PPX	Protein Phosphatase 4
PR	Progesterone Receptor
PRAS40	Proline-Rich AKT Substrate 40
pRB	Retinoblastoma Protein
pre-TCR	pre-T-Cell Antigen Receptor
PS	Phosphatidylserine
PSPs	Protein Serine/Threonine Phosphatases
PtdIns(3,4,5)-P3	phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and Tensin homolog, deleted on chromosome TEN
PTK	Protein Tyrosine Kinase
PTMs	Post-Translational Modifications
PTPs	Protein Tyrosine Phosphatases
p-value	probability Value
RB	Retinoblastoma-Associated Protein
RNA	Ribonucleic Acid
RPA2	Replication Protein A2

RPM	Revolutions per Minute
rpS6	S6 Ribosomal Protein
RSK	Ribosomal S6 Kinase
RT	Reverse Transcription
RT-qPCR	Real Time-quantitative Polymerase Chain Reaction
SCLIP	SCG10-Like Protein
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
Ser	Serine
SERMs	Selective Oestrogen-Receptor Modulators
SFKs	Src-Family Kinases
SHIP	Src Homology 2 (SH2) - Containing Inositol 5-Phosphatase
SIK	Salt-Inducible Kinase
siRNA	Small Interfering RNA
SMEK	Suppressor of MEK: Mitogen-activated Protein/
SMNs	Survival Motor Neurones
STAT	Signal Transducer and Activator of Transcription
TAE	Tris-Acetate-EDTA
TBS	Tris Buffered Saline

TBST	Tris Buffered Saline, 0.1% TWEEN® 20
TE	Tris EDTA
TGF-β	Transforming Growth Factor-beta
Thr	Threonine
TNBC	Triple-Negative Breast Cancer
TNF	Tumour Necrosis Factor
TNFR	TNF Family Receptor
TP53	Tumour Protein 53
TRADD	TNF Receptor-Associated Death Domain
TRAF2	Tumour Necrosis Factor Receptor Associated Factors
Tregs	regulatory T cells
TRiC	TCP-1 Ring Complex
Tyr	Tyrosine
UV radiation	Ultraviolet radiation
VEGF	Vascular Endothelial Growth Factor

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Chapter 1: Introduction

1.1 Cancer

Cancer is a group of diseases characterised by uncontrolled cell growth. Abnormalities in the genetic material of the cells are usually the cause of nearly all cancers. Although these abnormalities are usually somatic events caused by exposure to carcinogens, germ-line mutations can genetically predispose a person to cancer. Alterations in oncogenes, tumour suppressor genes and the recently identified microRNA genes also contribute to cancer (Croce, 2008). Most evidence indicates that the development of a malignant tumour is not often caused by one genetic change; in fact it is a result of a multistep process of dysregulation in several oncogenes, tumour suppressor genes, growth factors, receptors and their signalling pathways in cancer cells (Croce, 2008).

The process of oncogenesis is therefore a multistep process during which normal cells are transformed into cancer cells (Hanahan and Weinberg, 2011). These cells undergoes a series of changes at both cellular and genetic levels that eventually result in uncontrolled cell growth and division, inhibition of cell differentiation, and avoiding cell death, and thus forming a malignant tumour (Croce, 2008). Tumour cells are known to display important characteristics which are also recognised as the hallmarks of cancer (Hanahan and Weinberg, 2000). The hallmarks of cancer originally entailed six biological features that are obtained during the course of growth of human tumours. These hallmarks established a standard for rationalising the intricacies of neoplastic disease (Halperin et al., 2008). The hallmarks of cancer include the ability to sustain proliferative signals, avoid growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activation of invasion and metastasis (Hanahan and Weinberg, 2000). In the last decade, two

other hallmarks have been added which are energy metabolism reprogramming and resisting immune destruction (Hanahan and Weinberg, 2011) (Figure 1.1).

The first hallmark of cancer is sustaining proliferative signalling; cancerous cells have the ability to sustain chronic proliferation. Normal tissues are responsible for the release of signals that promote cell growth and maintain a homeostatic environment; On the other hand, tumour cells have the ability to deregulate the signals that control their destinies within the body (Hanahan and Weinberg, 2011). Many of the key signals are carried by growth factors which bind cell-surface receptors (Cheng et al., 2008). Cell surface growth factor receptors generally have intracellular tyrosine kinase domains which transmit signals through branched intracellular signalling pathways to regulate the process of progression through the cell cycle (Cheng et al., 2008). In most cases, these intracellular signals also affect other biological characteristics of the cell, for instance, cell survival and energy metabolism (Hanahan and Weinberg, 2011).

Evading growth suppression; a number of cancer-suppressing genes behave in a manner that limits the growth of cells (Stanbridge, 1976). For example, the retinoblastoma-associated protein (RB) and TP53 regulate control points involved in making decisions when undergoing multiplication or when activating senescence. RB incorporates information from sources arising both from within and from outside the cell that is responsible for controlling development and cell division (Burkhart and Sage, 2008). TP53 is a tumour suppressor gene which is responsible for controlling a variety of cellular functions including the cell cycle, DNA repair, senescence, and apoptosis (Aylon and Oren, 2011). TP53 mutation has been reported in almost all types of human cancer ranging from 10% abnormality in hematopoietic

malignancies (Peller and Rotter, 2003) up to 100% abnormality in high-grade serous carcinoma of the ovary (Ahmed et al., 2010).

Resisting cell death; classic examples of stresses that facilitate cell death resistance include the imbalances arising from over-expression of oncogenes as well as from DNA damage (Junttila and Evan, 2009). Cancer cells develop the ability to evade apoptosis even if the cells are grossly abnormal (Hanahan and Weinberg, 2000). Evading apoptosis by cancer cells is achieved through alterations that prevent detection of cell damage or abnormalities in regulatory mechanisms so that induction of cell death cannot occur (Hanahan and Weinberg, 2000). Thus there will be no activation of the proteins involved in the process of apoptosis, such as pro-apoptotic proteins in the Bcl-2 family (Adams and Cory, 2007).

Limitless replicative potential; cancer is not just a product of disrupted signalling. Human cells carry an autonomous programme which hinders their multiplication even in the face of disrupted signals from their surroundings (Hanahan and Weinberg, 2000). For a single cancer cell to grow into a tumour that can be seen, this programme must be overcome. Cells can normally undergo a restricted number of consecutive cell divisions so that growth slows down and stop finally in a state called senescence that is not reversible (Campisi et al., 2007). Sometimes cells are able to avoid senescence and undergo the second phase called crisis, a phenomenon that is associated with an increase in both rates of death and chromosomal abnormalities (Fouladi et al., 2000). In the crisis state, chromosomal translocations occur causing very many cells to die through apoptosis (Barillot et al., 2012). Cell senescence is established and maintained by at least two major tumour suppressor pathways; the p53/p21 and p16INK4a/pRB pathways (Campisi et al., 2007). Abnormalities in these pathways cause uncontrolled proliferation of the cells

that result in hyperplasia. In addition, cells gain some abnormal phenotypes that result from such genomic instability that allows unlimited proliferation of the cells, cell migration and metastasis (Hanahan and Weinberg, 2000). To avoid senescence the cancer cells are able to breach the in-built replication limit hard-wired into the cell. This is because they maintain their telomeres (Barillot et al., 2012) by the production of the enzyme telomerase. The enzyme adds telomeric DNA to the ends of the chromosomes. One defining feature of cancerous cells is the ability to undergo endless division devoid of exhaustion from generation to generation. This immortal nature of cancerous cells represents a significant step in the progression of tumours (Barillot et al., 2012).

Sustained angiogenesis; cells require oxygen and nutrients and the mechanism of removing waste products like carbon dioxide (Hanahan and Weinberg, 2011). The formation of new blood vessels meets these needs in a process known as angiogenesis. Tumours send signals to the neighbouring blood vessels thus inducing them to form extensions producing the supply and drainage channels needed. Angiogenesis is facilitated by vascular endothelial growth factor (VEGF) (Ferrara, 2009) that binds to VEGF receptors located on the endothelial cells and stimulates the formation of new blood vessels (Hanahan and Weinberg, 2011).

Activation of invasion and metastasis; growing tumours can produce cells that migrate outside the primary cluster .and also migrating to further locations where they create new clusters (Kim et al., 2009). The new clusters of cancerous tissues produced are metastases. Metastasis requires untethering of the extra cellular matrix bonds which thus allows for the free migration of cancer cells (Barillot et al., 2012). Immunoglobulins, as well as cadherins, facilitate the bonding of cells (Buxton and Magee, 1992), whereas integrins provide a bond between cells and extra cellular

matrix (Humphries et al., 2006). A key protein that mediates cell-cell adhesion is the E-cadherin. However, in migrating tumour cells, E-cadherin function is lost. In its place, N-cadherin is produced which assists tumour cells in sliding through the blood vessel wall while migrating (Barillot et al., 2012).

Reprogramming energy metabolism; cancer cells are defined by uncontrolled growth where the cells are required to duplicate all cellular materials the lipids, DNA and RNA, for the purposes of cell division. Tumour cells have to undergo adjustments to their metabolism to facilitate rapid growth. Tumour cells, therefore, ingest about 20 times more glucose when compared to the normal cells (Jones and Thompson, 2009). However, as a result the tumour cells discharge lactic acid. Malignant growths usually produce an environment with a lower oxygen concentration, thus activating the hypoxia stress response that is facilitated by hypoxia-inducible factors (HIFs) (Reitman and Yan, 2010). The HIFs play a major part in changing the metabolism for tumours from the usual aerobic respiration to glycolysis (Reitman and Yan, 2010).

Evading destruction by the immune system; cells are mostly monitored by the immune system whose role is to recognise and eliminate the vast majority of tumour cells. Tumours cells have different mechanisms of limiting immunological killing to evade eradication. For example by secreting TGF-β (Transforming Growth Factor-beta) or other immunosuppressive factors, the tumour cells inhibit the function of infiltrating CTLs (anti-tumour cytotoxic T-lymphocytes) and NK (natural killer) cells (Yang et al., 2010). In addition, evading immune destruction can be achieved by promoting the recruitment of regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) that suppress the actions of cytotoxic lymphocytes (Mougiakakos et al., 2010).

Another master characteristic of cancerous cells is genomic instability which is regarded as one of the major causes of many types of human cancer (Eshleman et al., 1995). Abnormalities in the process of cell division may result in various forms of genome alterations in the daughter cells either of a temporary or permanent nature. These abnormalities include various forms of mutations of specific genes, amplifications, deletions or rearrangements of chromosome segments, gain or loss of an entire chromosome and chromosomal translocation (Eshleman et al., 1995). Genomic instability arises from many different pathways, such as telomere damage, centrosome amplification, epigenetic modifications, and DNA damage from endogenous and exogenous sources (Shen, 2011). Most of these changes will end in cell death but sometimes a cell has acquired a mutation that enables it to grow faster, or survive longer and then these cells will outgrow and dominate the local tissue environment so that eventually such genomic instability can both initiate and influence the overall prognosis of cancer (Negrini et al., 2010).

The tenth key component of the tumour microenvironment is inflammation. It was perceived that cancer is linked to inflammation, and also many epidemiological studies have revealed that chronic inflammation predisposes to different forms of cancer. The origin of tumour-associated inflammation remains largely unknown. Key features of cancer-related inflammation are infiltration of white blood cells, tumour-associated macrophages, presence of cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines such as CCL2 and CXCL8, and the occurrence of tissue remodelling and angiogenesis (Mantovani et al., 2008). Some of these inflammatory mediators are considered to be mutagens that act by direct or indirect down-regulation of DNA repair pathways and cell cycle checkpoints resulting

in instability in the cancer cell genome and subsequent accumulation of genetic alterations (Mantovani et al., 2008).

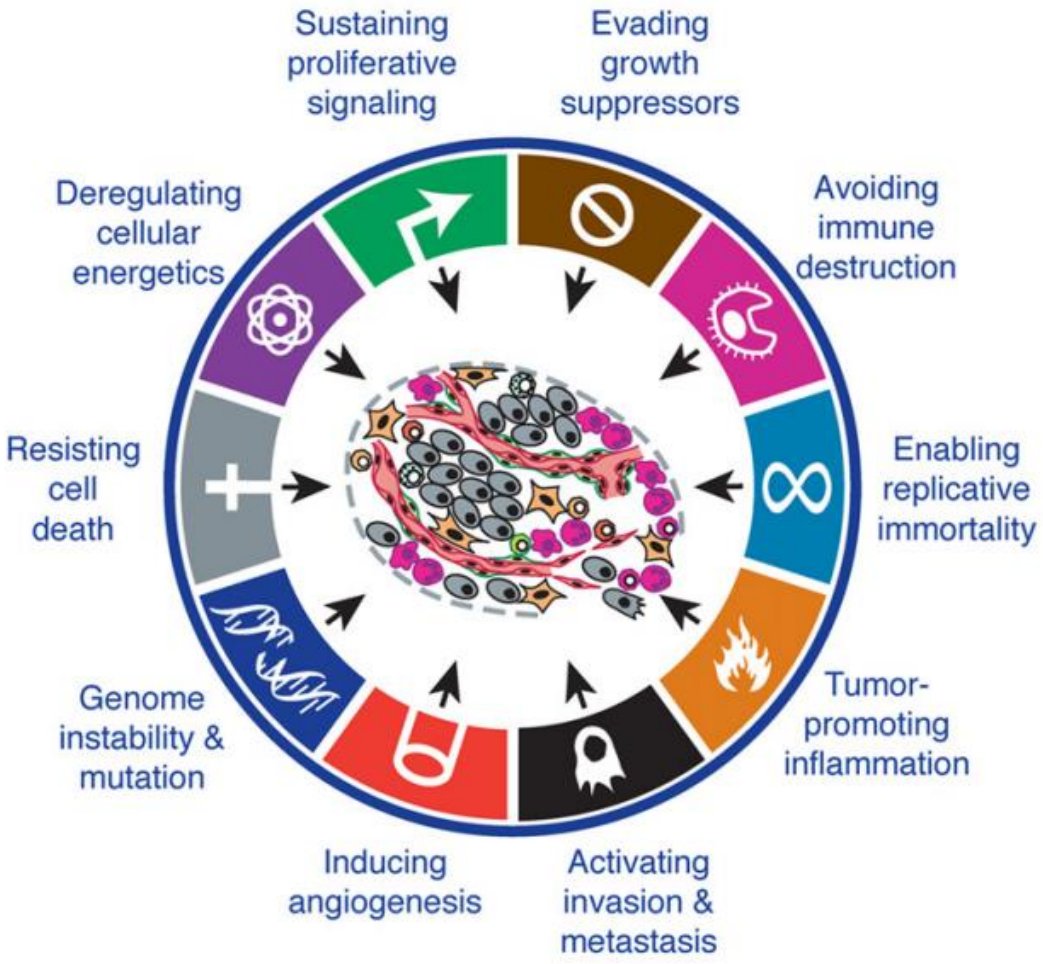


Figure 1.1: Hallmarks of cancer (Hanahan and Weinberg 2011).

1.2 Apoptosis

Apoptosis is the most common type of programmed physiological cell death (PCD) that is essential during embryonic development (Green and Evans, 2002). It also plays an important role in the maintenance of normal tissue homeostasis and the regulation of immune system function (Jacobson et al., 1997; Baehrecke, 2002). Abnormalities in this type of programmed cell death will result in various pathological problems. A decrease in apoptosis may lead to autoimmune disease and cancer, whereas an increase in apoptosis may lead to neurodegenerative disorders (Okada and Mak, 2004). Apoptosis is an irreversible process and once it begins it will unavoidably end with cell death (Kerr et al., 1972). The mechanisms of apoptosis are highly complex and sophisticated. To date, two main apoptotic pathways have been identified, that is, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Igney and Krammer, 2002) (Figure 1.2). The extrinsic pathway is usually initiated by extrinsic stimuli which are of two types; it is activated either by TNF (tumour necrosis factor), including other members of the family such as TRAIL (TNF-related apoptosis-inducing ligand), or by Fas-Fas ligand interactions, both types of stimulus act through receptors of the TNF family (TNFR) (Wajant, 2002). TNFα is a cytokine produced by activated macrophages and is regarded as the main stimulator of the extrinsic pathway of apoptosis. Binding of TNFα to the TNF1 receptor leads to the activation of the caspase (cysteine-aspartic proteases) cascade via the intermediate proteins; TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) (Chen and Goeddel, 2002). The death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. Fas (First apoptosis signal) receptor binds to a trans-membrane protein known as Fas ligand (FasL) and the

interaction between Fas and FasL results in the formation of the death-inducing signalling complex (DISC), which also contains FADD (Chen and Goeddel, 2002), this binding also results in activation of the caspase cascade, mainly caspase-8 and caspase-10. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residue within particular amino acid sequences. Once caspases are activated the process of apoptosis becomes irreversible and definitely ends with cell death. To date, ten major caspases have been identified and they are classified into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997). In some types of cells caspase-8 directly activates other members of the caspase family to activate apoptosis (Chen and Goeddel, 2002).

The second apoptotic pathway is the intrinsic pathway (also called the mitochondrial pathway) which is activated by intracellular signals generated as a result of cellular stress and based on the release of proteins from the inter-membrane space of the mitochondria. These signals either cause mitochondrial swelling through the formation of pores in the mitochondrial membrane, or they may increase the permeability of the mitochondrial membrane and result in the leakage of the apoptotic effectors which trigger the degradation of the cell (Gonzalez et al., 2010). Cytochrome c is released from mitochondria due to formation of channels in the outer mitochondrial membrane called the mitochondrial apoptosis-induced channel (MAC), cytochrome c binds with apoptotic protease activating factor – 1 (Apaf-1) and ATP which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9 which in turn activates the effector caspase-3 (Dejean et al., 2006).

The regulation of these mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory and Adams, 2002) which can be either pro-apoptotic such as Bax, Bak, Bid, Bad, Bim, Bik, and Blk or anti-apoptotic such as Bcl-2, Bcl-x, Bcl-XL, Bcl-w and BAG. Bcl-2 family proteins mainly regulate the release of cytochrome c from the mitochondria via alteration of mitochondrial membrane permeability as described above (Cory and Adams, 2002). Stimulation of both pathways of apoptosis results in the activation of caspases (Green, 2005). Due to these events, the apoptotic cell displays distinct characteristics that include rounding up of the cell, condensation of chromatin, fragmentation of the nucleus and shedding of apoptotic bodies; membrane bound vesicles containing cytoplasm and intact organelles (Edinger and Thompson, 2004).

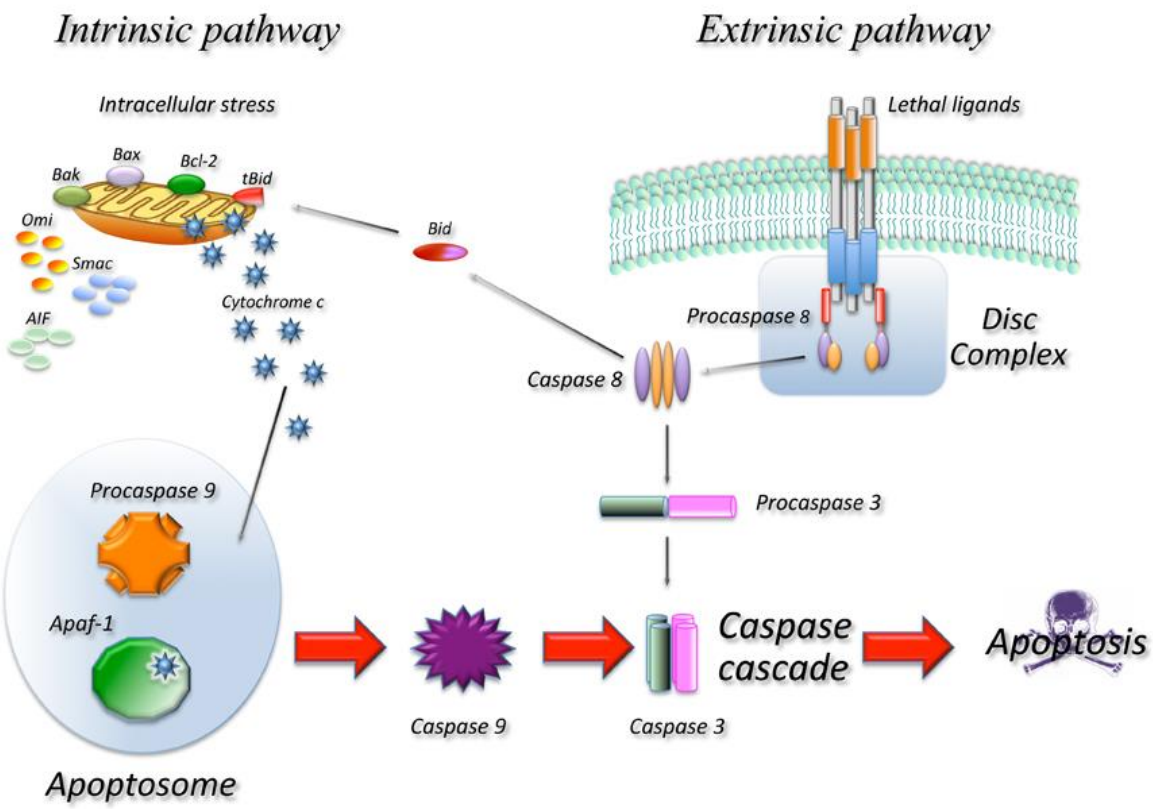


Figure 1.2: Figure shows the two main molecular pathways leading to apoptosis (Favaloro et al., 2012)

1.3 Breast cancer

Breast cancer is a complex disease and is produced through a multi-step biological process in which epithelial cells lining the ducts or lobules of the breast become abnormal and undergo malignant transformation. After lung cancer, breast cancer is considered the second most common cancer worldwide (Hutchinson, 2010) and is considered to be the leading cause of cancer deaths in women between 20 and 59 years old (Jemal et al., 2007; Hutchinson, 2010). The incidence rates of breast cancer are increasing, with high incidence in western developed countries. In the UK breast cancer is the second most common cancer in women after colorectal cancer and it accounts for about 30% of all cancers in women (Ferlay et al., 2010). The complexity of the pathogenesis of the disease, its heterogeneous clinical presentation with an increasing incidence of the development of chemotherapy resistant cases make its treatment challenging. During recent decades, early diagnosis and the use of adjuvant and neoadjuvant therapies has led to a considerable increase in survival rate. However every year, around 30-70% of breast cancer patients eventually develop recurrence and metastasis leading to their death (Sant et al., 2001; Clegg et al., 2002). Therefore, there is an urgent need to identify novel molecular targets in breast cancer to improve treatment options that could lead to a better prognosis for breast cancer patients (Jemal et al., 2007; Hutchinson, 2010).

1.3.1 Classification of breast cancer

There are different classifications of breast cancer that are based on different criteria. The purpose of breast cancer classification is to determine the suitable therapy for the patient and to predict the disease prognosis.

1.3.1.1 Classification according to receptor status

Four main molecular classes of breast cancer have been identified based on receptor expression status. These are oestrogen receptor positive (ER), progesterone receptor positive (PR), human epidermal growth factor receptor 2 positive (HER2) and triple negative breast cancer (Pusztai et al., 2006).

Oestrogen receptor positive breast cancer comprises about 75% of all breast cancer in which the growth of the tumour cells is stimulated by oestrogen. Oestrogen receptors (ERs) play a key role in controlling the growth and the development of normal breast tissue as well as the progression of breast cancer cells. Therefore blocking the function of oestrogen receptors represents the first molecular therapy target for the treatment of breast cancer (Schiff and Osborne, 2005). Many types of endocrine therapy that are now available act by inhibiting the function of ERs, for example, tamoxifen, which is a selective ER modulator that binds ERs and partially blocks their function. Another strategy for inhibiting ERs function is ovarian ablation to prevent long term exposure to oestrogen (Osborne et al., 2000). ERs are members of a large family of nuclear transcriptional regulators that are activated by steroid hormones (Parker, 1993). The ERs are of two isoforms α and β, both isoforms are expressed in normal mammary gland and the ER-α, but not ER-β, has

been shown to be crucial for normal mammary gland development (Bocchinfuso et al., 1997). Each ER isoform is encoded by a different gene (Mosselman et al., 1996) but, although both isoforms are similar to each other in their structure, they are rather different in their functions. The response of these isoforms to oestrogen agonists and antagonists is different because of difference in the C-terminal ligand-binding domains of the receptors (Pettersson et al., 2000). ER-α and ER-β are both found to be greatly increased in premalignant hyper proliferative breast lesions and in many breast cancers compared with normal mammary glands (Bocchinfuso et al., 1997), so ER expression is regarded as a prognostic marker of primary breast cancers. Positive ER-α status is usually associated with good prognosis (Allred et al., 2001). ER proteins consist of a DNA binding region that allows the receptors to bind to specific sequences in the promoters of oestrogen-regulated genes, and at least two transcriptional activation function regions (AFs) known as AF-1 and AF-2 that control the transcription of these genes. AF-2 contains the ligand binding domain that interacts with estradiol (Schiff et al., 2002). AF-1 shows basal activity but is enhanced by growth factor signalling (Kato et al., 1995) whereas; AF-2 is activated upon ligand with oestrogen but not with tamoxifen (Shiau et al., 1998).

About 65% of all oestrogen receptor positive breast cancers are also reported to express the progesterone receptor (PR) so that the tumour cells can grow in response to both oestrogen and progesterone hormones (Pusztai et al., 2006). PR is regarded as a positive prognostic marker in patients with ERα+ breast cancers (Blows et al., 2010). Although the functional role of PR signalling is still unclear, it was found that activation of PR may stimulate breast cancer cell growth in some women and in some model systems. On the other hand, progesterone treatment has been shown to have an anti-proliferative effect in ERα+ PR+ breast cancer cell lines

(Vignon et al., 1983). PR activation in ERα+ breast cancer can have an anti-tumourigenic effect and the use of PR agonists can have further clinical benefits in ERα+ breast cancer patients that have relapsed after treatment with ERα antagonists (Bines et al., 2014).

Breast cancers are usually assessed for the presence of ERα, PR and HER2 receptor expression in order to identify the histological subtype of the tumour and to guide the treatment options for the patients with breast cancer. ERα+ PR+ HER2- tumours tend to have the best prognosis (Dowsett et al., 2005). HER2 receptor positive breast cancer is observed in approximately 20% of invasive breast carcinomas and indicates a highly aggressive tumour and a poor prognosis (Slamon et al., 1987). The HER2 gene encodes a receptor tyrosine kinase that belongs to the epidermal growth factor receptor (EGFR) family that includes four members (EGFR/HER1, HER2, HER3 and HER4) (Yarden and Sliwkowski, 2001). Over-expression of HER2 stimulates the constitutive activation of growth factor signalling pathways, thus serving as an oncogene and driving tumour formation and maintenance (Gajria and Chandarlapaty, 2011). Studies have shown that there is phosphorylation and activation of the HER2 tyrosine kinase in human breast cancers which could be related to the co-expression of ligand-activated EGFR resulting in transactivation of the HER2 tyrosine kinase (Stern et al., 1988). When HER2 is normally expressed, ligands that bind to the HER receptors induce the formation of only a few HER2 heterodimers with a relatively weak response to the growth factors, resulting in normal growth of cells. However, when HER2 is over-expressed as in cancer cells, the cell is exposed to many ligands originating either in the stroma or in the tumour cells. This results in the formation of many HER2 heterodimers that persist at the cell surface and produce strong stroma-to-epithelium signalling with

enhanced responsiveness to growth factors and stimulation of cell growth with malignant cell formation (Rubin and Yarden, 2001).

The fourth molecular class is the triple negative breast cancer which forms about 10-17% of all breast cancer types. This tumour subgroup lacks expression of HER2, ER and PR and consequently does not respond to hormonal therapies and to HER2 targeted therapies (Hutchinson, 2010). Triple negative breast cancer is usually associated with poor prognosis and therefore the development of new systemic therapies for this subtype is greatly needed.

1.3.1.2 Classification according to gene expression

A considerable number of genes have been shown to be involved in the development of breast cancer. Mutations in BRCA1 (breast cancer associated gene 1) or BRCA2 (breast cancer associated gene 2) are responsible for the development of about 20-25% of all hereditary breast cancers (Easton, 1999) and about 5-10% of all breast cancers cases (Campeau et al., 2008). Women with a specific inherited mutation in one of these genes have an increased risk of development of breast and ovarian cancer (Antoniou et al., 2003). BRCA1 gene encodes a nuclear phosphoprotein that acts as a tumour suppressor gene by maintaining genomic stability (Wang et al., 2000). BRCA2 gene is also involved in maintaining genomic stability and it encodes a protein which has a role in many cellular processes such as DNA repair, cell replication, and the cell cycle (Apostolou and Fostira, 2013).

The tumour suppressor TP53 is also one of the genes involved in breast cancer. Compared to the general population, TP53 mutation carriers have about 18 to 60-

fold increased risk of development of early onset breast cancer (Walsh et al., 2006). The gene product of TP53 is the transcription factor P53 which is able to induce both arrest of the cell cycle in G_1 and apoptosis by activating several downstream genes (Goh et al., 2011). PTEN (Phosphatase and Tensin homolog, deleted on chromosome TEN) is a tumour suppressor on chromosome 10q23.3 that works by promoting apoptosis and G_1 cell cycle arrest (Carracedo et al., 2011). PTEN has been found to be mutated in breast cancer and in many other types of cancers (Carracedo et al., 2011). Some mutations in the tumour suppressor gene CHEK2 (Checkpoint kinase 2) are associated with increased risks of female breast cancer (Vahteristo et al., 2002). A particular germline mutation; CHEK2 c.1100delC is associated with a 2-fold increased risk of development of breast cancer (Vahteristo et al., 2002) and other cancers such as prostate cancer (Cybulski et al., 2004) and colorectal cancer (Meijers-Heiboer et al., 2003). The CHEK2 gene encodes CHEK protein which is a serine/threonine kinase that is activated in response to DNA damage and plays an important role in activating the DNA damage response (Stracker et al., 2009). An emerging target in the treatment of breast cancer is 3-phosphoinositide-dependent protein kinase-1 (PDK1), which is a protein encoded by the PDPK1 gene (Fyffe and Falasca, 2013). PDK1 is a critical kinase in oncogenic phosphoinositide 3-kinase (PI3K) signalling in breast cancer. PDK1 functions downstream of PI3K activation and phosphorylates and activates members of the AGC family of serine/threonine protein kinases such as protein kinase B (Akt), protein kinase C (PKC), p70 ribosomal protein S6 kinases, and serum glucocorticoid-dependent kinase (Fyffe and Falasca, 2013). These kinases are known to regulate cell metabolism, cell growth, cell proliferation, and cell survival. Changes in the expression and the activity of these kinases and the PDK1 enzyme have been linked

to human diseases including cancer (Raimondi and Falasca, 2011). Recent data have revealed that the modification of PDK1 can greatly affect the oncogenic phosphoinositide 3-kinase signalling pathway in breast cancer, suggesting that inhibition of PDK1 can inhibit breast cancer progression (Fyffe and Falasca, 2013).

1.3.2 Breast cancer chemotherapy and resistance

Many efforts have been made to study changes that occur in cancer cells which result in uncontrolled growth and multiplication; researchers have also developed new drugs or treatments that target these changes in cancer cells (Partridge et al. 2014). Most of these studies have focused on targeted therapy, that is, treatments that employ drugs or other reagents to attack specific types of cancer cells and cause less harm to normal cells. Such therapies can be used alongside less specific agents that attack all the cells in the body that are growing at a high rate as well as the cancer cells. Such targeted therapies have the potential to work in circumstances where conventional chemotherapeutic agents have failed (den Hollander et al., 2013). Moreover, number of targeted therapies might be combined with other treatments to improve their efficacy. These targeted therapies would therefore be expected to have less severe side effects as compared to less specific chemotherapies (den Hollander et al., 2013). For instance, some targeted reagents have been produced to target the HER2-positive breast cancers that tend to aggressively develop and spread (Nuciforo et al., 2015). These drugs include herceptin, perjeta, TDM-1 and tykerb; all of these are monoclonal antibodies except tykerb which is a kinase inhibitor (Nuciforo et al., 2015). Herceptin can also be combined with docetaxel, a traditional drug to treat women having metastatic breast

cancer that over-expresses HER2/neu protein (Barillot et al., 2012). For ER-positive breast cancer; endocrine treatment is of great therapeutic value, particularly treatment with tamoxifen which is an elective oestrogen-receptor modulator (SERM), raloxifene, aromatase inhibitors, and GnRH (Gonadotropin-releasing hormone) agonists (Lumachi et al., 2011).

Sledge et al (2015) argue that endocrine therapies along with other targeted therapies are of great importance for treating breast cancer patients. Targeted therapies can target the PI3K/Akt/mTOR1 signalling pathway and the cross-talk between growth factor receptors signalling and hormone receptors. Thus the human epidermal growth factor (HER2) inhibitors, mTOR1 inhibitors, PI3K/Akt inhibitors and DNA damaging agents improve the outcome when combined with endocrine therapies (Nagraj and Ma, 2015).

Triple negative breast cancer (TNBC) is a heterogeneous subtype of breast cancer characterized by absence of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 receptor (HER2) (Schneider et al., 2008). There is no targeted therapy specifically recommended for patients with TNBC, since it is not responsive to the common hormonal or endocrine treatments such as herceptin and tamoxifen. In this type of cancer, there is an insulin-like factor that stimulates proliferation of cells and promotes their survival through phosphorylation of growth receptors such as EGFR (epidermal growth factor receptor) and activates the adaptor proteins such as mitogen-activated protein kinase (Rakha et al., 2007). This type of cancer responds better to neoadjuvant treatments than other subtypes of breast cancer (Schmadeka et al., 2014). Neoadjuvant treatment is considered as the first line of treatment before the radical treatment which is usually radical surgery. It is given in the early stage of breast

cancer with the objective of reducing tumour size and limiting tumour extension. Neoadjuvant treatments could be in the form of chemotherapy, radiation therapy or hormone therapy (Liu et al., 2010). Anthracycline and taxane combinations are used as the first line treatment for advanced TNBC followed by capecitabine at the time of progression (Oakman et al., 2010). However, patients still have residual disease relapse and have extremely poor prognosis. Thus, there is a problem in treating TNBC especially as these cells frequently produce distal metastasis emphasising the urgent need for new therapies that might prolong the life span of patients suffering from triple negative breast cancer (Chu et al., 2012).

The extensive heterogeneity of breast cancer, being a heterogeneous disease, has stimulated a continuous drive to determine markers that might assist in predicting the response to certain therapies and disease prognosis. Predictive markers are factors associated with the sensitivity, resistance or response to a certain treatment or therapy which allow clinicians to predict therapeutic outcomes and so to decide future plan for the treatment of patients with breast cancer (Payne et al., 2008). Prognostic markers are regarded as indicators of tumour aggressiveness and invasiveness (Pankaj et al., 2010). Many molecular markers that have been studied have both prognostic and predictive values. The most common classical prognostic markers in breast cancer are Ki67, ER, PR and HER2. In addition, there are some genetic markers such as p53, p14ARF, cyclin D1, cyclin E, TBX2/3, BRCA1/2 and VEGF (Pankaj et al., 2010).

Ki67 is a cellular marker for proliferation that has been widely monitored as a prognostic factor for patients with early stage breast cancer (Scholzen and Gerdes, 2000). Ki67 protein can be detected during all phases of the cell cycle (G_1, S, G_2, and mitosis) but is absent in G_0. Ki67 antigen can be detected within the cell nucleus

during interphase while during mitosis most of the protein is relocated to the surface of the chromosomes (Scholzen and Gerdes, 2000).

The growth of breast cancer is often regulated by the female sex steroids, so determination of cellular concentrations of ER (oestrogen receptor) and PR (progesterone receptor) are useful prognosis markers and are of potential benefit in determining the likely effectiveness of anti-hormonal therapy in patient with breast cancer (Harris, 2007). ER and PR hormones work together to regulate and control growth, differentiation, and survival of mammary epithelial cells (Ross and Harbeck, 2005). ERα is responsible for mammary ductal elongation during puberty; PR and ERβ are responsible for lobular differentiation during lactation (Humphreys et al., 1997). Over-expression of PR indicates that the ER pathway is intact, even if the tumour is reported as ER-negative (Donegan, 1997). ERα-positive breast cancers are associated with slow tumour growth, low histological grade and thus a better overall prognosis (Ross and Harbeck, 2005). ER/PR-negative tumours are often associated with aggressive disease and such tumours frequently show amplification of HER2, c-Myc, and Int2 oncogenes and mutations of the p53 tumour suppressor gene (Ross and Harbeck, 2005).

1.4 Reversible phosphorylation and regulation of protein activity

Reversible protein phosphorylation is a key event in signal transduction pathways. The process of reversible protein phosphorylation is one of the most common mechanisms in controlling most if not all cellular processes (Denu et al., 1996). The function of nearly one-third of the proteins in a cell, including proteins that are identified as playing a critical role in breast cancer, are regulated via a change in their phosphorylation state. Phosphorylation of certain proteins serves as a switch in controlling key oncogenic pathways involved in regulating gene expression, cell proliferation, differentiation, apoptosis, migration and invasion (Virshup and Shenolikar, 2009). Consequently, phosphorylation plays a critical role in the regulation of cell physiology, and dysregulation of the mechanisms that control particular phosphorylation processes contributes to many diseases including cancer. Depending on the physiological requirements of the cell, proteins transiently change from phosphorylated to a dephosphorylated state and vice versa and such processes of reversible protein phosphorylation are specifically controlled by two groups of enzymes that counteract each other in their functions, that is, the protein kinases and protein phosphatases (Bennett et al., 2006). Protein kinases phosphorylate the proteins while phosphatases removes the phosphates (Sun and Wang, 2012; Mourtada-Maarabouni et al., 2003). Hence, kinases and phosphatases play critical role in many cellular processes and function as important checkpoint regulators.

1.5 Aberrant phosphorylation in breast cancer

Uncontrolled cell proliferation is a major feature of cancer and it is mostly due to dysregulation in the function of important proteins. The proteins involved are responsible for controlling cell growth and differentiation and their dysregulation is partly due to abnormalities in the process of reversible protein phosphorylation. Many hyperactive kinases have been implicated in the development of breast cancer (Nunes-Xavier et al., 2013). MAPKs are regarded as key players in the regulation of cell proliferation, cell growth, division, survival and differentiation and they are activated by phosphorylation (Ahn et al., 1990). The regulation of the MAPK signalling pathway is very important in the treatment of breast cancer and it is regarded as one of the targets for molecular therapy for breast cancer (Mester and Redeuilh, 2008). ERK1/2 (extracellular signal–regulated kinases 1/2) is one of the major MAPK pathway proteins and ERK1/2 abnormalities have been implicated in the development of breast cancer (Mester and Redeuilh, 2008). ERK1/2 are phosphorylated and activated by their specific MAPK kinases MEK1/2 that are dual threonine and tyrosine kinases and are regarded as essential components of MAPK signal transduction pathways. Mutations in MEK1 or MEK2 were first reported in ovarian cancer cell lines (Estep et al., 2007) and subsequently mutations have been reported in many other types of cancer such as melanoma, colorectal carcinoma and lung cancer (Marks et al., 2008). MKP1/DUSP1 (mitogen-activated protein kinase phosphatase-1/dual-specificity phosphatase-1) and MKP2/DUSP4 play crucial roles in the deactivation of MAPKs and have been found to be over-expressed in breast cancer cell lines and in malignant breast cancer samples (Wang et al., 2003). This has a substantial impact on the resistance of these cells to various chemotherapeutic agents (Pulido and Hooft van Huijsduijnen, 2008). Activation of PAM;

phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is also common in breast cancer (Paplomata and O'Regan, 2013). PAM is part of a major signalling pathway in controlling cellular proliferation and cell survival. Uncontrolled phosphorylation of various proteins in the PAM pathway has been reported to be the cause of initiation and progression of breast cancer as well as resistance to various breast cancer therapies such as endocrine therapy, human epidermal growth factor receptor 2 (HER2)-directed therapy and cytotoxic therapy in breast cancer (Paplomata and O'Regan, 2013). In this pathway, a central role is played by PI3K heterodimers. Activated PI3K converts phosphatidylinositol 4,5 biphosphate (PIP2) into phosphatidylinositol 3,4,5 phosphate (PIP3) which results in the membrane localization of phosphoinositol-dependent kinase-1 (PDK1) via its pleckstrin homology (PH) domain. Akt is also recruited to the lipid-rich plasma membrane by its PH domain and is phosphorylated at residue T308 by PDK1 and at S473 by an unidentified kinase (Chang et al., 2003). Akt can directly phosphorylate and activate the oestrogen receptors even in the absence of oestrogen and by that mechanism it stimulates oestrogen-independent growth and induces resistance to hormone therapy (Hernandez-Aya and Gonzalez-Angulo, 2011). Phosphatase and tensin homologue deleted on chromosome ten (PTEN) was the first phosphatase to be identified as a tumour suppressor through its inhibitory effect on the PAM pathway via dephosphorylation of PIP3 to PIP2 (Salmena et al., 2008). PTEN is found to be mutated and lost in several cancers including neuroglioma, endometrial, prostate, breast, thyroid and liver cancer (Li and Sun, 1997). PTEN mutations usually occur either in the catalytic domain (C2 domain) (Eng, 2003) or in ubiquitination sites (Trotman et al., 2007). Germline mutations in PTEN are the main cause of Cowden syndrome (CS) and patients with CS have high risk of breast cancer development

(Marsh et al., 1998). It has been reported that PTEN expression level plays an important role in the response of breast cancer cells to hormone therapy, particularly tamoxifen. Down-regulation of PTEN expression results in loss of inhibition of PI3K/Akt. On the other hand, PTEN over-expression sensitizes breast cancer to tamoxifen. Therefore, breast cancer patients with a positive expression of PTEN and ER have a good prognosis and longer survival (Campbell et al., 2001). INPP4B (inositol polyphosphate 4-phosphatase type II) is another tumour suppressor which is also involved in dephosphorylation of PIP3 to PIP2. INPP4B deficiency has been reported in patients with aggressive basal-like breast carcinomas (Fedele et al., 2010). Liver kinase B1 (LKB1) is a serine/threonine kinase which is upstream of AMP-activated protein kinase (5' adenosine monophosphate-activated protein kinase). Liver kinase B1 negatively regulates the mTOR signalling pathway and inactivation of the LKB1-AMPK pathway has been implicated in breast cancer (Dupuy et al., 2013). Over-activation of the mTOR signalling pathway significantly contributes to the initiation and development of different malignancies including breast, prostate, lung, melanoma, bladder, brain, and renal carcinomas (Xu et al., 2014). Mutations in tumour suppressor PTEN are the most common cause of constitutive activation of mTOR signalling pathway. mTOR is a serine/threonine protein kinase that consists of two complexes, mTORC1 (mTOR complex 1) (mTOR-raptor complex 1) and mTORC2 (mTOR complex 2) (mTOR-rictor complex 2) (Brown et al., 1994). mTORC1 regulates translation by phosphorylating its various downstream proteins particularly S6K1 (Ribosomal protein S6 kinase beta-1) and 4E-BP1 (4E-binding protein 1) (Fingar et al., 2002). mTORC2 regulates cellular proliferation and metabolism by regulating proteins such as IGF-IR (insulin-like growth factor 1 receptor), InsR (insulin receptor), Akt/PKB and SGK (serum- and

glucocorticoid-induced protein kinase) (Sarbassov et al., 2005; García-Martínez et al., 2008). mTORC1 is the main target of rapamycin analogs but mTORC2 can be also inhibited if sufficient dose of rapamycin is applied (Sarbassov et al., 2006). mTOR is activated from upstream growth factor receptors, such as the PI3K and AMPK. mTOR complex 1 activates its substrate; p70-S6kinase which in turn activates ER through its phosphorylation at Ser167 that leads to ligand-independent ER activation (Yamnik and Holz, 2010). A high level of phosphorylated (and therefore activated) Akt, phosphorylated mTOR, and phosphorylated 4E-BP1 (which is one of mTORC1 substrates) has been reported in 40% of all invasive ductal carcinomas of the breast (Meric-Bernstam and Esteva, 2005). mTOR activation via PI3K/Akt signalling pathway causes resistance of some of ERα + breast cancer cells to endocrine therapy (O'Regan and Hawk, 2011). Rapamycin inhibits mTOR signalling and restores sensitivity to tamoxifen in laboratory models of resistance (Beeram et al., 2007). It was hypothesised that activated mTOR drives ligand-independent ERα signalling and short circuits the ligand-dependent pathway that is most sensitive to inhibition by endocrine therapies (Shrivastav et al., 2014).

The activities of ER (oestrogen receptor), PR (progesterone receptor) and HER2 receptors (human epidermal growth factor receptor 2) are modulated by the reversible phosphorylation of their tyrosine residues (Hynes, 2000), and this constitutes a major regulatory mechanism of their activities. Aberrant activation of these receptors leads to multiple signalling cascades and plays a vital role in the initiation, development and progression of breast cancer (Hynes, 2000). The protein tyrosine kinase (PTK) families HERs and the non-receptor Src-family kinases (SFKs) have been directly implicated in the development and progression of breast cancer (Hynes, 2000), and their downstream signalling is mainly mediated via the activation

of a number of effector pathways that involve serine/threonine (Ser/Thr) kinases including MAP kinase (MAPK), the phosphoinositide-3 kinase (PI3K)/ Akt/mammalian target of rapamycin (mTOR) and the JAK/STAT (Janus kinase/ Signal Transducer and Activator of Transcription) signalling pathways (Knutson et al., 2012).

Studies have shown that phosphorylation events act as key regulators of the activity of PR hormone receptors. PR-B isoform has been found to be phosphorylated on Ser294 by mitogen activated protein kinase (MAPK) and cyclin dependent kinase2 (CDK2), significantly affecting receptor stability, localisation, transcriptional activity, and promoter selectivity (Knutson et al., 2012).

ERα (oestrogen receptors alpha), like many other proteins, can undergo post-translational modifications (PTMs) (Le Romancer et al., 2011) which could be in the form of phosphorylation, acetylation, methylation and ubiquitination. These PTMs regulate the activity of ERα (Le Romancer et al., 2011). Investigations have revealed that phosphorylated ERα has been detected in many breast tumour biopsy samples (Murphy et al., 2011). A negative correlation between the phosphorylation status of ERα isoform at the serine118 residue and the response of breast cancer patient to tamoxifen has also been found, suggesting that phosphorylated ERα could be used as a marker for the resistance to tamoxifen therapy (Chen et al., 2013). A positive correlation between serine phosphorylation of the β isoform of ER and the progression of breast cancer has been reported (Meerasahib and Asseri, 2011).

The activity of HER2 receptors is also regulated by the phosphorylation status of their tyrosine residues. Such phosphorylation constitutes a major regulatory

mechanism that has a great impact on the initiation, development and progression of breast cancer (Hynes, 2000).

1.6 Protein phosphatases

The phosphorylation status of the protein components that are regulated by phosphatases determines the hypo activity or hyper activity of the PI3K/Akt/mTOR pathway (Hinds and Sanchez, 2008). In contrast to protein kinases, a relatively smaller number of protein phosphatase genes are known to be present in biological systems (Virshup and Shenolikar, 2009). Phosphatases are classified into two major classes, protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSPs) (Alonso et al., 2004). An emerging subclass of the protein tyrosine phosphatase class is the dual-specificity phosphatases (DUSPs) which consists of a heterogeneous group of protein phosphatases that can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within the substrate (Bennett et al., 2006). Protein serine/threonine phosphatases (PSPs) are further divided into three sub-families, phosphoprotein phosphatases (PPPs), metal dependent protein phosphatases (PPMs) and aspartate based phosphatases (Mumby and Walter, 1993, Barford, 1996; Moorhead et al., 2009).

In the cell, these phosphatases can exist in active monomeric forms which consist of an active catalytic subunit alone, an active dimeric form (comprising of catalytic and regulatory subunit) or multimeric enzymes consisting of a small number of catalytic subunits combining with many regulatory and scaffold subunits (Virshup and

Shenolikar, 2009). It is these combinations that regulate the activity of the phosphatases and allow the coordination and control of many vital cellular functions.

The mammalian serine/threonine phosphatase (PPP) group consists of the most abundant protein phosphatases and includes PP1, Ca2+-dependent PP2B, Mg2+-dependent PP2C, PP2A and PP2A-like phosphatases such as PP6, PP5, PP7 and PP4 (Olsen et al., 2006). Members of the type 2A group of phosphatases, PP2A, PP4, and PP6, are the most closely related in sequence (Olsen et al., 2006). They are sensitive to inhibition by low doses of okadaic acid or fostriecin which distinguishes them from PP1 (Moorhead et al., 2009). Protein phosphatase type 2A or PP2A is one of the major serine/threonine protein phosphatases in all eukaryotic cells; it makes up 0.3–1% of the total cellular protein in the mammalian cell (Ruediger et al., 1991). PP2A consists of a core dimer made up of a catalytic subunit PP2Ac (36-KDa) that forms a complex structure with a scaffold regulatory subunit (PR65/A), combined with one of the regulatory subunits PR55/B, PR61/B, PR72/B or PR110/B (Olsen et al., 2006). PP2A has a well-established role as a regulator of the cell cycle and apoptosis and in many other cellular processes (Eichhorn et al., 2009). The function of PP2A in the regulation of major metabolic process, gene expression, apoptosis, cell proliferation and cancer is now well established (Reviewed by Seshacharyulu et al., 2013). It has been reported to act as tumour suppressor or tumour promoter depending on the cell type (Bhardwaj et al., 2011). Loss, mutations and abnormal expression of PP2A scaffold and regulatory subunits have been observed in various human cancers including lung, breast, skin, prostate and colon cancer, highlighting its role as a tumour suppressor (Bhardwaj et al., 2011; Pandey et al., 2013). On the other hand, some of PP2A subunits have also been implicated

in tumour initiation, progression and metastasis, underlining its role as an oncogene (Seshacharyulu et al., 2013).

Protein Phosphatase PP4 is another phosphatase within the PPP family and shares about 65% homology with PP2A. This PP2A-like phosphatase is also known to regulate number of important biological processes independently of PP2A. Although the consequences of the alteration in kinase functions in diseases and cancer are now well established (Manning et al., 2009); the role of specific phosphatases in these processes is less understood.

1.7 Protein phosphatase 4

Protein Phosphatase 4 is a ubiquitous and highly conserved serine/threonine phosphatase that belongs to the PPP family. Protein phosphatase 4 is also known as PP4, PPP4, PPX, PP4c and PPH3. PP4 causes dephosphorylation of the targeted proteins at the serine or threonine site and by that it regulates a variety of cellular functions independently of other protein phosphatases in the PPP family. PP4 shares about 65% homology with PP2A (Helps et al., 1998). Like other members of the PPP families, PP4 exists as a holoenzyme composed of a highly conserved catalytic subunit (PP4c) and one or more regulatory subunits and this difference in the assembly accounts for the ability of PP4 to regulate a wide range of cellular biological processes (Cohen et al., 2005). These PP4 component polypeptides include PP4 regulatory subunit 1 (PP4R1), PP4 regulatory subunit 2 (PP4R2), PP4 regulatory subunit 3 alpha (PP4R3 α), PP4 regulatory subunit 3 beta (PP4R3 β), PP4 regulatory subunit 4 (PP4R4), and alpha 4/ immunoglobulin (CD79A) binding protein 1 (α 4/ IGBP1) (Hastie et al., 2000; Carnegie et al., 2003; Gingras et al.,

2005; Chen et al., 2008). All of these regulatory subunits bind specifically to PP4c but not to the structurally related PP2A catalytic subunit (PP2Ac), except α4 that binds to both PP4c and PP2Ac (Murata et al., 1997; Chen et al., 1998). Current interest in PP4 has been increased after the discovery of its involvement in a number of important cellular processes. Some of these functions include centrosome maturation in *Drosophila melanogaster, Caenorhabditis elegans* and humans (Helps et al., 1998; Sumiyoshi et al., 2002; Martin-Granados et al., 2008), involvement in the nutrient sensing pathway and conferring resistance to the anti-cancer DNA-binding agents cisplatin and oxaliplatin in *Saccharomyces Cerevisiae* (Bertram et al., 2000). In mammalian cells, PP4 has also been implicated in apoptosis (Mourtada-Maarabouni et al., 2003; 2008), DNA repair (lee et al., 2010), tumour necrosis factor (TNF)-alpha signalling (Zhou et al., 2002), activation of c-Jun N-terminal kinase MAPK8 (Zhou et al., 2002), regulation of histone acetylation (Zhang et al., 2005), DNA damage checkpoint signalling (Nakada et al., 2008), NF-kappa-B activation (Hu et al., 1998) and cell migration (Martin-Granados et al., 2008).

1.7.1 PP4 catalytic subunit (PP4c)

The PP4c gene was mapped to human chromosome 16 p11.2, a location that has been linked to many of the translocational mutations associated with acute leukaemia (Bastians et al., 1997). Human PP4c was first isolated and described in 1992 by Brewis and Cohen who cloned its cDNA from a human teratocarcinoma library and named it as PPX. Human PP4c protein consists of a 307 amino acid sequence and has a size of 35 kDa. It is highly conserved during the course of mammalian evolution with human and *Drosophila* PP4 sharing 91% amino acid

identity (Brewis and Cohen, 1992). Human PP4c is 100% identical to mouse protein and differs from that of rabbit by only two amino acids (Brewis and Cohen, 1992). Such a high degree of conservation suggests that PP4c is an essential protein that may be involved in many essential cellular processes and that its function is tightly regulated *in vivo.* PP4c is mainly localized to the centrosomes where intense staining had been shown by immunofluorescence (Hastie et al., 2000), but it is also found elsewhere in the nucleus and at a lower level in the cytoplasm (Hastie et al., 2000).

Although PP4c shares about 65% amino acid identity with PP2A alpha and PP2A beta catalytic subunits, its regulatory subunits are very distinct from those of PP2A, suggesting that PP4 plays a different role in the regulation of cellular functions from PP2A (Brewis et al., 1993).

1.7.2 Functions of PP4c

The catalytic subunit of PP4 (PP4c) is crucial to the regulation of many major cellular functions and it appears that a minimal expression level of this enzyme is necessary for cell survival and proliferation (Mourtada-Maarabouni and Williams, 2008). PP4c plays a vital role in the process of microtubule organization (Toyo-oka et al., 2008), spliceosomal assembly via interaction with the survival motor neurones (SMNs) complex (Carnegie et al., 2003), and tumour necrosis factor signalling, where it is reported to be involved in TNF alpha-induced activation of JNK (Mihindukulasuriya. et al., 2004). In addition, PP4c gene deletion in the T-cell lineage resulted in abnormal thymocyte development and maturation, indicating that PP4c is essential for thymocyte development and for pre-T-cell antigen receptor (pre-TCR) signalling (Shui et al., 2007). It is also reported to regulate the activity of histone deacetylase 3

(HDAC3), one of four members of the human class I HDACs which regulate gene expression by deacetylation of histones and non-histone proteins (Zhang et al., 2005). PP4c is also involved in the DNA damage response. PP4c dephosphorylates ɣ- H2AX that is generated as a response to DNA replication (Chowdhury et al., 2008). Evidence has suggested that PP4c controls neural progenitor cell proliferation and differentiation in mouse neocortex by regulating the phosphorylation status of nuclear distribution protein nudE-like1 (Ndel1) (Xie et al., 2013). Deletion of PP4c in the early onset of neurogenesis resulted in a defect in the differentiation of cortical progenitor cells into neurons (Xie et al., 2013).

PP4c expression was reported to be altered in different tissues in murine embryo, indicating a potential role in embryonic development (Hu et al., 2001). Indeed, knockout mice studies have confirmed a vital and non-redundant role for PP4c in tissue development (Shui et al., 2007). Deletion of PP4c is reported to lead to embryonic lethality of mice (Shui et al., 2007). PP4c knockout mice showed a disruption in pro-B-cell differentiation leading to a complete absence of mature B-cells indicating an essential role of PP4c in B-cells development (Su et al., 2013).

Such pleiotropic and opposing functions of PP4c have been related to the existence of different PP4c complexes that have different compositions as a result of interaction of PP4c with its different regulatory subunits (Cohen et al., 2005; Brechmann et al., 2012). Therefore, substrate specificities, subcellular localisations, activities and functions of PP4c depend largely on the composition of its different complexes (Figure 1.3).

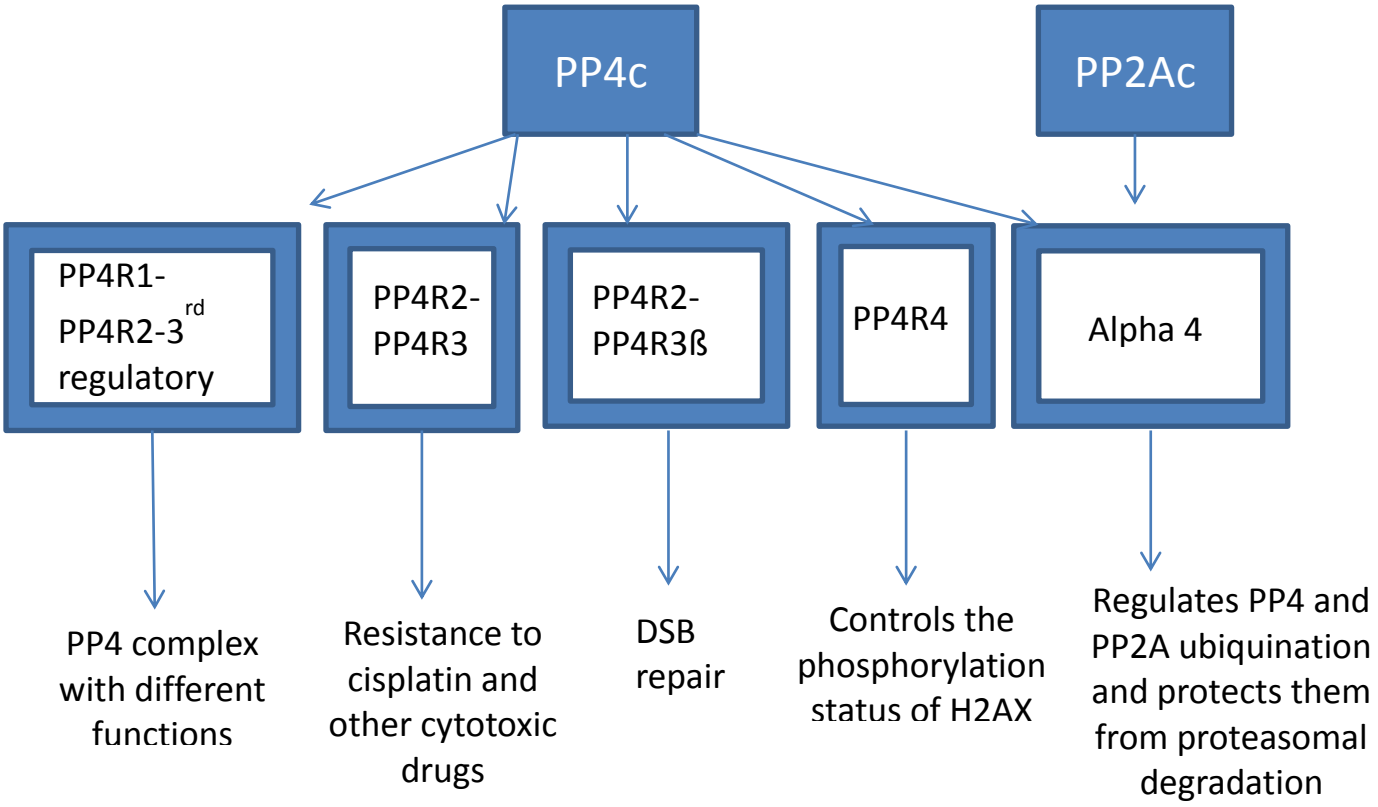


Figure 1.3: Wide variation in the function of PP4 enzyme complexes that results from combination of PP4c with different regulatory subunits. Alpha 4 subunit interacts with both PP4c and PP2Ac. All other PP4c regulatory subunits are specific for PP4c

1.7.3 Role of PP4c in apoptosis

Many proteins important in controlling apoptosis are reversibly phosphorylated and the activity of these proteins is determined by their phosphorylation status (Martin et al., 2008). These proteins include several members of the Bcl-2 family, including Bcl-2 itself (Deng et al., 1998), Bad (Chiang et al., 2001), Bid (Desagher et al., 2001) and Bik (Verma et al., 2001) as well as Forkhead transcription factor (FKHLR) (Brunet et al., 1999) and caspase 9 (Cardone et al., 1998). The function of the serine/threonine phosphatases PPP family in both intrinsic and extrinsic apoptotic pathways has been well documented (Figure 1.4) (Reviewed by Sun and Wang, 2012). As shown in figure 1.4, to date most of the studies have focused on PP2A and PP2B, and only a few studies directly implicate PP1, PP4, PP5, PP6, and PP7 members in cell death (Sun and Wang, 2012). However, a number of emerging pieces of evidence strongly support an important role for PP4 in the regulation of different signalling pathway involved in apoptosis.

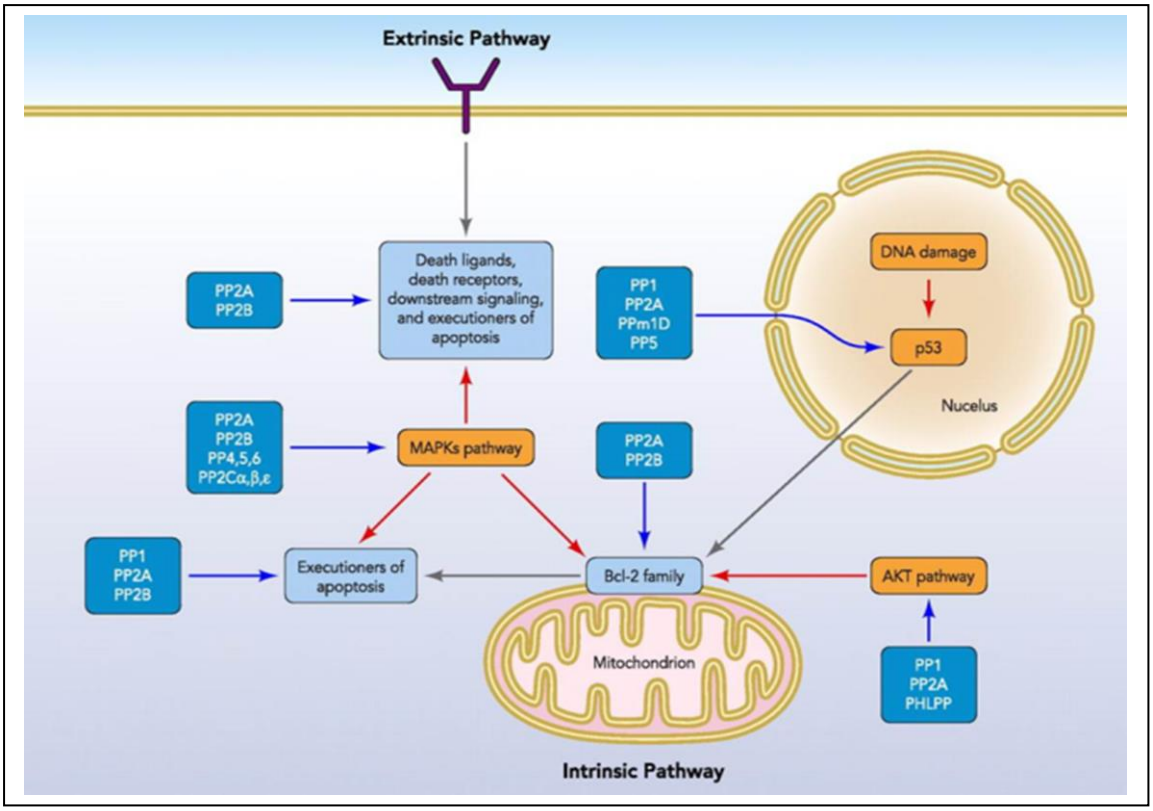


Figure 1.4: A diagram shows the involvement of different protein serine/threonine phosphatases in both intrinsic and extrinsic apoptotic pathways (Sun and Wang, 2011)

The initial experiments that implicated PP4c in controlling apoptosis in murine W7.2c thymoma cells involved the isolation of partial cDNA sequence of PP4c by an unbiased functional screen for genes regulating apoptosis (Mourtada-Maarabouni et al., 2003). W7.2c cells transfected with this partial cDNA in a retroviral expression vector had down-regulated PP4c and were resistant to both dexamethasone and UV radiation, suggesting that PP4c plays an important pro-apoptotic role in T-lymphocytes (Mourtada-Maarabouni et al., 2003). These observations were further supported by demonstrating that over-expression of PP4c induced apoptosis and inhibited cell proliferation in HEK 293T human embryonic cells and in both leukemic T-cell lines and primary human peripheral blood T-cells (Mourtada-Maraabouni and Williams, 2008, 2009). On the other hand, decreasing PP4c levels using siRNAs stimulated cell proliferation in these cells and protected against cell death induced by a number of apoptotic stimuli (Mourtada-Maraabouni and Williams, 2008; 2009). Proteomic analysis has shown that changes in PP4c expression levels affect the phosphorylation status of many proteins involved in apoptosis and cell proliferation, including activating transcription factor 2 (ATF2), cofilin 1, double-stranded RNA-dependent protein-serine kinase (PKR), c-Fos, heat shock 25 kDa protein (Hsp25), heat shock 27 kDa protein beta 1 (Hsp27), Akt1 (PKB), STAT3 (signal transducer and activator of transcription 3), ERK1 and ERK2 (extracellular signal-regulated kinase 1 and 2). Two critical apoptosis regulators, the crucial BH3-only pro-apoptotic Bcl-2 family protein Bad and PEA15 (Phosphoprotein Enriched in Astrocytes), were also identified to be significantly over-phosphorylated when PP4c expression was suppressed. PEA15 is a member of the death effector domain (DED) protein family which are known to control both cell proliferation and apoptosis (Krueger et al., 2005). A further study showed that the control of PP4-induced apoptosis in human

T-cells is partly mediated through dephosphorylation of PEA15 (Mourtada-Maarabouni et al., 2009). PEA15 is involved in the signalling pathways mediated by ERK1/2 and Akt and its function to increase or to decrease apoptosis is regulated by its phosphorylation (Krueger et al., 2005). Increasing evidence is now available to support a role for PP4c in MAPK–mediating apoptosis (Rose et al., 2010). The level of PP4c expression affects the phosphorylation status of ERK1 and ERK2 (Mourtada-Maarabouni et al., 2008) as well as PEA15, which is also involved in regulating ERK1 and ERK2 (Mourtada-Maarabouni et al., 2008, Krueger et al., 2005). PP4c is also involved in the TNFα-induced activation of JNK (Mihindukulasuriya et al., 2004), supporting a role for PP4c in regulating this pathway.

1.7.4 Role of PP4c in cancer

PP4c gene has been mapped to the middle region of the 16p11.2 locus, a region that has been reported to be involved in invasive breast tumours (Stange et al., 2006). The 16p11.2 locus has been also linked to many of the translocational mutations associated with acute leukaemia (Bastians et al., 1997). The fact that PP4c plays an important role in microtubule growth/organization (Toyooka et al., 2008), cell proliferation (Mourtada-Maarabouni and Williams, 2008; 2009) apoptosis (Mourtada-Maarabouni et al., 2003; Mourtada-Maarabouni and Williams, 2008; 2009) and tumour necrosis factor signalling (Mihindukulasuriy et al., 2004) indicates that PP4c is involved in different regulatory functions that determine cell fate.

PP4c acts as a positive regulator of the JNK-1 MAP kinase in prostate carcinoma cell lines (PC-3 and LNCaP) (Inostroza et al., 2005). It has also been reported to act

as a positive regulator of nuclear factor-kappa B (NF-κB) activity in human cervical carcinoma SiHa (Yeh et al., 2004). Whereas in T-lymphocytes; PP4c was found to have the opposite effect and to negatively regulate the activity of NF-kB (Brechmann et al., 2012). Indeed, negative regulation of the activity of NF-κB in T- lymphocytes has been linked to the PP4c-PP4R1 complex (Brechmann et al., 2012). One of the major causes of many types of human cancer is genomic instability which leads to an increase in the mutation rate and accumulation of genetic changes resulting in the transformation of a normal cell into a cancerous cell (Eshleman et al., 1995). Studies have shown that PP4c over-expression reduces mutation in the indicator gene, hypoxanthine phosphoribosyl transferase **(**hprt**),** whereas PP4c down- regulation increases its mutation frequency (Mourtada-Maarabouni and Williams, 2008; 2009). These observations support the role of PP4c in the induction of apoptosis after DNA damage and support the hypothesis that PP4c may function as a tumour suppressor gene.

Another major cause of oncogenesis in humans is the failure of the DNA repair systems that prevent the fixation of pre-mutagenic lesions in the genome. Accumulation of these lesions is very important for carcinogenesis (Shimada and Nakanashi, 2013). DNA damage results in the initiation of the DNA damage response (DDR) and the activation of cell cycle check points, allowing the cell to deal with the damage. Cell responds either by arresting the cell cycle, inducing senescence and apoptosis, or initiation of an appropriate DNA damage repair pathway (Shimada and Nakanashi, 2013). DNA damage repair can occur by two major pathways, homologous recombination repair (HR), which is highly accurate, and non-homologous end joining repair (NHEJ) which is more prone to error (Davis and Chen, 2012). Both pathways require activation of large number of protein

kinases, including ATM (Ataxia-Telangiectasia Mutated), ATR (Rad3-Related), DNA-PK (DNA dependant protein kinase), CHK1 (Checkpoint kinase 1) and CHK2 (Checkpoint kinase 2). Activities of these kinases are tightly regulated by phosphorylation and therefore their effects are reversed by the activation of various phosphatases (Lee et al., 2011). PP4 has been proved to be an important regulator of the NHEJ pathway (Lee et al., 2010). The dimeric PP4 complex, PP4c-PPP4R2, regulates the phosphorylation status of the replication protein A2 (RPA2). RPA2 phosphorylation is essential in the DNA double strand break (DSB) repair pathway (Davis and Chen, 2012). In response to a variety of DNA-damaging agents, including amptothecin, hydroxyurea and UV, ATR and DNA-PKcs phosphorylate RPA2. The PP4 complex; PP4c-PPP4R2 dephosphorylates the phosphorylated RPA2 and so regulates its role in the DNA-damage response (Lee et al., 2010). Results show that deletion of PP4c or PP4R2 causes an increase in hyperphosphorylated RPA2 (Lee et al., 2010). Dephosphorylation of RPA2 is essential for the continuation of post damage DNA synthesis and this in turn allows the cell to restart the cell cycle (Lee et al., 2010). In response to DNA double-stranded breaks, the histone H2A variant H2AX is rapidly phosphorylated by ATR to produce gamma -H2AX (Chowdhury et al., 2008). Gamma-H2AX functions to stabilise cell-cycle checkpoint proteins and DNA repair factors at the break site (Chowdhury et al., 2008). Another PP4 phosphatase complex containing PP4c, PP4R2, and PP4R3β was reported to dephosphorylate gamma-H2AX generated during DNA replication, a process required for DNA damage repair (Chowdhury et al., 2008).

While many studies have supported a role for PP4c as a tumour suppressor, others have suggested that PP4c may act as an oncogene (Wang et al., 2008; Weng et al., 2012). PP4c was reported to be over-expressed in human breast and lung tumours

and inhibition of its expression sensitised breast and lung cancer cells to cisplatin treatment (Wang et al., 2008). PP4c was also reported to be over-expressed in patients with pancreatic ductal adenocarcinoma (PDAC) and this over-expression was associated with distant metastasis in patients with stage II PDAC and was also associated with poor prognosis (Weng et al., 2012). It is therefore essential to further investigate the role of PP4c in these cancers and determine the significance of its over-expression in these tumours.

1.7.5 PP4c-interacting proteins

The PP4 holoenzyme occurs in different complexes of the PP4c with one or more regulatory subunits and the interaction between PP4c-regulatory subunits and PP4c has been reported to be essential to the mode of action of the enzyme (Cohen et al., 2005). To date, a number of PP4c-interacting proteins have been identified (see Figure 1.3 above).

1.7.5.1 PP4 regulatory subunit 1 (PP4R1)

Also known as MEG1, PP4R1 and PP4 (Rmeg). PP4R1 was the first regulatory subunit to be identified (Kloeker and Wadzinski, 1999; Wada et al., 2001). The size of its polypeptide is 105 kDa and its gene is located on chromosome18 p11.22. Initial studies have shown that PP4R1 interacts with PP4c forming a stable complex (Kloeker and Wadzinski, 1999; Wada et al., 2001). Further independent studies using proteomic approaches have provided further evidence for a specific interaction between PP4R1 and PP4c (Chen et al., 2008; Gingras et al., 2005). Recently, this

specific interaction has been confirmed to be occurred in human T-cells (Brechmann et al., 2012). T-cell receptor or tumour necrosis factor receptor 1(TNFR1) stimulation leads to the phosphorylation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) kinase (IKK complex) which is regarded as the core element of the NF-κB cascade. Mostly NF-kB exists in an inactive form in the cytoplasm due to its binding to the inhibitory kB proteins (IkBs). Phosphorylation of the IKK complex is followed by the proteasomal degradation of IkB resulting in the translocation of NF-κB proteins to the nucleus, where they bind to DNA and activate the transcription of NF-κB-regulated genes (Hayden and Ghosh, 2008). Brechmann et al (2012) have shown that PP4R1 potentiates the activity of PP4c and allows its association with IKK leading to its dephosphorylation and consequently the inhibition of NF-κB activity. Their work indicated that in the absence of PP4R1, PP4c is unable to dephosphorylate the IKK complex, resulting in unrestrained IKK phosphorylation and increased NF-kB activity (Brechmann et al., 2012). Inactivation of NF-κB by PP4R1 can be also induced by inhibiting TRAF2 (tumour necrosis factor receptor associated factor) and TRAF6. PP4R1 interacts with TRAF2 and TRAF6 in a RING domain-dependent manner and prevents activation of NF-κB (Hadweh et al., 2014). It was reported that PP4R1 plays an important role in promoting the growth of tumour cells in hepatocellular carcinoma (HCC). This role of PP4R1 in HCC cells is due to activation of two major mitogen-activated protein kinase signalling cascades p38 and c-Jun N-terminal kinase (JNK) (Wu et al., 2015).

1.7.5.2 PP4 regulatory subunit 2 (PP4R2)

The second PP4c-regulatory subunit is PP4R2 with a molecular mass of 50 kDa (Hastie et al., 2000). The gene that encodes PP4R2 is located on chromosome 3p13 (Hastie et al., 2000). Immunocytological studies revealed that like PP4c, PPP4R2 is predominantly located in centrosomes in human cells suggesting that it may be involved in localization of PP4c to this site (Hastie et al., 2000). Studies have suggested that PPP4-R1 and PPP4-R2 may act as core regulatory subunits forming PP4c-R1 and PP4c-R2 complexes and it was suggested that these complexes may attach to a third variable subunit to form different PP4 complexes with different functions (Carnegie et al., 2003; Janssens and Goris, 2001). Indeed, Gemin 4, a 120 kDa protein component of the survival of motor neuron (SMN) complex was identified as another possible variable subunit associating with the human PP4c–R2 complex (Carnegie et al., 2003). Association of both PP4R1 and PP4R2 with PP4c was reported to significantly decrease the activity of PP4c and this could be a consequence of general inhibition of the catalytic subunit by one of the regulatory subunits or because the regulatory subunits narrow PP4c substrate specificities (Hastie et al., 2000; Kloeker and Wadzinski, 1999). PP4c-PP4R2 complex regulates the phosphorylation status of the replication protein A2 (RPA2). RPA2 phosphorylation is essential in the DNA double strand break (DSB) repair pathway (Davis and Chen, 2012). PP4R2 affects the differentiation and survival of mouse motor-neuronal cell line NSC-34 through its interaction with survival of motor neuron protein (SMN) and it protects these cells from DNA damage-induced apoptosis (Bosio et al., 2012). PP4R2 and PP4R3 interact specifically with PP4c, but not with the related phosphatases in the PPP family like PP2Ac or PP6c, to form a multimeric complex called PP4cs (protein phosphatase 4, cisplatin-sensitive complex) which is

involved in cisplatin sensitivity (Gingras et al., 2005). PP4R2 was found to be over-expressed in human primary breast tumours but not in breast cancer cell lines (Wang et al., 2008).

1.7.5.3 PP4 regulatory subunit 3 (PP4R3)

PP4R3 is of two types; PP4R3α and PP4R3β (also termed respectively SMEK1 and SMEK2) (Gingras et al., 2005; Chowdhury et al., 2008) SMEK (suppressor of MEK : Mitogen-activated Protein/Extracellular Signal-regulated Kinase) plays a role in different molecular processes which include enhancing hepatic gluconeogenesis *in vivo* (Yoon et al., 2010), the regulation of MEK in *Dictyostelium* cells (Mendoza et al., 2005) and dephosphorylation of γ-H2AX (gamma-histone 2A variant X) in mammalian cells (Chowdhury et al., 2008). In *Dictyostelium;* PP4R3/SMEK specifically translocates from the cytoplasm to the nucleus in response to starvation (Mendoza et al., 2005). Since SMEK over-expression increased the nuclear accumulation of PP4c, it was suggested that SMEK may regulate the localisation of PP4c to the nucleus (Mendoza et al., 2005). In yeast and mammalian cells, PP4R3/SMEK forms a complex with PP4c and PP4R2 resulting in a trimer PP4c-PP4R2-PP4R3 that has a conserved role in resistance to cisplatin and other cytotoxic agents (Hastie et al., 2000; Gingras et al., 2005; Keogh et al., 2006). Whereas, another heterotrimeric PP4 complex PP4c-PP4R2-PP4R3β has been reported to be involved in DNA double strand break repair (DSB) (Lee et al., 2010). PP4R3α (SMEK1) was found to be over-expressed in human primary breast tumours but not in breast cancer cell lines (Wang et al., 2005). It was reported that the expression of SMEK1 is reduced in ovarian and in cervical tumour patients as well

as in the tumour cell lines (Dong et al., 2012). SMEK1 sensitizes ovarian cancer cells (OVCAR-3) to gemcitabine and enhanced the inhibitory effect of gemcitabine on cell migration. SMEK1 reduced cell proliferation by induction of cell cycle arrest at the G_1–G_0 phase, and increased expression of p53 and p21 with reduced expression of the anti-apoptotic Bcl-2 and Bcl-xL proteins (Dong et al., 2012). A combination of SMEK1 and gemcitabine reduced the phosphorylation of important proteins downstream of PI3K signalling pathway such as PDK1 and Akt, and also proteins that are targeted by the mTOR complex cascade such as p70S6K and 4E-BP1 (Byun et al., 2012). Also SMEK1 sensitized OVCAR-3 to paclitaxel by inducing cell cycle arrest at G_1 or G_2 phase and promoting paclitaxel-induced apoptosis by enhancing caspase-3 activation and Poly (ADP-ribose) polymerase (PARP) cleavage (Kim et al., 2014).

1.7.5.4 PP4 regulatory subunit 4 (PP4R4)

Using affinity purification and mass spectrometry, another PP4c interacting protein was characterised known as PP4R4/KIAA1622 (Chen et al., 2008). Although PP4R4 shares weak sequence homology with the scaffolding subunit A of the PP2A holoenzyme, it interacts specifically with PP4c and not with PP2Ac or other phosphatases, to form a stable cytosolic complex (Chen et al., 2008). As for PP4R1 and PP4R2, PP4R4 is likely to interact with PP4c in a direct manner and independently of other subunits (Chen et al., 2008). This is not the case for PP4R3 isoforms which depend on PP4R2 for association with PP4c (Chen et al., 2008; Chowdhury et al., 2008). PP4R4 and PP4R1 shares some homology with the A subunit of PP2A in containing HEAT repeats. The HEAT repeat is a tandem repeat

of 37-47 amino acids occurring in a number of cytoplasmic proteins, including huntingtin, elongation factor 3 (EF3), PP2A and the yeast PI3-kinase TOR1 (Andrade and Bork, 1995). While the PP2A-A subunit contains 15 HEAT repeats, PP4R1 contains 14 and PP4R4 contains 3 (Chen et al., 2008; Kloeker et al., 2003). Mutation and deletion in these HEAT repeat domains have been shown to prevent PP4c binding to PP4R1 (Chen et al., 2008).

1.7.5.5 Alpha 4/ immunoglobulin (CD79A) binding protein 1 (α4/ IGBP1)

In contrast to all PP4c interacting proteins described previously, α4 is the only regulatory subunit of PP4c that binds to both PP4c and PP2Ac (Hastie et al., 2000; Kloeker et al., 2003). α4 has been reported to play a role in the regulation of PP2Ac ubiquitination (McConnell et al., 2010). The protein prevents PP2Ac ubiquitination and so protects PP2Ac from proteasomal-mediated degradation (Kong et al., 2009; McConnell et al., 2010). Depletion of α4 leads to a rapid decline in the levels of PP2A, PP4 and PP6 catalytic subunit and to the inhibition of the dephosphorylation of their substrates (Kong et al., 2009).

1.8 PEA15 (phosphoprotein enriched in astrocytes)/ PED (phosphoprotein enriched in diabetes)

PEA15 is a member of the death effector domain (DED) protein family that is known to regulate cell proliferation, autophagy, glucose metabolism and apoptosis (Condorelli et al., 1999). PEA15 is predominantly expressed in the central nervous system, particularly in astrocytes and it is composed of an N-terminal death effector domain (ERK binding site) and two phosphorylation sites (Ser^{104} and Ser^{116}) at the C terminal tail (Kubes et al., 1998). PEA15 is implicated in the regulation of many signalling pathways involved in cancer progression and tumourigenesis. Some studies have reported PEA15 as a tumour suppressor others have reported it as a tumour promoter and this depend on its phosphorylation status which acts as a switch that turns PEA15 from tumour suppressor to tumour promoter (Glading et al., 2007; Bartholomeusz et al., 2008). PEA15 gene is amplified in breast cancer as well as in other cancers (Wei, 2015), but other studies have shown that PEA15 levels are significantly reduced in breast tumour tissues and in metastatic breast cancer cells (Glading et al., 2007). High PEA15 expression is associated with good prognosis and a high survival rate in women with ovarian carcinomas so PEA15 is regarded as a good prognostic marker in ovarian cancer (Bartholomeusz et al., 2008).

In its un-phosphorylated form, PEA15 binds to the mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2 (ERK1/2) and acts as a cytoplasmic anchor by sequestering ERK1/2 in the cytoplasm and preventing its nuclear localisation (Formstecher et al., 2001). Phosphorylation of PEA15 leads to the release of ERK1/2 resulting in the activation of the nuclear transcription factor Elk-1 and promotion of cell proliferation (Formstecher et al., 2001) (Figure 1.5). Phosphorylation of PEA15 also promotes its binding to FADD (Fas-associated

protein with death domain) via its DED domain (death effector domain) preventing FADD-mediated activation of caspases and formation of DISC (death-inducing signalling complex) resulting in an inhibition of apoptosis (Ramos et al., 1998). A number of kinases have been reported to be involved in the phosphorylation of PEA15 including Akt which phosphorylates PEA15 at Ser^{116} and stabilizes its anti-apoptotic activity (Araujo et al., 1993). It has been reported that phosphorylation of PEA15 at Ser^{116} resulted from activation of the Akt pathway by activated H-Ras through activation of Ras-MAPK/ERK signalling pathway (Sulzmaier et al., 2012). Phosphorylation of PEA15 at Ser^{116} is also required for apoptosis inhibition in glucose-deprived glioblastoma cells (Eckert et al., 2008). Other kinases that have been implicated in the phosphorylation of PEA15 are (PKC) protein kinase C which phosphorylates PEA15 at Ser^{104} (Araujo et al., 1993), Ca2+/calmodulin-dependent protein kinase (CaMKK) and AMP-activated protein kinase (AMPK) (Fiory et al., 2009; Hindupur et al., 2014). PEA15 phosphorylation at Ser^{104} by PKC blocks ERK binding both *in vitro* and *in vivo* (Renganathan et al., 2005) and eliminates PEA15-mediated inhibition of HeLa cell invasion (Glading et al., 2007). In addition, PEA15 phosphorylation at Ser^{104} is necessary for PEA15 mediated regulation of glucose metabolism (Condorelli et al., 2001). However, the phosphatases that are involved in the dephosphorylation of PEA15 have yet to be identified.

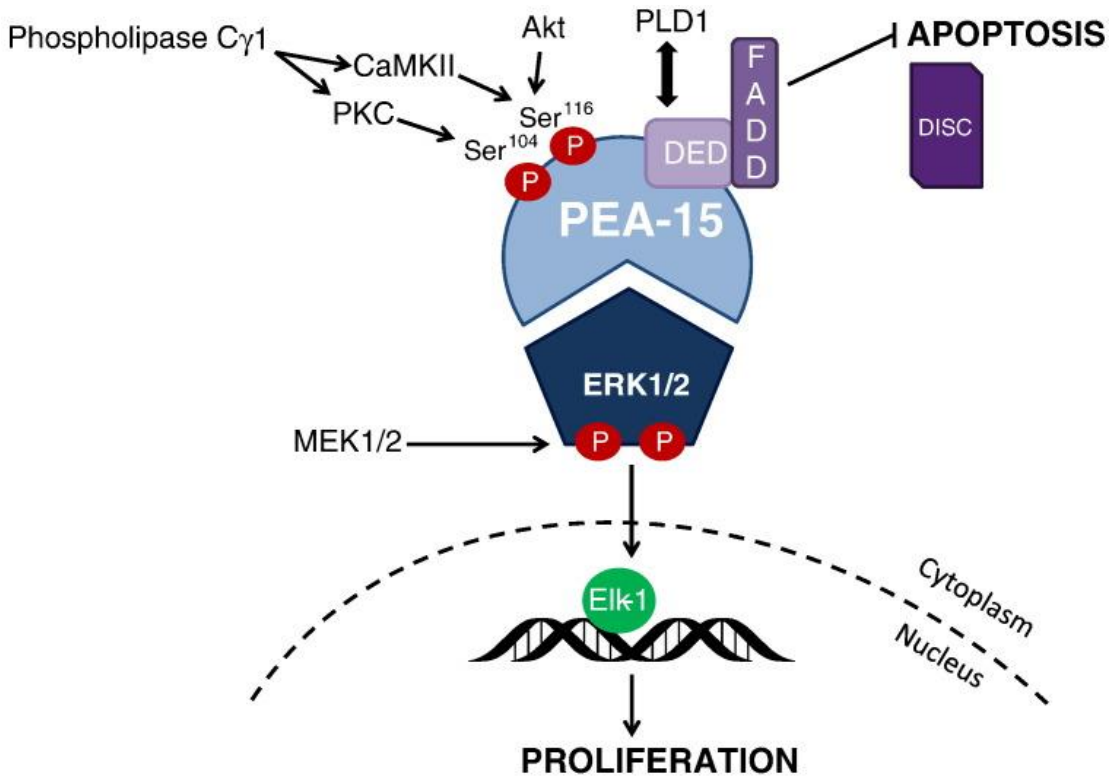


Figure 1.5: The effect of the phosphorylated form of PEA15 on cell proliferation and apoptosis. (Adapted from phosphoprotein enriched in astrocytes PEA15: A potential therapeutic target in multiple disease states) (Greig and Nixon, 2014)

Aims of the study

After proving the role of the process of reversible phosphorylation in controlling functions of most of the proteins that are involved in the signal transduction pathways of normal cells in general and of cancer cells in particular, and also after the implication of many protein kinases in the pathogenesis and the progression of many human cancers; investigation into the roles played by phosphatases, in particular PP4 catalytic subunit and its regulatory subunits, is essential. Evidence presented so far suggest that PP4c may play an important role in human malignancies. However, the expression and the function of PP4 in the tumuorigenesis and progression of human cancers including breast cancer remain unclear. Some studies previously suggested that PP4c may act as an oncogene whereas other studies revealed a pro-apoptotic role of PP4c and suggested that it may act as a tumour suppressor. The present work aimed to investigate the effects of alteration of PP4c expression on breast cancer cell growth and behaviour using two different breast cancer cell lines that are hormone positive breast cancer cells, including the MCF7 cell and triple negative breast cancer cells including MDA-MB-231 and Hs578T. Furthermore, the present study is intended to demonstrate how PPc down-regulation in these cells affects the response of these cells to various chemotherapeutic agents.

Activation of the PI3K/Akt/mTOR pathway can elicit changes in cell growth, division, apoptosis and cell migration and this has a great impact on tumour growth and patients' prognosis. The present work focuses on determining signalling pathways and proteins that are targeted by PP4c in inducing its function; the study also focuses on the identification of PP4c-interacting proteins that are crucial in

determining the function of the holoenzyme and its impact on breast cancer cell survival. Results obtained may open a new window toward a potential therapeutic approach for breast cancer.

2.1 Materials

2.1.1 Cell culture

The human breast cancer cell lines MCF7, MDA-MB-231 and Hs578T were purchased from ATCC-LGC Promochem (Teddington, UK). R-10 medium (RPMI-1640 (# R0883), L-glutamine (# G7513), sodium pyruvate (# S8636), HEPES buffer (# H0887), gentamicin (# G1272), Phosphate-buffered saline (PBS) (# 101584397) and ampicillin (# A0166) were from Sigma-Aldrich Company Ltd. (Gillingham, UK). Foetal bovine serum (FBS) (# FB-1001S) was from Biosera (East Sussex, UK). Trypsin/EDTA (# T3924) was from Invitrogen (Waltham, USA). Mr. Frosty™ Freezing Container (# 5100-0001) was from Fisher Scientific (Loughborough, UK).

2.1.2 Cell health and viability assays

Trypan blue stain (# T8154), acridine orange hemi (zinc chloride) salt (# A6014) and crystal violet stain (# HT90132) were from Sigma. Muse® Count & Viability Assay Kit (# MCH 100102), Muse® Annexin V and Dead Cell Assay Kit (# MCH100105) and Muse® Cell Cycle Assay Kit (# MCH100106) were from Merck Millipore (Frankfurter, Germany). The Cell Titer 96® AQueous One Solution Reagent (# G3582) was from Promega (Southampton, UK).

2.1.3 Real time RT-PCR (RT-qPCR) (Real-Time Quantitative Reverse Transcription PCR)

2.1.3.1 Gel electrophoresis

Agarose (Genetic Analysis Broad Separation Range for DNA/RNA/Genetic Analysis Grade) (# BP1356) was from Fisher Scientific. Ethidium bromide (# H5041) was from Promega. 10 X TAE buffer (Tris-acetate-EDTA) (# 15558-026) was from Invitrogen. HyperLadder™ 1kb (**#** BIO-33053) and DNA loading buffer (# BIO-37045) were from Bioline (London, UK).

2.1.3.2 Reverse transcription

TRIsure™ (**#** BIO-38032) was from Bioline. Omniscript RT Kit (# 205111) was from Qiagen (Crawley, UK). The kit includes 200 units Omniscript reverse transcriptase, 150 µl 10 x buffer RT, 100 µl dNTP Mix (Deoxyribonucleotide triphosphate) (contains 5 mM each dNTP) and 1.1 ml RNase-free water. RQ1 DNAase buffer (# M6101), RQ1 RNase-Free DNase (# M6101) and RQ1 DNAse stop solution (# M6101) were from Promega. Random primer (# 48190-011) and RNAse inhibitor (# N8080119) were from Invitrogen.

2.1.3.3 RT-qPCR

SensiFast Probe™ Hi-ROX kit (# bio-82020) was from Bioline. TaqMan gene expression assays; housekeeping gene expression assays and gene specific expression assays were obtained from Applied Biosystems (Warrington, UK). Yeast

total RNA (Ribonucleic acid, transfer from baker's yeast) (# R8508) was from Sigma. TissueScan™ Cancer and Normal Tissue cDNA Arrays (# BCRT102) was from OriGene (Rockville, USA).

2.1.4 Transient transfection

HiPerFect Transfection Reagent (# 301705), EndoFree Plasmid Maxi Kit (# 12362), PP4c-siRNA8, PP4R2-specific siRNAs, SMEK1 and SMEK2-specific siRNAs were purchased from Qiagen. Silencer™ siRNA Labeling Kit - Cy™3 (# 1632), (-)siRNA, PP4c-siRNA1, PP4c-siRNA2 and PEA15-specific siRNAs were from Ambion, Life Technologies Ltd. (Paisley, UK). Opti-MEM I (# 51985-026) was from Invitrogen. Amaxa® Cell Line Nucleofector® Kit V (# VCA-1003), Amaxa® Cell Line Nucleofector® Kit L (# VCA-1005) and pmaxGFP™ Vector (# D-00066) were from Lonza Biosciences (Verviers, Belgium). Ingenio® Kit (# 50115) and TransIT®-BrCa Transfection Reagent (# 50115) were from Mirus Bio LLC. (Madison, USA). NanoJuice® Transfection Kit (# 71902) was from Merck Millipore (Hertfordshire, UK) GeneJammer (# 204132) was from Agilent Technologies (Santa Clara, USA). EcoR1 (# R0101S) and Xhol (# R0146S) were from New England Biolabs (Hitchin, UK).

2.1.5 Western blotting

Laemmli buffer (# S3401), Protease Inhibitor Cocktail (# P8340), bovine serum albumin (BSA) (# A4503), TWEEN® 20 (# P9416) , RIPA buffer (Radio Immuno Precipitation Assay buffer) (# R-0278), monoclonal anti β-actin clone AC-74 purified

mouse immunoglobulin (# A-2228) and anti-goat IgG (whole molecule) peroxidase conjugates (# A-5420) were from Sigma. Bio-Rad Protein Assay Dye Reagent Concentrate (# 5000006), 7.5% Mini-PROTEAN® TGX™ Precast Protein Gels (# 4561021), 10 x Tris Glycine SDS running buffer (# 161-0772), Precision Plus Protein™ Dual Color Standards (**#** 1610374) and Clarity™ Western ECL Blotting Substrate (# 170-5060) were from Bio-Rad Laboratories Ltd, (Hempstead, UK). Immune-blot PVDF (Polyvinylidene fluoride) transfer membrane (Hybond-P) (# RPN303F) was from Amersham BioSciences UK Ltd. (Buckinghamshire, UK). 10 x Tris Buffered Saline (TBS) (# BP2471500) was from Fisher Scientific. PPX (C-18) (# sc-6118), SMEK1 (C-20) (# sc-244184) and SMEK2 (D-13) (# sc-169368) were from Santa Cruz Biotechnology (Heidelberg, Germany). PEA15 antibody (# 8682) was from Cell Signalling Technology (Danvers, USA). Anti-PED/PEA15 [$_{p}S^{116}$] (# 44-836G) was from Biosource International (Camarillo, CA, USA). PP4R2 polyclonal antibody (# ENT3834) was from Elabscience Biotechnology Co. Ltd. (Sydney, Australia). Goat anti-rabbit IgG (H&L), F(ab')2 Fragment (HRP) conjugate (# A24531) was from Novex ThermoFisher Scientific. Polyclonal goat anti-mouse immunoglobulin (# P0447) was from Dako UK Ltd. (Cambridge, UK).

2.1.6 Chemotherapeutic drugs

Cisplatin (# 155663-27), doxorubicin (# 25316-40-9), 5-fluorouracil (# F6627) and 10% dimethyl sulfoxide (DMSO) (#D5879) were from Sigma. Rapamycin (# 553210-100UG) was from Calbiochem- EMD Millipore (Watford, UK). Everolimus (# E-4040) was from LC Laboratories (Woburn, MA). BEZ235 (# S1009-SEL), AZD8055 (#

003022-USB) AZD5363 (# S8019-SEL) and LY294002 (# S1105-SEL) were from Stratech Scientific (Newmarket, UK).

2.1.7 Akt signalling pathway

PathScan® Akt Antibody Array Kit (Chemiluminescent Readout) (# 9474) was from Cell Signalling Technology.

2.2 Methods

2.2.1 Cell culture

The human breast cancer cell lines MCF7 (Soule et al., 1973), MDA-MB-231 (Cailleau et al., 1974), Hs578T (Hackett et al., 1977), T47D (Keydar et al., 1979), MDA-MB-361 (Brinkley et al., 1980) and the non-tumourigenic epithelial cell line (MCF10) (Soule et al., 1990) were generated from secondary stocks of cells which had been frozen down within two weeks receipt from the ATCC. Cells were cultured in RPMI medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 10% foetal bovine serum and 50 µg/ml gentamicin at 37°C in a humidified incubator with 5% CO_2. Cell lines were replaced with fresh stocks after a maximum culture period of two months. Cells were examined daily under a light microscope to check for growth and infection. At a confluence of about 80%, cells were split in a ratio of 1:10. To this end the cell culture medium was removed from the flask and the cells washed with phosphate-buffered saline (PBS), the supernatant was discarded and 2 ml of 0.25% trypsin/EDTA solution were added and the flask was returned to the incubator for 5 minutes to facilitate detachment of the cells. An equal volume of medium was added to the flask to inactivate the trypsin and the content of the flask was transferred to a 15 ml centrifuge tube and centrifuged at 1500 rpm at 25°C in order to pellet the cells. The supernatant was discarded and the cell pellet was re-suspended in fresh RPMI medium and seeded with a confluence of 10% into new flasks.

2.2.2 Freezing and thawing of the breast cancer cell lines

For long term storage of the cells, about 1 x 10^6 cells were harvested and re-suspended in 1 ml of cryoprotectant medium (40% FBS, 50% complete RPMI and 10% DMSO). Cell suspension was transferred into a cryotube and cooled slowly in Mr. Frosty™ freezing container and stored at -80°C to be transferred and stored at -140°C in liquid nitrogen for long term storage. For thawing cryopreserved cells, cells were rapidly thawed at 37°C and immediately re-suspended in 10 ml of RPMI to dilute the DMSO. Afterwards, cells were centrifuged at 1500 rpm for 5 minutes and re-suspended in an appropriate volume of fresh culture medium.

2.2.3 Cell count and cell viability assays

2.2.3.1 Vital dye exclusion assay

Cell viability was determined by trypan blue exclusion analysis using a haemocytometer. Dead cells absorb trypan blue dye through their damaged membrane so they selectively appear to have blue colour whereas the intact membrane of the live cell excludes this dye and the cell looks bright under the microscope. 10 µl of the cell suspension was mixed with an equal volume of 0.4% trypan blue stain. The cell suspension was applied to the edge of the chamber between the coverslip and the groove in the chamber. Cell suspension was allowed to be drawn into the chamber by capillary action. The haemocytometer grid was visualised under the microscope and viable cells and non-viable cells were counted. To calculate cell concentration per ml, the following equation was used:

Average Number of Cells in Four Large Squares X Dilution Factor X 10^4

Cell viability was calculated as a % using the following equation:

(Viable cell count / Total cell count) X 100

2.2.3.2 Muse® Count & Viability Assay

Total and viable cell counts were also determined by flow cytometry using the Muse® Count & Viability Assay kit and the mini flow cytometer the Millipore Muse. Muse® Count & Viability Assay is based on the use of two DNA-binding dyes, the nuclear dye stains only the nucleated cells and detects the viable cells, and it also detects unstained debris. Another dye is the viability dye which brightly stains dying and dead cells. A stained cell sample was prepared by mixing cell suspension with Muse® Count & Viability reagent at a 20-fold dilution (for example, 20 µL of cell suspension into 380 µL of Muse® Count & Viability reagent). Cells were allowed to stain for a minimum of five minutes at room temperature and were counted using the Millipore Muse cell analyser.

2.2.3.3 MTS cell viability assay

MTS cell viability assay is a colorimetric method for determining the number of viable cells in different cell survival assays. CellTiter 96® AQueous One Solution reagent is a reagent that contains a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an

electron coupling reagent (phenazine ethosulfate; PES). PES enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The assay is based on the reduction of MTS tetrazolium compound by viable cells to generate a coloured formazan product that is soluble in cell culture media. 20 µl of CellTiter 96® AQueous One Solution reagent was pipetted into each well of a 96-well plate containing the cells in 200 µl of culture medium. The plate was incubated at 37°C for three hours in a humidified 5% CO_2 atmosphere before assessment of cell viability by using Wallac Victor 1420 Multilabel Counter which measures sample absorbance readings at 490 nm (A490), the readings were corrected for the appropriate medium plus drug blank values. Absorbance is proportional to the number of viable cells.

2.2.4 Determination of apoptosis

2.2.4.1 Muse® Annexin V and Dead Cell Assay

Apoptosis was measured by flow cytometry using Millipore Muse cell analyser and Muse® Annexin V and Dead Cell Assay kit. Muse® Annexin V and Dead Cell Assay based on the use of a single reagent and two stains that are differentiate live, dead and apoptotic cells. Annexin V-PE stain detects phosphatidylserine (PS) molecules that are trans-located to the external membrane of apoptotic cells in the early apoptotic pathway (van Genderen et al., 2008) while 7-AAD satin (7-amino-actinomycin D) is excluded from live and healthy cells so it reflects the late-stage apoptotic and dead cells. A stained cell sample was prepared by mixing 100 µL of Muse® Annexin V and Dead Cell reagent with 100 µL of cells in suspension. The mixture was incubated for 20 minutes in dark place and at room temperature before data acquisition using the Millipore Muse cell analyser.

2.2.4.2 Acridine orange staining and fluorescence microscopy

Apoptosis was routinely determined by assessment of nuclear morphology by fluorescence microscopy after staining with acridine orange (25 µg/ml). After trypsinisation the cells were centrifuged at 1500 rpm and at 25°C in order to pellet the cells. The supernatant was discarded and the cell pellet was re-suspended in 250 µl RPMI medium, 20 µL of cell suspension were mixed with 20 µL of (25 µg /ml) acridine orange dye. The mixture was put on the slide and was covered with a cover slip and was examined under Nikon Eclipse E400 Binocular fluorescence microscope from Mazurek Optical Services ltd. Cells containing condensed or fragmented chromatin were scored as apoptotic cells.

2.2.5 Cell cycle analysis

Cell cycle profile was analysed by nuclear propidium iodide staining and flow cytometry using Muse® Cell Cycle Assay Kit and the Millipore Muse cell analyser. Muse® Cell Cycle Assay Kit uses reagent includes the nuclear DNA intercalating stain propidium iodide (PI). The assay is based on measuring G_0/G_1, S, and G_2/M phase distributions. Cells in the G_0/G_1 phase will have the faintest staining with PI as they have 2N DNA while cells in S phase because they are in process of DNA synthesis so they have anywhere from 2N to 4N DNA. Cells in G_2/M phase will stain twice as brightly compared to the cells in the G_0/G_1 phase because they will have 4N the DNA. 24 hours post-transfection with plasmid DNA or 72 hours post-transfection with siRNAs, Cells were harvested by trypsinisation and were plated in fresh medium at 5×10^5 cells/well in a 6-well plate. Following incubation for 24 hours, cells(~ one million) were suspended in 200 µl phosphate buffered saline (PBS), and fixed in 1 ml ice cold

(70% ethanol / 30% PBS) while vortexing at medium speed. Cells were incubated at –20°C for at least three hours prior to staining. Following the incubation, the cells were centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and the cell pellet was re-suspended in 200 µl of Muse™ Cell Cycle reagent. Cells were incubated for 30 minutes in the dark before data acquisition using the Millipore Muse cell analyser.

2.2.6 Anchorage-dependent clonogenic assay

Long term survival of the transfected cells was studied by assessing the ability of these cells to grow and to form colonies on plastic surface. Cells were seeded at a density of 1 x 10^3 cell/well in triplicate in a 6-well plate and covered by 2 ml RPMI medium containing 10% conditional growth medium. Cells were incubated in a humidified incubator at 37°C in 5% CO_2 and 95% air for 2-3 weeks. Number of the colonies was counted after staining the colonies with crystal violet stain (0.5% (w/v) in methanol) for 10 minutes.

2.2.7 Migration assay (wound scratch assay)

Wound scratch assay was used for migration study. Cells were transfected with gene specific siRNAs and plated in a 6-well plate. After the cells being 80% confluent, 10 µl pipet tip was used to create a longitudinal scratch of the cell monolayer. The cells were washed twice with PBS and 2 ml of the growth media were added. The distance between the two edges of the wound was measured at zero time and then every 18

hours using Leica DMIRB inverted fluorescence microscope. The % of the wound closure was calculated as following:

$$\frac{(\text{Pre-migration})_{\text{area}} - (\text{Migration})_{\text{area}}}{(\text{Pre-migration})_{\text{area}}} \times 100$$

2.2.8 Induction of cell death and cell survival assays

At 72 hours post-siRNA transfection, cells were trypsinised, and then seeded at a density of 500 cell/well in 0.1 ml RPMI medium in a 96-well plate or at a density of 1.6 x 10^5 cell/well in 0.8 ml RPMI medium in a 12-well plate. After four hours, an equal volume of medium containing the appropriate drug or vehicle, (final concentrations: 5-fluorouracil (5-FU; 175 µM), doxorubicin (0.2 µM), rapamycin (1 µM), everolimus (10 nM), cisplatin (5 µg/ml), BEZ235 (100 nM), AZD8055 (50 nM), AZD5363 (10 mM), LY294002 (10 µM) or vehicle (0.25% dimethyl sulphoxide) was added. Cells were cultured for 72 hours post-treatment, and then adherent cells were trypsinised and combined with non-adherent cells for analysis of cell survival. Cell viability was determined by Muse® Count & Viability Assay kit and MTS assay. For the latter, sample absorbance readings which reflect number of viable cells were measured at 490 nm (A490) using Wallac Victor 1420 Multilabel Counter and were corrected for the appropriate medium plus drug blank values.

2.2.9 RNA isolation

Total RNA was isolated from the parental cells and the transfected cells in order to determine gene expression; using TRIsure™. TRIsure™ is a ready-to-use reagent combines a blend of phenol and other components; it is used for the isolation of total RNA from cells and tissues because of its ability to maintain the integrity of the extracted RNA, while disrupting cells and subsequently dissolving cell components. Cells were pelleted at 1500 rpm for 5 minutes at room temperature. Cells were lysed with 1 ml of TRIsure™ per 5 x 10^6 cells and 800 µl of TRIsure™ for small quantities of cells (10^2-10^6). After homogenising the cells with TRIsure™ reagent, chloroform was added (1/5 volume of TRIsure™) for example 0.2 ml of chloroform for each 1 ml TRIsure™. The samples were shaken vigorously for 15 seconds before being incubated for 5 minutes at room temperature. Samples were spun at maximum speed of 13000 rpm for 15 minutes at 4°C. This step allows the homogenate to separate into a clear upper aqueous layer which contains the RNA, an interphase and a red lower organic layer which contains the DNA and proteins. The aqueous upper phase was transferred into new tube and the RNA was precipitated by adding ice cold isopropanol (70% of aqueous phase). The samples were vortexed and incubated for 10 minutes at room temperature. The samples were centrifuged at 13000 rpm for 15 minutes and at 4ºC. The supernatant was removed and the precipitated RNA was then washed with 1 ml of 75% ethanol per each 1 ml of TRIsure™. The samples were centrifuged at 8000 rpm for 5 minutes at 4°C. The pellets were air dried for 10 minutes before being re-suspended in 15 µl of RNAase free water. The concentration and purity of the extracted RNA was determined using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). A sample of 260/280 ratio of absorbance value of less than 1.8 and

260/230 ratio of absorbance value of less than 2.0 was discarded because this may indicate presence of protein contamination.

2.2.10 Plasmid preparation

Plasmid contains the gene under study; pcDNA3.1-PP4c expression constructs was expanded from frozen glycerol bacterial stocks (Mourtada-Maarabouni and Williams, 2008; 2009). A small piece of the frozen cells was chipped off and was added to a starter culture of 5 ml Lysogeny broth (LB) which is nutritionally rich medium primarily used for the growth of bacteria. LB consists of 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl; dissolved in 800 ml distilled water. Plasmids carry antibiotic resistance genes and this provides antibiotic resistance to the bacteria carrying them. 50 µg/ml of ampicillin was added to LB to avoid growth of bacteria that doesn't contain plasmid. The culture was incubated at 37°C for about eight hours with vigorous shaking (approximately 300 rpm). The starter medium was diluted into selective LB medium in a ratio of 1/500 then incubated at 37°C for 16 hours with vigorous shaking (approximately 300 rpm). Plasmid DNA was extracted from the culture using EndoFree Plasmid Maxi Kit. The culture medium was placed in 50 ml sterile centrifuge tube and centrifuged at 1500 rpm for 15 minutes at 4°C to harvest the bacterial cells. The supernatant was discarded and 10 ml of lysis buffer (P1) (with added RNase) was added and mixed by vortex.10 ml of lysis buffer 2 (P2) was added and mixed by inverting the tube for 6 times. The lysate was incubated at room temperature for five minutes and 10 ml of chilled P3 was added to enhance precipitation of the DNA, the mixture mixed by inverting the tube 6 times. The mixture was then transferred into a barrel of QIAfilter which was prepared by screwing the cap onto the outlet nozzle of the

QIAfilter Maxi Cartridge.The QIAfilter cartridge was placed in a convenient 50 ml tube and the mixture was incubated for 10 minutes at room temperature. During this period a white precipitate containing protein, genomic DNA and detergent was formed and floated to the top of the cartridge. The plunger was inserted and the lysate filtered. 2.5 ml of buffer ER (endotoxin removal) were added to the filter and mixed by inverting the tube 10 times then the mixture was incubated on ice for 30 minutes. A Qiagen tip 500 was equilibrated with 10 ml of Buffer QBT and allowed to drain by gravity. The filtered lysate was then added to the column and also allowed to drain by gravity and this followed by washing the QIAGEN-tip by 30 ml of Buffer QC twice, then 15 ml Buffer QN to elute the DNA. DNA was precipitated by adding 10.5 ml (0.7 volumes) room-temperature isopropanol; the mixture was mixed and centrifuged immediately at 12500 rpm for 30 minutes at 4°C and the supernatant was carefully decanted. The DNA pellet was washed with 5 ml endotoxin-free 70% ethanol to remove precipitated salt and to replace isopropanol with the more volatile ethanol, making the DNA easier to re-dissolve. The mixture was centrifuged at 12500 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was allowed to dry in air and then dissolved with 50 µl of sterile endotoxin-free buffer TE (Tris EDTA).

2.2.11 DNA digestion with restriction endonucleases

A diagnostic digest was carried out to verify the size of the isolated plasmid DNA (pcDNA3.1-PP4c). 2 µl of DNA sample was incubated with 40 units of EcoR1 and 20 units of XhoI restriction enzymes in the presence of 2 µl buffer D and 15 µl of molecular biology water. The reaction was allowed to incubate for one hour at 37°C before analysis by agarose gel electrophoresis.

2.2.12 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to determine the quality of the extracted RNA and digested DNA. 1 g of 1% agarose (Genetic Analysis Broad Separation Range for DNA/RNA/Genetic Analysis Grade) powder was poured into microwavable flask along with 100 ml of 1 x TAE which is a buffer solution containing a mixture of Tris base, acetic acid and EDTA. The mixture was microwaved for three minutes or until the agarose is completely dissolved. The agarose solution was allowed to cool down for five minutes before adding a fluorescent tag (nucleic acid stain); ethidium bromide to a final concentration of approximately 0.5 µg/ml. The agarose was poured slowly into a gel tray with the well comb in place. The newly poured gel was placed at 4°C for 15 minutes or at room temperature for 30 minutes, until it has completely solidified. Once solidified, the comb was removed and the agarose gel was placed into the gel box (electrophoresis unit) which has been filled with 1 x TAE (or TBE) until the gel is completely covered. 5 µl of HyperLadder™ 1kb were carefully loaded into the first slot of the gel. 1 µg of each sample was loaded with 4 µl of 5 x DNA loading buffer in a consideration of keeping a ratio of 1:5 before being loaded in the next slots of the gel. The gel was run at 150 V until the dye line is approximately 80% of the way down the gel. The ethidium bromide-stained DNA or RNA was visualised using White/ UV Transilluminators, UVP.

2.2.13 Real time RT-PCR

2.2.13.1 Reverse transcription

Reverse transcription of RNA was carried out to produce complementary DNA (cDNA) which is double-stranded DNA synthesized from a single stranded RNA. Qiagen Omniscript Reverse Transcription (RT) kit was used for the purpose of efficient and sensitive reverse transcription. Initially, a volume containing 1 µg of RNA was made up to a total volume of 7 µl with nuclease free water. An initial DNAase treatment was carried out by adding 1 µl of RQ1 DNAase buffer and 2 µl of RQ1 RNase-Free DNase; the mixture was gently mixed and then incubated at 37°C for 30 minutes. The samples were put immediately in ice and 1 µl of RQ1 DNAse stop solution was added to stop the activity of the DNAase. The samples were incubated at 10°C for 10 minutes. The reverse transcription step was performed by preparing master mix solution (Table 2.1).

Component	Volume/reaction	Final concentration
10 x Buffer RT	2 µl	1 x
dNTP Mix (5 mM each dNTP)	2 µl	0.5 mM each dNTP
Oligo-dT 15 primer (10 µM)	2 µl	1 µM
RNase inhibitor (10 units/ µl)	1 µl	10 units (per 20 µl reaction)
Omniscript Reverse Transcriptase	1 µl	4 units (per 20 µl reaction)
RNase-free water	5 µl	
Total volume	**13 µl**	**-**

Table 2.1: Components of the reverse transcription reaction. Reverse transcription step was performed by preparing master mix solution. An appropriate amount of each reagent applied in Omniscript kit was mixed and the volume was completed to 13 µl by RNase-free water

The total volume with 7 µl of our RNA sample will be 20 µl. The samples were incubated at 37°C for 60 minutes. The final concentration of cDNA obtained at the end of this procedure was 50 ng/ µl. Stocks of 5 ng/ µl were made by diluting the cDNA using nuclease-free water and the aliquots were stored at -80°C.

2.2.13.2 RT-qPCR

A real-time PCR was conducted using SensiFast Probe Hi-ROX kit and TaqMan gene expression assays (Table 2.2).

Name of the gene	Abbreviation	Assay	Accession number	Catalogue number
Eukaryotic 18S ribosomal RNA	18S	TaqMan Gene Expression Assays	NC_000005.10	Hs99999901_m1
5'-aminolevulinate synthase 1	ALAS1; ALAS; MIG4; ALAS3; ALASH; ALAS-H	TaqMan Gene Expression Assays	NC_000003.12	Hs00167441_m1
Protein phosphatase 4 catalytic subunit	PP4; PPX; PP-X; PP4C; PPH3; PPP4	TaqMan Gene Expression Assays	NC_000016.10	Hs00427262_m1
Phosphoprotein enriched in astrocytes 15	PEA15	TaqMan Gene Expression Assays	NC_000001.11	Hs00269428_m1
Protein phosphatase 4 regulatory subunit 1	PPP4R1; MEG1; PP4R1; PP4(Rmeg)	TaqMan Gene Expression Assays	NC_000018.10	Hs00933337_m1
Protein phosphatase 4 regulatory subunit 2	PP4R2; PPP4R2	TaqMan Gene Expression Assays	NC_000003.12	Hs00752559_m1
SMEK 1, (suppressor of mek1)	Smek1; Ppp4r3a; BC064465; mKIAA2010; 1110034C04Rik	TaqMan Gene Expression Assays	NC_000078.6	Hs00215697_m1

SMEK 2, (suppressor of mek1)	Smek2; Ppp4r3b; AW011752; AW557776; mKIAA1387	TaqMan Gene Expression Assays	NC_000077.6	Hs01106682_m1
Protein phosphatase 4 regulatory subunit 4	PPP4R4; PP4R4; CFAP14; KIAA1622	TaqMan Gene Expression Assays	NC_000014.9	Hs00372980_m1

Table 2.2: TaqMan gene expression assays. The table shows specific gene name and its abbreviation, assay accession number and the catalogue number

Each PCR reaction contained 1 µl (50 ng) of cDNA, 10 µl Sensifast, 1 µl TaqMan gene expression assays and 8 µl nuclease free water in a final volume of 20 µl. A standard curve was included with each run to allow relative quantitation. A standard curve was made by preparing serial dilution of 0.1–30 ng cDNA (prepared from mixture of breast cancer cells cDNA) (Table 2.3).

Number of Standard	cDNA concentration (ng)	Standard (µl)	Diluent (µl)
1	30	50	116.7
2	10	60	120
3	3	50	116.7
4	1	60	120
5	0.3	50	116.7
6	0.1	60	120

Table 2.3: Serial dilution used to prepare standard curve for RT-qPCR. A standard curve was made by preparing serial dilution of 0.1–30 ng cDNA (prepared from mixture of breast cancer cells cDNA)

Nuclease-free water containing 100 ng/µl yeast tRNA was used as a diluent; it acts as a carrier molecule that adheres to nucleic acid binding sites in the micro centrifuge tube and prevents binding of the nucleic acid of interest. For each assay; a standard curve of threshold cycle (Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to 18S mRNA. cDNA prepared from cells transfected with pcDNA3.1 empty vector was used as a control for PP4c over-expression and cDNA prepared from cells transfected with (-)siRNA (siRNA with scrambled sequence) was used as a control for PP4c down-regulation. In addition, cDNA prepared from MCF10 cells was used as a control for determining relative PP4c expression in different types of breast cancer cell lines.

RT-qPCR was also performed on TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. The array is ready-to-use panel of cDNA samples derived from patients with breast cancer of different stages, grades and hormone receptors expression together with cDNA samples derived from healthy individuals. The panel contains 48 samples covering 5 normal samples as a control and 45 breast cancer samples. The samples were collected from females 31-84 year of age with breast cancer of grade 6-9 according to Nottingham grading system. The tumour ranges from carcinoma in situ to metastatic ductal or lobular adenocarcinoma of the breast. The panel contains samples of different stages; 11-stage I, 8-IIA, 6-IIB, 8-IIIA, 2-IIIB, 4-IIIC, 4-IV. The panel contains samples show different hormone receptor expression; some are oestrogen positive, progesterone positive, oestrogen/progesterone positive, HER2 positive, triple positive or triple negative. For each assay, a standard curve of threshold cycle (Ct) value versus log input standard

cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to ALAS1 mRNA.

2.2.14 RNA interference by siRNA

2.2.14.1 siRNA labelling

siRNA labelling was carried out for subcellular localization of siRNA, stability, and to determine the transfection efficiency. This method tracks cells that receive siRNA during transfection and the transfection efficiency correlated with down-regulation of the target protein. siRNA labelling was performed by using Silencer™ siRNA Labelling Kit - Cy™3. Proper volume of water, 10 X labelling buffer, (20 µM) siRNA and Cy3 were mixed in 0.5 ml Eppendorf (Table 2.4). The Eppendorf was covered with tin foil and was heated at 37°C for one hour. The final concentration obtained was 7.68 µM. The prepared samples were stored at -80°C.

Substance	Volume/ µl
H2O	81.3
10 X labelling buffer	5
siRNA (20 µM)	19.2
Cy3	7.5

Table 2.4: siRNA labelling using Silencer™ siRNA Labelling Kit - Cy™3. The table shows the volume of the reagents used for the preparation of the mixture for siRNA labelling

2.2.14.2 Optimisation of siRNA transfection using HiPerFect transfection reagent

HiPerFect transfection reagent was a new transfection reagent that replaced the RNAifect, optimisation of the transfection conditions for this reagent was carried out using Cy3 labelled scrambled siRNA. MCF7, MDA-MB-231 and Hs578T breast cancer cells were plated at a concentration of 0.6 x 10^5 cell/well in a 6-well plate with 2.5 ml growth medium and were incubated at 37°C for 30 minutes. siRNA complex was prepared in 0.5 ml Eppendorf tube by mixing 6 µl of 7.68 µM of siRNA labelled with Cy3 and 94 µl Opti-MEM I. Opti-MEM I is a reduced serum media which is ideal for cationic lipid transfection. Opti-MEM I is buffered with HEPES and sodium bicarbonate; and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors. The mixture was mixed by vortexing before adding the HiPerFect transfection reagent in a three different volume (3, 6, 9 µl). Cells were incubated at 37°C for 72 hours. 72 hours following transfection, the cells were trypsinised and centrifuged at 1500 rpm for six minutes, the supernatant was discarded and the cell pellet was re-suspended in 20 µl of RPMI medium, 5 µl of the cell suspension were placed on a slide, covered by a cover slip and viewed under the microscope. The number of the cells in a field of vision was counted using the light microscope, several fields of view were examined until a minimum of 200 cells had been counted and the transfection efficiency was established by determining the percentage of the red fluoresced cells that are successfully transfected with the siRNA labelled with Cy3 using Nikon Eclipse E400 Binocular fluorescence microscope with FITC filter.

The optimisation of siRNA transfection results using HiPerFect transfection reagent showed that the three different volumes of HiPerFect transfection reagent resulted in

high transfection efficiency of 95% in the three cell lines. Furthermore, the three different volumes of the transfection reagent did not affect the cell viability.

2.2.14.3 Gene silencing using HiPerFect transfection reagent

MCF7, MDA-MB-231 and Hs578T breast cancer cells were transfected with PP4c-specific siRNAs, also MCF7 and MDA-MB-231 were transfected with two different PEA15-specific siRNAs using HiPerFect transfection reagent. Cells with mock transfection (no siRNA, HiPerFect transfection reagent been added only) as well as cells been transfected with (-)siRNA (siRNA with scrambled sequence) have been used as a control. Three different PP4c-specific siRNAs termed as PP4s1, PP4s2, PP4s8 and two different PEA15-specific siRNAs termed PEA15s1 and PEA15s2 were used (Table 2.5).

Gene name	siRNA	Target exon	Catalogue number	Company
PP4c	PP4c siRNA1 PP4c siRNA2 PP4c siRNA8	Exon 7 Exon 4 Exon 6	# 105835 # 105834 S102658698	Ambion Ambion Qiagen
PEA15	PEA15 siRNA1 PEA15 siRNA2	Exon 4 Exon 2	n137203 n43349	Ambion, Life Technologies Ltd
PP4R2	PP4R2 siRNA5 PP4R2 siRNA7	Exon 1 Exon 3	SI04131974 SI04186266	Qiagen Qiagen
SMEK1	SMEK1 siRNA2 SMEK1 siRNA5	Exon 3 Exon 4	SI04294955 SI05069960	Qiagen Qiagen
SMEK2	SMEK2 siRNA1 SMEK2 siRNA5	Exon 1 Exon 3	SI04165119 SI05096112	Qiagen Qiagen

Table 2.5: Target gene silencing was achieved by using pre-designated siRNAs. The table shows the target gene name, gene specific siRNAs, specific siRNA targeted exons, specific siRNA catalogue number and the provider companies

Cells were trypsinised and seeded at 1.6 x 10^5 in a 6-well plate, 2.5 ml of growth medium were added and the cells were incubated at 37°C for 30 minutes. siRNA complex was prepared in 0.5 ml Eppendorf tube by mixing 6 µl of 2 µM of either (-)siRNA or gene-specific siRNAs and 94 µl Opti-MEM I, the mixture was mixed by vortexing then 9 µl of HiPerFect transfection reagent was added and mixed by gentle pipetting for five minutes. The mixture was incubated at room temperature for 10 minutes before being added in a drop wise manner whilst swirling the plate. Cells were harvested at 72 hours post-transfection, and re-plated for assessment of cell survival after a further 48 hours.

2.2.15 Plasmid DNA transfection

2.2.15.1 Optimisation of the transfection

Different commercially available chemical transfection reagents such as GeneJammer, TransIT®-BrCa and Nanojuice were used to optimise the transfection of MCF7, MDA-MB-231 and Hs578T breast cancer cells. A plasmid expressing the green fluorescent protein (GFP) was used to determine the transfection efficiency. 24 hours post-transfection, the transfected cells were trypsinised and centrifuged at 1500 rpm for six minutes and re-suspended in 20 µl of RPMI medium, 5 µl of the re-suspended cells were placed on a slide and were covered with a cover slip and viewed under the microscope. The number of the cells in a field of vision was counted using the light microscope, several fields of view were examined until a minimum of 200 cells had been counted and the transfection efficiency was established by determining the percentage of cells expressing the green fluorescent protein using Nikon Eclipse E400 Binocular fluorescence microscope with FITC filter.

GeneJammer was used for the transfection of MCF7 and MDA-MB-231 breast cancer cells at a ratio of 3:1 and 6:1 (GeneJammer reagent: DNA) and resulted in a transfection efficiency of less than 20% in both types of the cells after 24 hours of the transfection. Different ratios of NanoJuice® Transfection Kit reagent were also used for the transfection of MCF7 cells and it was also resulted in a low transfection efficiency of 25% at 24 hours post-transfection.

TransIT®-BrCa transfection reagent was proved to be the method of choice for MCF7 transfection where the transfection efficiency was 40% after 24 hours of the transfection and 70% after 48 hours of the transfection.

Nucleofection using the Amaxa® Cell Line Nucleofector® Kit V in MDA-MB-231 cells and Amaxa® Cell Line Nucleofector® Kit L in Hs578T cells resulted in very high transfection efficiency which was 75% and 80% respectively at 48 hours post-transfection.

2.2.15.2 Plasmid DNA transfection using Amaxa® Cell Line Nucleofector® Kit V and Kit L

MDA-MB-231 and Hs578T cells were nucleofected with plasmids using Amaxa® Cell Line Nucleofector® Kit V and Amaxa® Cell Line Nucleofector® Kit L respectively. The cells were sub-cultured two days before nucleofection. On the day of the nucleofection, 2 x 10^6 cells were re-suspended in 100 µl of Amaxa® Cell Line Nucleofector® Kit V or Amaxa® Cell Line Nucleofector® Kit L. 2 µg of plasmid DNA (pcDNA3.1-PP4c) or 2 µg pmaxGFP® Vector were added to the cells. Cell/DNA suspension was transferred into nucleofector certified cuvettes and was nucleofected

using Amaxa™ Nucleofector™ II. Program X-013 was used for MDA-MB-231 and program T-30 was used for Hs578T. Cells were recovered and plated in 1.5 ml RPMI medium in a 6-well plate. The plate was incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. 24 hours after transfection the transfected cells were trypsinised, counted and re-plated for 48 hours before assessment of the cell survival.

2.2.15.3 Transfection of MCF7 using TransIT®-BrCa transfection reagent

TransIT®-BrCa transfection reagent was used for the transfection of MCF7 breast cancer cell line. 24 hours before the transfection the cells were trypsinised, counted and plated at a density of 2.5 x 10^5 cells per transfection in 2.5 ml of growth media in a 6-well plate. Cells were incubated in a humidified incubator at 37°C and 5% CO_2. 24 hours after incubation, the transfection complex was prepared by mixing 250 µl 0f Opti-MEM I and 2.5 µg of plasmid DNA (pcDNA3.1-PP4c) followed by the addition of 5 µl of TransIT®-BrCa transfection reagent. The mixture was incubated at room temperature for 15 minutes before being added to the cells in a drop wise manner whilst swirling the plate. The plate was incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. 24 hours after transfection, the transfected cells were trypsinised, counted and re-plated in a 6-well plate to be incubated for a further 48 hours before assessment of cell survival.

2.2.16 Western blotting

Western blotting was performed to determine the expression level of target protein. The efficiency of PP4c over-expression, PP4c down-regulation, PEA15 down-regulation as well as PP4c-specific regulatory subunits such as PP4R2, SMEK1 and SMEK2 down-regulation was assessed by performing western blotting analysis on the proteins that are extracted from the transfected cells.

2.2.16.1 Protein extraction

Protein extraction was performed using Laemmli sample buffer (12.5 ml of 1.0 MTris-HCl, Ph 6.8), 40 ml; 10% SDS, 37.7g glycerol, 1ml β-mercaptoethanol (electrophoresis grade), 100 µg bromophenol blue). Cells were trypsinised and counted, 1 x 10^6 cells were placed on ice, washed twice with PBS and re-suspended in 20 µl of a mixture of (1:100); (protease inhibitor :RIPA buffer). RIPA buffer consists of 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0. 20 µl of sample buffer; Laemmli 2 x concentration was then added. The samples were incubated at 95°C for 10 minutes and centrifuged at 1000 rpm for 10 minutes before being subjected to SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis.

2.2.16.2 Bradford protein quantification assay

Bradford protein assay is a simple and accurate procedure for determining the concentration of the proteins in a solution. It is a colorimetric method based on the

colour change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein (Reisner et al., 1975). Serial dilution of 0-0.5 mg/ml has been prepared from BSA (bovine serum albumin) (Table 2.6).

BSA mg/ml	Volume of 0.5 mg/ml stock / µl	Volume of H2O/ µl
0.5	**500**	**0**
0.4	**400**	**100**
0.3	**300**	**200**
0.2	**200**	**300**
0.1	**100**	**400**
0 (Blank)	**0**	**500**

Table 2.6: BSA serial dilution used for Bradford protein assay. Serial dilution of 0-0.5 mg/ml BSA was prepared and used as a standard curve for protein quantification

Duplicate of 10 µl of each BSA dilution has been pipetted in a 96-well plate. Protein samples have been prepared from the cultured cells. The media was removed and the cells were washed once with ice-cold 1 X PBS. After removal of PBS, 0.5 ml of ice-cold 1 X cell lysis buffer was added for each plate (10 cm in diameter) and was incubated on ice for 5 minutes. Cells were transferred to an appropriate tube and been kept on ice. Cells were microcentrifuged at maximum speed for 10 minutes at 4°C and the supernatant was transferred to a new tube. The supernatant is the cell lysate. Lysate was used immediately or stored at –80°C in single-use aliquots. The unknown samples of protein have also been diluted in a ratio of 1:10 using molecular biology water. 10 µl of each unknown sample has been pipetted in a duplicate in the 96-well plate. 200 µl of BI0-Rad protein reagent has been added to the standard samples and the unknown samples and the plate was incubated for five minutes before being read using BioTek™ ELx800™ Absorbance Microplate Readers at 630 nm.

2.2.16.3 One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)

One-dimensional, sodium dodecyl sulphate; SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5% Mini-PROTEAN® Precast gels and Trans-Blot® SD Semi-Dry Transfer Cell. Two gels were placed in a BIO-RAD Western rig and the tank filled to above the gel with running buffer; 1 X TGS (Tris 25 mM, Glycine 192 mM, 0.1%SDS, pH 8.3). 5 µl of the protein marker (Precision Plus Protein™ Dual Color Standards) was pipetted into the first well and 50 µg of the prepared protein samples were pipetted in the following wells. The gel was run at 150 volts until the samples had run the length of the gel. The gel was then removed

from the mould and allowed to equilibrate in transfer buffer (25 mM Tris 190 mM glycine 20% methanol) for 10 minutes before being placed in a cassette holder against an equal sized piece of immune-blot PVDF transfer membrane with a transfer buffer wet six pieces of filter paper pads that were placed on the outer aspect of the gel and the membrane in a sandwich like pattern. The cassette was closed and run at 38 mA /gel for two hours to allow transfer of the proteins into the membrane. After this time the membrane was removed and washed with TBST (Tris-buffered saline, 0.1% TWEEN® 20) for five minutes. The membrane was incubated with the blocking buffer (5% dried skimmed milk; (MARVEL) in TBS-T) with gentle agitation at room temperature for one hour to prevent nonspecific binding of the detection antibodies during subsequent steps. The membrane was subsequently washed three times in TBS-T for 15 minutes. The membrane was then incubated with a primary antibody that is specific for the target protein (Table 2.7).

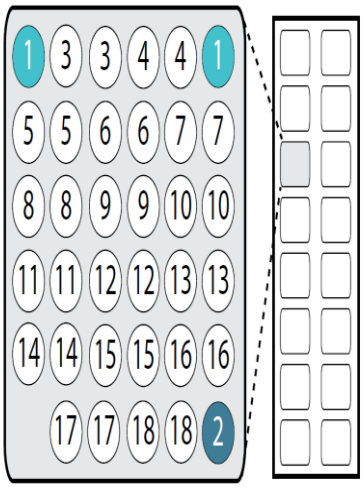
Primary antibody	Target	Clonality	Secondary antibody	Catalogue number	Company
anti-PP4c (PPX/PP4 (C-18)	PP4c	Polyclonal	Anti-goat IgG-HRP conjugate	sc-6118	Santa Cruz Biotechnology
anti-PEA-15 antibody	Total PEA-15	Polyclonal	Anti-rabbit IgG-HRP conjugate	#8682	Cell Signalling Technology
Anti-PED/PEA [pS116]	PED/PEA [pS116]	Polyclonal	Anti-rabbit IgG-HRP conjugate	#44-836G	Biousource
Anti-PP4R2	PP4R2	Polyclonal	Anti-rabbit IgG-HRP conjugate	#ENT3834	Elabscience
Anti-SMEK1	SMEK1	Polyclonal	Anti-goat IgG-HRP conjugate	sc-244184	Santa Cruz Biotechnology
Anti-SMEK2	SMEK2	Polyclonal	Anti-goat IgG-HRP conjugate	sc-169368	Santa Cruz Biotechnology
anti-β-actin antibody	β-actin	Monoclonal	Anti-mouse IgG-HRP conjugate	A-2228	Sigma

Table 2.7: Antibodies used in western blot analysis. The table shows the names of different primary antibodies, their target proteins, clonality, suitable secondary antibodies, their catalogue numbers and the productive companies

The primary antibody was diluted with 5% milk and the incubation period of the primary antibody depends on the clonality of the antibody; monoclonal antibodies were incubated with the membrane for one hour whereas polyclonal antibodies were incubated for overnight at 4°C. Three further washes with TBS-T were performed before applying the secondary antibody which was also diluted with 5% milk and incubated with the membrane for one hour. Three further washes with TBS-T were performed before scanning. The membrane was incubated with the enhanced chemiluminescence (ECL) Clarity™ Western ECL Blotting Substrate for 12 minutes. Enhanced chemiluminescence (ECL) is an indirect detection method that uses horseradish peroxidase (HRP)-labelled secondary antibodies. HRP is an enzyme that oxidize substrate using hydrogen peroxide as an oxidizing agent, yields a characteristic change that is detectable by spectrophotometric methods (Veitch, 2004). Signals were visualized utilizing the Odyssey® Imager (Li-Cor). Light is the signal that the film or digital imager detects (Mathews et al., 2009). Densitometric analysis carried out using the associated Image Studio™ Software (version 3.1).

2.2.17 Detection of the expression of the phosphorylated proteins in the Akt pathway

Analysis of the expression of the phosphorylated proteins in the Akt pathway was carried out using the PathScan® Akt Antibody Array kit (Chemiluminescent Readout) (Figure 2.1).



	Target	Phosphorylation Site
1	Positive Control	N/A
2	Negative Control	N/A
3	Akt	Thr308
4	Akt	Ser473
5	S6 Ribosomal Protein	Ser235/236
6	AMPKα	Thr172
7	PRAS40	Thr246
8	mTOR	Ser2481
9	GSK-3α	Ser21
10	GSK-3β	Ser9
11	p70 S6 Kinase	Thr389
12	p70 S6 Kinase	Thr421/Ser424
13	Bad	Ser112
14	RSK1	Thr421/Ser424
15	PTEN	Ser380
16	PDK 1	Ser241
17	Erk1/2	Thr202/Tyr204
18	4E-BP1	Thr37/46

Figure 2.1: Target map of the PathScan® Akt Signalling Antibody Array Kit (Chemiluminescent Readout)

PathScan® Akt Antibody Array Kit allows for the detection of sixteen phosphorylated proteins belonging to the Akt signalling network these proteins are: Akt Thr308, Akt Ser473, S6 Ribosomal Protein Ser235/236, AMPKa Thr172, PRAS40 Thr246, mTOR Ser2481, GSK-3a Ser21, GSK-3b Ser9, p70 S6 Kinase Thr389, p70 S6 Kinase Thr421/Ser424, Bad Ser112, RSK1 Thr421/Ser424, PTEN Ser380, PDK 1 Ser241, Erk1/2 Thr202/Tyr204, 4E-BP1 Thr37/46 .Protein samples were prepared and quantification of the extracted protein was done using Bradford protein assay. Cell lysates were diluted to 1 mg/ml using array diluent buffer before performing the assay. After affixing a multi-well gasket to the glass slide, 100 µl of array blocking buffer was added to each well and the gasket was covered with sealing tape. After incubation for 15 minutes at room temperature on an orbital shaker, 75 µl of the diluted lysate were added to each well and the gasket was incubated for two hours at room temperature on an orbital shaker. The gasket was washed three times with 1 X array wash buffer and incubated for five minutes at room temperature on an orbital shaker. 75 µl of 1 X detection antibody cocktail were added to each well and the gasket was incubated for one hour at room temperature on an orbital shaker. After washing 4 X 5 minutes with 100 µl 1 X array wash buffer; 75 µl of 1 X DyLight 680®-linked Streptavidin were added to each well and the gasket was incubated for 30 minutes at room temperature on an orbital shaker, another washing 4 X 5 minutes with 100 µl 1 X array wash buffer was performed. The slide was removed from the multi-well gasket and was washed once with 10 ml deionized water for ten seconds. Signals were visualized utilizing the Odyssey® Imager (Li-Cor) and densitometric analysis carried out using the associated Image Studio™ Software (version 3.1).

2.2.18 Statistical analysis

Data are presented as the mean ± SEM; the number of observations (n) refers to different transfected samples or separate cultures. Data analysis was done either by an unpaired Student's t-test or by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests (MCT). Additional analysis has been done on some of data (noted in the text) using Pearson's correlation coefficient where the coefficient value is considered to be significant if it ranges between -1.00 and 1.00. 1 is total positive linear correlation, 0 is no linear correlation, and -1 is total negative linear correlation. The closer the value of R to +1 is the stronger the positive linear correlation. Statistical analyses were performed using GraphPad Prism6 and Origin 6.1. p-value of < 0.05 was considered to be statistically significant.

Chapter 3:The protein phosphatase 4-PEA15 axis regulates the survival of breast cancer cells

3.1 Introduction

Accumulating evidence has demonstrated that the catalytic subunit of PP4 (PP4c) plays important and complex roles in apoptosis, cell proliferation and cancer. Over-expression of PP4c results in an increase in cell death and a decrease in cell proliferation in mouse thymoma cells (Mourtada-Maarabouni et al., 2003), human embryonic kidney cell line (HEK 293T) (Mourtada-Maarabouni et al., 2003) and in both leukemic T-cells and untransformed human peripheral blood T-cells (Mourtada-Maarabouni and Williams, 2009). Down-regulation of PP4c causes an increase in the rate of cell proliferation and conferred resistance to a number of apoptotic stimuli in human leukemic T-cells and untransformed human peripheral blood T-cells (Mourtada-Maarabouni and Williams, 2009). Down-regulation of PP4c is also reported to have a strong influence on gene mutation rate in leukemic T-cells, which is crucial to oncogenesis (Mourtada-Maarabouni and Williams, 2009). On the other hand, reduction in endogenous PP4c expression induced apoptosis in A549 and HeLa cells (Theobald et al., 2013). Increased expression of PP4c has been observed in breast cancer, lung cancer, pancreatic ductal adenocarcinoma and colorectal carcinoma (Wang et al., 2008; Weng e al., 2012). The interaction of PP4c with different regulatory proteins which results in the formation of different PP4 complexes with distinct subcellular locations and diverse substrates could explain the different effects of PP4c observed on cell growth and apoptosis. Overall, these observations suggest that PP4c dysfunction may be important in the development

and progression of cancer and highlight the importance of further studies to characterise its role in breast cancer.

Proteomic analysis has shown that changes in PP4c expression level in human embryonic kidney (HEK 293T) cells affected the phosphorylation status of many proteins involved in apoptosis and cell proliferation, including the critical apoptosis regulator phosphoprotein enriched in astrocytes 15 kD (PEA15) (Mourtada-Maarabouni and Williams, 2008). Further analysis has confirmed the interaction between PP4c and PEA15 and showed that the PP4c-induced apoptosis in normal lymphocytes and T-leukemic cells is partly mediated through the direct or indirect dephosphorylation of PEA15 (Mourtada-Maarabouni and Williams, 2009). PEA15 is a member of the death effector domain (DED) protein family known to regulate cell proliferation, autophagy, and apoptosis (Dontu et al., 2003; Kim et al., 2012). It is implicated in the dysregulation of many signalling pathways involved in cancer progression and tumourigenesis and has been described to act as both tumour suppressor and tumour promoter dependent on its phosphorylation status (Glading et al., 2007; Bartholomeusz et al., 2008; Gawecka et al., 2012). PEA15 gene is amplified in breast cancer as well as in other cancers (Wei, 2015). Studies have also shown that PEA15 levels are significantly reduced in breast tumour tissues and its unphosporylated form is more potent than the wild-type form in suppressing tumourigenicity in breast cancer (Xie et al., 2015). While a number of kinases has been reported to be involved in the phosphorylation of PEA15 including Akt, Ca2+/calmodulin-dependent protein kinase (CaMKII) and AMP-activated protein kinase (AMPK) (Fiory et al., 2009; Hindupur et al., 2014), the dephosphorylation of PEA15 is much less understood.

In this chapter, we have further investigated the role of PP4c in breast cancer. First, we have examined the expression status of PP4c in breast cancer tissue samples and in breast cancer cell lines. Second, we have investigated the hypothesis that modulation of PP4c expression level in breast cancer cells affects their survival and proliferation. Finally, we have explored the interaction between PP4c and PEA15 and the involvement of PEA15 in mediating the functional responses to the modulation of PP4c level in breast cancer cells.

3.2 Methods

For PP4c over-expression; MCF7, MDA-MB-231 and Hs578T breast cancer cells were transfected with pcDNA3.1 empty victor as a control or with pcDNA3.1 encoding the catalytic subunit of PP4 (PP4c). TransIT®-BrCa transfection reagent was used for the transfection of MCF7 (as described in section 2.2.15.3). Amaxa® Cell Line Nucleofector® Kit V and Amaxa® Cell Line Nucleofector® Kit L were used for the transfection of MDA-MB-231 and Hs578T respectively (as described in section 2.2.15.2). For PP4c down-regulation MCF7, MDA-MB-231 and Hs578T cells were mock transfected (no siRNA, only the transfection reagent has been added) or transfected with (-)siRNA (scrambled siRNA) as a control or the cells were transfected with siRNAs to different PP4c sequences using HiPerFect transfection reagent. For PEA15 down-regulation MCF7 and MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PEA15-specific siRNAs using HiPerFect transfection reagent (as described in section 2.2.14.3). After confirmation of over-expression 24 hours after transfection or down-regulation 72 hours after transfection by mean of western blotting analysis (as described in section 2.2.16), cell viability and cell functions were assessed. Culture viability was assessed by Muse® Count & Viability Assay Kit and vital dye stain (as described in sections 2.2.3.2 and 2.2.3.1), apoptosis was assessed by Muse® Annexin V and Dead Cell Assay Kit and acridine orange stain (as described in sections 2.2.4.1 and 2.2.4.2), cell cycle was assessed by Muse® Cell Cycle Assay Kit (as described in section 2.2.5), cell migration was studied using wound scratch assay (as described in section 2.2.7), Long term survival of the transfected cells was studied by assessing colony forming ability of the cells (as described in section 2.2.6).

PEA15 phosphorylation was examined by western blot analysis on proteins extracted from cells transfected with PP4c-specific siRNAs. The role of PEA15 in mediating the effects of PP4c was investigated in cells transfected with PEA15-specific siRNAs and pcDNA3.1-PP4c.

To assess the expression level of PP4c mRNA; real time RT-PCR, TaqMan gene expression assays (assay codes Hs99999901_m1 for 18S, Hs00427262_m1 for PPP4c and Hs00167441_m1 for ALAS1) were employed with cDNA prepared from different types of parental breast cancer cell lines including MCF10, MCF7, MDA-MB-231, MDA-MB-361, T47D and Hs578T cells and also tissue sample cDNA using TissueScan™ Cancer and Normal Tissue cDNA Arrays (as described in section 2.2.13). TissueScan™ Cancer and Normal Tissue cDNA Arrays contains samples of different breast cancer stages, stage I; the tumour is 2cm or less in size and there is no tumour spread outside the breast, stage II; there is no tumour or a tumour of 2cm or less in size with the involvement of 1 to 3 axillary lymph nodes or lymph nodes near the breastbone or the tumour is 2-5 cm in size with no lymph nodes involvement, stage III; no tumour is seen in the breast or the tumour may be of any size with the involvement of 4 to 9 axillary lymph glands or the lymph glands near the breastbone or the tumour is larger than 5 cm and small clusters of breast cancer cells are in the lymph nodes or the tumour is more than 5 cm and has spread into up to 3 lymph nodes in the armpit or to the lymph nodes near the breastbone, stage IV; the tumour can be any size, the lymph nodes may or may not be involved, the cancer has spread (metastasised) to other parts of the body such as the bones, lungs, liver or brain (http://www.cancerresearchuk.org/about-cancer/type/breastcancer/treatment/number-stages-of-breast-cancer; American Joint Committee on Cancer, 2002). For each assay, a standard curve of threshold cycle

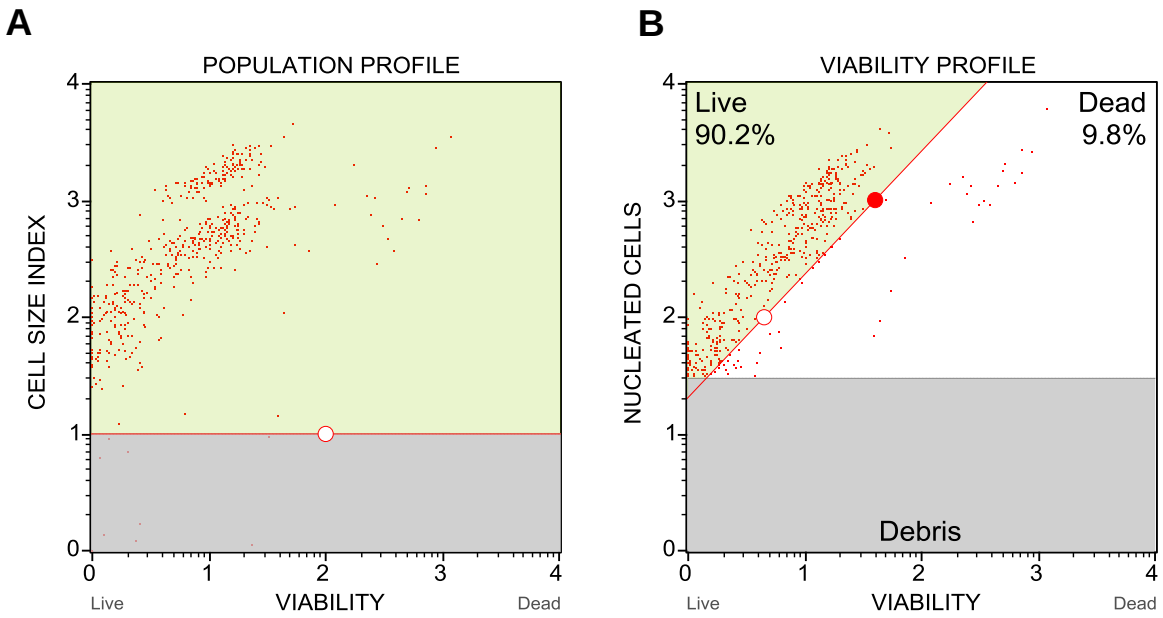
(Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to 18S or ALAS1 mRNA.

3.3 Results

3.3.1 Development of the assays used

3.3.1.1 Muse® Count & Viability Assay

Total and viable cell counts were determined by vital dye exclusion assay (as described in section 2.2.3.1) and by flow cytometry using Muse® Count & Viability Assay kit and the mini flow cytometer (Muse® Cell analyser) (as described in section 2.2.3.2) (Figure 3.1).



Figures 3.1: An example of the results obtained using Muse® Count & Viability Assay kit. After trypsinisation of the cells, the cells were mixed with Muse® Count & Viability reagent at a 20-fold dilution. The cells were allowed to stain for a minimum of five minutes at room temperature and were counted using Muse® Cell analyser. **A)** Viability vs cell size. **B)** Viability versus nucleated cell plot

3.3.1.2 Muse® Annexin V and Dead Cell Assay

Apoptosis was measured by acridine orange staining and fluorescence microscopy (as described in section 2.2.4.2) and by flow cytometry using Muse® Cell Analyser and Muse® Annexin V and Dead Cell Assay kit (as described in section 2.2.4.1) (Figure 3.2).

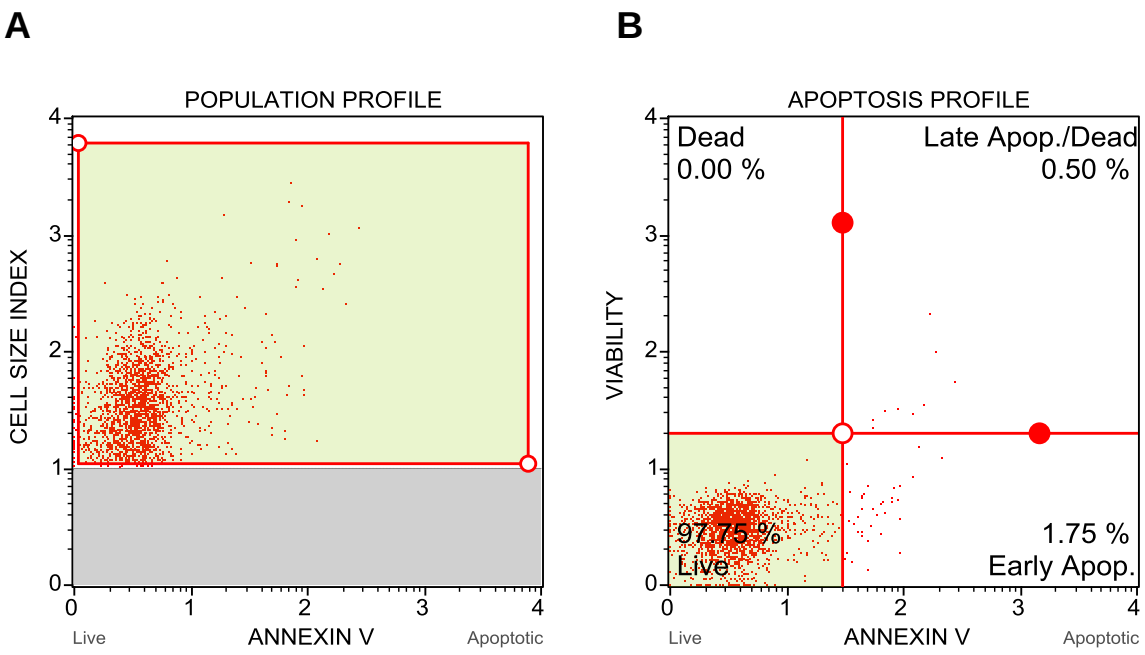


Figure 3.2: An example of the results obtained using Muse® Annexin V & Dead Cell Assay kit. After trypsinisation of the cells, 100 µL of Muse® Annexin V and Dead Cell reagent were mixed with 100 µL of cells in suspension. The mixture was incubated for 20 minutes in dark place and at room temperature before data acquisition using the Muse® Cell analyser. **A)** Annexin V versus cell size. **B)** Viability vs Annexin V cell plot. The events in four quadrants as follows: Lower-left quadrant: viable cells, not undergoing detectable apoptosis. Lower-right quadrant: cells in the early stages of apoptosis. Upper-right quadrant: cells in the late stages of apoptosis or dead. Upper-left quadrant: cells that have died not through the apoptotic pathway

3.3.1.3 Cell cycle analysis

Cell cycle profile was analysed by nuclear propidium iodide staining and flow cytometry using Muse® Cell Cycle Assay kit and the Muse® Cell analyser (as described in section 2.2.5) (Figure 3.3).

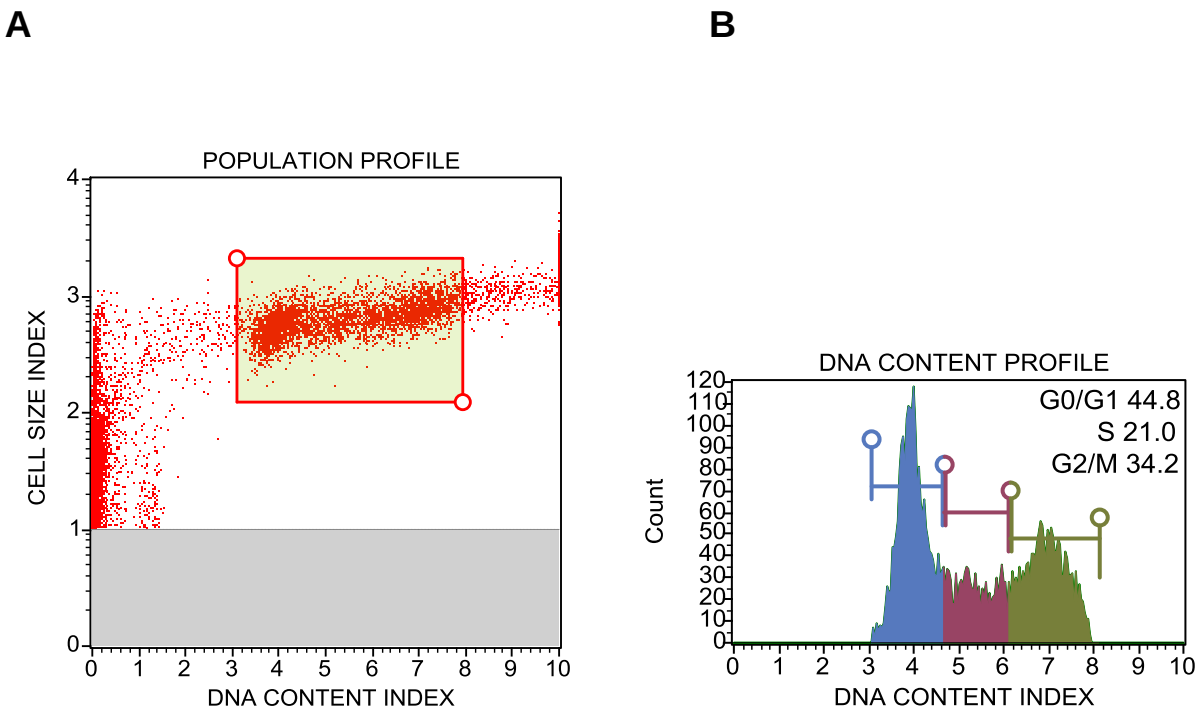


Figure 3.3: An example of the results obtained with the Muse® Cell Cycle reagent. (~ one million) log-phase cells were suspended in 200 µl phosphate buffered saline (PBS) and were ethanol fixed overnight . The cells were centrifuged for five minutes at 2000 rpm. The supernatant was discarded and the cell pellet was re-suspended in 200 µl of Muse™ Cell Cycle reagent. Cells were incubated for 30 minutes in dark before data acquisition using the Muse® Cell analyser. **A)** DNA content vs the cell size index. **B)** The distribution of the cell cycle phases (G_0/G_1, S and G_2/M) in histogram format

3.3.1.4 DNA Digestion with restriction endonucleases

A diagnostic digest was carried out to verify the size of the isolated plasmid DNA (pcDNA3.1-PP4c) (as described in section 2.2.11) (Figure 3.4).

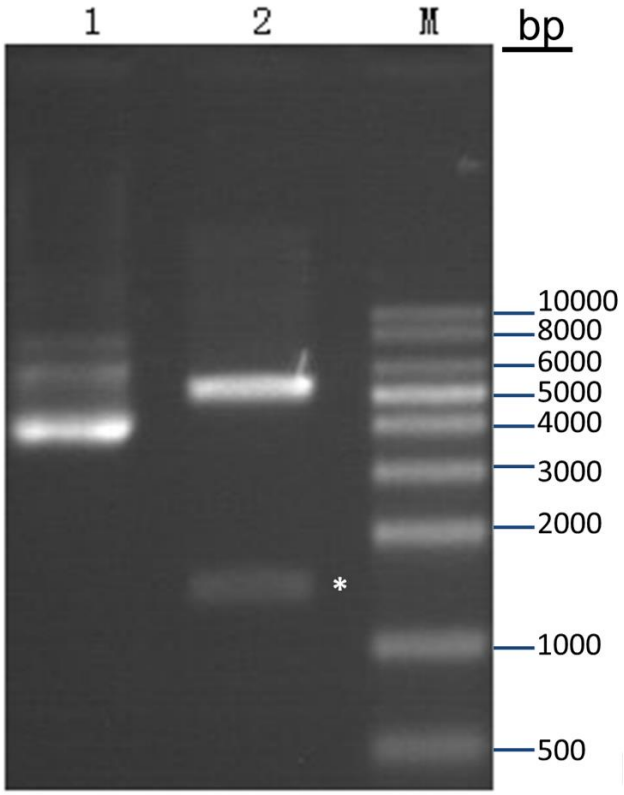


Figure 3.4: Restriction digests for pcDNA3.1-PP4c. A diagnostic digests on the isolated plasmid using ECOR1 and XhoI restriction enzymes. Lane M, DNA ladder. Lane 1, undigested plasmid. Lane 2, plasmid digested with two restriction enzymes. As verified by sequence analysis (MWG). * corresponds to a band of 1500 bp, PP4c

3.3.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to determine the quality of the extracted RNA and digested DNA (as described in section 2.2.12) (Figure 3.5).

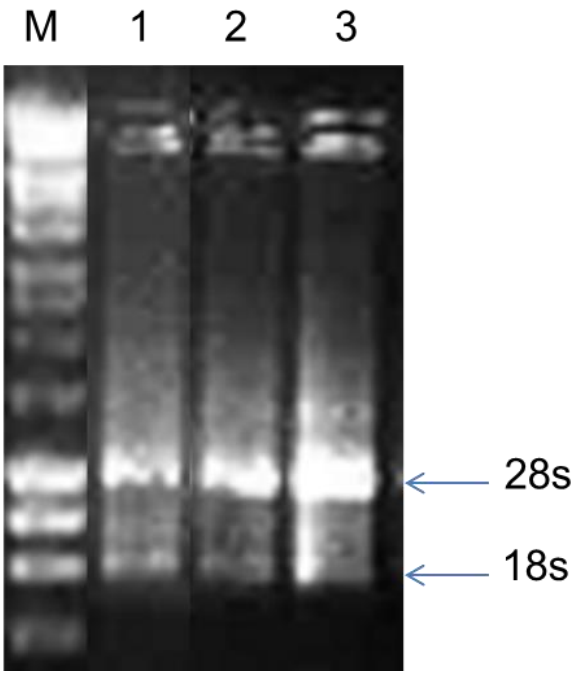


Figure 3.5: Gel electrophoresis of RNA samples. M: HyperLadder™ 1kb. Lanes 1) RNA isolated from MCF7 cell line. Lane 2) RNA isolated from MDA-MB-231 cell line. Lane 3) RNA isolated from Hs578T cell line. The upper band shows 28S rRNA and the lower band shows 18S rRNA

3.3.1.6 RT-qPCR

Real-time PCR was conducted using SensiFast Probe Hi-ROX kit and TaqMan gene expression assays (as described in section 2.2.13). A standard curve was generated by preparing serial dilution of 0.1–30 ng cDNA prepared from mixture of breast cancer cells, the standard curve of threshold cycle (Ct) value versus log input standard cDNA was constructed by linear regression and the equation of the line was used to calculate input amounts of samples from their respective Ct values (Figure 3.6).

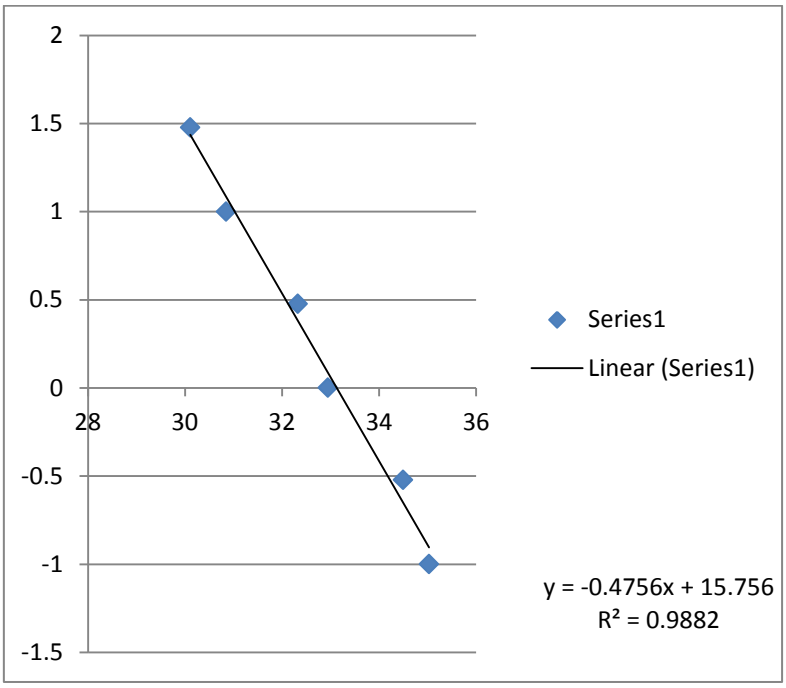


Figure 3.6: RT-qPCR standard curve. A linear regression represents threshold cycle (Ct) value versus log input standard cDNA. The equation of the line was used to calculate input amounts of samples from their respective Ct values. R2 is > 0.9 which provides good confidence in correlating two values and reflects efficient PCR

3.3.1.7 Optimisation of siRNA transfection using HiPerFect transfection reagent

Optimisation of the transfection conditions for HiPerFect transfection reagent was carried out using Cy3 labelled scrambled siRNA (as described in section 2.2.14.2). The transfection efficiency was established by determining the percentage of the red fluoresced cells that are successfully transfected with the siRNA labelled with Cy3 using Nikon Eclipse E400 Binocular fluorescence microscope with FITC filter (Figure 3.7). Three different volumes of HiPerFect transfection reagent that are 3 µl, 6 µl and 9 µl were used and the results showed that the three different volumes resulted in high transfection efficiency of 95% in MCF7, MDA-MB-231 and Hs578T breast cancer cell lines (Table 3.1). Furthermore, the three different volumes of the transfection reagent did not affect the cell viability (Table 3.1).

Cell line + volume of the transfection reagent	Transfection efficiency after 72 hours of the transfection	Cell viability No. of viable cells counted / total cells counted (viable and dead) x 100
MCF7		
3 µl	95%	98%
6 µl	95 %	98 %
9 µl	95 %	98 %
MDA-MB-231		
3 µl	95%	98%
6 µl	95%	98%
9 µl	95%	98%
Hs578T		
3 µl	95%	98%
6 µl	95%	98%
9 µl	95%	98%

Table 3.1: Optimisation of siRNA transfection using HiPerFect transfection reagent. Three different volumes of HiPerFect transfection reagent were used for the optimisation of the transfection in MCF7, MDA-MB-231 and in Hs578T cells. All the three volumes show high transfection efficiency of 95% after 72 hours of the transfection and with no associated effect on cell viability

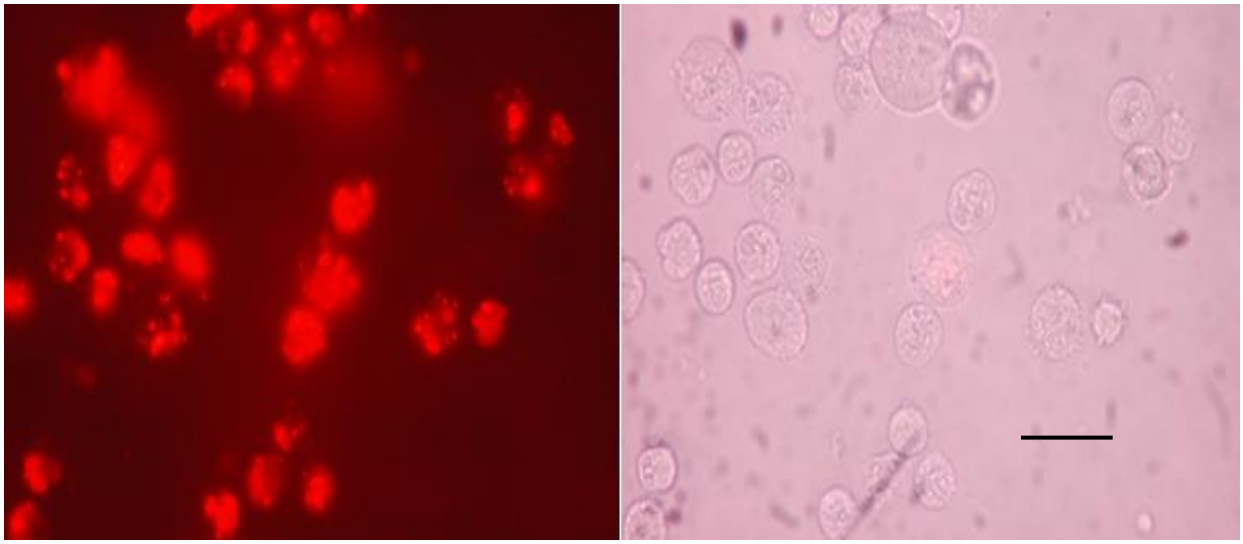


Figure 3.7: A representative image of efficient transfection using HiPerFect transfection reagent in MCF7 cells. MCF7 breast cancer cells were transfected with Cy3 labelled siRNA using HiPerFect transfection reagent. The transfection efficiency was 95% at 72 hours post-transfection. Scale bar, 20 μm.

3.3.1.8 Optimisation of plasmid DNA transfection

A plasmid expressing the green fluorescent protein (GFP) was used to determine the transfection efficiency (as described in section 2.2.15.1). The transfection efficiency was established by determining the percentage of cells expressing the green fluorescent protein using Nikon Eclipse E400 Binocular fluorescence microscope with FITC filter (Figure 3.8).

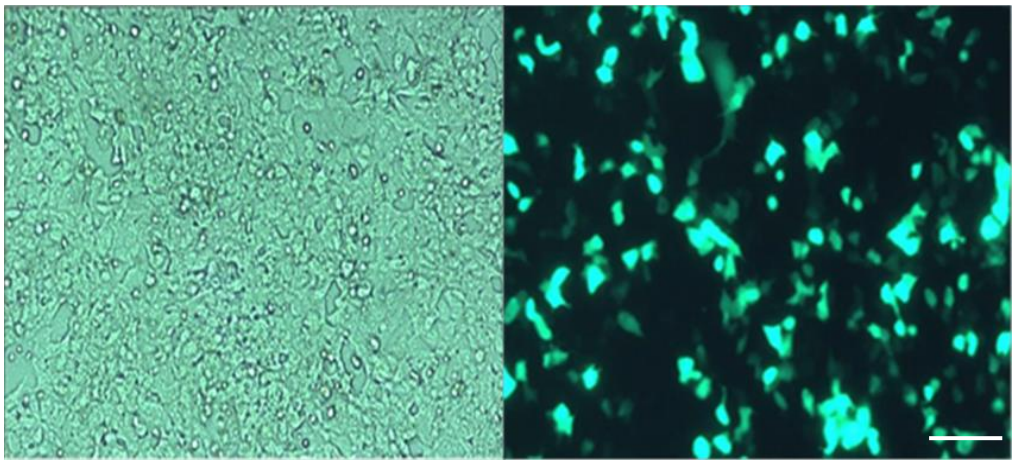


Figure 3.8: Transfection efficiency of MCF7 cells using TransIT®-BrCa transfection reagent. Representative images showing transfected MCF7 cells expressing GFP. The transfection efficiency was 70% at 48 hours post-transfection. Scale bar, 50 μm.

3.3.1.9 Western blotting

Cells were harvested 24 hours after transfection in case of PP4c up-regulation and 72 hours after transfection in case of PP4c down-regulation for determination of target protein expression. Western blotting was performed using one-dimensional, sodium dodecyl sulphate; SDS polyacrylamide gel electrophoresis (SDS-PAGE) (as described in section 2.2.16). The blotted PVDF membrane (Figure 3.9) was incubated with the enhanced chemiluminescence (ECL) Clarity™ Western ECL blotting substrate for 12 minutes. Signals were visualized utilizing the Odyssey® Imager (Li-Cor), densitometric analysis carried out using the associated Image Studio™ Software (version 3.1).

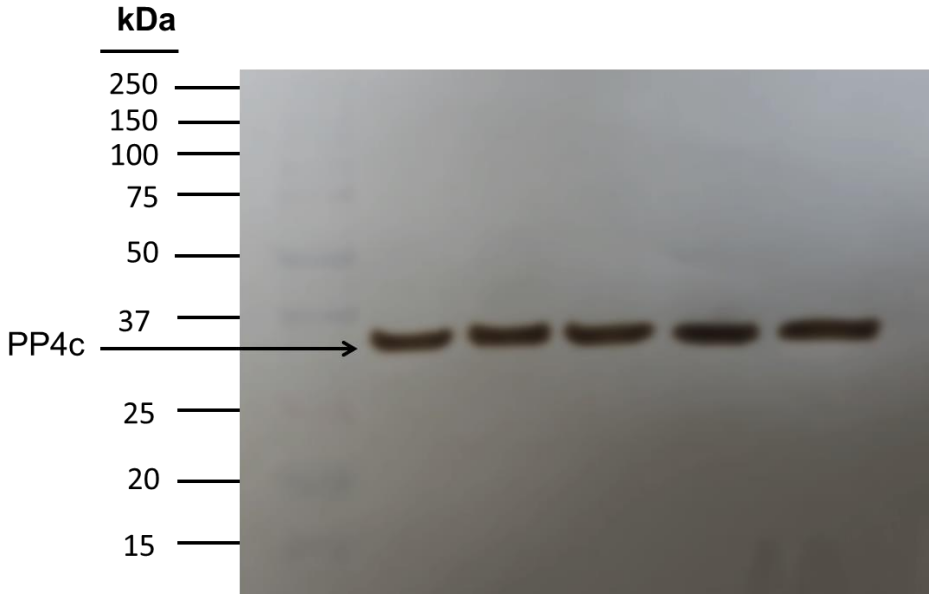


Figure 3.9: An example of blotted PVDF (polyvinylidene fluoride) transfer membrane (Hybond-P) showing bands of PP4c protein that are correspond with 35 kDa size.

3.3.2 PP4c is over-expressed in breast cancer cell lines

Real time RT-PCR; TaqMan gene expression assays was performed on cDNA prepared from different types of parental breast cancer cell lines including MCF10 breast cancer cell line which is a non-tumourigenic epithelial cell line that is widely used as an *in vitro* model for studying normal breast cell function and transformation (Soule et al., 1990). MCF10 is a cell line and might not reflect the situation in normal breast cells. MCF10 was used as a control to assess the expression level of PP4c mRNA in tumourigenic types of breast cancer cell lines. Data were collected using ABI PRISM® 7000 Sequence Detection System software and a standard curve of cycle threshold (Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to 18S mRNA expression of each cell line (Figure 3.10A). PP4c was found to be over-expressed in MCF7 (Soule et al., 1973) and in T47D cells (Keydar et al., 1979) these are oestrogen and progesterone receptor positive cells, MCF7 cells show the highest expression level of PP4c among other types of breast cancer cell lines. PP4c was also found to be over-expressed in MDA-MB-231 (Cailleau et al., 1974) and in Hs578T (Hackett et al., 1977) cells which are triple negative breast cancer cells. We have also demonstrated that PP4c is over-expressed in MDA-MB-361 cells that are oestrogen/HER2 positive cells and progesterone receptor negative cells (Neve e al., 2006) (Figure 3.10A).

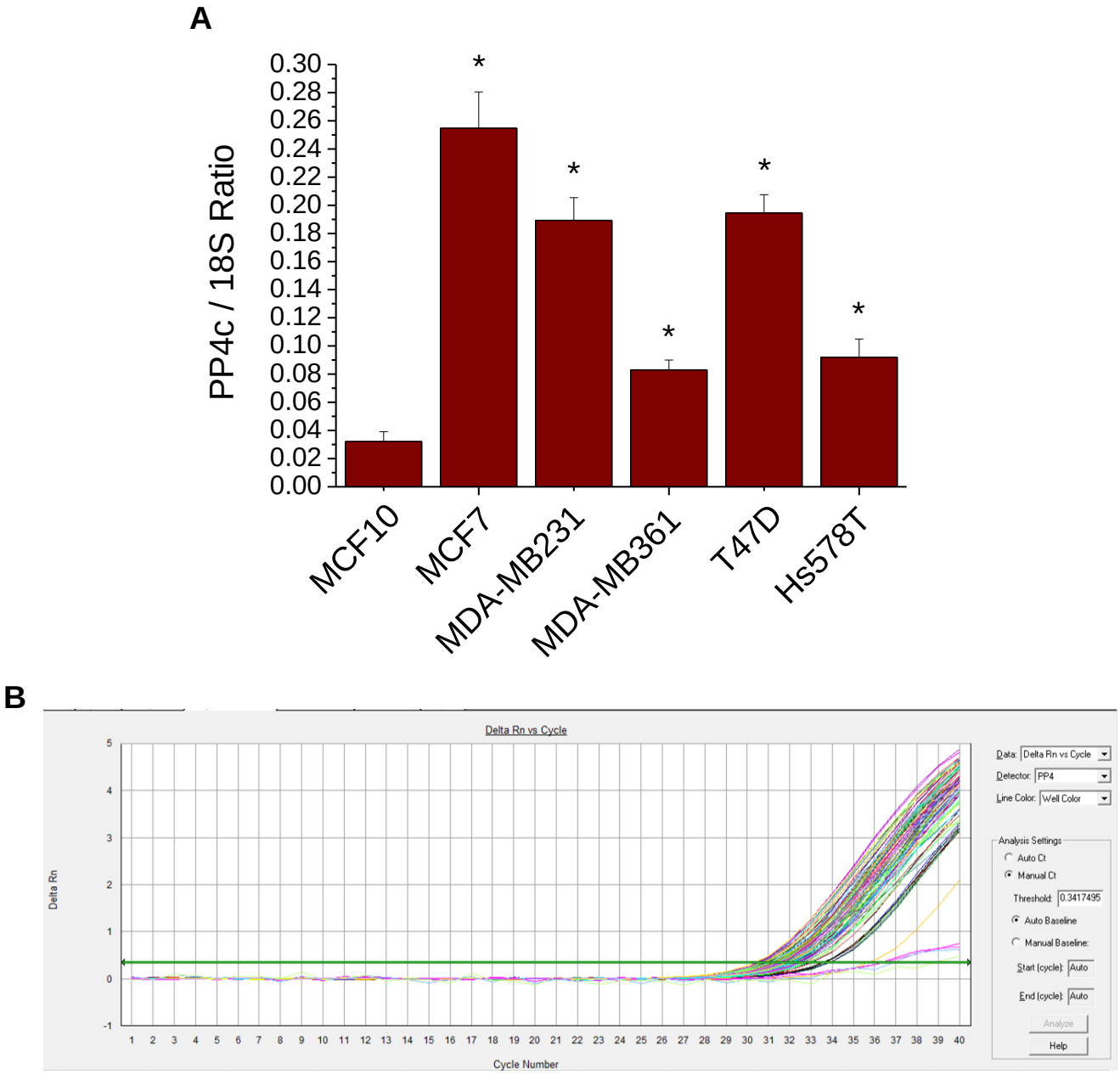


Figure 3.10: A) PP4c is over-expressed in hormone positive and in triple negative breast cancer cell lines. Real time RT-PCR; TaqMan gene expression assays was performed on cDNA prepared from different types of parental breast cancer cell lines. Data were collected using ABI PRISM® 7000 Sequence Detection System software and a standard curve of cycle threshold (Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to 18S mRNA expression of each cell line. (n=4). *P < 0.05 versus MCF10 cells (one-way ANOVA and Bonferrroni's MCT). **B)** An example of the amplification curve.

3.3.3 PP4c is over-expressed in all stages of breast cancer

Real time RT-PCR; TaqMan gene expression assays was performed on cDNA derived from tissue samples of patients with breast cancer of different stages, grades and hormone expression using TissueScan™ Cancer and Normal Tissue cDNA Arrays. Input amounts of samples were calculated from their respective threshold cycle (Ct) values, using standard curves generated with each assay. Data were expressed relative to ALAS1 mRNA. PP4c was found to be over-expressed in all stages of breast cancer in comparison to samples of normal breast tissue cDNA (Figure 3.11).

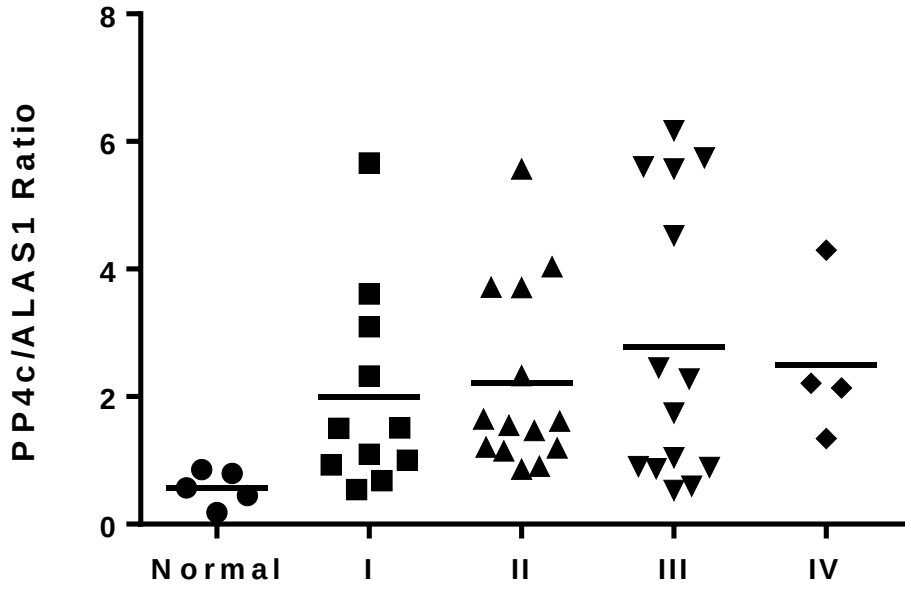


Figure 3.11: PP4c is over-expressed in all stages of breast cancer. Real time RT-PCR; TaqMan gene expression assays was performed on cDNA derived from tissue samples of patients with breast cancer using TissueScan™ Cancer and Normal Tissue cDNA Arrays. (n=3). Data were expressed relative to ALAS1 mRNA. *P < 0.05 versus normal samples (one-way ANOVA and Bonferrroni's MCT)

3.3.4 Transient expression of PP4c enhances apoptosis and decreases the survival of breast cancer cells

To examine the effects of increased PP4c expression on breast cancer cell survival, MCF7 cells were transiently transfected with pcDNA3.1 empty vector or pcDNA3.1 expression plasmid containing full length cDNA encoding PP4c. The influence of PP4c over-expression on cell survival and apoptosis was examined under basal conditions.

MCF7 pcDNA3.1-PP4c transfection caused 60-70% increase in PP4c protein level as revealed by western blotting analysis 24 hours post-transfection (Figure 3.12). This had significant effects on the cell survival and basal apoptosis level. Compared to cultures transfected with pcDNA3.1 empty vector, both total and viable cell counts in cultures over-expressing PP4c were considerably decreased at 48 hours post-transfection compared to cultures transfected with empty vector, as assessed by FACS analysis and vital dye staining (Figure 3.13A, 3.13B). Cells over-expressing PP4c also showed a 2.5 fold increase in apoptotic cells as assessed by annexin V and acridine orange dye (Figure 3.13C, 3.13D) and showed a significant decrease in clonogenic activity (Figure 3.14). In order to determine whether the growth suppression produced by PP4c was due to apoptosis, to cell cycle arrest, or to both, a cell cycle analysis was performed using propidium iodide staining and flow cytometry. The results revealed a significant increase in the proportion of cells in sub-G_0 in PP4c over-expressing cultures, suggesting an increase in the apoptosis rate, and a consistently lower percentage of cells in G_1 and S phases suggesting reduced cell proliferation in PP4c over-expressing cultures (Figure 3.15)

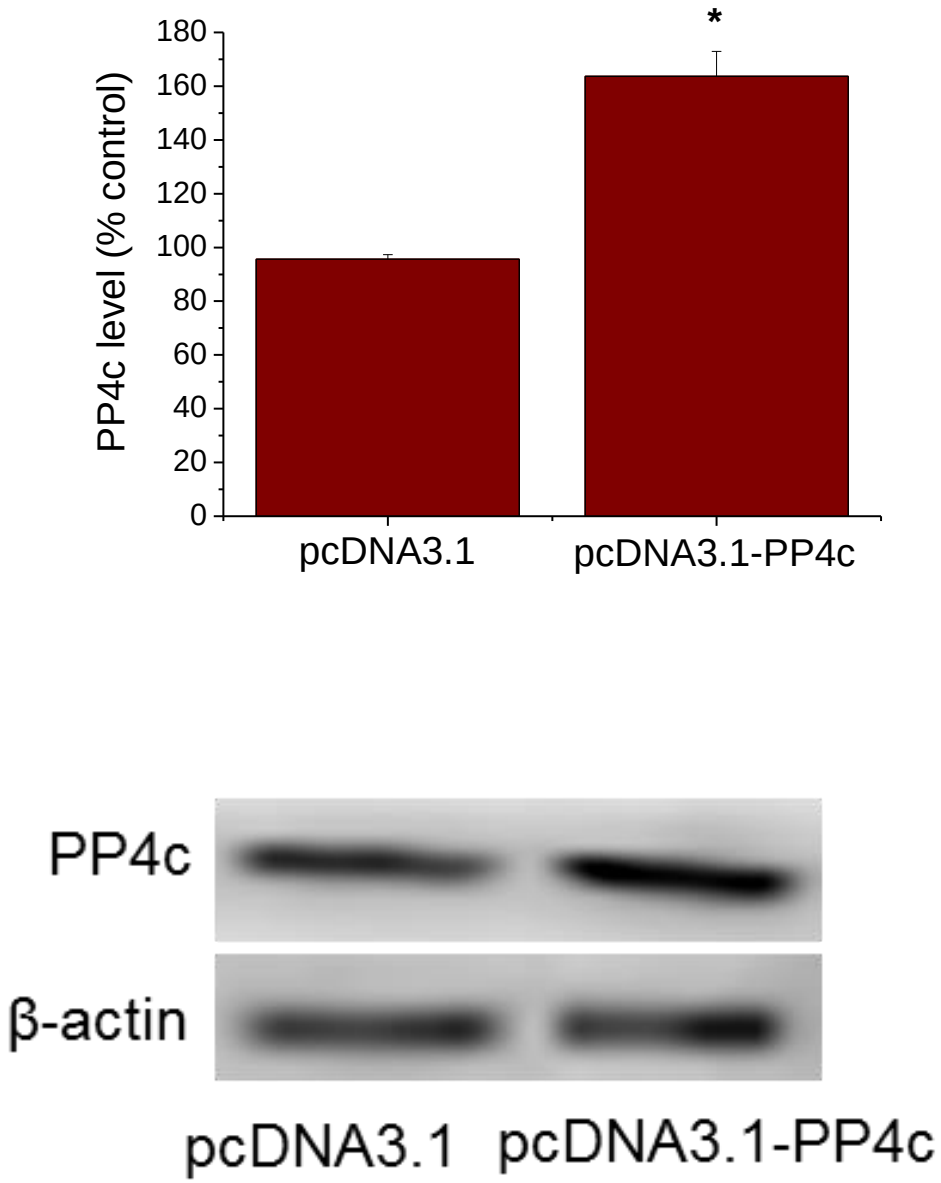


Figure 3.12: Cellular levels of PP4c protein in MCF7 cells as determined by western blotting analysis 24 hours after transfection, β -actin was used as a loading control. A representative autoradiograph is presented below the bar chart. Protein bands were visualised by enhanced chemiluminescence (ECL) Clarity™ Western ECL Blotting Substrate. Signals were visualized utilizing the Odyssey® Imager (Li-Cor) and densitometric analysis carried out using the associated Image Studio™ Software (version 3.1). PP4c protein expression levels were normalised with that of β-actin. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

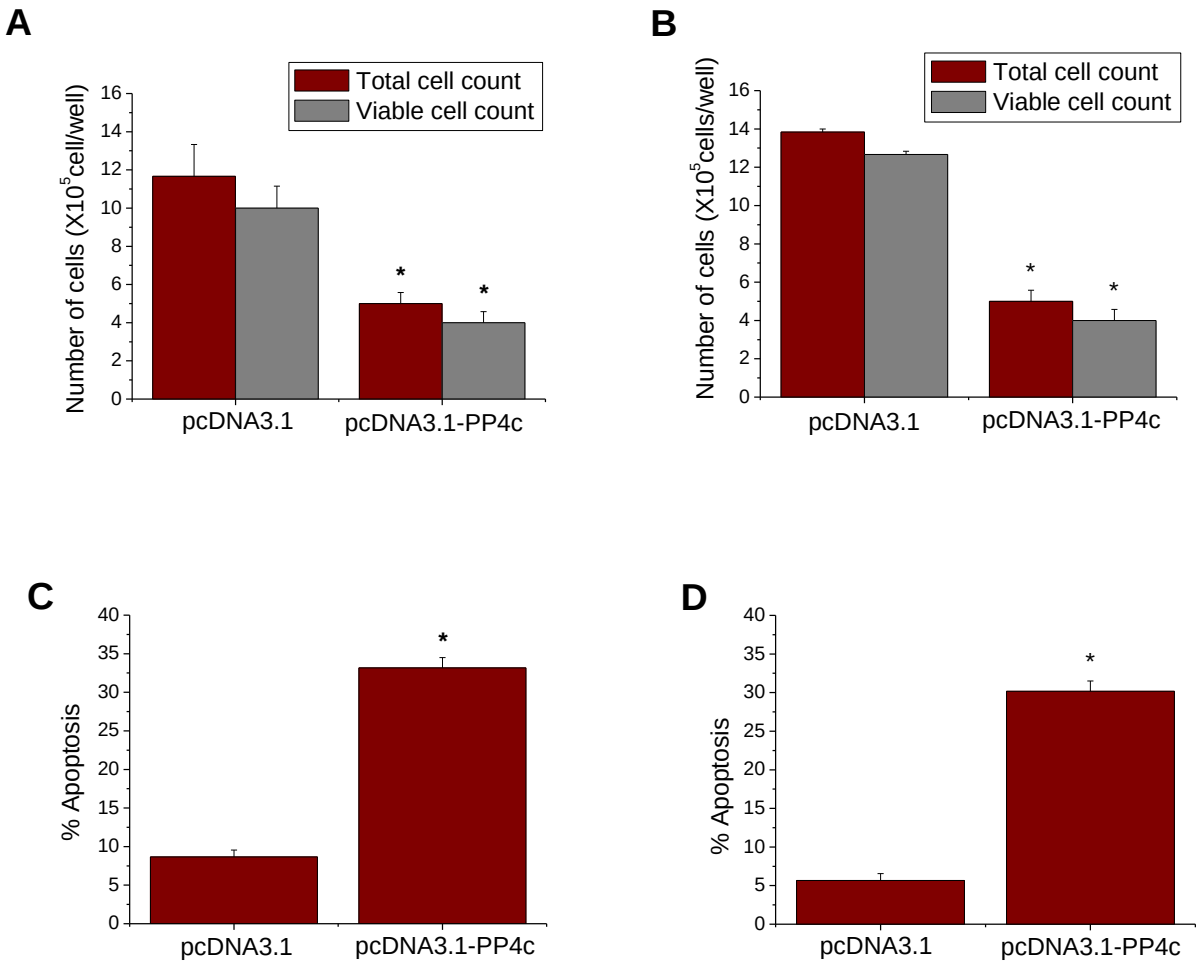
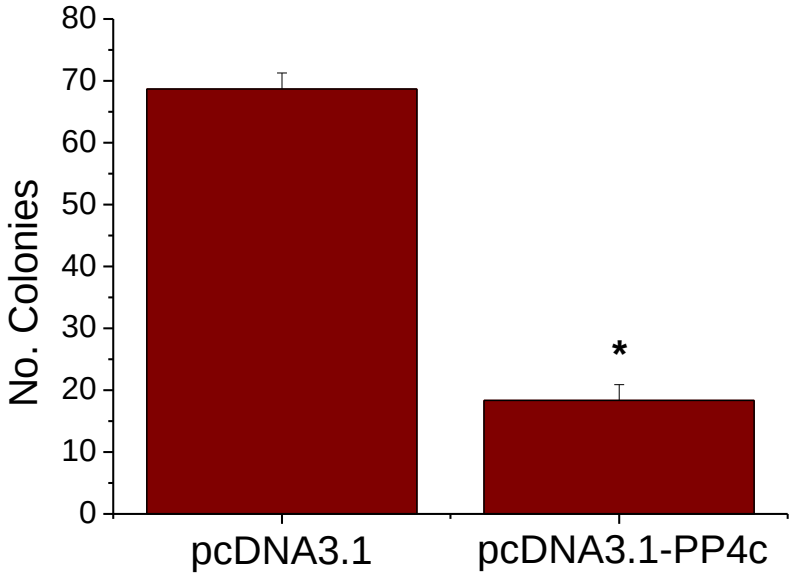


Figure 3.13: Transient expression of PP4c decreases cell survival and enhances spontinuous apoptosis of MCF7 breast cancer cells. MCF7 were transfected with pcDNA3.1 empty vector or pcDNA3.1-PP4c expression vector. Cell survival and apoptosis were examined 48 hours after transfection under basal condition. **A)** Total and viable cell counts as determined by FACS analysis using Muse® Count & Viability Assay kit. **B)** Total and viable cell counts as determined by vital dye stain. **C)** The proportion of apoptotic cells as assessed by FACS analysis using Muse® Annexin V and Dead Cell Assay. **D)** The proportion of apoptotic cells as assessed by acridine orange dye. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)



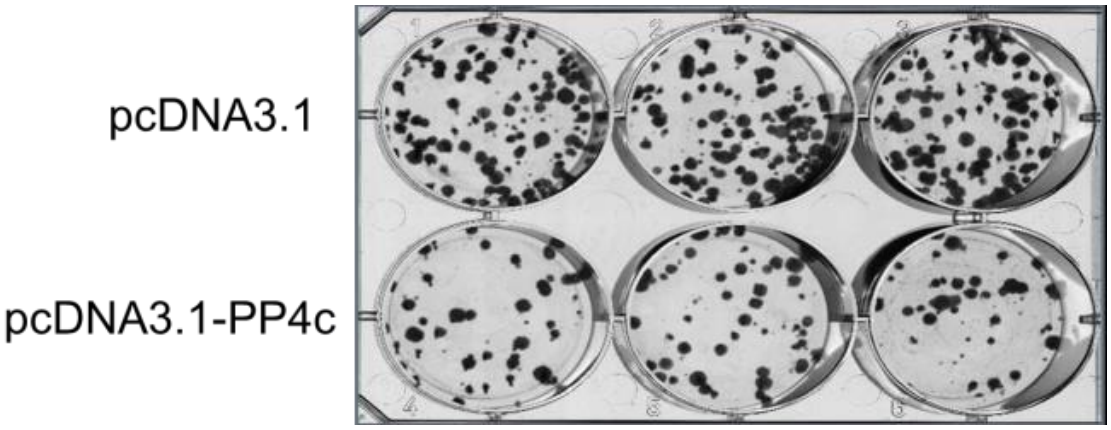


Figure 3.14: Clonogenic assay demonstrates that long term survival of MCF7 cells is compromised after transfection with PP4c construct. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The average of three wells of pcDNA3.1 of the 1st, 2nd, 3rd and 4th experiment was 75, 70, 68 and 65 respectively compared with the average of three wells of pcDNA3.1-PP4c of the 1st, 2nd, 3rd and 4th experiment which was 27, 18, 17, and 19 respectively. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

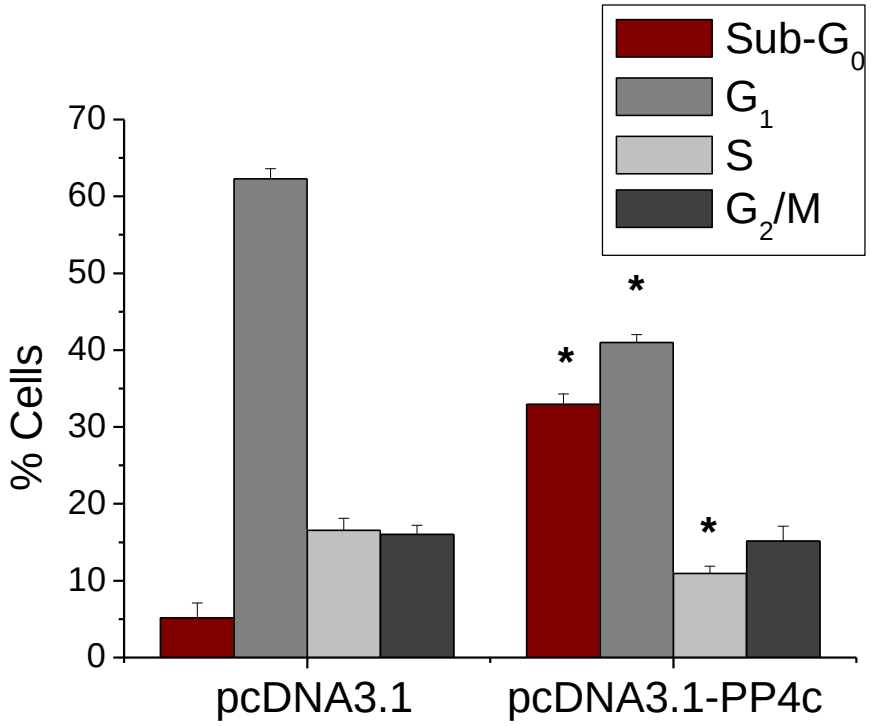


Figure 3.15: PP4c over-expression reduces proliferation of MCF7 breast cancer cells. Cell cycle analysis was performed using Muse™ Cell Cycle reagent. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

3.3.5 Transient expression of PP4c enhances apoptosis and decreases the survival of triple negative breast cancer cells

To examine the effects of increased PP4c expression on triple negative breast cancer cell survival. MDA-MB-231 and Hs578T cells were transiently transfected with pcDNA3.1 empty vector as a control or with a pcDNA3.1 expression plasmid containing full length cDNA encoding PP4c. The influence of PP4c over-expression on cell survival and apoptosis was examined under basal conditions.

Transfection of the triple-negative MDA-MB-231 and Hs578T cells with plasmid encoding PP4c and the increase in PP4c protein levels as determined by western blotting analysis after 24 hours of the transfection (Figure 3.16) and (Figure 3.20) respectively; caused a significant reduction in total and viable cell numbers of MDA-MB-231 cells (Figure 3.17A, 3.17B) and of Hs578T cells (Figure 3.21A) also there was a substantial increase in basal apoptosis of MDSA-MB-231 cells (Figure 3.17C, 3.17D) and of Hs578T cells (Figure 3.21B). Over-expression of PP4c also caused a significant reduction in the clonogenic activity of MDA-MB-231 cells (Figure 3.18) and of Hs578T cells (Figure 3.22). Cell cycle analysis revealed an increase in the proportion of cells in sub-G_0 fraction in MDA-MB-231 cells transfected with pcDNA3.1-PP4c which was associated with a decrease in the percentage of cells in G_1, S and G_2/M phases (Figure 3.19). Thus, the results confirmed that the triple-negative MDA-MB-231 and Hs578T cells are sensitive to the increase in PP4c protein level, as for the oestrogen receptor positive-MCF7 cells.

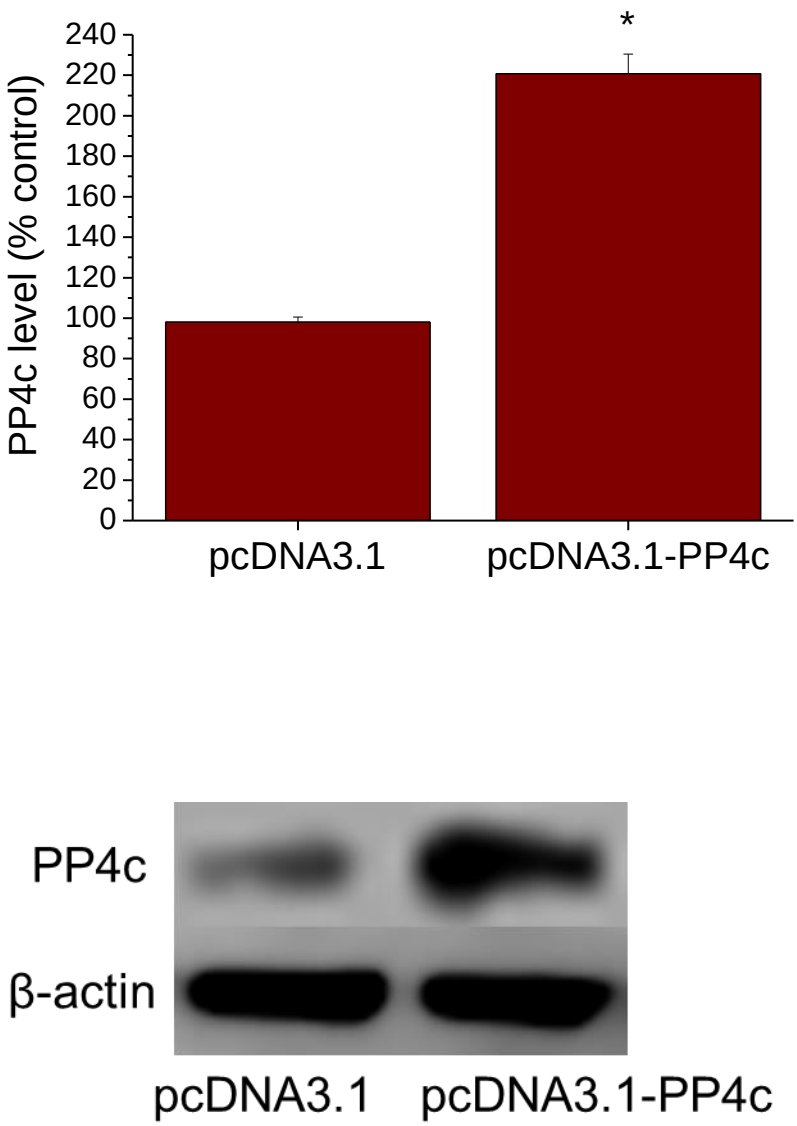


Figure 3.16: Cellular levels of PP4c protein in MDA-MB-231 cells were determined 24 hours post-transfection by western blotting analysis, β -actin was used as a loading control. A representative autoradiograph is presented below the bar chart. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4c protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

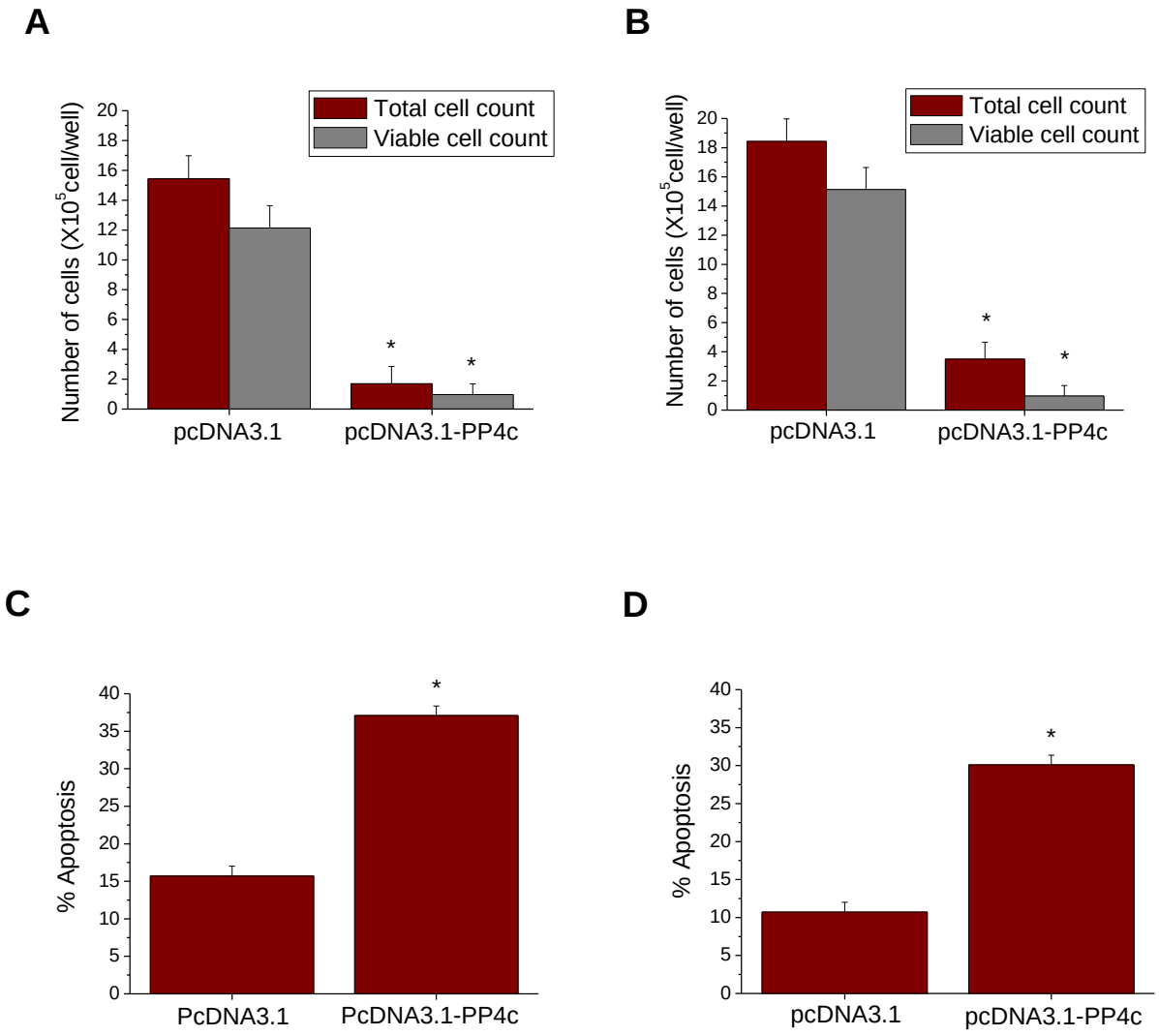
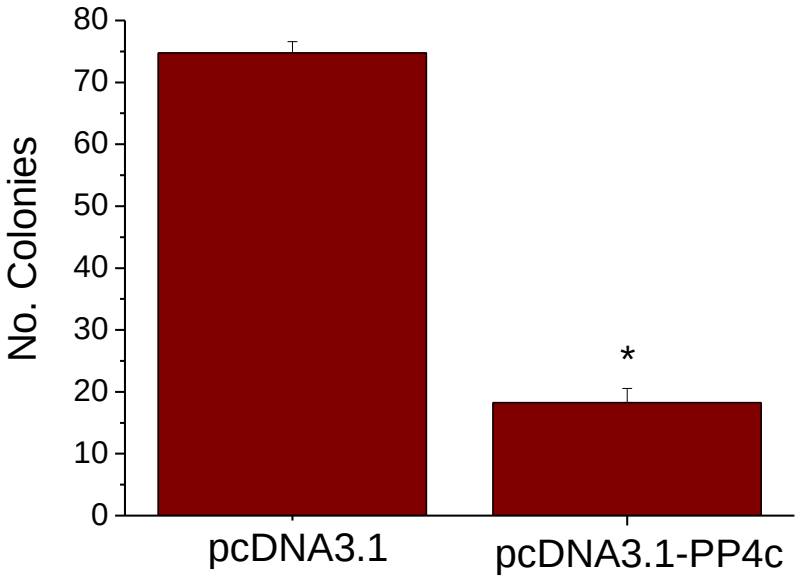


Figure 3.17: PP4c induces apoptosis and reduces short term survival in triple-negative-MDA-MB-231 cells. MDA-MB-231 were transfected with pcDNA3.1 empty vector or pcDNA3.1-PP4c expression vector. Cell survival and apoptosis were examined 48 hours after transfection under basal condition. **A)** Total and viable cell counts as determined by FACS analysis using Muse® Count & Viability Assay kit. **B)** Total and viable cell counts as determined by vital dye stain. **C)** The proportion of apoptotic cells as assessed by FACS analysis using Muse® Annexin V and Dead Cell Assay. **D)** The proportion of apoptotic cells as assessed by acridine orange dye. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)



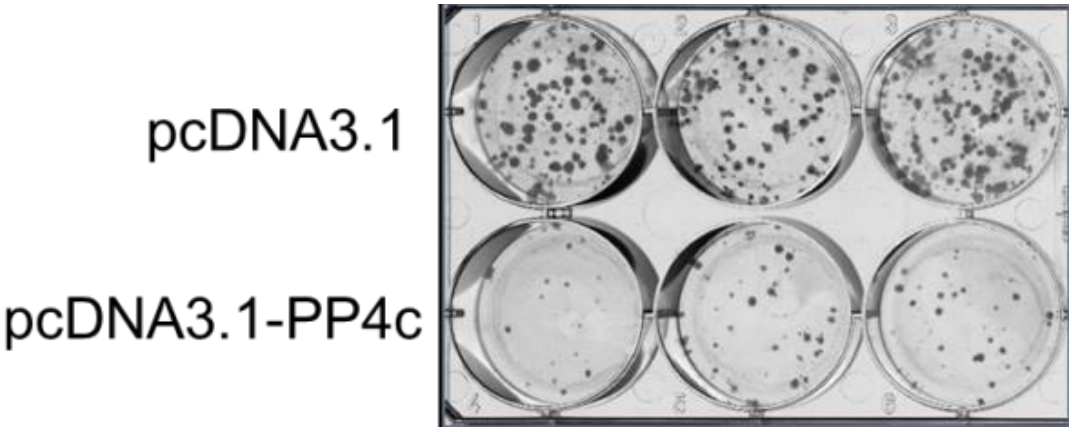


Figure 3.18: Clonogenic assay demonstrates that long term survival of MDA-MB-231 cells is compromised after transfection with PP4c construct. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The average of three wells of pcDNA3.1 of the 1st, 2nd, 3rd and 4th experiment was 75, 71, 72 and 80 respectively compared with the average of three wells of pcDNA3.1-PP4c of the 1st, 2nd, 3rd and 4th experiment was 13, 20, 21 and 22

respectively. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

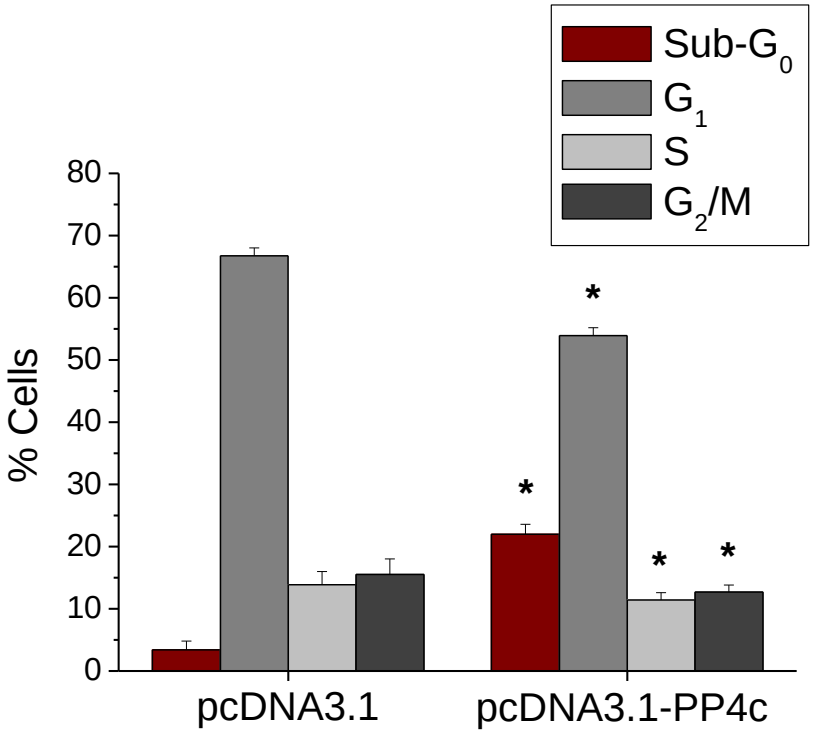


Figure 3.19: PP4c over-expression reduces proliferation of MDA-MB-231 breast cancer cells. Cell cycle analysis was performed using Muse™ Cell Cycle reagent. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

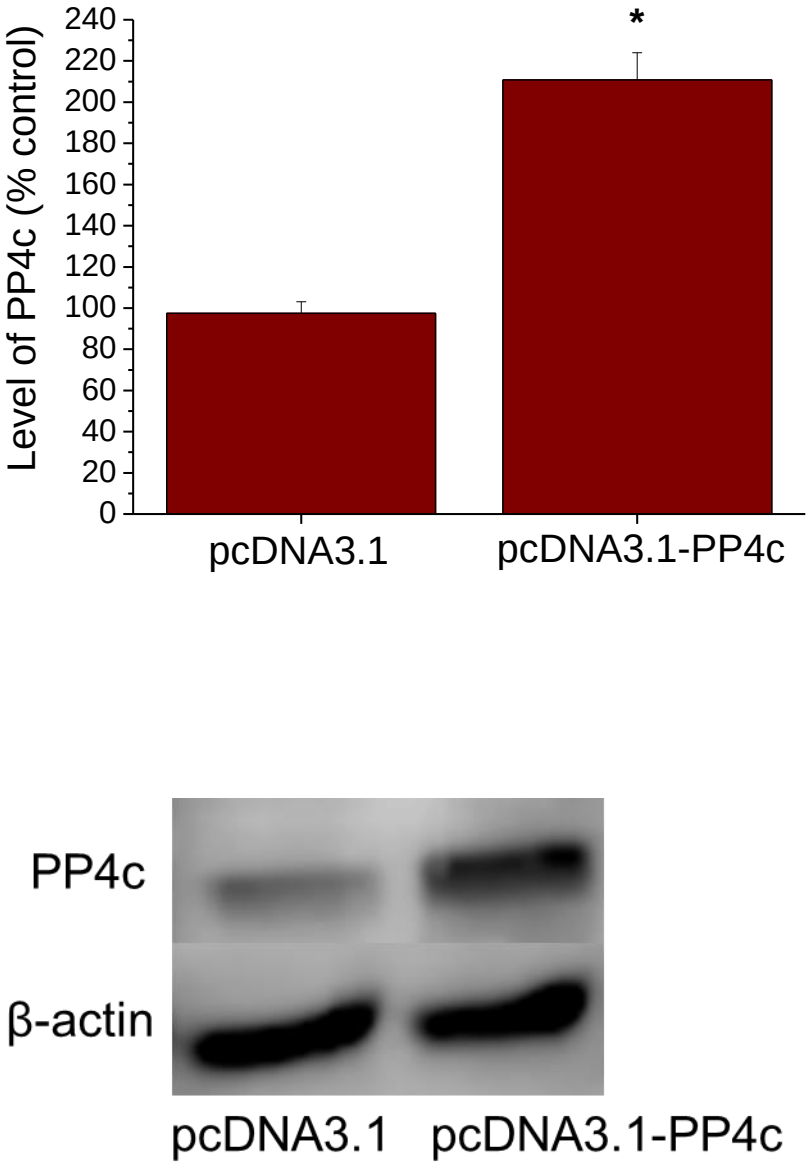


Figure 3.20: Cellular levels of PP4c protein in Hs578T cells were determined 24 hours post-transfection by western blotting analysis, β -actin was used as a loading control. A representative autoradiograph is presented below the bar chart. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4c protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

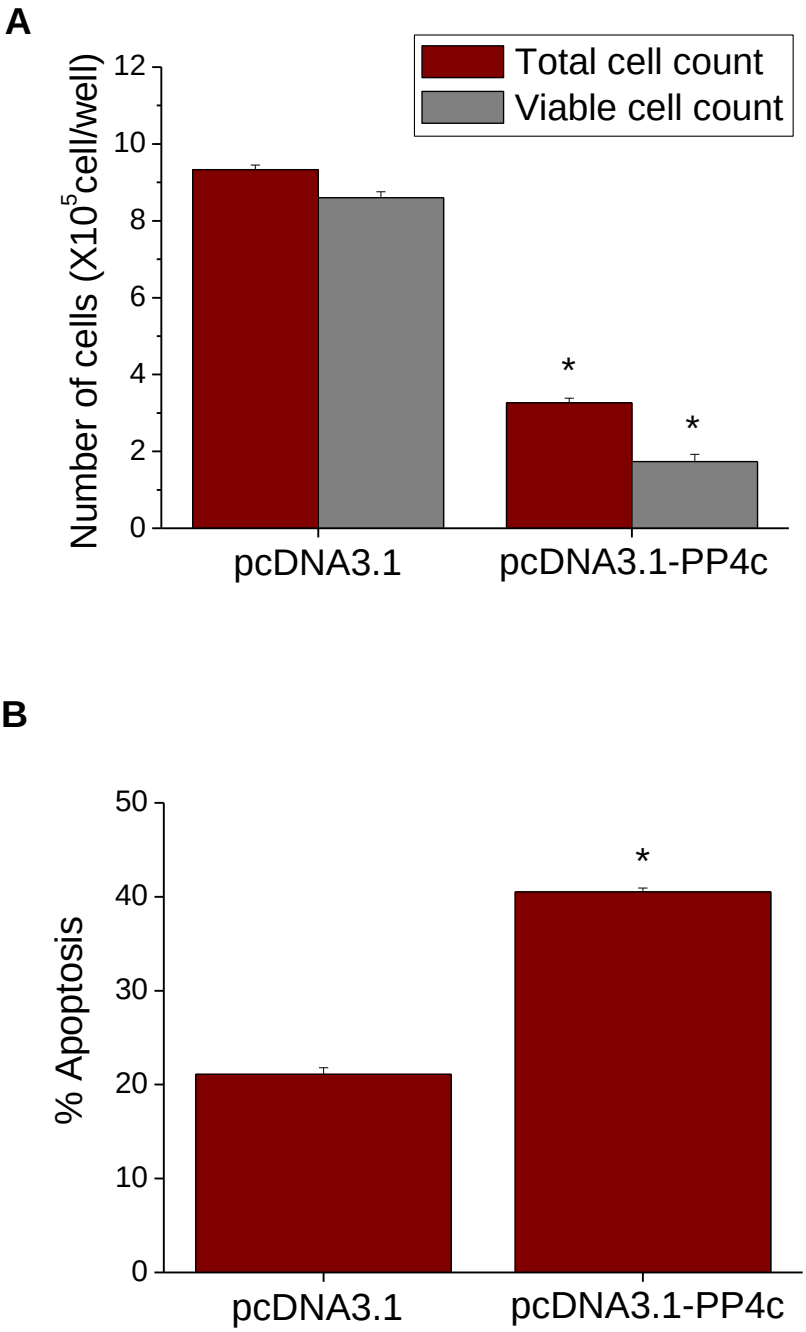
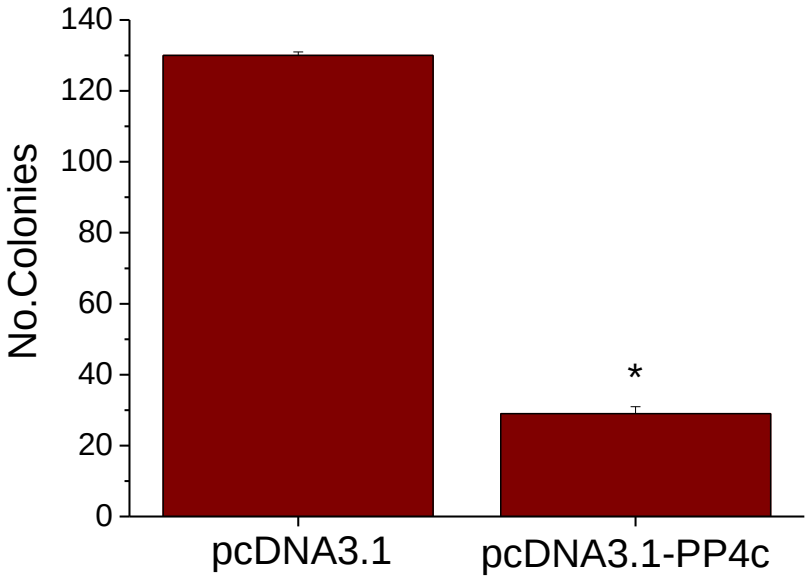


Figure 3.21: PP4c induces apoptosis and reduces short term survival in triple-negative-Hs578T cells. Hs578T cells were transfected with pcDNA3.1 empty vector or pcDNA3.1-PP4c expression vector. Cell survival and apoptosis were examined 48 hours after transfection under basal conditions. **A)** Total and viable cell counts as determined by FACS analysis using Muse® Count & Viability Assay kit. **B)** The proportion of apoptotic cells as assessed by FACS analysis using Muse® Annexin V and Dead Cell Assay. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)



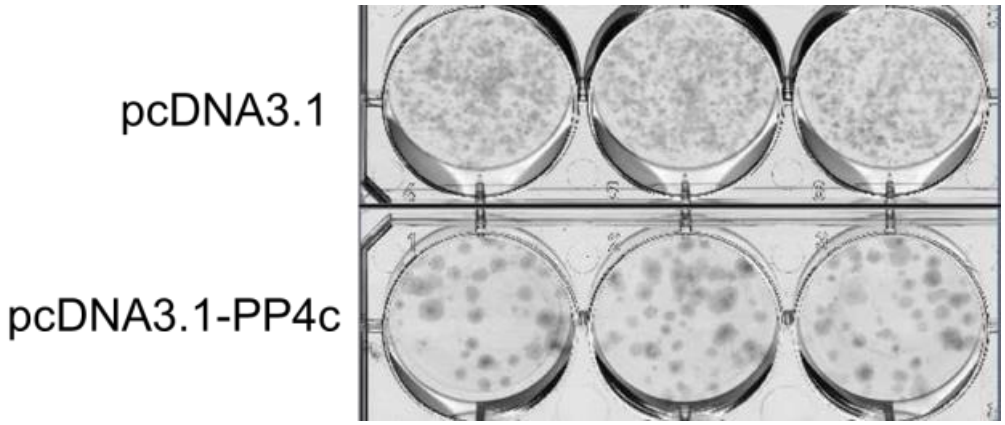


Figure 3.22: Clonogenic assay demonstrates that long term survival of Hs578T cells is compromised after transfection with PP4c construct. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 alone (one-way ANOVA and Bonferrroni's MCT)

3.3.6 PP4c silencing increases cell survival and attenuates basal apoptosis in MCF7 breast cancer cells

In order to investigate PP4c function further in MCF7 cells, specific PP4c siRNAs; PP4s1, PP4s2 were used to reduce endogenous PP4c expression in these cells. This strategy is particularly important since the effects of over-expression of any protein should be confirmed by independent methods in order to exclude possible artefacts. The efficiency of PP4c knockdown was determined by immunoblotting 72 hours post-transfection and the influence of PP4c silencing on long and short term cell survival and apoptosis was examined under basal conditions.

Both PP4c-targeted siRNAs (PP4s1 and PP4s2) reduced PP4c protein levels by 50-60% in MCF7 cells (Figure 3.23). PP4c knockdown caused a significant increase in total and viable cell numbers (Figure 3.24A, 3.24B) and a reduction in the level of basal apoptosis (Figure 3.24C, 3.24D). PP4c knockdown considerably increased the clonogenic activity of MCF7cells (Figure 3.25). FACS analysis revealed a decrease in the percentage of cells in sub-G_0 and an increase in S and G_2/M populations in the cells transfected with PP4s1 and PP4s2 siRNAs compared with the cells transfected with the control (-)siRNA suggesting a reduction in apoptosis and an induction of cell proliferation in cultures with PP4c down-regulation (Figure 3.26).

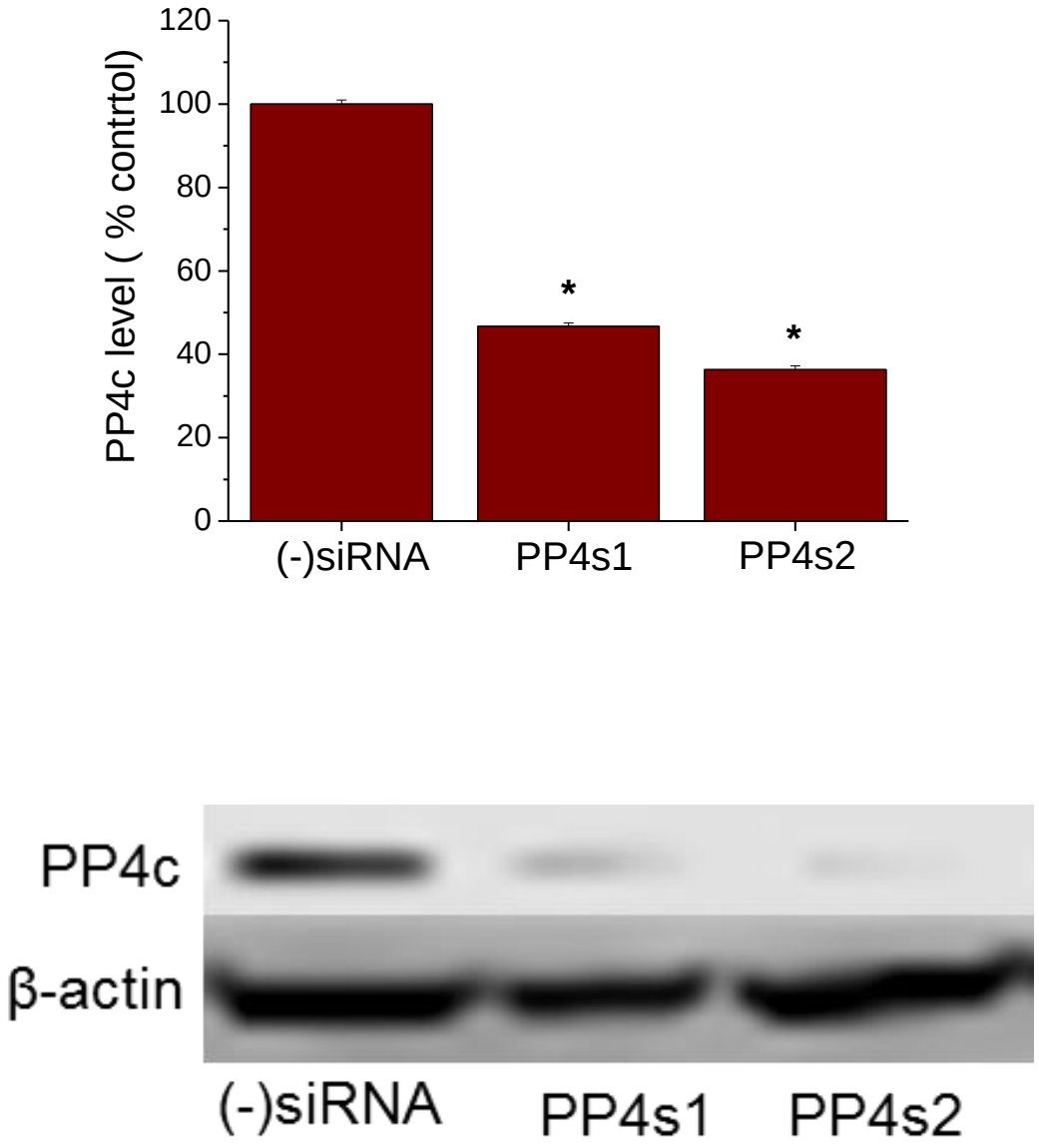


Figure 3.23: Cellular levels of PP4c protein in MCF7 cells was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. Signals were visualized utilizing the Odyssey® Imager (Li-Cor) and analysed by densitometry. A representative autoradiograph is presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4c protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

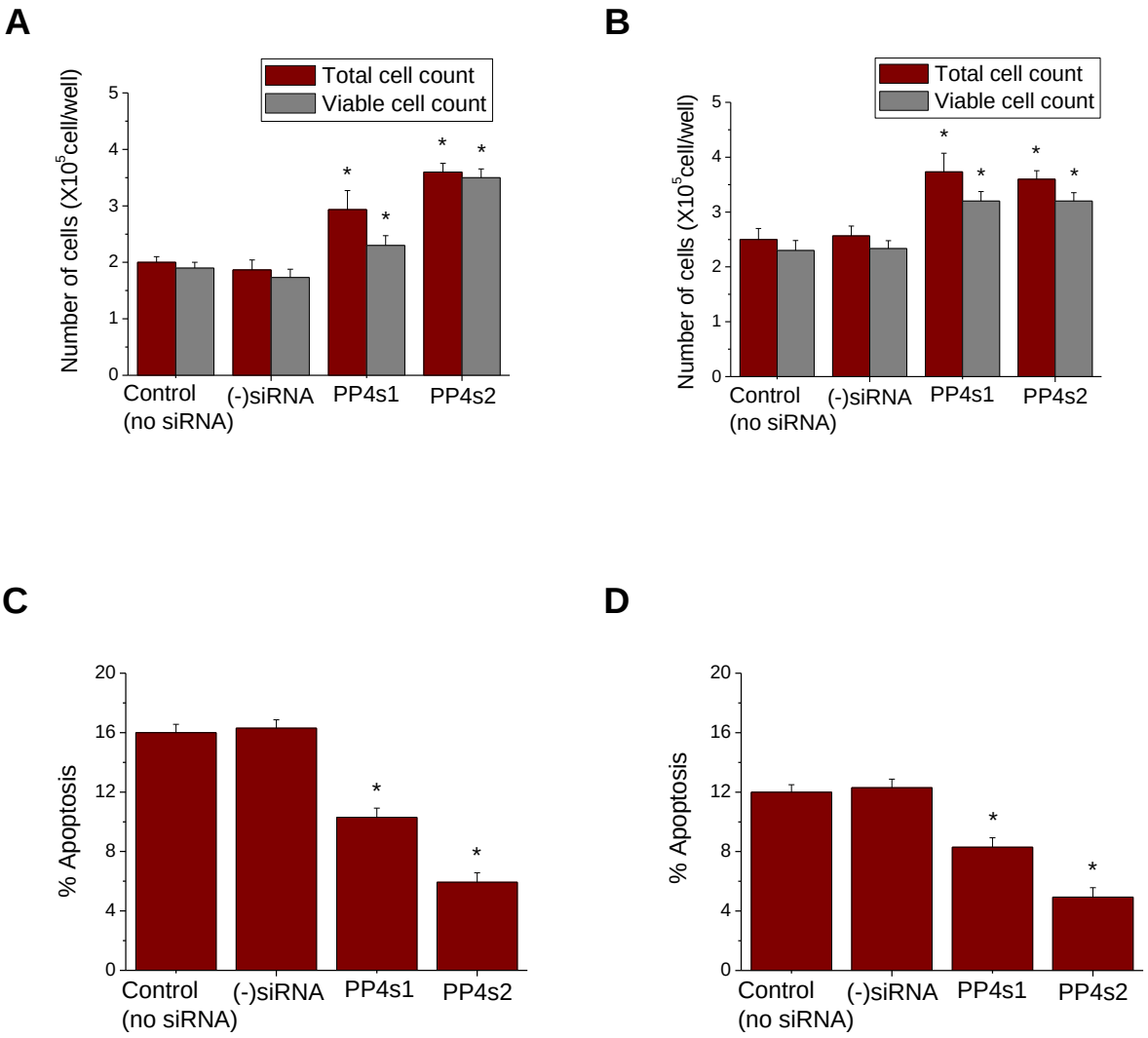


Figure 3.24: PP4c-specific siRNAs inhibit basal apoptosis and increase short term survival of MCF7 cells. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNA. Cells were harvested at 72 hours post-transfection, and re-plated for assessment of cell survival after a further 48 hours. **A)** Total and viable cell numbers as determined by FACS analysis using Muse® Count & Viability Assay kit. **B)** Total and viable cell numbers as determined by vital dye stain. **C)** The level of basal apoptosis as determined by FACS analysis using Muse® Annexin V and Dead Cell Assay. **D)** The level of basal apoptosis as determined by acridine orange dye. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

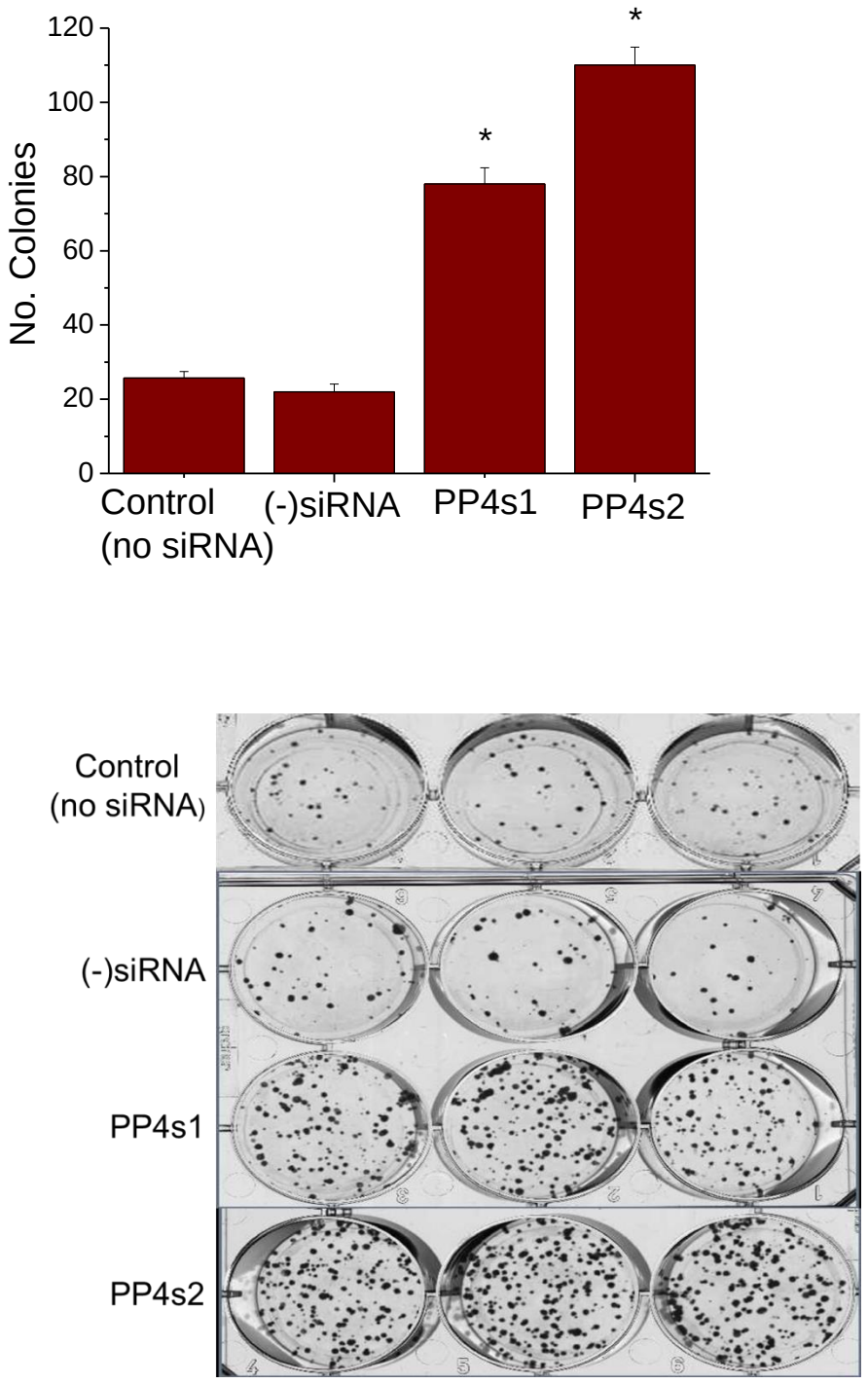


Figure 3.25: Clonogenic assay demonstrates that PP4c silencing enhances long term survival of MCF7 cells. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

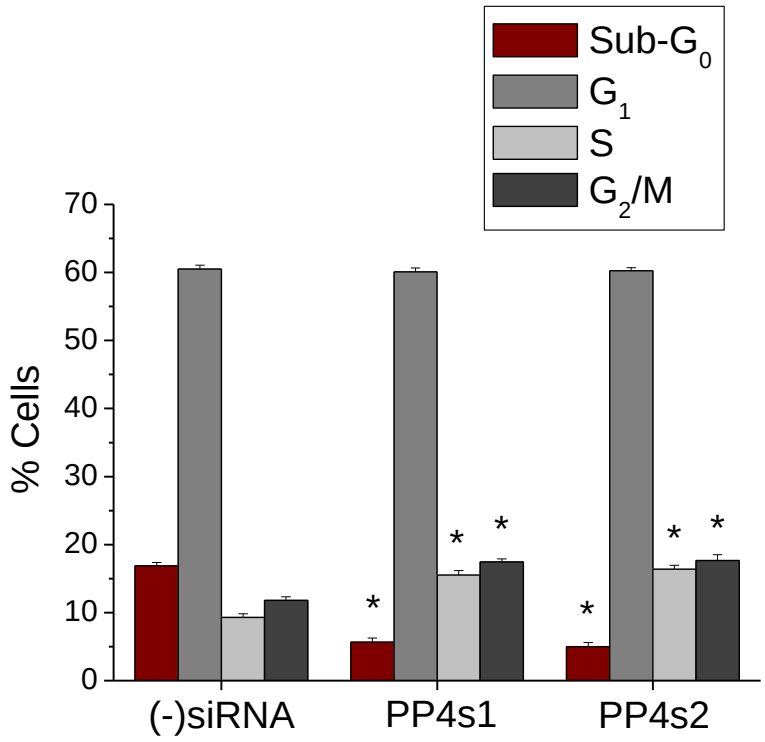


Figure 3.26: Cell cycle analysis by propidium iodide staining of fixed cells and fluorescence flow cytometry revealed that PP4c down-regulation affected the cell cycle profile of MCF7 cells. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

3.3.7 PP4c silencing increases cell survival and attenuates basal apoptosis in triple negative breast cancer cells

The effect of PP4c silencing in triple negative breast cancer cells-MDA-MB-231 and Hs578T cells was examined using PP4s2 and PP4s8. Both PP4s2 and PP4s8 caused a 80-90% decrease in the endogenous expression of PP4c protein in MDA-MB-231 cells (Figure 3.27) and the reduction was 50-60% in Hs578T cells (Figure 3.31). Reduction of PP4c protein levels in MDA-MB-231and Hs578T cells caused an increase in total and viable cell numbers (Figure 3.28A, 3.28B and Figure 3.32A) respectively and protected the cells from spontaneous apoptosis (Figure 3.28C, 3.28D) for MDA-MB-231 and (Figure 3.32B) for Hs578T cells. PP4c silencing also significantly promoted the clonogenic activity and long term survival of MDA-MB-231 and Hs578T cells, as shown by the increase in the number of colonies formed by the cells transfected with PP4c-specific siRNAs (Figure 3.29) and (Figure 3.33) respectively. PP4c down-regulation caused an alteration in the cell cycle profile of MDA-MB-231 cells. Cultures of PP4c silenced cells showed a substantial reduction in the proportion of cells in sub-G_0 fraction, confirming that the apoptosis rate in these cells is indeed decreased (Figure 3.30). The proportion of cells in G_1 phase in cultures with reduced PP4c expression was consistently lower than that in the control cultures and the percentage of cells in S and G_2/M phases is consistently higher than that of the control cultures suggesting that PP4c down-regulation may promote acceleration in G_1 progression i.e increased proliferation rate (Figure 3.30).

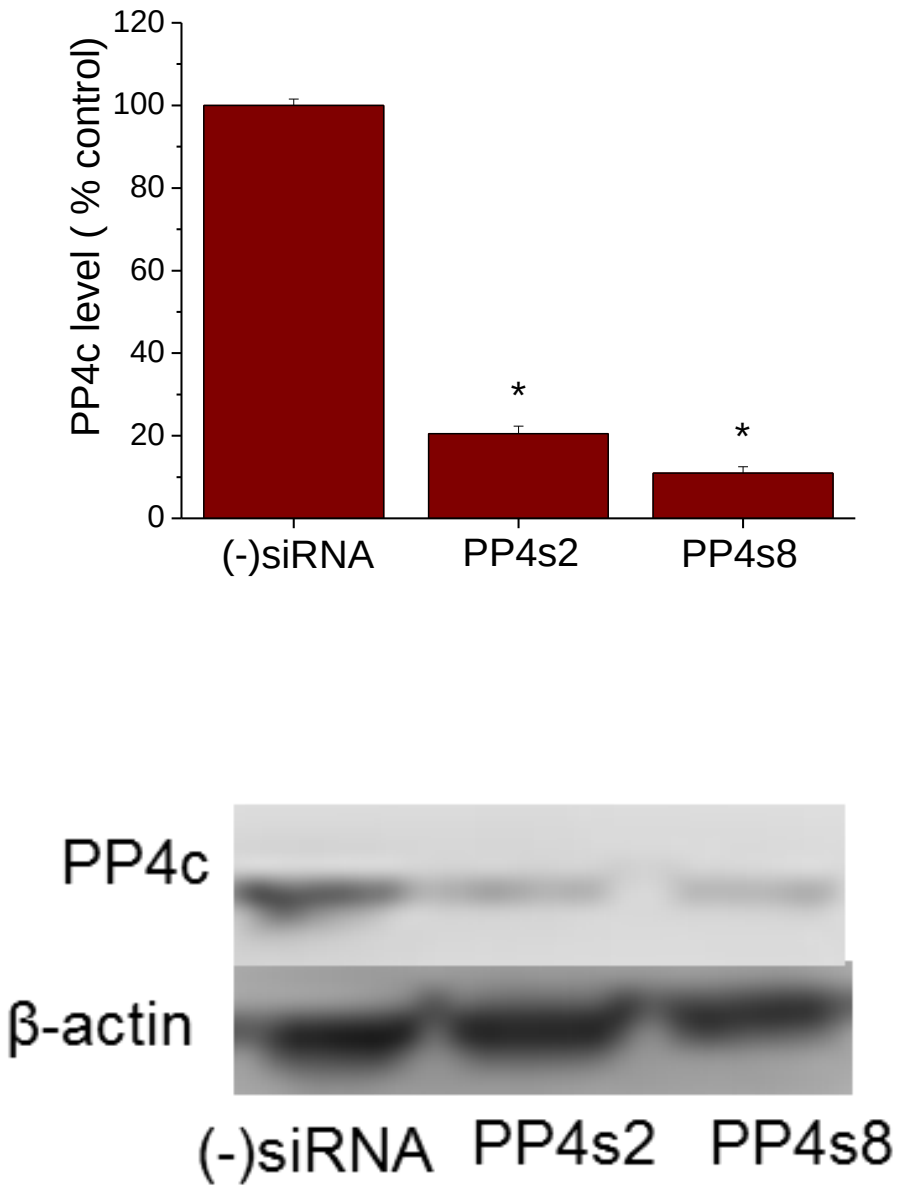


Figure 3.27: Expression level of PP4c protein in MDA-MB-231 cells was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. Signals were visualized utilizing the Odyssey® Imager (Li-Cor) and analysed by densitometry. A representative autoradiograph is presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4c protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

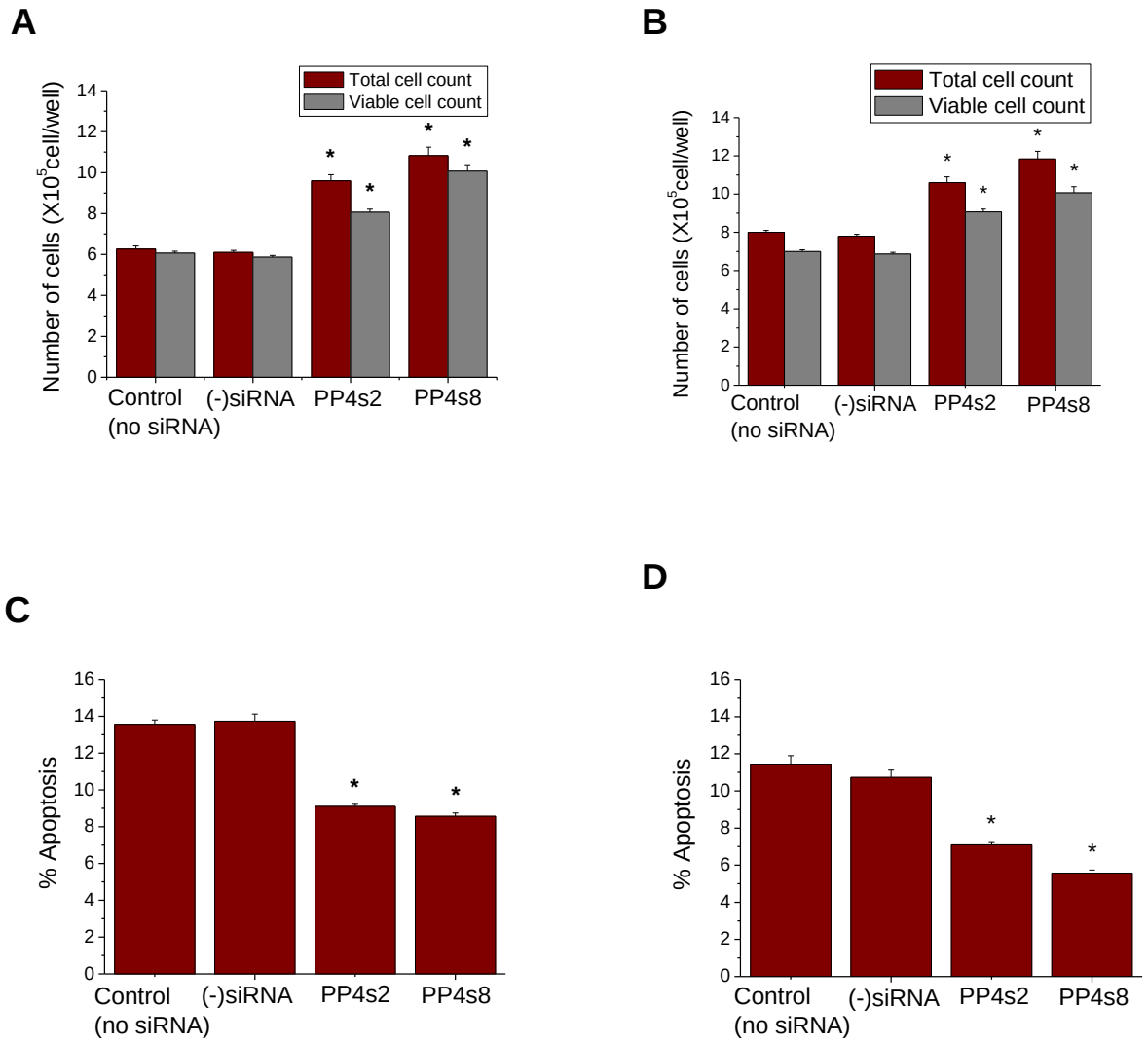


Figure 3.28: PP4c-specific siRNAs inhibit basal apoptosis and increase short term survival of MDA-MB-231 cells. MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNA. Cells were harvested at 72 hours post-transfection, and re-plated for assessment of cell survival after a further 48 hours. **A)** Total and viable cell numbers as determined by FACS analysis using Muse® Count & Viability Assay kit. **B)** Total and viable cell numbers as determined by vital dye stain. **C)** The level of basal apoptosis as determined by FACS analysis using Muse® Annexin V and Dead Cell Assay. **D)** The level of basal apoptosis as determined by acridine orange dye. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

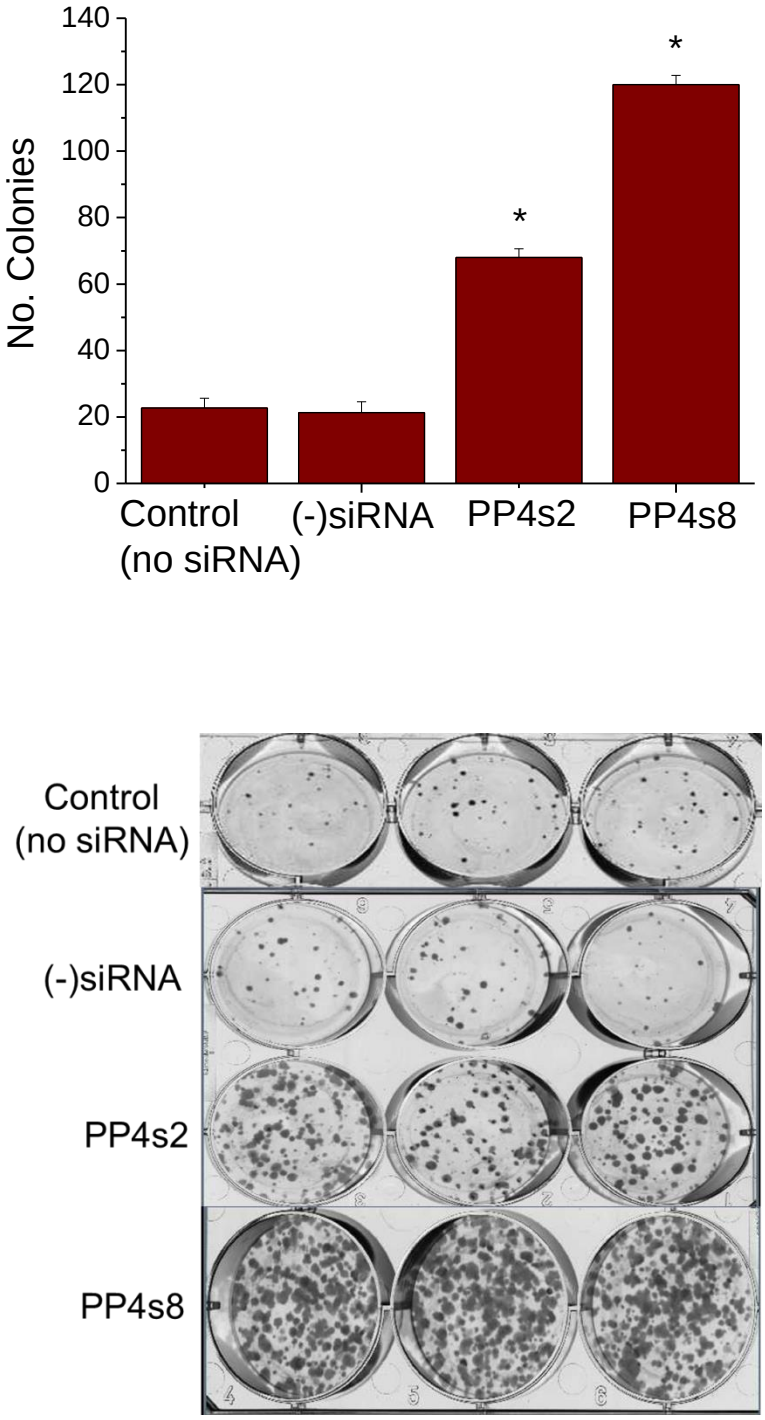


Figure 3.29: Clonogenic assay demonstrates that PP4c silencing enhances long term survival of MDA-MB-231 cells. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

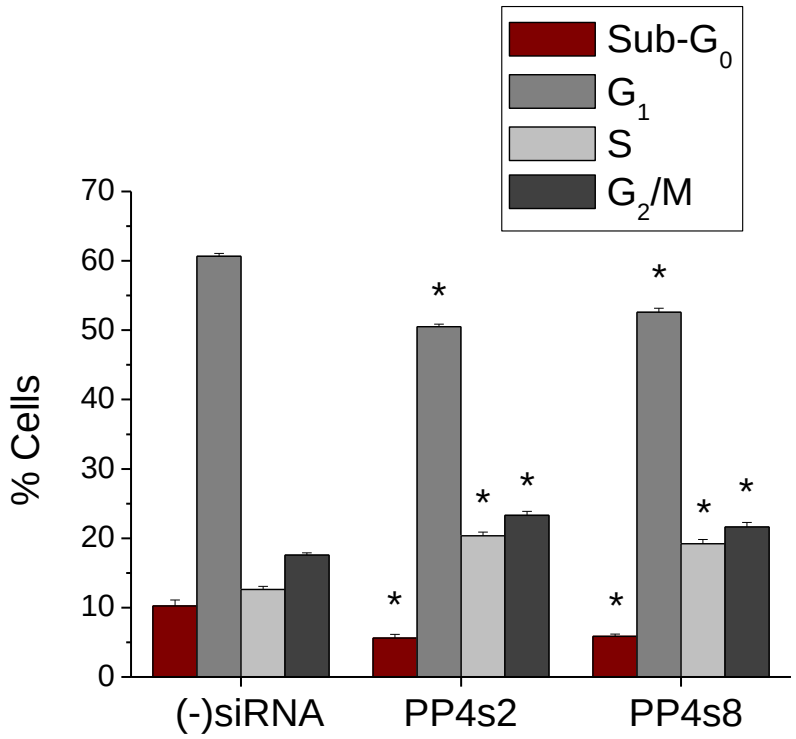


Figure 3.30: Cell cycle profiles of (-)siRNA-transfected MDA-MB-231 cells and PP4s2 and PP4s8 siRNA-transfected MDA-MB-231 cells were determined by propidium iodide staining of fixed cells and fluorescence flow cytometry. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

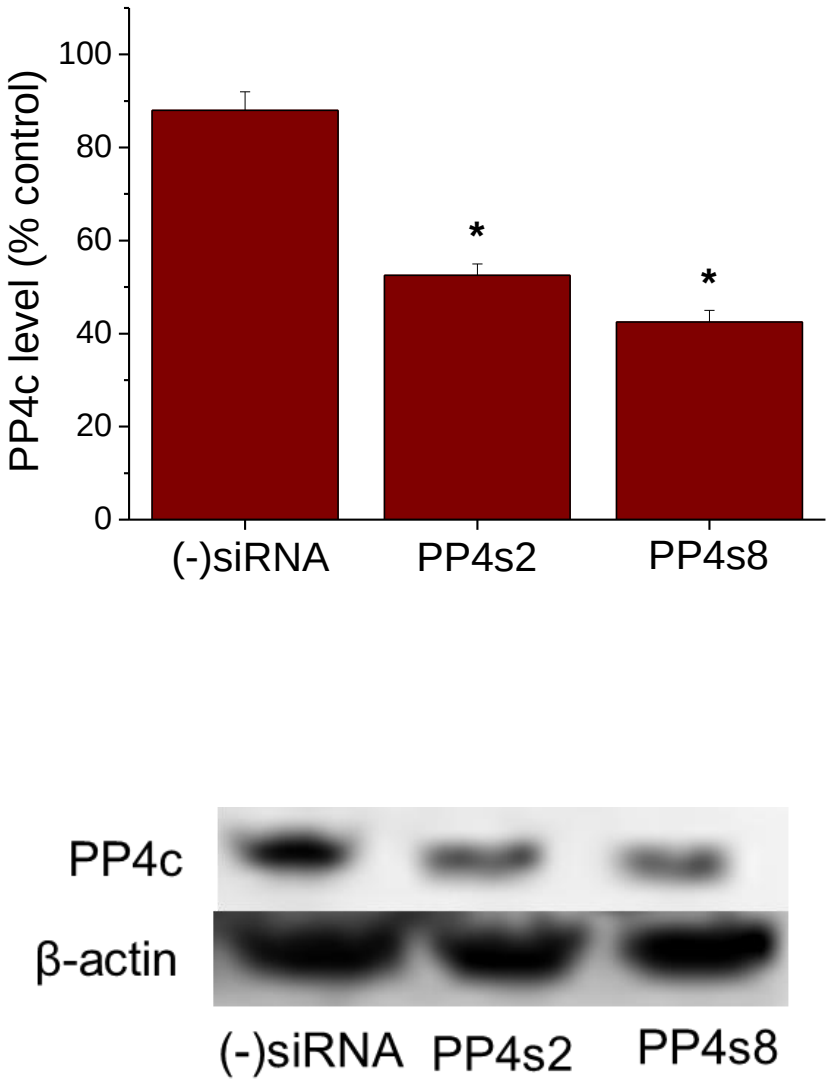


Figure 3.31: Cellular levels of PP4c in Hs578T cells as determined 72 hours post-transfection by western blotting. Anti-β-actin antibody was used to reveal β-actin as a loading control. Signals were visualised utilizing the Odyssey® Imager (Li-Cor) and subjected to densitometric analysis. A representative autoradiograph is presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4c protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

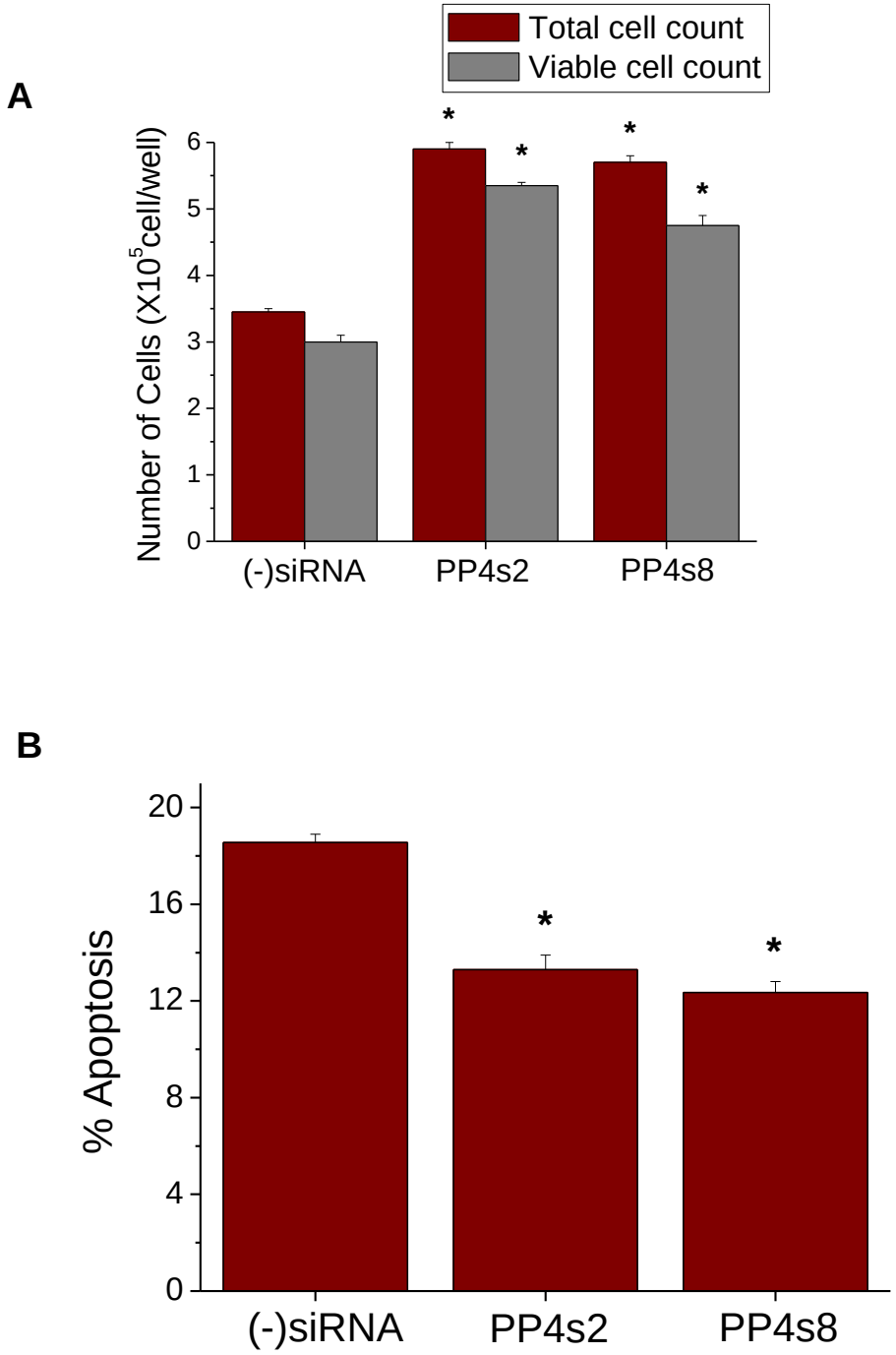


Figure 3.32: siRNA-mediated PP4c Knockdown reduces basal apoptosis and enhances survival of Hs578T breast cancer cells. **A)** Total and viable cell numbers as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** The proption of basal apoptosis as assessed by Muse® Annexin V and Dead Cell Assay. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

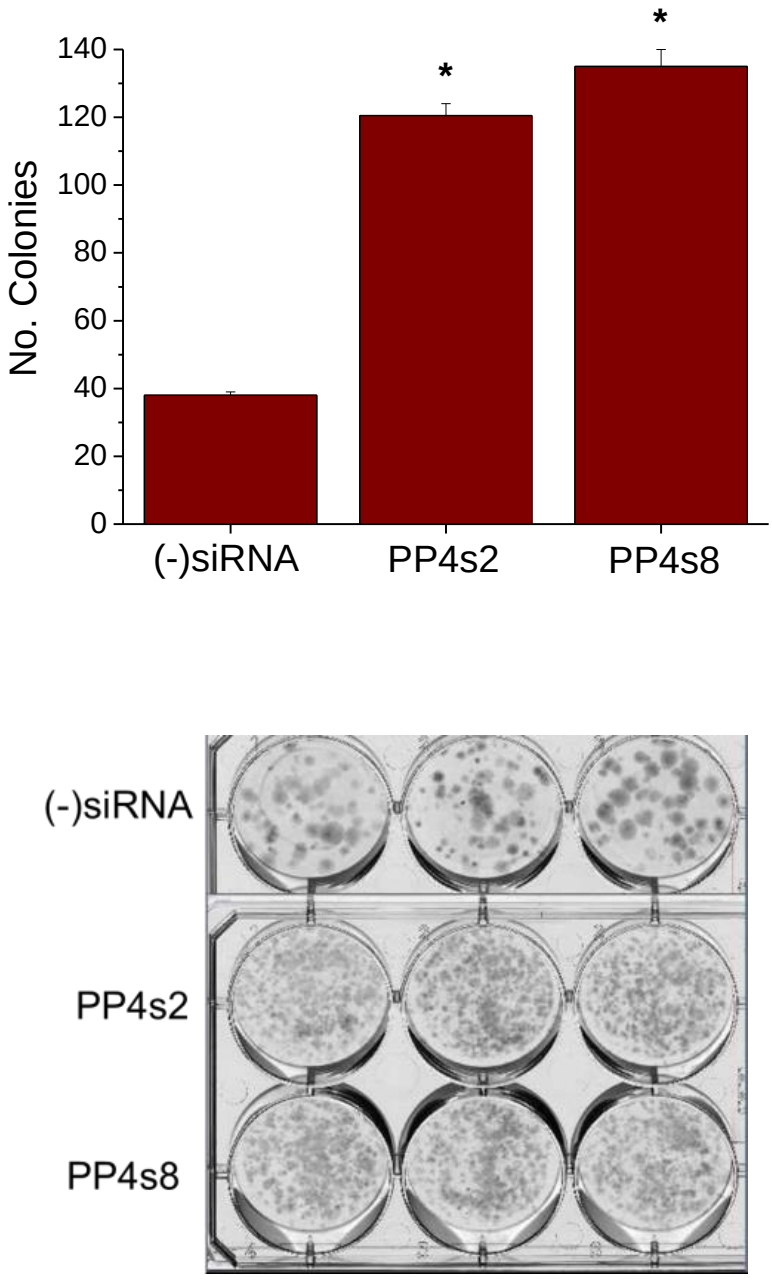


Figure 3.33: siRNA-mediated PP4c Knockdown enhances long term survival of Hs578T breast cancer cells. An example image of a clonogenic assay plate is shown. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

3.3.8 PP4c silencing promotes migration in breast cancer cells

The effect of PP4c silencing on cell migration was also assessed using an *in vitro* scratch assay. RNA mediated silencing of PP4c promoted cell migration in both MCF7 and MDA-MB-231 cells (Figure 3.34 and Figure 3.35) respectively. PP4c down-regulation was found to increase MCF7 cell migration by up to 30% at 36, 54 and 72 hours compared with mock transfected cells and (-)siRNA transfected cells (Figure 3.34). Consistent with these observations, PP4c knockdown in MDA-MB-231 cells showed a significant increase in cell migration ability by 30% at 18 hours and by 20% at 36 hours and 54 hours (Figure 3.35). Overall, the data indicates that PP4c down-regulation in breast cancer cells provide these cells with an advantage in proliferation and migration, with implications for progression and metastasis.

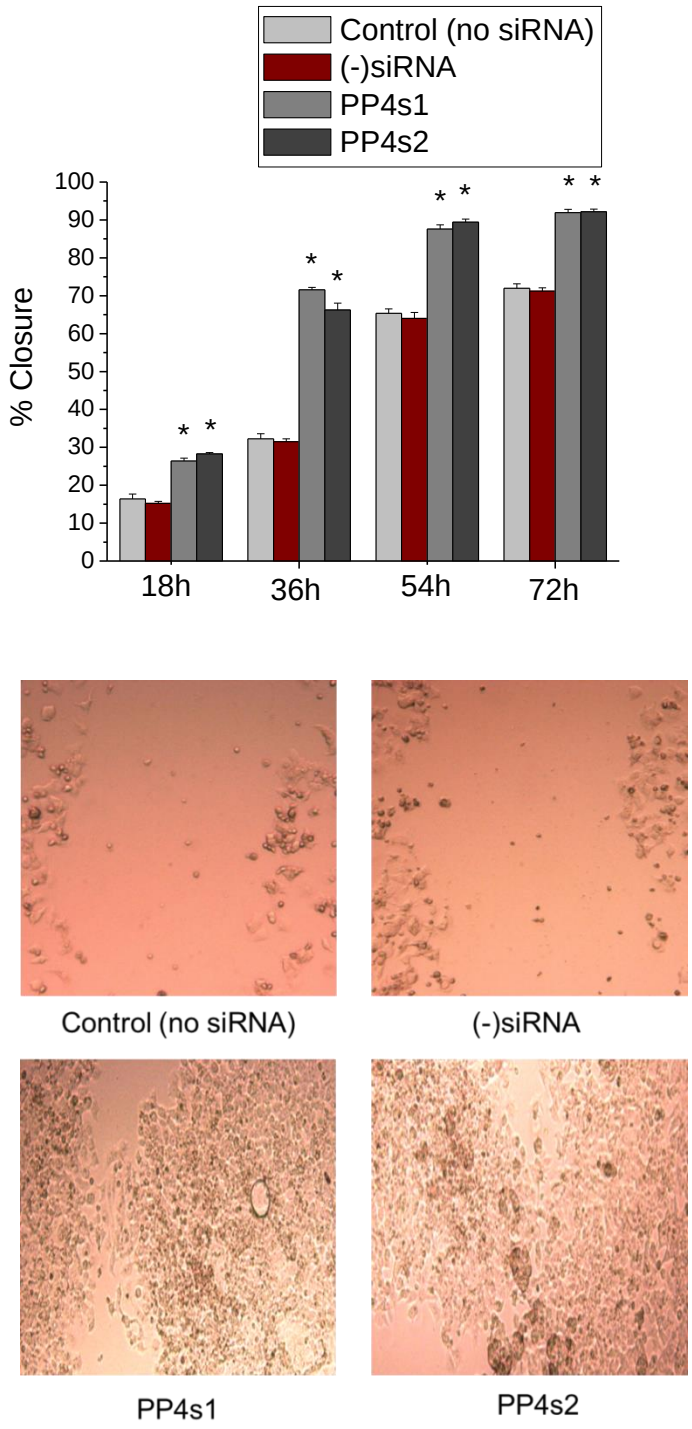


Figure 3.34: PP4c down-regulation promotes the migration ability of MCF7 breast cancer cell line. The effect of PP4c silencing on cell migration was assessed using an *in vitro* scratch assay and the cell migration was presented as % wound closure. Data was presented as the means ± S.E. (n=4). Representative images of the wound closure at 72 hours were presented below the bar graph. *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

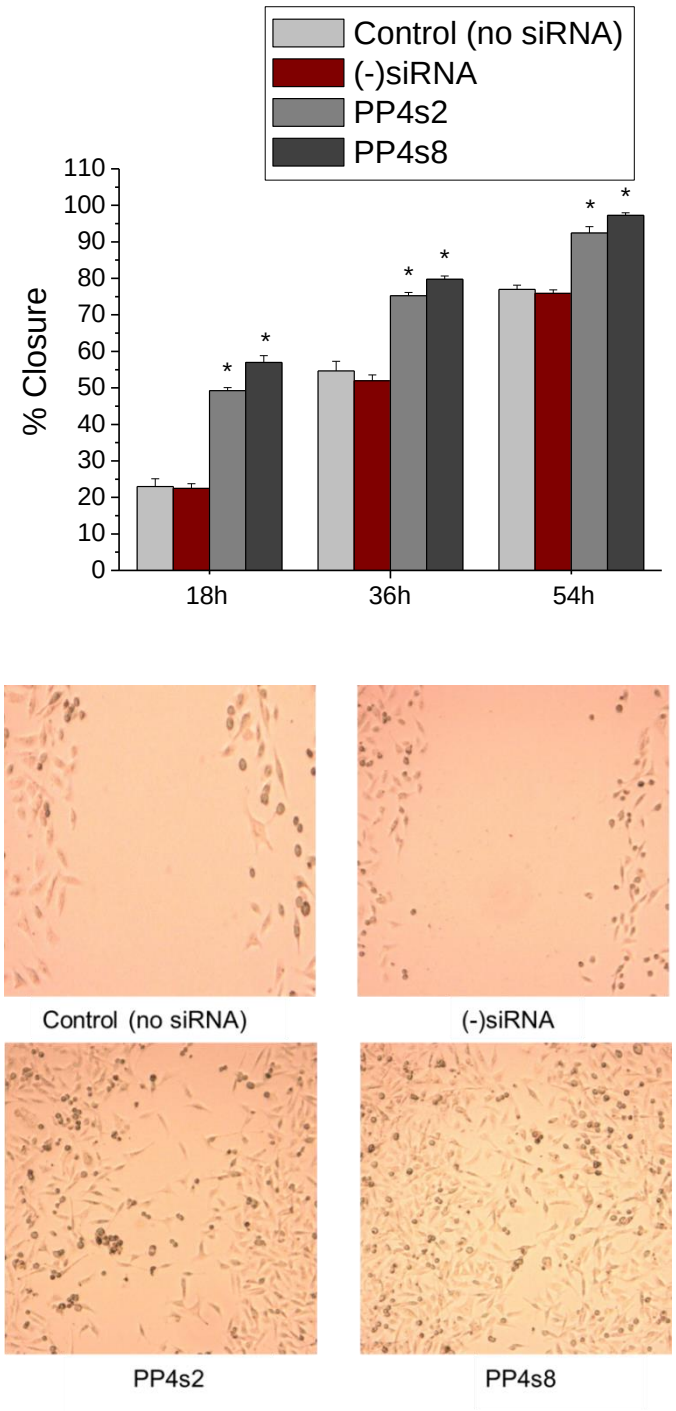


Figure 3.35: PP4c down-regulation promotes the migration ability of MDA-MB-231 breast cancer cell line. The effect of PP4c silencing on cell migration was assessed using an *in vitro* scratch assay and the cell migration was presented as % wound closure. Data was presented as the means ± S.E. (n=4). Representative images of the wound closure at 54 hours were presented below the bar graph *P < 0.05 versus cells with mock transfection and cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

3.3.9 PP4c down-regulation leads to an increase in the PEA15 Ser^{116} phosphorylation

Phosphoprotein enriched in astrocytes 15 kD (PEA15) is one of the proteins reported to be regulated, directly or indirectly, through dephosphorylation by PP4c (Glading et al., 2007; Fiory et al., 2009). Previous studies have shown that over-expression of PP4c in human embryonic kidney HEK 293T cells correlates with dephosphorylation of PEA15 at Ser^{116}, while PP4c silencing results in a significant increase in the level of phosphorylated PEA15 at Ser^{116} (Mourtada-Maarabouni and Williams, 2008). In order to investigate whether PP4c regulates the phosphorylation level of PEA15 in breast cancer cells, the status of PEA15 phosphorylation was measured in the cells transfected with PP4c-specific siRNAs using an antibody that specifically recognises the phosphorylated form of PEA15 at Ser^{116}. MCF7 and MDA-MB-231 cells were transfected with two different PP4c-specific siRNAs or with (-)siRNA as a control. Cells were harvested at 72 hours post-transfection and the proteins were extracted from the tansfected cells. The effect of PP4c down-regulation on the phosphorylation of PEA15 was assessed by western blot analysis using phosphospecific anti-PEA15 antibody. Western blot analysis revealed that PEA15 phosphorylation state significantly increased when PP4c expression was suppressed in both MCF7 and MDA-MB-231 cells. Both PP4c targeted siRNAs caused up to 120% increase in the phosphorylated form of PEA15 at Ser^{116} in MCF7 (Figure 3.36). PP4c down-regulation in MDA-MB-231 cells resulted in 220% (for PP4s2) and 240% (for PP4s8) elevation in the level of phosphorylated PEA15 at Ser^{116} (Figure 3.37).

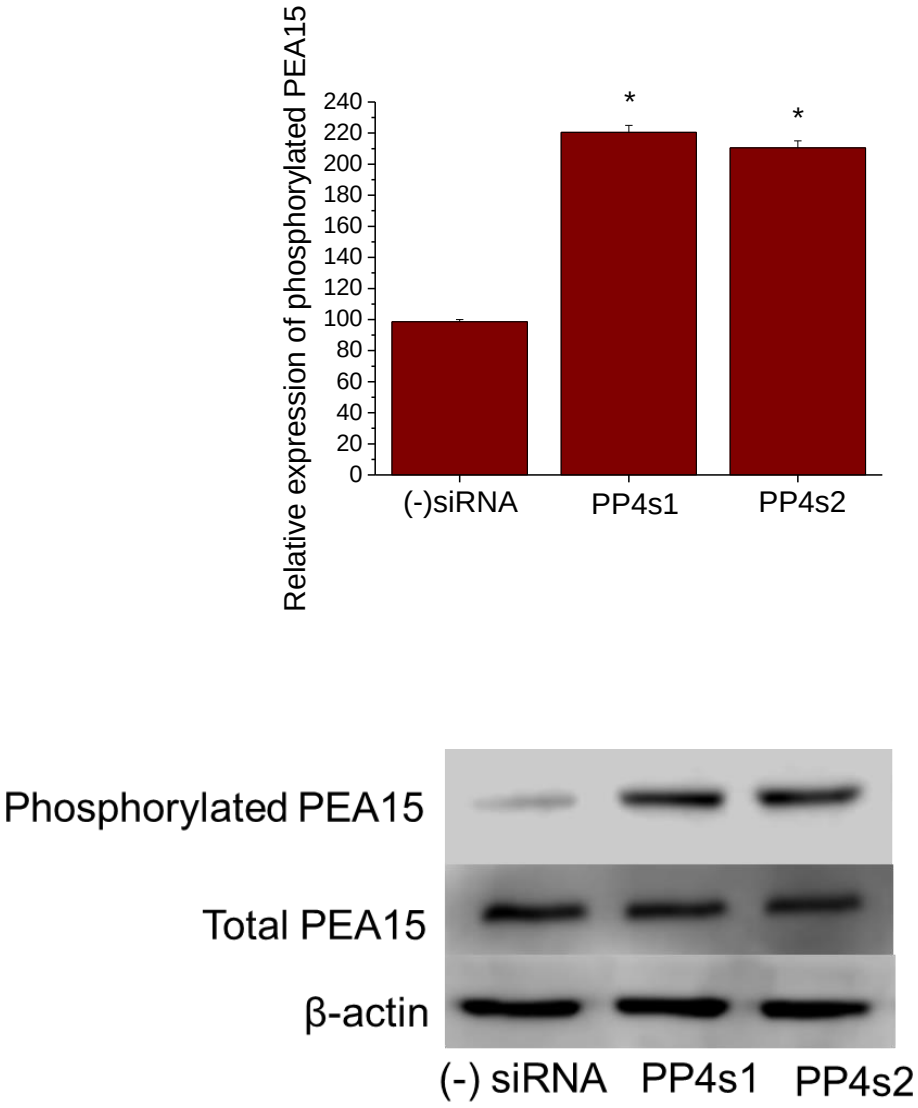


Figure 3.36: PP4c down-regulation is associated with an increase in the phosphorylation of PEA15 at Ser[116] in MCF7 cells. MCF7 cells were transfected with PP4c-specific siRNAs or (-)siRNA. Cells were harvested at 72 hours post-transfection and the effect of PP4c down-regulation on the phosphorylation of PEA15 was assessed by western blot analysis using phospho-specific anti-PEA15 antibody. Quantification of phosphorylated PEA15 was determined by densitometry relative to total PEA15 protein and β–actin and results are expressed as relative percent change compared to the control (-siRNA). The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

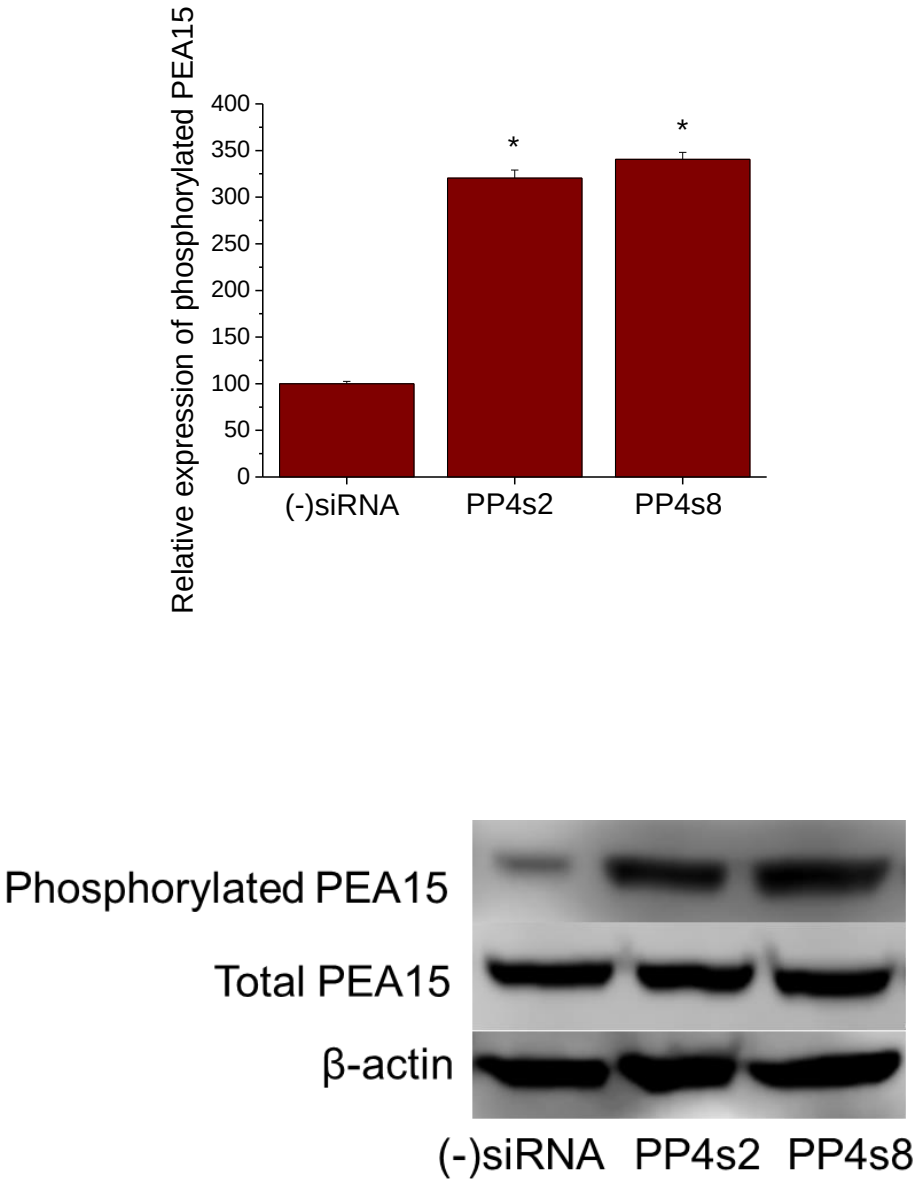


Figure 3.37: PP4c down-regulation is associated with an increase in the phosphorylation of PEA15 at Ser^{116} in MDA-MB-231 cells. MDA-MB-231 cells were transfected with PP4c-specific siRNAs or (-)siRNA. Cells were harvested at 72 hours post-transfection and the effect of PP4c down-regulation on the phosphorylation of PEA15 was assessed by western blot analysis using phospho-specific anti-PEA15 antibody. Quantification of phosphorylated PEA15 was determined by densitometry relative to total PEA15 protein and β–actin and results are expressed as relative percent change compared to the control (-siRNA). The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

3.3.10 PP4c effects on cell survival, migration and apoptosis are mediated at least partly by PEA15

The control of apoptosis and cell proliferation mediated by PP4c in leukemic and primary human T-cells was found to be mediated at least partly through the dephosphorylation of PEA15 at Ser^{116} (Mourtada-Maarabouni and Williams, 2008 and 2009). Further experiments were carried out in order to investigate whether PEA15 plays a major role in mediating the apoptosis and growth inhibitory effects of PP4c. Two PEA15-specific siRNAs were used to down-regulate PEA15 expression in breast cancer cells before studying the effects of modulation of PP4c expression on the viability of these cells. Down-regulation of PEA15 in MCF7 cells was assessed 72 hours post-transfection which showed that both PEA15-specific siRNAs were effective in causing a reduction in PEA15 protein expression by 55-80% (Figure 3.38). In agreement with previous studies that are conducted in HMECs (Human Mammary Epithelial Cells) (Hindupur et al., 2014) our results revealed that PEA15 down-regulation in MCF7 cells resulted in a significant decrease in the number of viable cells and cell viability as assessed by FACS analysis and vital dye stain (Figure 3.39A, 3.39B, 3.39C) and a significant increase in basal apoptosis level as confirmed by Muse® Annexin V and Dead Cell Assay (Figure 3.39D). PEA15 down-regulation also reduced long term survival and the colony forming ability of MCF7 breast cancer cells (Figure 3.40). Consistent with these observations, PEA15 silencing in MDA-MB-231 cells also resulted in a significant reduction in viable cell number and in cell viability (Figure 3.42A, 3.42B, 3.42C), an increase in basal apoptosis (Figure 3.42D) and a reduction in long term survival and colony forming ability of these cells (Figure 3.43) suggesting an oncogenic role for PEA15 in both oestrogen receptor-positive and triple negative breast cancer (TNBC) cell lines.

To investigate the significance of PEA15 in mediating PP4c functions; control cells (cells transfected with (-)siRNA) and cells transfected with PEA15-specific siRNAs were transiently transfected with pcDNA3.1-PP4c or pcDNA3.1. In MCF7; over-expression of PP4c in the cells transfected with (-)siRNA caused 35% reduction in viable cell number and an increase in basal apoptosis (Figure 3.44A, 3.44B) confirming the results obtained earlier in this chapter. While down-regulation of PEA15 in the cells transfected with vector only caused 50% decrease in the number of viable cells, over-expression of PP4c in the cells with PEA15 down-regulation did not have any additional effects on the viable cell number and apoptosis level (Figure 3.44A, 3.44B). These findings were also confirmed in the TNBC-MDA-MB-231 cells. Over-expression of PP4c in (-)siRNA transfected cells decreased the number of viable cells by more than 40% and enhanced the level of basal apoptosis (Figure 3.45A, 3.45B). Down-regulation of PEA15 in the cells transfected with vector only significantly decreased the number of viable cells and over-expression of PP4c in the cells with PEA15 down-regulation did not cause an additional decrease in the number of viable cells and increase in the level of apoptosis (Figure 3.45A, 3.45B). These findings suggested that down-regulation of PEA15 abolished PP4c-mediated loss of cell viability in MCF7 and in MDA-MB-231 breast cancer cell lines.

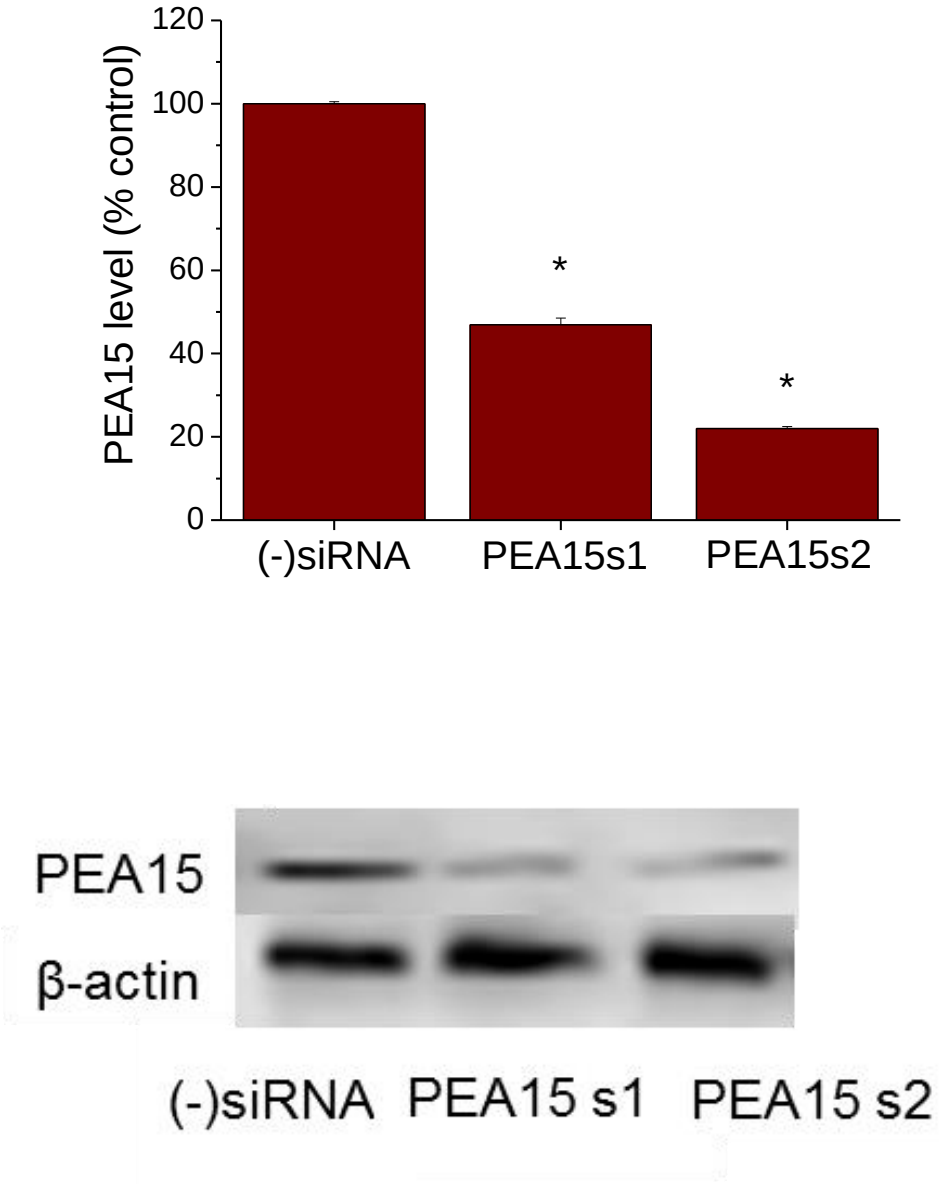


Figure 3.38: Expression of PEA15 protein levels in MCF7 cells were determined by western blotting. An equivalent loading was demonstrated using anti-β-actin antibody. Signals were visualised utilizing the Odyssey® Imager (Li-Cor) and analysed by densitometry. A representative autoradiograph is presented below the bar graph. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT).

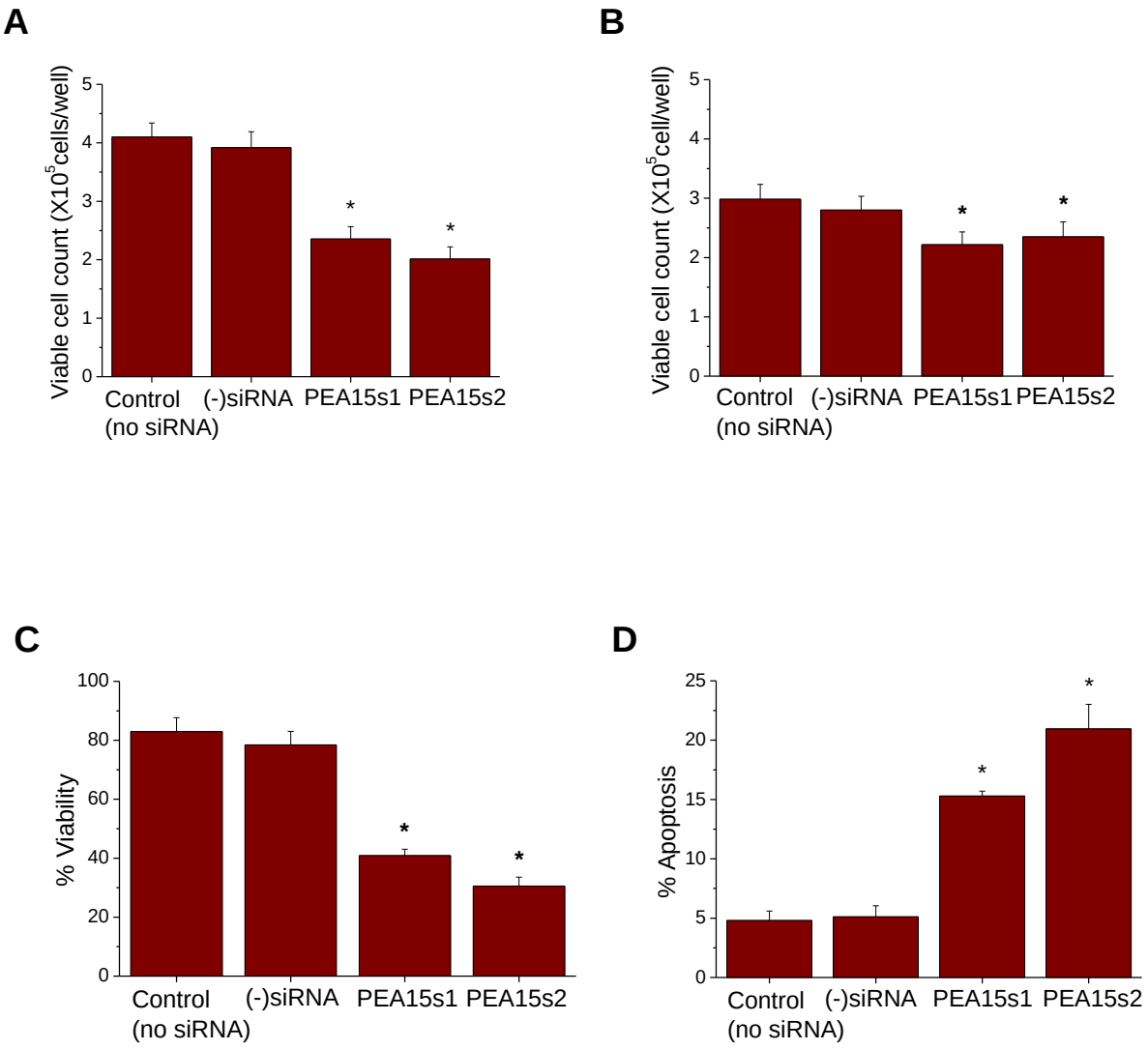


Figure 3.39: PEA15 down-regulation enhances apoptosis and reduces viability of MCF7 cells. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with two different PEA15-specific siRNAs. 72 hours post-transfection, cells were harvested to assess PEA15 protein expression level and cells were re-plated for assessment of cell survival after a further 48 hours. **A)** Viable cell number as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** Viable cell number as assessed by vital dye stain. **C)** Cell viability as assessed by FACS analysis. **D)** The proportion of apoptotic cells as determined by Muse® Annexin V and Dead Cell Assay. Results are represented as means ± S.E. from four independent experiments. *P < 0.05 compared with mock transfected and (-)siRNA transfected cultures (one-way ANOVA and Bonferrroni's MCT)

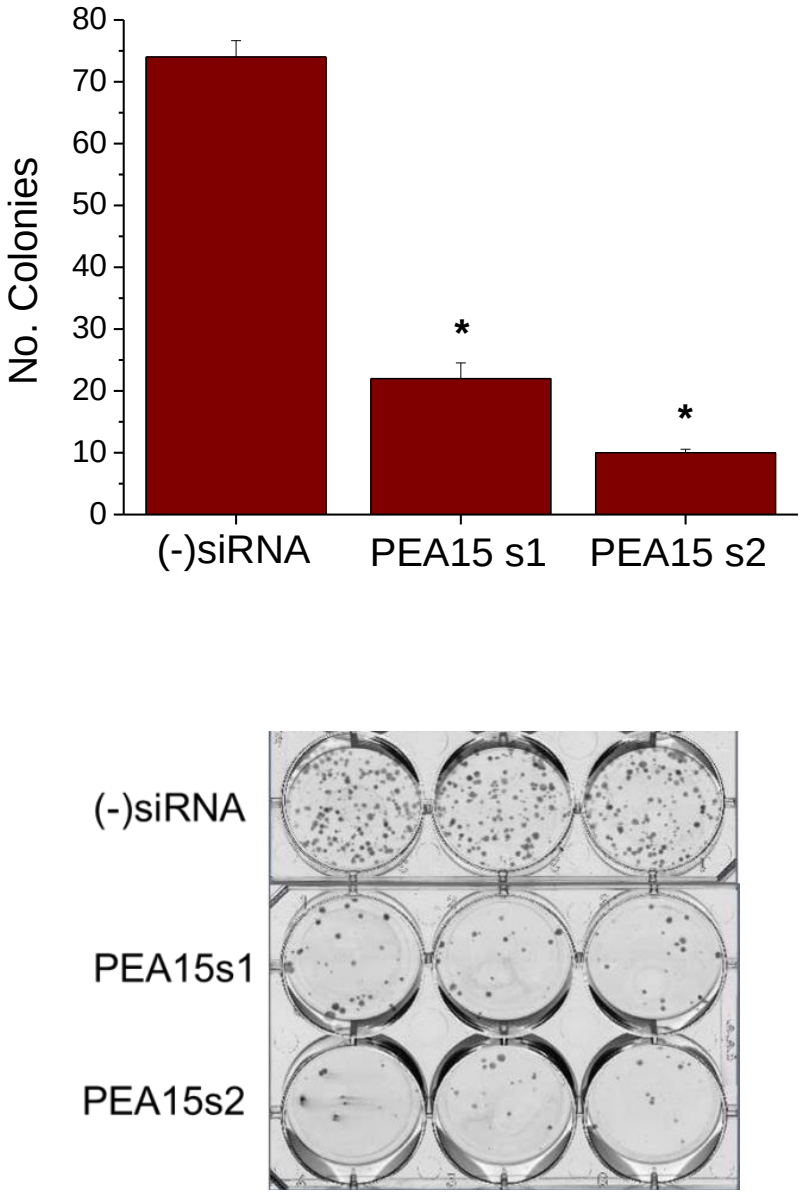


Figure 3.40: PEA15 down-regulation reduces long term survival and colony forming ability of MCF7 cells. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 compared with (-)siRNA transfected cells (one-way ANOVA and Bonferrroni's MCT)

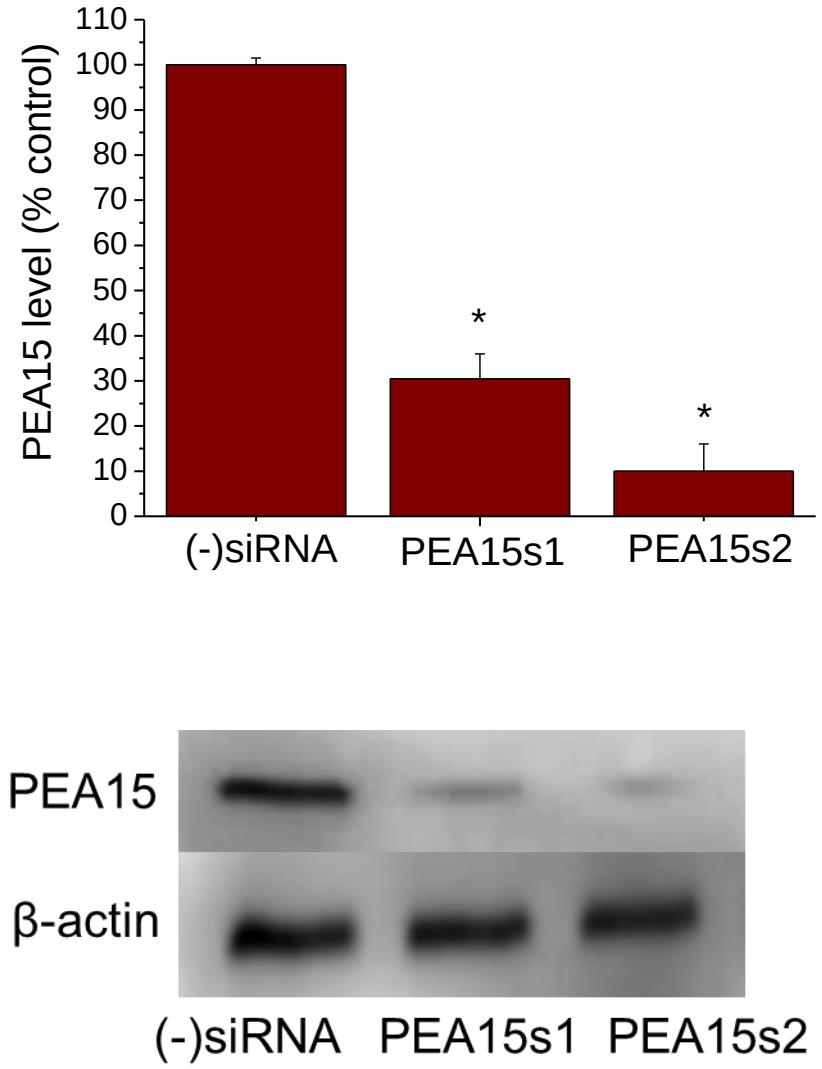


Figure 3.41: Expression level of PEA15 protein in MDA-MB-231 cells was determined by western blotting and equivalent loading was demonstrated using anti-β-actin antibody. Signals were visualised utilizing the Odyssey® Imager (Li-Cor) and analysed by densitometry. A representative autoradiograph is presented below the bar graph. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PEA15 protein expression levels were normalised with that of β-actin. *P < 0.05 compared with (-)siRNA transfected cells (one-way ANOVA and Bonferrroni's MCT)

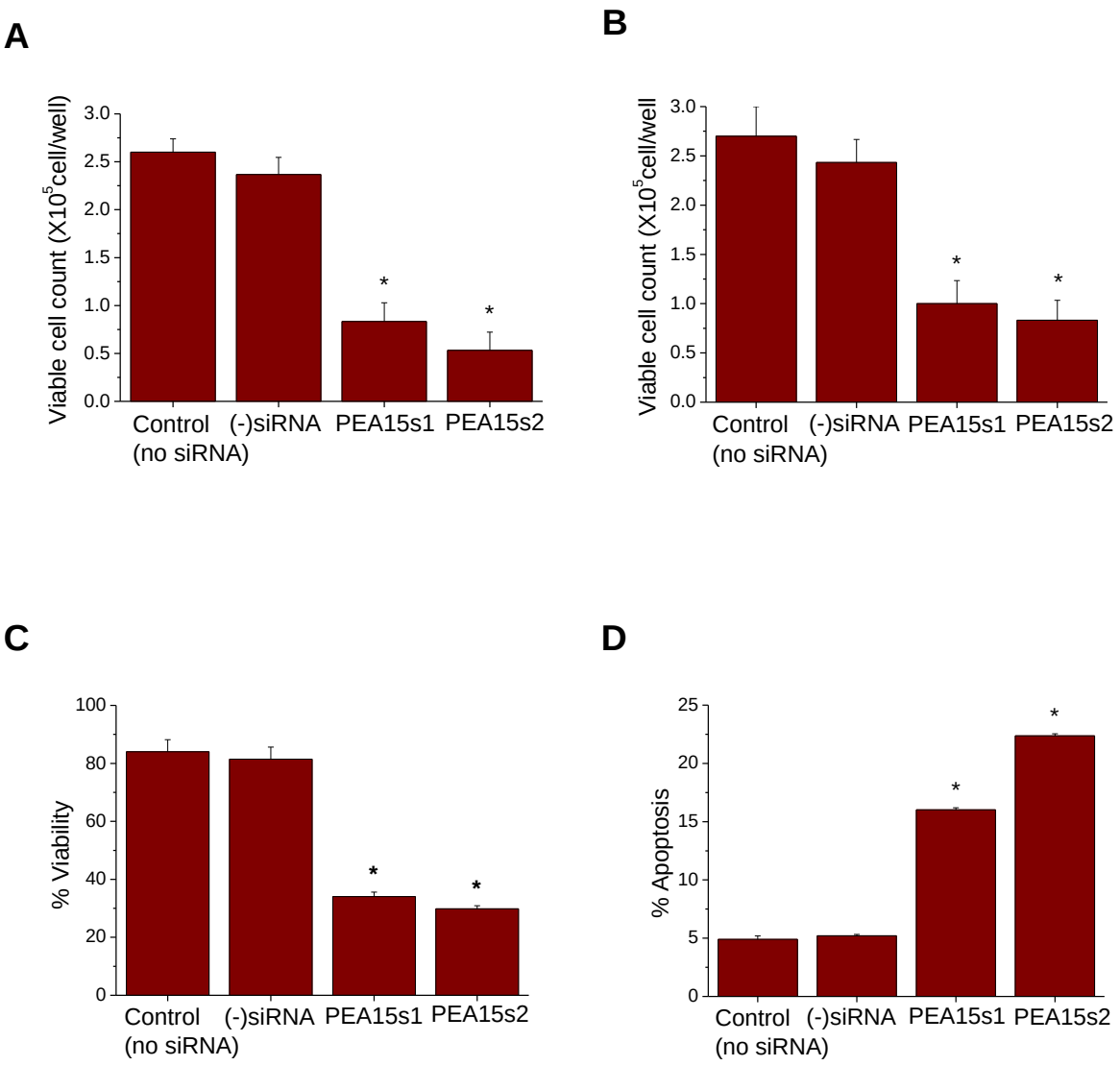


Figure 3.42: PEA15 down-regulation enhances apoptosis and reduces viability of MDA-MB-231 cells. MDA-MB-231 cells were were mock transfected or transfected with (-)siRNA as a control or with two different PEA15-specific siRNAs. 72 hours post-transfection, cells were harvested to assess the expresion of PEA15 protein and were re-plated for assessment of cell survival after a further 48 hours. **A)** Viable cell number as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** Viable cell number as assessed by vital dye stain. **C)** Cell viability as assessed by FACS analysis. **D)** The proportion of apoptotic cells as determined by Muse® Annexin V and Dead Cell Assay. Results are represented as means ± S.E. from four independent experiments. *P < 0.05 compared with mock transfected and (-)siRNA transfected cultures (one-way ANOVA and Bonferrroni's MCT)

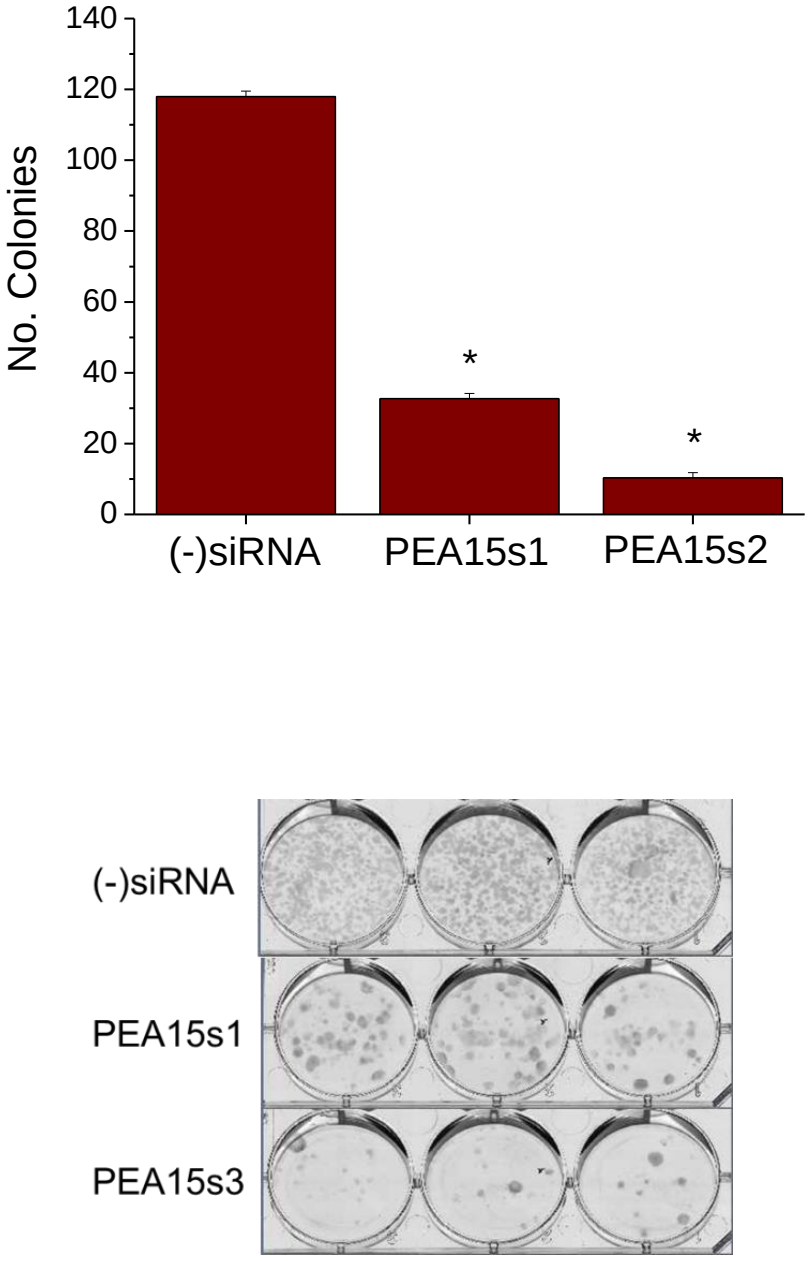


Figure 3.43: PEA15 down-regulation reduces long term survival and colony forming ability of MDA-MB-231 cells. An example image of a clonogenic assay plate after crystal violet staining is shown below the bar chart. The bar graphs represent means ± S.E. from four independent experiments. $P < 0.05$ compared with (-)siRNA transfected cells (one-way ANOVA and Bonferrroni's MCT)

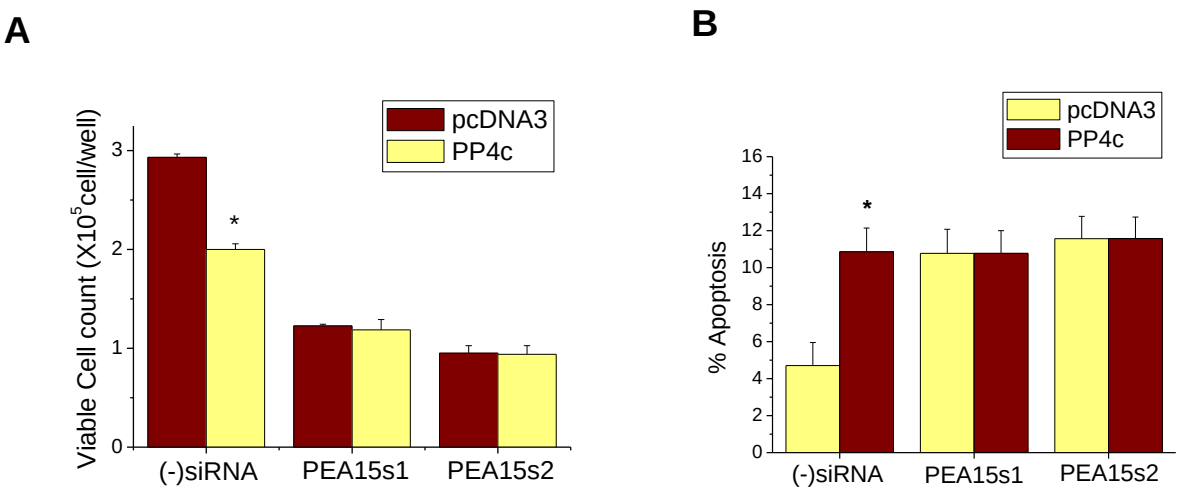


Figure 3.44: Down-regulation of PEA15 abolishes PP4c-mediated loss of cell viability in MCF7 breast cancer cell line. Cells were transfected with (-)siRNA or PEA15-specific siRNAs to down-regulate PEA15, 72 hours post-transfection all cultures (cells transfected with (-)siRNA and cells transfected with PEA15-specific siRNAs) were transiently transfected with pcDNA3.1-PP4c or pcDNA3.1. Cell survival and apoptosis were assessed after 48 hours. **A)** Total and viable cell counts as assessed by FACS using Muse® Count & Viability Assay kit. **B)** The proportion of total apoptosis as assessed by FACS using Muse® Annexin V and Dead Cell Assay. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared with (-)siRNA. *P < 0.05 compared with pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

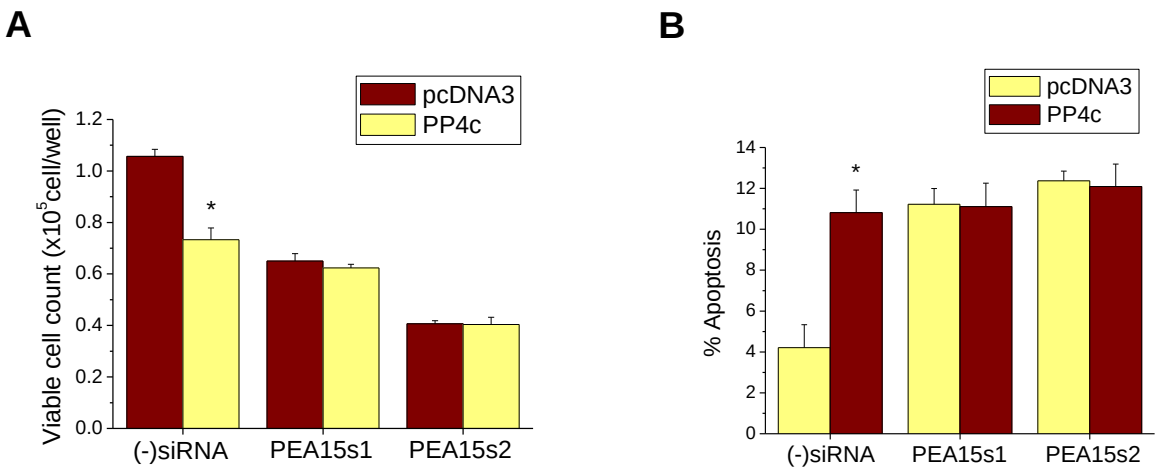


Figure 3.45: Down-regulation of PEA15 abolishes PP4c-mediated loss of cell viability in MDA-MB-231 breast cancer cell line. MDA-MB-231 cells were transfected with (-)siRNA or PEA15-specific siRNAs to down-regulate PEA15, 72 hours post-transfection all cultures (cells transfected with (-) siRNA and cells transfected with PEA15-specific siRNAs) were transiently transfected with pcDNA3.1-PP4c or pcDNA3.1. Cell survival and apoptosis were assessed after 48 hours. **A)** Total and viable cell counts as assessed by FACS using Muse® Count & Viability Assay kit. **B)** The proportion of total apoptosis as assessed by FACS using Muse® Annexin V and Dead Cell Assay. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared with (-)siRNA. *P < 0.05 compared with pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

3.4 Discussion

Reversible protein phosphorylation, controlled by the opposing operation of protein kinases and phosphatases, regulates many cellular processes. Disturbance to the well balanced function of kinases and phosphatases contributes to the development and progression of various cancers including breast cancer. Many kinases are now characterised to be oncogenic and changes in their activities have been linked to the pathogenesis and progression of breast cancer. However, the roles played by the phosphatases are much less clear and less studied, although, logically, they must be of great importance through their ability to counteract the activities of the kinases (Nunes-Xavier et al., 2013). Indeed, phosphatases such as PP2A (Cohen et al., 2005; Van and Goris, 2003), phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Src homology 2 (SH2) containing inositol 5-phosphatase (SHIP) are recognised as potential therapeutic targets due to their tumour suppressor activities (Zhang and Clarit, 2012; Mourtada-Maarabouni and Williams, 2009). In this study we have found that PP4c is over-expressed in different types of hormone positive and hormone negative breast cancer cells and this confirmed previous studies which demonstrated that PP4c is over-expressed in different types of cancer such as breast, lung (Wang et al., 2008) and pancreatic cancer, where it is associated with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma (Weng et al., 2012). In addition, PP4c was found to be over-expressed in human colorectal cancer and this correlates with more aggressive tumour and poor patient prognosis (Li et al., 2015). In our study we have demonstrated that PP4c is over-expressed in triple negative breast cancer cells-MDA-MB-231, progesterone/HER2 positive cells-MDA-MB-361 and in oestogen/progesterone positive cells-MCF7 and T47D, these results are correspond

with the results obtained by Wang et al., 2008. Here we demonstrate, for the first time, that the Ser/Thr phosphatase PP4 regulates the survival, proliferation and migration of oestrogen/progestrone receptor-positive breast cancer and TNBC cells. We further demonstrate that these effects are mediated in part by modulation of the phosphorylation state of PEA15, pointing to the existence of a PP4c-PEA15 axis that controls breast cancer cell fate.

The results in this chapter provide strong evidence that the serine/threonine phosphatase PP4 regulates the survival and proliferation of oestrogen/progestrone receptor-positive and TNBC breast cancer cells. Our study revealed that endogenous level of PP4c is of critical importance for the survival and growth of breast cancer cells. Modulation of the expression level of the catalytic subunit of PP4 causes significant and specific effects on the survival and proliferation of breast cancer cells. An increase in PP4c protein levels in both oestrogen/progesterone receptor-positive and TNBC cells was associated with a consistent and a significant decrease in both short and long term viability and stimulated apoptosis in the absence of extracellular stimuli. We have also shown that PP4c knockdown caused an increase in the rate of cell proliferation and migration in both cell types. Together, the data support an important role for PP4c in maintaining the delicate balance between cell survival and cell death in a range of breast cancer cell types and point to a tumour suppressor function for this protein in breast cancer.

Indeed PP4c has been reported to negatively regulate the survival of other cell types, including both leukemic T-cells and untransformed human peripheral blood T-cells (Mourtada-Maarabouni and Williams, 2009), in keeping with our findings here. Studies of a wide range of cell types other than breast epithelial cells have revealed that PP4 regulates an increasing number of cellular functions. The pleiotropic effects

of PP4c have been related to the existence of different PP4 complexes that have different compositions as a result of the interaction of PP4c with its different regulatory subunits (Cohen et al., 2005; Brechmann et al., 2012). The enzyme is involved in the regulation of microtubule growth and organization at the centrosomes (Carnegie et al., 2003), centrosome maturation in mitosis and meiosis and in the DNA damage response (Hastie et al., 2000; Chowdhury et al., 2005). Recent evidence suggested that PP4c controls neural progenitor cell proliferation and differentiation in the mouse neocortex by regulating the phosphorylation status of nuclear distribution protein nudE-like1 (Ndel1) (Xie et al., 2013). PP4c interacts with and down-regulates insulin receptor substrate 4 (IRS4) following tumour necrosis factor-alpha (TNF-α) stimulation leading to the inhibition of the anti-apoptotic function of IRS4 (Mihindukulasuriya et al., 2004). Such evidence is entirely consistent with the findings from the present studies and suggests potential downstream mechanisms underlying regulation of cell survival by PP4c in a cell context-dependent manner.

On the other hand, the exact role of PP4c in relation to cancer is not clear, with some studies suggesting a tumour suppressor role, while others support an oncogenic role. For example, our results showed an increase in PP4c expression level in breast cancer cell lines and in breast cancer tissue samples. Other studies have also reported an increase in PP4c expression level in human breast and lung tumours (Wang et al., 2008) and inhibition of PP4c expression increased the sensitivity of breast and lung cancer cells to cisplatin treatment, suggestive of an oncogenic function (Wang et al., 2008). PP4c is also over-expressed in pancreatic ductal adenocarcinoma (PDAC) and is associated with poor prognosis (Weng et al., 2012). Also PP4c has been reported to be expressed constitutively in prostate cancer cell

lines (PC-3 and LNCaP) where it acts as a positive regulator of the MAP kinase JNK-1 (Inostroza et al., 2005). Other studies have however shown that PP4c over-expression in HEK 293T (human embryonic kidney cells), T-leukemic cells and primary lymphocytes causes an increase in apoptosis, an inhibition of cell proliferation by inducing cell cycle arrest in G_1 and a significant decrease in the mutation rate, and correspondingly, that decreased PP4c protein expression increases the rate of cell proliferation and protects the cells against apoptosis induced by a range of stimuli (Mourtada-Maarabouni and Williams, 2008; Mourtada-Maarabouni et al., 2003)**.** These and present findings together support a tumour suppressor role for PP4c.

To resolve these apparently conflicting views, further information is required about the functional activity of PP4c in breast and other cancers, especially since this is dependent on the expression of regulatory subunits. For example, the regulatory subunit PP4R1 is down-regulated in a subset of malignant T-lymphocytes derived from patients with a severe form of cutaneous T-cell lymphoma, resulting in inactive PP4c. This in turn results in constitutive IKK/NF-kB signalling, suggesting that PP4R1-PP4c complex serves as a negative regulator of IKK activity (Brechmann et al., 2012). It is therefore important to elucidate the functional status of the catalytic subunit in breast cancer and further investigate the precise roles of other individual regulatory subunits and their potential functions during transformation. The possibility of the existence of an endogenous inhibitor should also be investigated, since an endogenous inhibitor of the closely related phosphatase PP2A, has been identified in cells undergoing blast crisis in chronic myeloid leukaemia (Neviani et al., 2005).

The key finding in this study was that PP4c influenced the phosphorylation status of PEA15, especially since PEA15 is itself implicated in the regulation of cell

proliferation and apoptosis (Mourtada-Maarabouni and Williams, 2008). Proteomic analysis has shown that changes in PP4c expression affect the phosphorylation status of many proteins involved in apoptosis and cell proliferation including PEA15 (Mourtada-Maarabouni and Williams, 2008). PEA15 is a multi-functional protein that has been implicated in the regulation of major intracellular processes including proliferation and apoptosis and its function is tightly regulated by its phosphorylation at two serine residues, Ser^{104} and Ser^{116} (Mourtada-Maarabouni and Williams, 2008). The non-phosphorylated form of PEA15 binds to the extracellular signal-regulated kinase 1/2 (ERK1/2) preventing its nuclear accumulation, leading to the inhibition of cell proliferation (Glading et al., 2007). Whereas, the phosphorylated form of PEA15 at Ser^{116} binds to Fas-associated death domain protein (FADD) via its DED domain, preventing FADD-mediated activation of caspases, the formation of the death inducing signalling complex (DISC) and leading to the inhibition of the extrinsic apoptotic pathway (Glading et al., 2007; Wei, 2015; Neviani et al., 2005). Both Calcium/calmodulin-dependent protein kinase II (CaMKII) and Akt phosphorylate PEA15 at Ser^{116} (Sulzmaier et al., 2012; Renganathan et al., 2005). More recently, AMPK was also reported to act as upstream kinase acting on PEA15 in both normal and cancerous breast epithelial cells (Hindupur et al., 2014). Phosphatases play as important a role as kinases in regulating the phosphorylation state of PEA15. In this regard, a loss of PTEN function commonly seen in tumour cells is associated with an increased PEA15 phosphorylation at Ser^{116} and an inhibition of Fas-mediated apoptosis (Hayashi et al., 2012). However, evidence suggests that PTEN does not dephosphorylate PEA15 directly, but it modulates its phosphorylation level by controlling Akt activity (Hayashi et al., 2012). In this study, we showed that PP4c also regulates the phosphorylation of PEA15 at Ser^{116}**.** Down-

regulation of PP4c which stimulated proliferation and inhibited apoptosis resulted in significant elevation in the phosphorylation of PEA15 at Ser^{116}. Down-regulation of PEA15 was found to inhibit cell growth and to reduce viable cell number and cell viability in both MCF7 and in MDA-MB-231 cell lines (Figure 3.39 and Figure 3.42) respectively. Crucially, PP4c had no effect on apoptosis in cells with prior knockdown of PEA15 expression, suggesting that the induction of apoptosis by PP4c is mainly mediated through PEA15 and pointing to the existence of a PP4c-PEA15 axis that controls breast cancer cell fate. Consequently, PP4c may be involved in mediating the switch of PEA15 from a tumour promoter to a tumour suppressor. Recent studies have shown that AMPK directly phosphorylates PEA15 at Ser^{116}, thereby converting it to a tumour promoter, resulting in increased survival and anchorage-independent growth of normal and breast cancer cells, both *in vivo* and *in vitro* (Hindupur et al., 2014). Thus, PP4c which dephosphorylates PEA15 may counteract the effects of AMPK and switch the activity of PEA15 from a tumour promoter to a tumour suppressor. In this regard, the balance in the activities of AMPK and PP4c are likely to be crucial in determining the phosphorylation status of PEA15 and consequently, the development and progression of breast cancer. The interaction between AMPK, PEA15 and PP4c may therefore be critical in the development and progression of breast cancer. The results presented in this chapter also highlight the potential therapeutic value of PEA15. Targeting PEA15 and its interactions, or the use of PEA15 inhibitors as therapeutics, may open a window of opportunities to treat breast cancer.

Chapter 4: The interaction between PP4c and its regulatory subunits in breast cancer

4.1 Introduction

In the previous chapter we have elucidated the role of PP4c in the regulation of survival, proliferation and migration of oestrogen/progesterone receptor-positive-MCF7 and triple negative breast cancer cells-MDA-MB-231 and Hs578T cells, also we have pointed to the importance of the PP4c-PEA15 axis which plays at least a partial role in mediating PP4c function as a regulator of breast cancer cell fate.

Our study confirmed the tumour suppressor activity of PP4c protein in breast cancer whereas other studies showed the oncogenic activity of PP4c (Weng et al., 2012; Li et al., 2015). This diverse function of PP4c could be related to the corresponding regulatory subunits in the PP4 holoenzyme. The PP4 holoenzyme occurs in different assemblies of the catalytic subunit (PP4c) and one or more regulatory subunits and it is the interaction between the different regulatory subunits of PP4c that is reported to be essential to the mode of action of the PP4 enzyme (Cohen et al., 2005). Several PP4c interacting proteins have been identified. These include PP4 regulatory subunit 1 (PP4R1), PP4 regulatory subunit 2 (PP4R2), PP4 regulatory subunit 3 alpha (PP4R3α), PP4 regulatory subunit 3 beta (PP4R3β), PP4 regulatory subunit 4 (PP4R4), and alpha 4/ immunoglobulin (CD79A) binding protein 1 (α4/ IGBP1) (Hastie et al., 2000; Carnegie et al., 2003; Gingras et al., 2005; Chen et al., 2008). With exception of α4/ IGBP1, all the identified regulatory subunits bind specifically to PP4c but not to its structurally related PP2A (Murata et al., 1997; Chen et al., 1998). In this chapter we have studied the expression of PP4c regulatory subunits in breast cancer tissue samples; we have investigated the correlation between PP4c and its

regulatory subunits in these breast cancer tissue samples. Also we have investigated the effect of down-regulation of some of these regulatory subunits on the tumour suppressor activity of PP4c in breast cancer cell lines.

4.2 Methods

RT-PCR, TaqMan gene expression assays (assay codes Hs00167441_m1 for ALAS1, Hs00427262_m1 for PPP4c, Hs00933337_m1 for PPPR1, Hs00752559_m1 for PPPR2, Hs00215697_m1 for SMEK1, Hs01106682_m1for SMEK2, Hs00372980_m1 for PPPR4) were employed in a ready-to-use panel of cDNAs derived from tissue samples of healthy individuals and from patients with breast cancer using TissueScan™ Cancer and Normal Tissue cDNA Arrays. RT-PCR was also performed on cDNAs samples prepared directly from MCF7 and MDA-MB-231 cells having PP4c over-expression or PP4c down-regulation to quantify the level of mRNA of PP4c regulatory subunits (as described in section 2.2.13). Input amounts of samples were calculated from their respective threshold cycle (Ct) values, using standard curve generated with each assay. Data were expressed relative to 18S or ALAS1 mRNA.

For PP4c over-expression; MCF7 and MDA-MB-231 breast cancer cells were transfected with pcDNA3.1 empty victor as a control or with pcDNA3.1 encoding the catalytic subunit of PP4 (PP4c). MCF7 cells were transfected using TransIT®-BrCa transfection reagent (as described in section 2.2.15.3) whereas Amaxa® Cell Line Nucleofector® Kit V was used for the transfection of MDA-MB-231 (as described in section 2.2.15.2). For PP4c down-regulation; MCF7 and MDA-MB-231 cells were transfected with (-)siRNA as a control or with PP4c-specific siRNAs or siRNAs to different PP4R2, SMEK1 and SMEK2 sequences using HiPerFect transfection reagent (as described in section 2.2.14.3). After confirmation of over-expression 24 hours after transfection or down-regulation 72 hours after transfection by mean of western blotting analysis (as described in section 2.2.16) cell viability and cell functions were assessed. Cell viability was assessed by Muse® Count & Viability

Assay kit (as described in section 2.2.3.2) and apoptosis was assessed by Muse® Annexin V and Dead Cell Assay kit (as described in section 2.2.4.1).

4.3 Results

4.3.1 PP4c intra-cellular level affects mRNA expression level of its regulatory subunits in breast cancer cells

In previous chapter we have demonstrated that PP4c is over-expressed in hormone positive and in triple negative breast cancer cell lines as well as in all stages of breast cancer in breast cancer tissue samples. This study may explain the wide range difference in the role of PP4c as an oncogene or anti-oncogene as that could be related to the changes in the expression of its corresponding regulatory subunits. In order to examine the effect of PP4c over-expression on the mRNA expression level of its regulatory subunits; MCF7 and MDA-MB-231 cells were transfected with pcDNA3.1 empty vector or pcDNA3.1-PP4c expression vector. Transfected cells were harvested 24 hours post-transfection and PP4c over-expression was confirmed by western blotting analysis. RNA was extracted from the transfected cells and real time PCR was performed on cDNA directly prepared from the extracted RNA. It was found that in both MCF7 and MDA-MB-231 cells mRNA expression of all PP4c regulatory subunits increased in cultures expressing high PP4c compared to the control, except PP4R4 which showed a reduced expression in cultures transfected with pcDNA3.1-PP4c compared to the control cultures that are transfected with pcDNA3.1 empty vector. For instance in MCF7, mRNA expression of PP4R1 and PP4R2 in cultures with PP4c over-expression was increased by 43.9% and 45.8%, respectively, in comparison with control cultures (Figure 4.1A). Similarly, PP4c over-expression increased the expression level of SMEK1 and SMEK2 by 46.8% and 234.6% respectively compared to the control samples (Figure 4.1A). However, in MCF7, PP4c over-expression decreased the expression level of PP4R4 mRNA by 98.7% (Figure 4.1A).

PP4c over-expression in MDA-MB-231 cells resulted in an increase in the expression level of PP4R1, PP4R2, SMEK1, and SMEK2 mRNA by 331.5%, 352.3%, 40%, and 401.2%, respectively, with a reduction in the expression level of PP4R4 mRNA by 97% compared to the control cultures (Figure 4.2A).

To examine the effect of PP4c down-regulation on the expression level of mRNA of PP4c regulatory subunits, MCF7 and MDA-MB-231 cells were transfected with PP4c-specific siRNAs or control (-)siRNA. The cells from both types of cultures were gathered and down-regulation was confirmed by western blotting analysis 72 hours post-transfection. RNA and cDNA were prepared from the transfected cells. Real time PCR was performed to check the level of mRNA of PP4c regulatory subunits. In MCF7 cells PP4c silencing decreased mRNA expression level of all PP4c regulatory subunits comparing to the control cells. The expression level of PP4R1, PP4R2 and PP4R4 mRNA was gone down by 42.7%, 80% and 78.2 respectively, compared to the control samples (Figure 4.1B). Likewise, the expression level of SMEK1 and SMEK2 mRNA was greatly reduced by 95.7% and 93.2%, respectively in cells with silenced PP4c compared to cells having wild-type expression level of PP4c (Figure 4.1B).

In MDA-MB-231 cells, PP4c down-regulation decreased mRNA expression of PP4R1, PP4R2, SMEK1 and SMEK2 by 98%, while the reduction was 96.6% in the case of PP4R4 (Figure 4.2B). These data indicated the great importance of endogenous level of PP4c on the expression level of its regulatory subunits mRNA in both hormone positive and triple negative breast cancer cells.

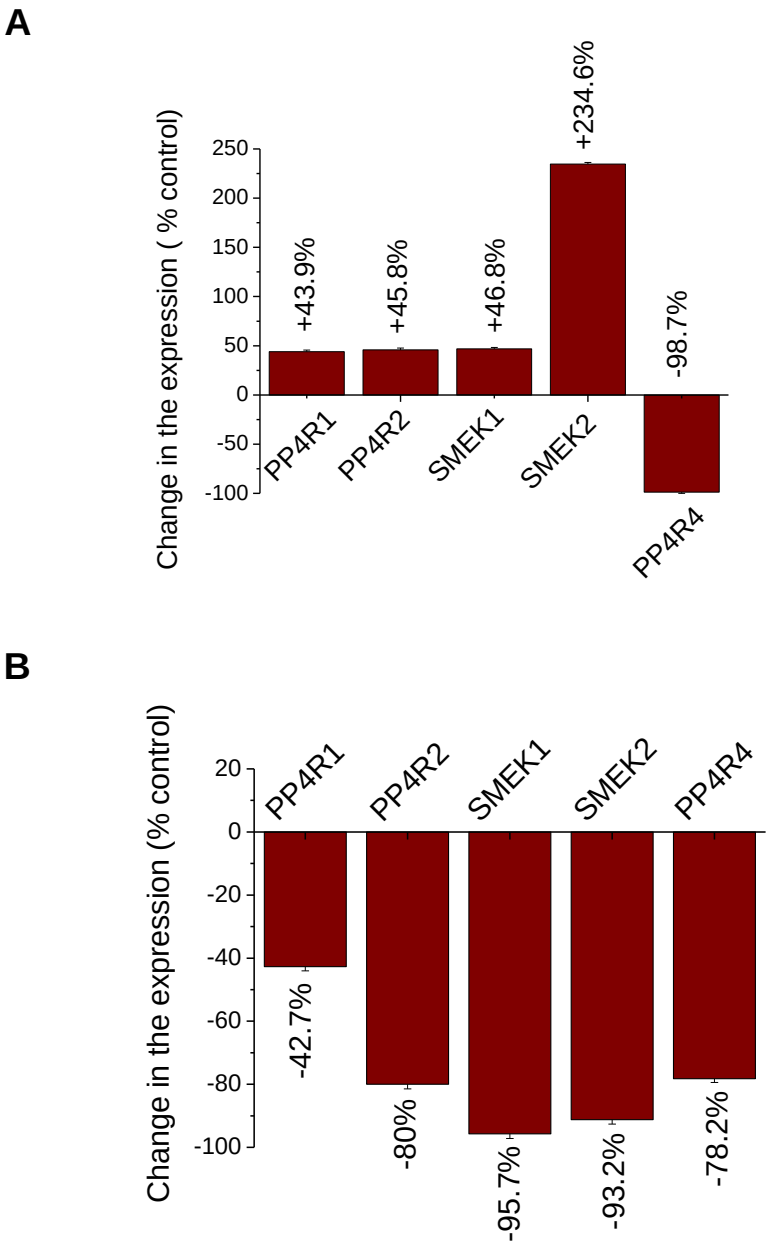


Figure 4.1: PP4c over-expression and down-regulation changes the expression level of PP4c regulatory subunits mRNA in MCF7 cells. Real-Time PCR (qPCR) was performed on the cDNA prepared from MCF7 cells transfected with pcDNA3.1 empty vector (control for A) or pcDNA3.1-PP4c expression vector in case of PP4c over-expression and on cells transfected with (-)siRNA (control for B) or PP4c-specific siRNAs in case of PP4c down-regulation **A)** Effect of PP4c over-expression on the expression level of PP4R1, PP4R2, SMEK1, SMEK2 and PP4R4 mRNA. **B)** Effect of PP4c down-regulation on the expression level of PP4R1, PP4R2, SMEK1, SMEK2 and PP4R4 mRNA. The bar graphs represent means ± S.E from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 (A) or (-)siRNA (B) (one-way ANOVA and Bonferrroni's MCT)

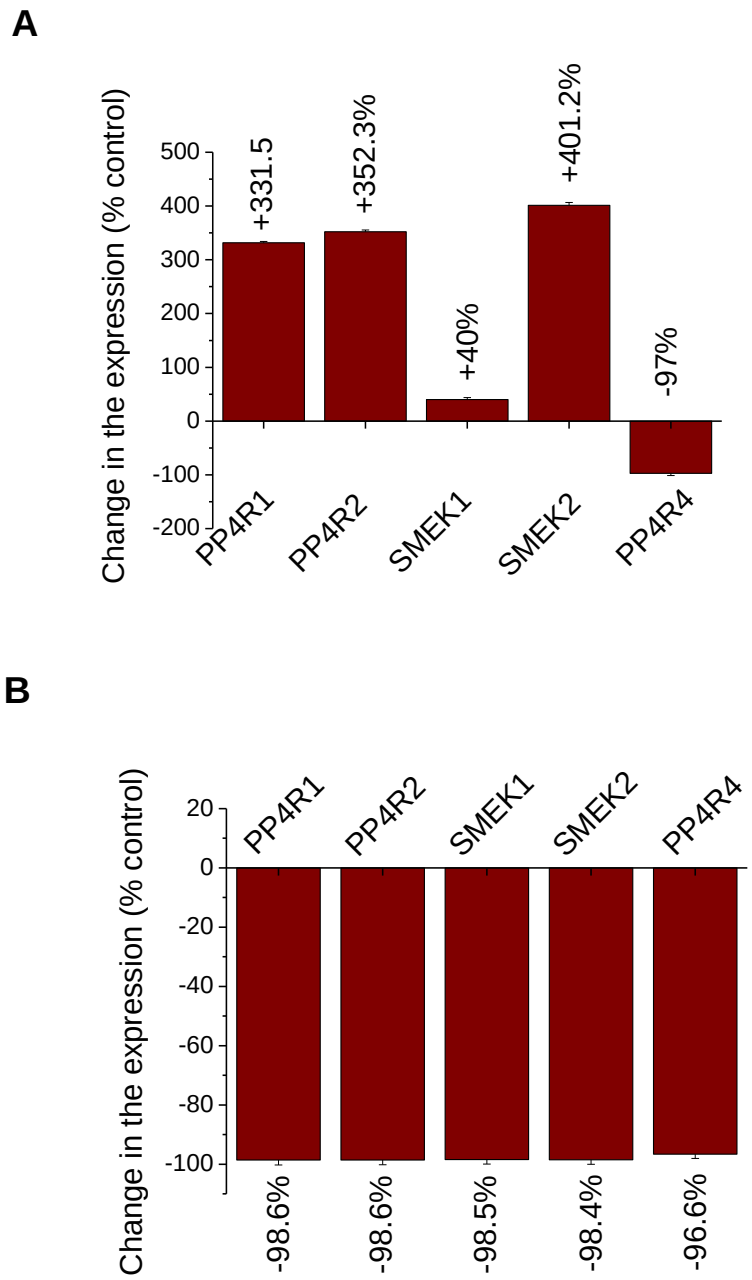


Figure 4.2: PP4c over-expression and down-regulation changes the expression level of PP4c regulatory subunits mRNA in MDA-MB-231 cells. Real-Time PCR (qPCR) was performed on the cDNA prepared from MDA-MB-231 cells transfected with pcDNA3.1 empty vector (control for A) or pcDNA3.1-PP4c expression vector in case of PP4c over-expression and on cells transfected with (-)siRNA (control for B) or PP4c-specific siRNAs in case of PP4c down-regulation. **A)** Effect of PP4c over-expression on the expression level of PP4R1, PP4R2, SMEK1, SMEK2 and PP4R4 mRNA. **B)** Effect of PP4c down-regulation on the expression level of PP4R1, PP4R2, SMEK1, SMEK2 and PP4R4 mRNA. The bar graphs represent means ± S.E from four independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 (A) or (-)siRNA (B) (one-way ANOVA and Bonferrroni's MCT)

4.3.2 Effect of breast cancer on PP4c regulatory subunits' mRNA expression

In Chapter 3 we have found that PP4c is over-expressed in all stages of breast cancer in breast cancer tissue samples compared to the samples from healthy individuals, so it was hypothesized that mRNA expression level of PP4c regulatory subunits differs in patients with breast cancer compared to the healthy individuals. To test this hypothesis TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II; ready-to-use panel of cDNA derived from tissues of breast cancer was analysed, the panel also contains control cDNA samples gathered from normal tissue of healthy individuals. qPCR assay was performed to quantify the expression level of mRNA of all PP4c regulatory subunits in normal samples and in breast cancer tissue samples present in the cDNA panel. For each assay, a standard curve of threshold cycle (Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to a housekeeping gene ALAS1 mRNA. The results showed that there was no significant difference in the expression level of PP4R1 mRNA in all stages of breast cancer collectively compared to that in normal tissue samples (Figure 4.3A, B). In addition there was no significant change in PP4R1 mRNA expression level in stage I, II and IV of breast cancer compared to the normal tissue samples, apart from tendency of reduced expression of PP4R1 mRNA in stage III (Figure 4.3C). In case of PP4R2 there was tendency of reduced mRNA expression level in breast cancer tissue samples compared to the normal tissue sample (Figure 4.4A, B). Also there was no significant reduction in PP4R2 mRNA expression in stage I, II, and III with no significant increase in stage IV (Figure 4.4C). In SMEK1 (Figure 4.5 A, B) and SMEK2 (Figure 4.6 A, B) there was no difference in mRNA expression level between

breast cancer tissue samples and normal tissue samples. Results showed fluctuation in SMEK1 (Figure 4.5C) and SMEK2 (Figure 4.6C) mRNA expression level in stage I, II, III and IV of breast cancer compared to that in normal tissue samples. In case of PP4R4 there was tendency of increased expression level of PP4R4 mRNA in breast cancer tissue samples in all stages of breast cancer collectively compared to the normal tissue samples (Figure 4.7 A, B). Furthermore, the results showed fluctuation in PP4R4 mRNA expression in stage I, II, III and IV of breast cancer compared to that in normal tissue samples (Figure 4.7 C). Failure to get a statistical significance in in PP4c regulatory subunits mRNA expression between breast cancer tissue samples and normal tissue samples could be due to the wide range in the readings of mRNA expression level in the breast cancer tissue samples in each stage of breast cancer added to the minimum number of the control samples, which is only five compared to 43 breast cancer samples.

A heat map showing the characteristics of each sample in TissueScan™ Cancer and Normal Tissue cDNA Arrays regarding the tumour stage, the hormone receptors expression status including oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), the expression of PP4c, PP4c regulatory subunits; PP4R1, PP4R2, SMEK1, SMEK2, PP4R4 as well as PEA15 (% the mean of the control samples of each gene) was generated (Figure 4.8). The heat map showed that PP4c was highly expressed in almost all breast cancer tissue samples regardless of the disease stage and hormone receptors expression pattern while PP4R1, PP4R2, SMEK1, SMEK2, PP4R4 and PEA15 showed low expression in more than 2/3 of the breast cancer tissue samples regardless of the tumour stage and hormone receptor expression status. However, in few breast cancer tissue samples PP4c, PP4c regulatory subunits and PEA15 was found to have normal

expression. This was only in one case for PP4c, PEA15 and PP4R1, in four cases for PP4R2, and in two cases for SMEK1. This indicates the variation in the expression of PP4c, PP4c regulatory subunits and PEA15 in breast cancer which may have substantial implications for the behaviour and prognosis of breast cancer in different patients.

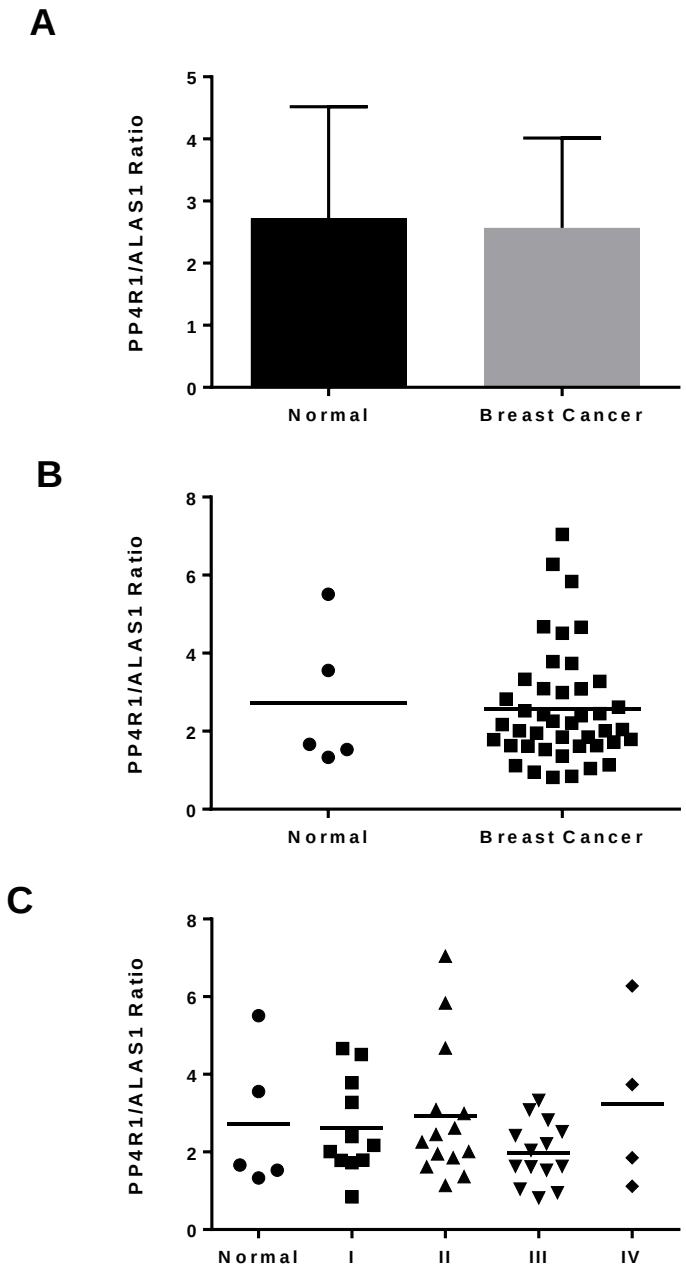


Figure 4.3: PP4R1 mRNA expression level in breast cancer tissue samples. qPCR assay was performed on cDNA from TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. **A, B)** PP4R1 mRNA expression level in all stages of breast cancer collectively compared to the normal tissue samples. **C)** The expression level of PP4R1 mRNA in every stage of breast cancer compared to the normal tissue samples. The bar graphs represent means ± S.E. from four independent experiments. Means were not statistically significantly different, $P > 0.05$ (one-way ANOVA and Bonferrroni's MCT)

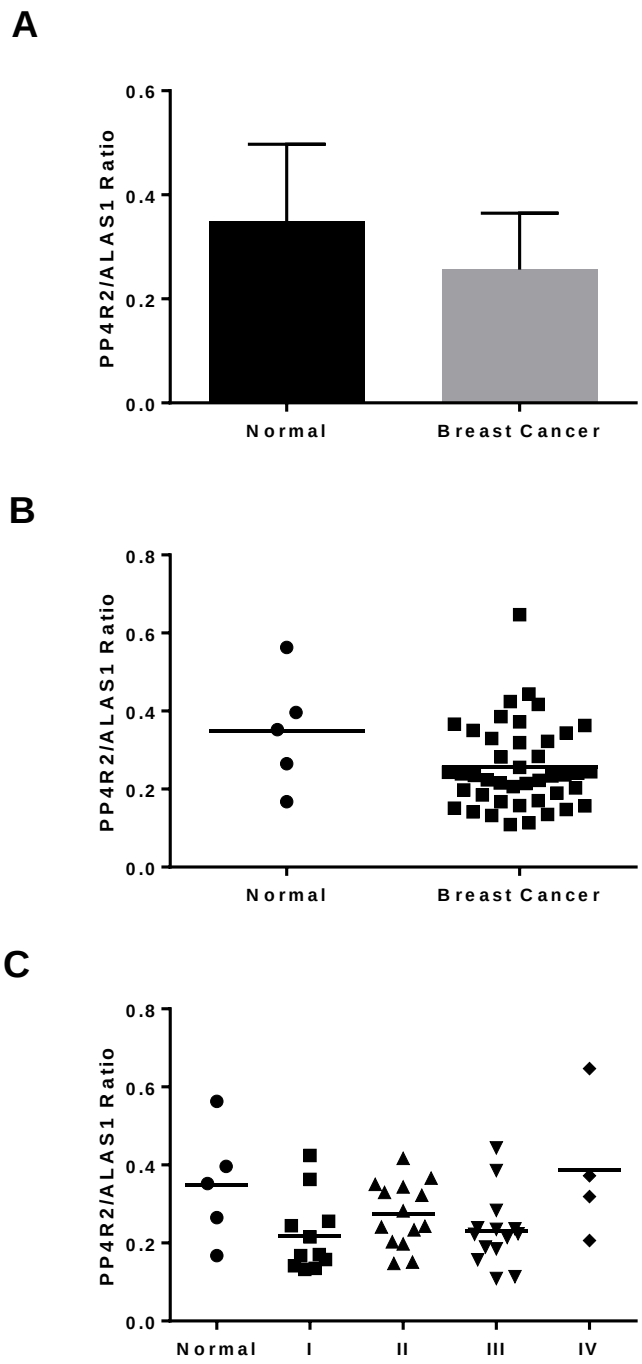


Figure 4.4: PP4R2 mRNA expression level in breast cancer tissue samples. qPCR assay was performed on cDNA from TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. **A, B)** PP4R2 mRNA expression level in all stages of breast cancer collectively compared to the normal tissue samples. **C)** The expression level of PP4R2 mRNA in every stage of breast cancer compared to the normal tissue samples. The bar graphs represent means ± S.E. from four independent experiments. Means were not statistically significantly different, $P > 0.05$ (one-way ANOVA and Bonferrroni's MCT)

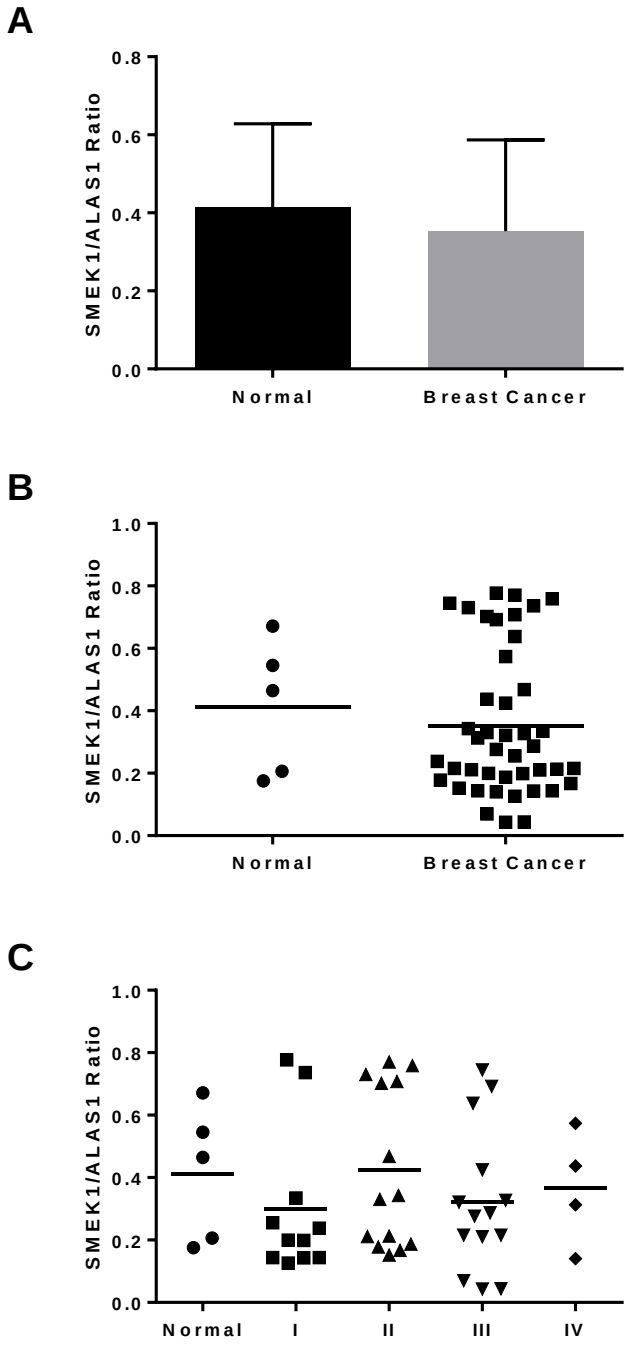


Figure 4.5: SMEK1 mRNA expression level in breast cancer tissue samples. qPCR assay was performed on cDNA from TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. **A, B)** SMEK1 mRNA expression level in all stages of breast cancer collectively compared to the normal tissue samples. **C)** The expression level of SMEK1 mRNA in every stage of breast cancer compared to the normal tissue samples. The bar graphs represent means ± S.E. from four independent experiments. Means were not statistically significantly different, $P > 0.05$ (one-way ANOVA and Bonferrroni's MCT)

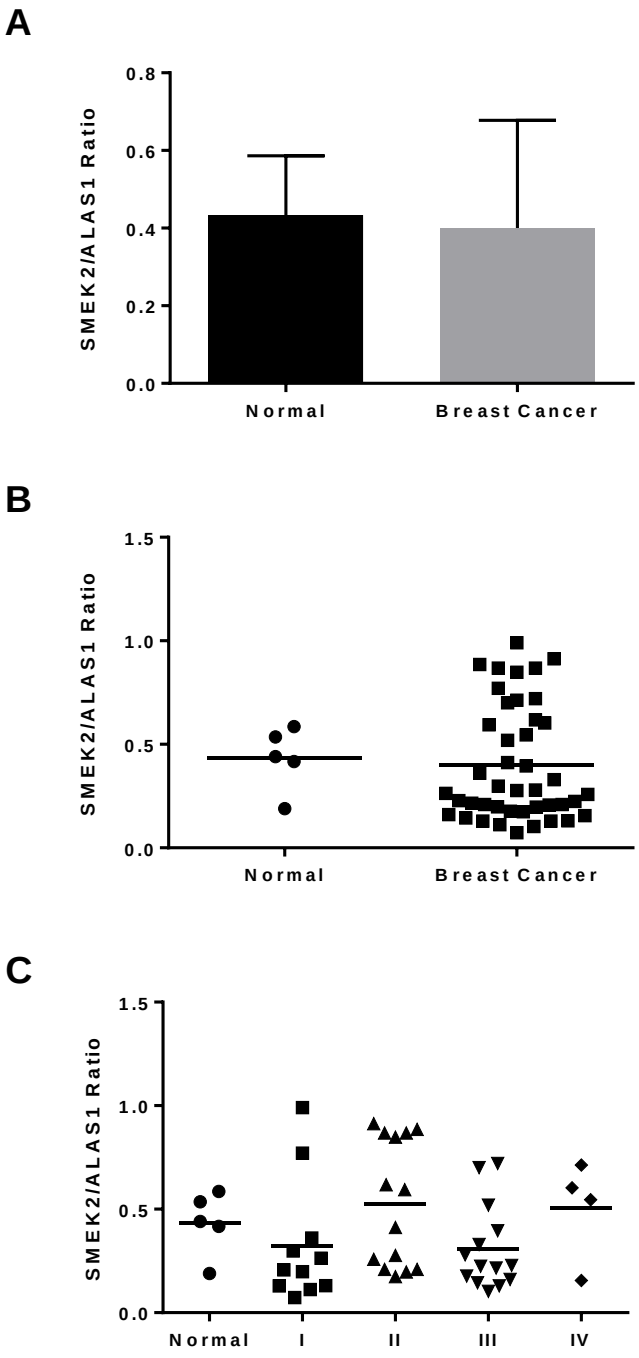


Figure 4.6: SMEK2 mRNA expression level in breast cancer tissue samples. qPCR assay was performed on cDNA from TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. **A, B)** SMEK2 mRNA expression level in all stages of breast cancer collectively compared to the normal tissue samples. **C)** The expression level of SMEK2 mRNA in every stage of breast cancer compared to the normal tissue samples. The bar graphs represent means ± S.E. from four independent experiments. Means were not statistically significantly different, $P > 0.05$ (one-way ANOVA and Bonferrroni's MCT)

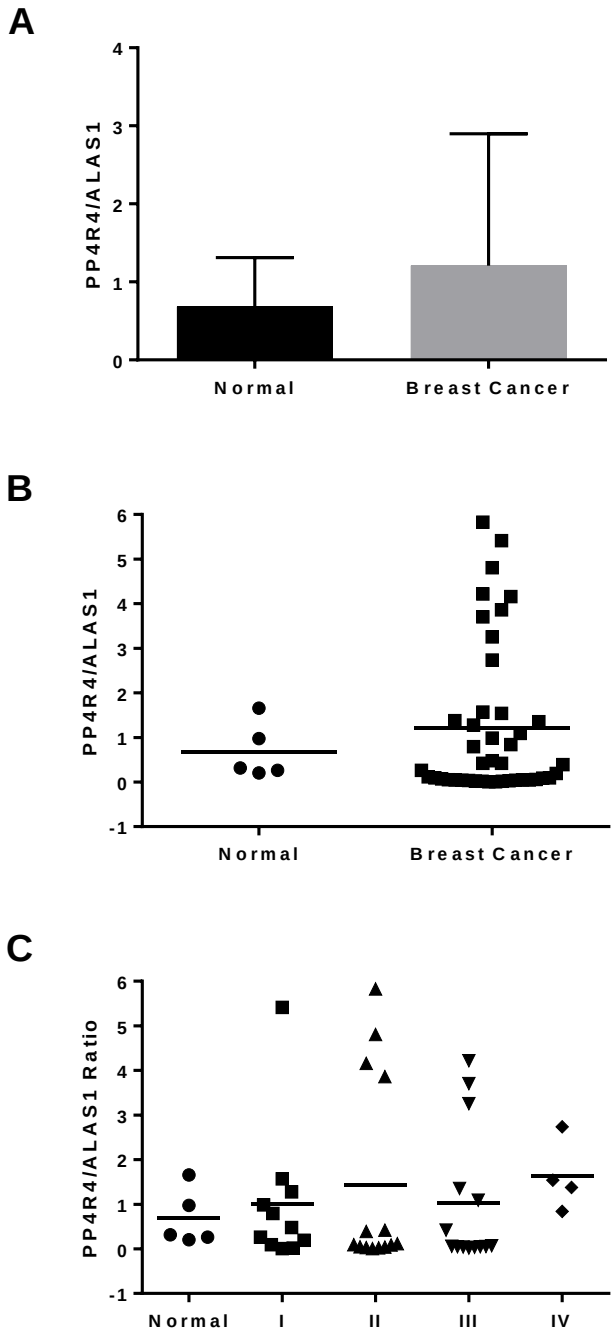


Figure 4.7: PP4R4 mRNA expression level in breast cancer tissue samples. qPCR assay was performed on cDNA from TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. **A, B)** PP4R4 mRNA expression level in all stages of breast cancer collectively compared to the normal tissue samples. **C)** The expression level of PP4R4 mRNA in every stage of breast cancer compared to the normal tissue samples. The bar graphs represent means ± S.E. from four independent experiments. Means were not statistically significantly different, $P > 0.05$ (one-way ANOVA and Bonferrroni's MCT)

High expression (> mean % control + SEM)
Normal expression (within the range of mean % control +/- SEM)
Low expression (< mean % control -SEM)

Sample No.	Minimum Stage Group	Oestrogen receptor (ER) by IHC from pathology report	Progesterone receptor (PR) by IHC from pathology report	HER2 (ERBB2) by IHC from pathology report	PP4c	PP4R1	PP4R2	SMEK1	SMEK2	PP4R4	PEA15
1	I	Not applicable	Not applicable	Not applicable	219.03	140.18	81.36	48.37	65.95	202.13	127.57
2	I	Not applicable	Not applicable	Not applicable	618.90	172.72	56.86	57.97	50.49	43.51	68.18
3	I	Positive	Positive	Negative	140.81	167.13	120.95	189.45	179.2	161.91	71.33
4	I	Negative	Negative	Negative	200.22	31.34	55.92	62.30	61.24	41.10	160.16
5	I	Negative	Negative	Not reported	185.48	66.04	52.59	71.51	169.31	52.55	66.22
6	I	Positive	Positive	Negative	422.43	136.34	141.37	179.54	80.45	45.42	51.99
7	I	Positive	Positive, weak	Positive, strong	300.70	68.83	85.15	35.03	83.77	53.18	60.36
8	I	Positive	Positive, strong	Negative	87.01	63.85	44.1	34.98	56.95	59.39	47.90
9	I	Positive	Positive, strong	Negative	140.52	68.38	71.98	30.80	70.12	261.58	77.40
10	I	Positive	Positive, weak	Negative	464.22	66.20	47.29	34.88	45.95	212.85	65.80

11	I	Positive	Positive, weak	Negative	301.62	54.39	94.91	48.73	117.99	164.34	42.02
12	IIA	Positive	Positive	Negative	407.24	146.28	152.02	187.93	201.86	344.05	138.34
13	IIA	Negative	Negative	Positive, weak(2+)	342.33	56.96	87.54	134.09	70.86	55.1	167.62
14	IIA	Negative	Positive	Negative	243.65	260.76	84.18	73.73	118.76	60.45	170.11
15	IIA	Positive	Equivocal	Negative	310.01	134.52	79.82	51.93	73.76	271.57	81.34
16	IIA	Negative	Negative	Positive, strong	465.59	59.92	67.56	45.55	40.42	44.08	70.21
17	IIA	Positive	Negative	Positive	329.51	50.53	80.98	172.74	125.65	59.92	175.75
18	IIA	Positive	Positive	Negative	294.39	70.712	49.12	43.48	77.18	61.27	60.47
19	IIA	Negative	Negative	Positive, strong	213.66	130.65	148.91	171.20	125.76	201.01	117.28
20	IIB	Positive	Positive	Negative	323.30	68.54	77.98	70.66	62.26	64.81	66.65

21	IIB	Negative	Negative	Positive, weak(2+)	230.49	63.68	80.40	37.07	48.63	57.85	150.81
22	IIB	Negative	Negative	Negative	181.8	42.07	65.79	51.55	60.08	47.06	66.89
23	IIB	Positive	Positive, weak	Negative	239.93	173.20	116.76	185.15	124.33	64.21	154.25
24	IIB	Equivocal Not reported	Equivocal	Positive	172.59	62.19	114.60	178.15	206.1	55.93	55.75
25	IIB	Not reported	Not reported	Not reported	242.20	54.44	50.24	40.70	68.29	293.09	57.68
26	IIIA	Negative	Negative	Positive, weak(2+)	490.38	60.40	63.14	69.98	64.95	57.74	70.78
27	IIIA	Positive	Positive	Negative	181.2	59.70	74.52	103.65	132.27	242.55	56.85
28	IIIA	Positive	Positive	Negative	205.29	144.10	148.58	181.59	80.74	217.85	61.23
29	IIIA	Positive	Positive	Negative	148.92	63.35	78.88	78.40	76.64	302.95	45.75
30	IIIA	Positive	Positive	Negative	454.81	60.29	37.89	40.63	120.26	59.21	59.36
31	IIIA	Positive	Positive	Negative	140.09	30.36	71.45	155.62	62.88	50.26	94.93

32	IIIA	Positive, focal	Positive, focal	Negative	348.04	63.25	84.61	67.28	63.47	47.46	77.38
33	IIIA	Positive	Positive	Negative	209.25	104.66	78.23	51.28	53.13	182.24	56.79
34	IIIB	Not reported	Not reported	Negative	178.64	65.65	36.27	56.9	60.01	65.62	45.70
35	IIIB	Not reported	Not reported	Not reported	86.15	56.56	79.71	60.37	124.02	56.93	64.13
36	IIIC	Positive	Positive	Negative	233.88	38.60	147.97	168.89	67.54	225.17	164.51
37	IIIC	Positive	Positive	Borderline	98.738	61.83	74.04	69.85	71.94	63.39	132.12
38	IIIC	Not reported	Not reported	Not reported	173.73	59.85	52.27	52.52	77.38	49.97	154.12
39	IIIC	Positive	Positive	Negative	213.90	35.14	61.73	52.58	141.14	51.61	73.21
40	IV	Not reported	Not reported	Not reported	242.02	68.60	144.01	76.35	140.40	160.39	62.08
41	IV	Positive	Positive	Negative	327.4	138.37	76.43	106.58	126.97	130	140
42	IV	Negative	Positive	Not reported	460	232.44	215.77	140	65.95	256.16	123.5

43	IV	Positive	Positive	Positive, weak(2+)	268.66	41.42	68.86	34.14	76.23	257.2	44.70

Figure 4.8: Heat map shows tumour stage, hormone receptor expression status and mRNA expression level of PP4c, PP4c regulatory subunits and PEA15 of each sample in the TissueScan™ Cancer and Normal Tissue cDNA Arrays (Breast Cancer cDNA Array II). High and low expression are defined as greater or lower respectively than the mean of the % control of the specific gene +/-SEM

4.3.3 Positive correlation between mRNA expression level of PP4c and some of its regulatory subunits in breast cancer

Previous results showed that there is fluctuation in mRNA expression of PP4c regulatory subunits in breast cancer tissue samples compared to the normal samples. It is therefore interesting to find which of the regulatory subunits play a key role in regulating the function of PP4c as a regulator of cell growth and apoptosis in breast cancer. In order to investigate this, the correlation between mRNA expression level of PP4c and that of PP4c-regulatory subunits was tested. qPCR assay was performed on cDNA applied in TissueScan™ Cancer and Normal Tissue cDNA Arrays. For each assay, a standard curve of threshold cycle (Ct) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective Ct values. Data were expressed relative to a housekeeping gene ALAS1 mRNA. Results showed that there was significant modest positive correlation between mRNA expression level of PP4c and PP4R2; P = 0.0007, R = 0.489 (Figure 4.9A), PP4c and SMEK1; P = 0.0001, R = 0.553 (Figure 4.9B) and also between PP4c and SMEK2; P = 0.0085, R = 0.426 (Figure 4.9C) as indicated by performing Pearson's correlation coefficient. The coefficient value is considered to be significant if it ranges between -1.00 and 1.00. The closer the value of R to +1 is the stronger the positive linear correlation. Modest positive correlation is considered when R value is 0.40 to 0.69 (Fowler and Cohen, 1990). There was no correlation between mRNA expression level of PP4c and PP4R1; P = 0.1061, R = 0.241 (Figure 4.10A) or between PP4c and PP4R4; P = 0.1254, R = 0.231 (Figure 4.10B). This suggested that PP4R2, SMEK1 and SMEK2 may play a critical role in the regulation of PP4c function in breast cancer.

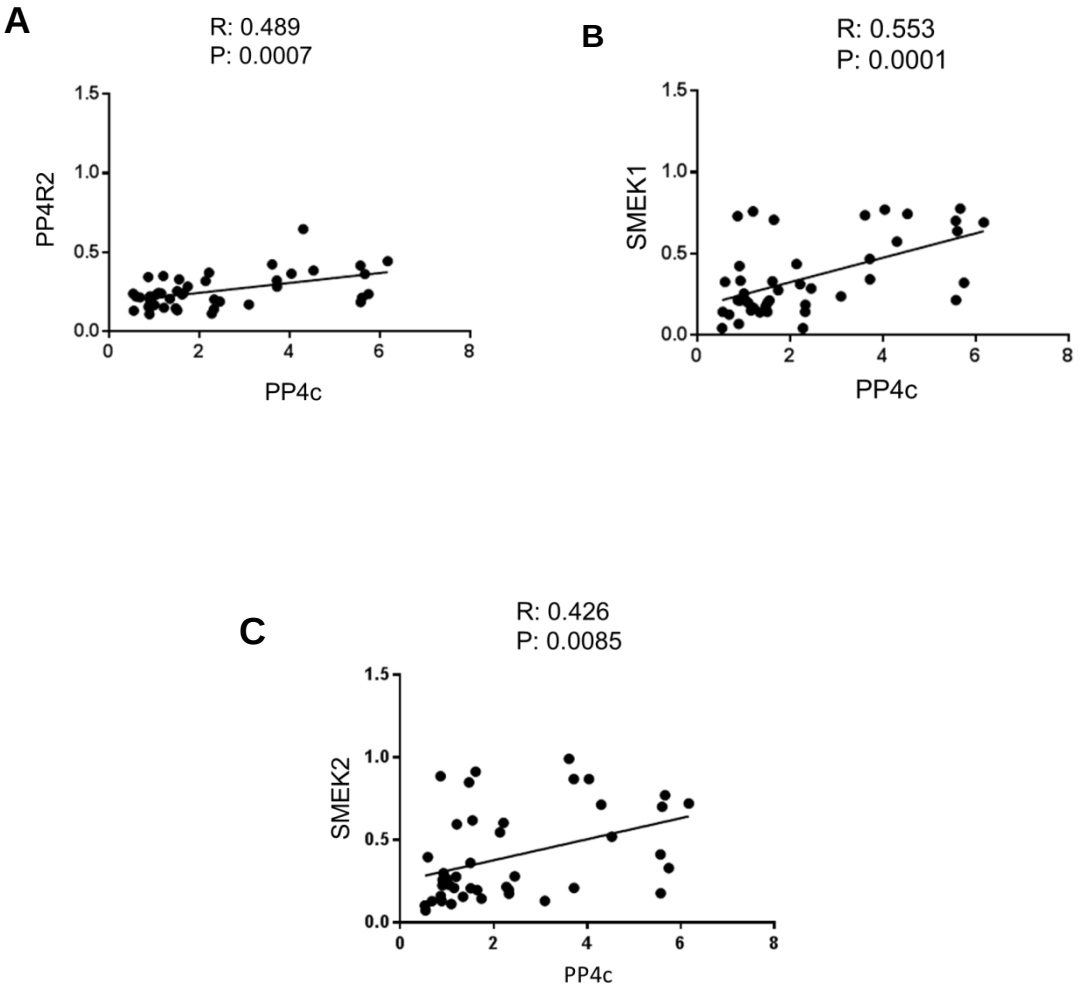


Figure 4.9: Positive correlation between mRNA expression level of PP4c and some of its regulatory subunits in breast cancer tissue samples. qPCR assay was performed on TissueScan™ Cancer and Normal Tissue cDNA Arrays to assess mRNA expression level of PP4c and its regulatory subunits. **A)** Correlation between PP4c and PP4R2. **B)** Correlation between PP4c and SMEK1. **C)** Correlation between PP4c and SMEK2. (n=4). *P < 0.05. P value and the R value show that there is a significant modest correlation as indicated by performing Pearson's correlation coefficient using GraphPad Prism 7. The closer the value of R to +1, the stronger the positive linear relationship

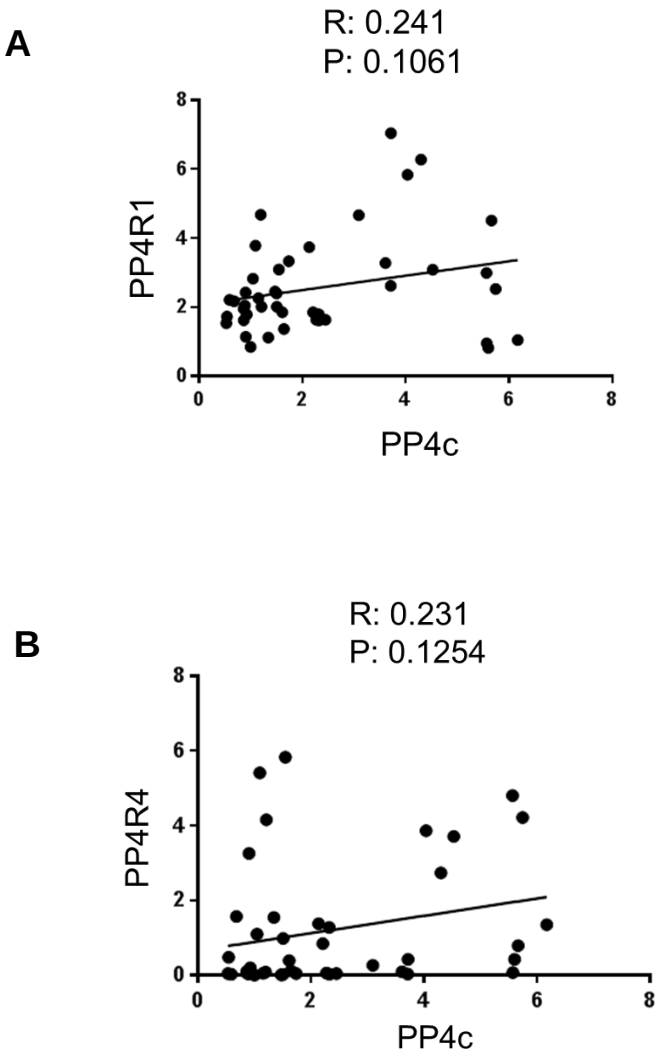


Figure 4.10: Correlation between mRNA expression of PP4c and PP4R1 and PP4R4. qPCR assay was performed on TissueScan™ Cancer and Normal Tissue cDNA Arrays to assess the expression level of PP4c and its regulatory subunits mRNA. **A)** Correlation between PP4c and PP4R1. **B)** Correlation between PP4c and PP4R4. (n=4). *P > 0.05. P value and the R value show that there is no statistically significant correlation as indicated by performing Pearson's correlation coefficient using GraphPad Prism 7

4.3.4 PP4c function in breast cancer is substantially regulated by some of its regulatory subunits

According to the above results, there is a modest positive correlation in mRNA expression between PP4c and PP4R2, PP4c and PP4R3 (SMEK1 and SMEK2). Therefore, PP4R2, SMEK1 and SMEK2 were selected to verify the effects of their down-regulation on the function of PP4c in hormone positive cells-MCF7 and in triple negative cells-MDA-MB-231.

4.3.4.1 PP4R2 silencing attenuates cell survival and increases apoptosis in MCF7 cells and has no effect on MDA-MB-231 cells

The effect of PP4R2 down-regulation on the survival of MCF7 and MDAMB-231 cells has been investigated. Two specific PP4R2 siRNAs, PP4R2s5 and PP4R2s7, were used to reduce endogenous PP4R2 expression in MCF7 and in MDA-MB-231 cells. Cells were transfected with (-)siRNA as a control or with PP4R2-specific siRNAs. Cells were harvested and the efficiency of PP4R2 knockdown was determined by immunoblotting 72 hours post-transfection. Cells were re-plated and the influence of PP4R2 silencing on the short term cell survival and apoptosis was examined under basal conditions after a further 48 hours by FACS analysis using Muse® Count & Viability Assay kit and by Muse® Annexin V and Dead Cell Assay kit respectively. In MCF7 PP4R2s5 and PP4R2s7 siRNAs reduced PP4R2 protein levels by 58% and 67% respectively (Figure 4.11). PP4R2 knockdown caused a significant reduction in viable cell number compared to the control (Figure 4.12A) also the percentage of total apoptosis was significantly increased in cells transfected with PP4R2-specific siRNAs compared to cultures transfected with (-)siRNA (Figure 4.12B). The data

indicated that PP4R2 knock-down decreased viable cell number and increased basal apoptosis in MCF7 breast cancer cells. In MDA-MB-231 cells PP4R2s5 and PP4R2s7 siRNAs reduced the expression level of PP4R2 proteins by 70% and 75% respectively (Figure 4.13). In MDA-MB-231 cells, PP4R2 down-regulation had no effect on cell survival and apoptosis under basal conditions (Figure 4.14A, B).

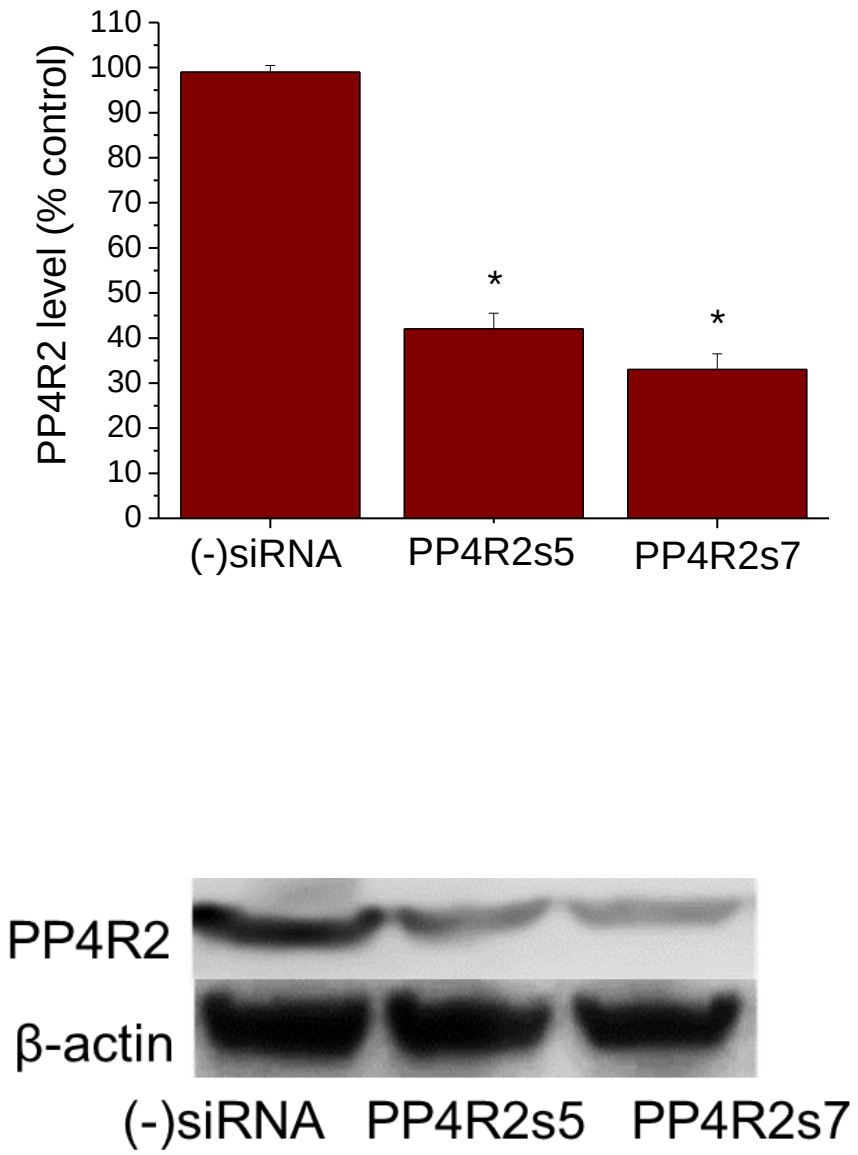


Figure 4.11: Expression level of PP4R2 protein in MCF7 with PP4R2 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4R2 protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

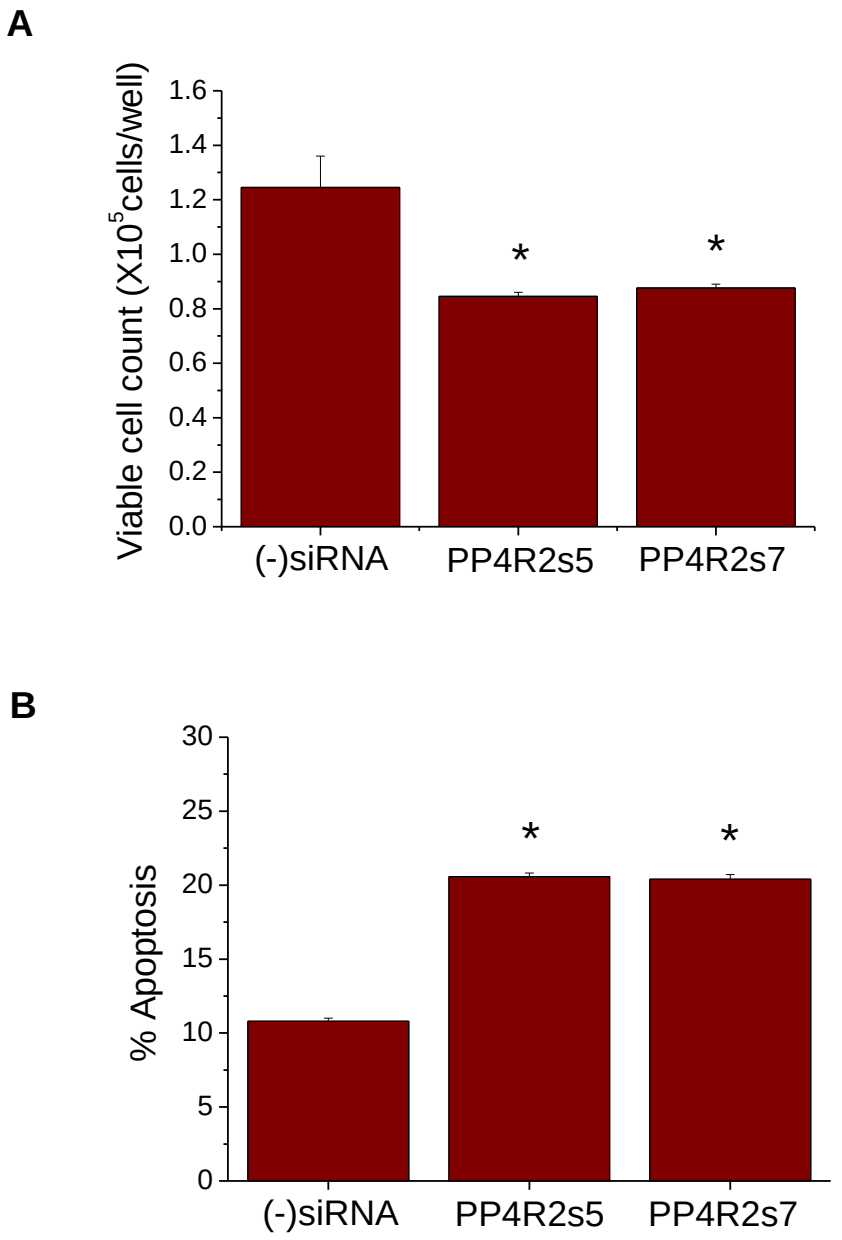


Figure 4.12: PP4R2 down-regulation reduces viable cell count and increases basal apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or PP4R2-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis under basal condition. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

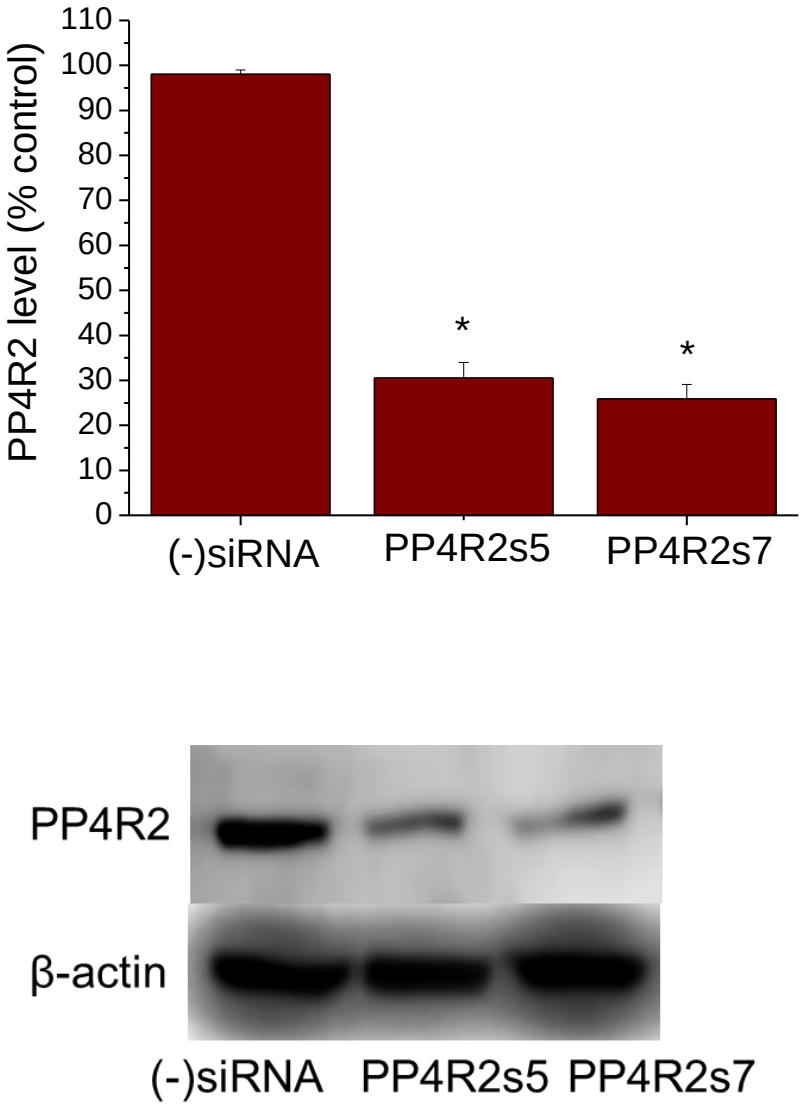


Figure 4.13: Expression level of PP4R2 protein in MDA-MB-231 cells with PP4R2 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. PP4R2 protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

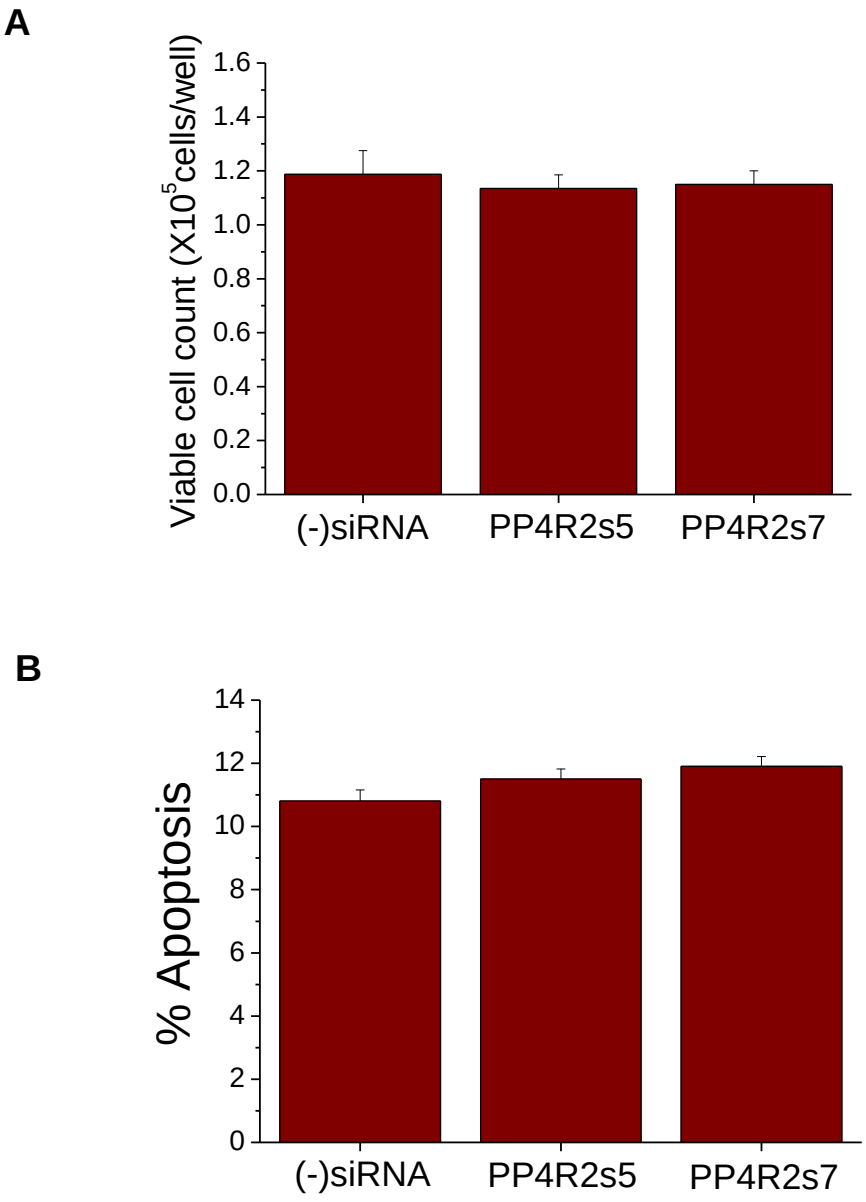


Figure 4.14: PP4R2 down-regulation has no effect on cell viability and on basal apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with PP4R2-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P > 0.05 versus cells transfected with (-)siRNA control (one-way ANOVA and Bonferrroni's MCT)

4.3.4.2 PP4R2 regulates the function of PP4c in MCF7 and in MDA-MB-231 breast cancer cells

In order to examine the significance of PP4R2 in mediating the effect of PP4c in controlling breast cancer cell survival and apoptosis, MCF7 and MDA-MB-231 cells were transfected with (-)siRNA or PP4R2-specific siRNAs, 72 hours post-transfection; PP4R2 down-regulation was confirmed by western blotting analysis, the control cells that are transfected with (-)siRNA as well as cultures transfected with PP4R2-specific siRNAs were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. In MCF7 cells PP4c over-expression reduced viable cell count of the cells transfected with (-)siRNA by 50% compared to the control (cells transfected with pcDNA3.1) this confirmed the results obtained earlier in Chapter 3 (Figure 4.15A). PP4R2 down-regulation reduced viable cell number by about 33.4% compared to the control culture transfected with (-)siRNA and pcDNA3.1. On the other hand, PP4c over-expression in cultures transfected with PP4R2-targeted siRNA-PP4R2s5 and PP4R2s7 resulted in 66.7% reduction in viable cell count compared to the cells transfected with pcDNA3.1 and the reduction was about 50% compared to the cells expressing PP4c over-expression alone without PPR2 down-regulation (Figure 4.15A). This indicates that combinational effect of PP4c over-expression and PP4R2 down-regulation in the term of decreasing cell viability was greater than that of PP4c over-expression or PP4R2 down-regulation on its own.

Similarly, in MCF7 cells, PP4c over-expression in (-)siRNA transfected cultures increased the percentage of basal apoptosis by 50% compared to the pcDNA3.1 transfected cultures, confirming the results obtained in Chapter 3 (Figure 4.15B).

However, further 10% increase in the percentage of basal apoptosis was observed in the cells having combined PP4c over-expression and PP4R2 down-regulation compared to the cells having PP4c over-expression state alone or PP4R2 down-regulation state alone (Figure 4.15B). This supports the results obtained earlier, and is indicative of the fact that combination of PP4c over-expression and PP4R2 down-regulation has greater impact on the cell survival compared to the effect of PP4c over-expression or PP4R2 down-regulation on its own.

In MDA-MB-231 cells PP4c over-expression in cultures transfected with (-)siRNA reduced viable cell count by 50% compared to the control (cells transfected with pcDNA3.1) this confirmed the results obtained earlier in Chapter 3 (Figure 4.16A). Whereas, PP4c over-expression in the cells transfected with PP4R2-targeted siRNAs (PP4R2s5 and PP4R2s7) resulted in 75% reduction in viable cell count compared to the control (cells transfected with (-)siRNA and pcDNA3.1) and there is 25% further reduction in viable cell count compared to the cells having PP4c over-expression alone without PPR2 down-regulation (Figure 4.16A). This indicates that combinational effect of PP4c over-expression and PP4R2 down-regulation in the term of decreasing cell viability was greater than that of PP4c over-expression on its own in MDA-MB-231.

Similarly, the data showed that in (-)siRNA transfected cultures PP4c over-expression caused an increase in the level of apoptosis by about 50% compared to the (-)siRNA and pcDNA3.1 transfected cultures, However, PP4c over-expression caused 8% more increase in basal apoptosis in the cells having PP4R2s5 and PP4R2s7 mediated down-regulation compared to the cells having PP4c over-expression alone (Figure 4.16B). Data showed that combined PP4c over-expression and PP4R2 down-regulation in MDA-MB-231 cells resulted in more induction of

apoptosis than that obtained by PP4c over-expression alone. Above data support the importance of PP4R2 down-regulation in promoting PP4c over-expression effects on hormone receptor-positive and on triple negative breast cancer cells.

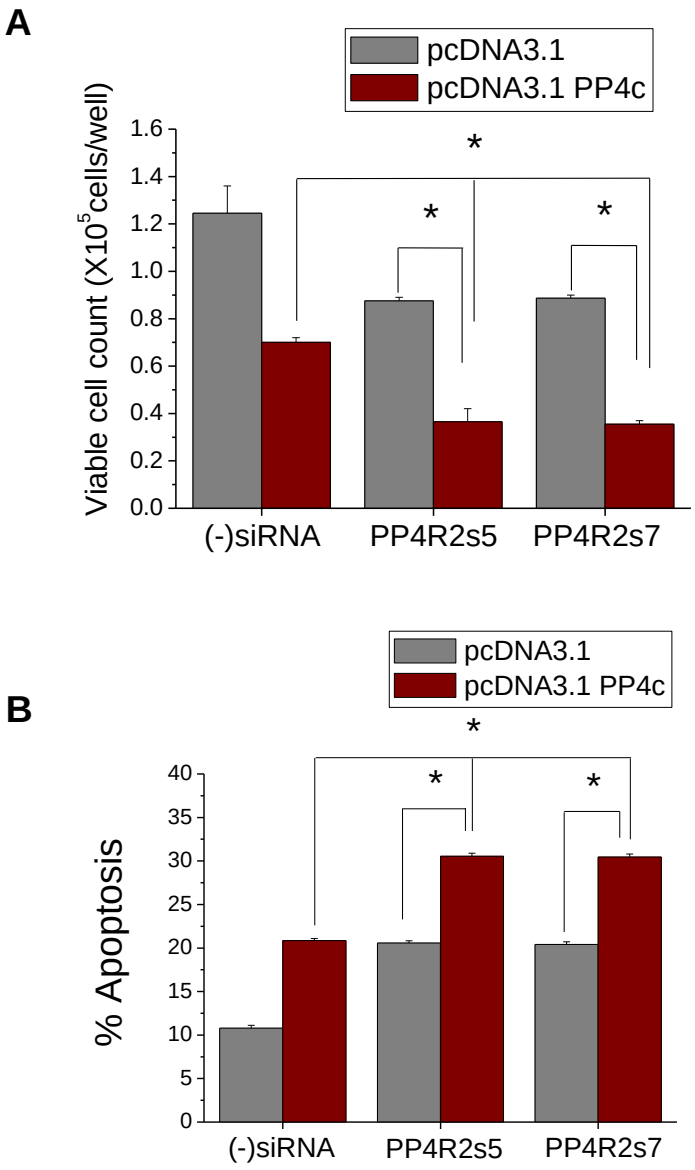


Figure 4.15: PP4R2 down-regulation increases PP4c over-expression mediated reduction in viable cell count and promotion of basal apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or with PP4R2-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1 empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared with (-)siRNA or pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

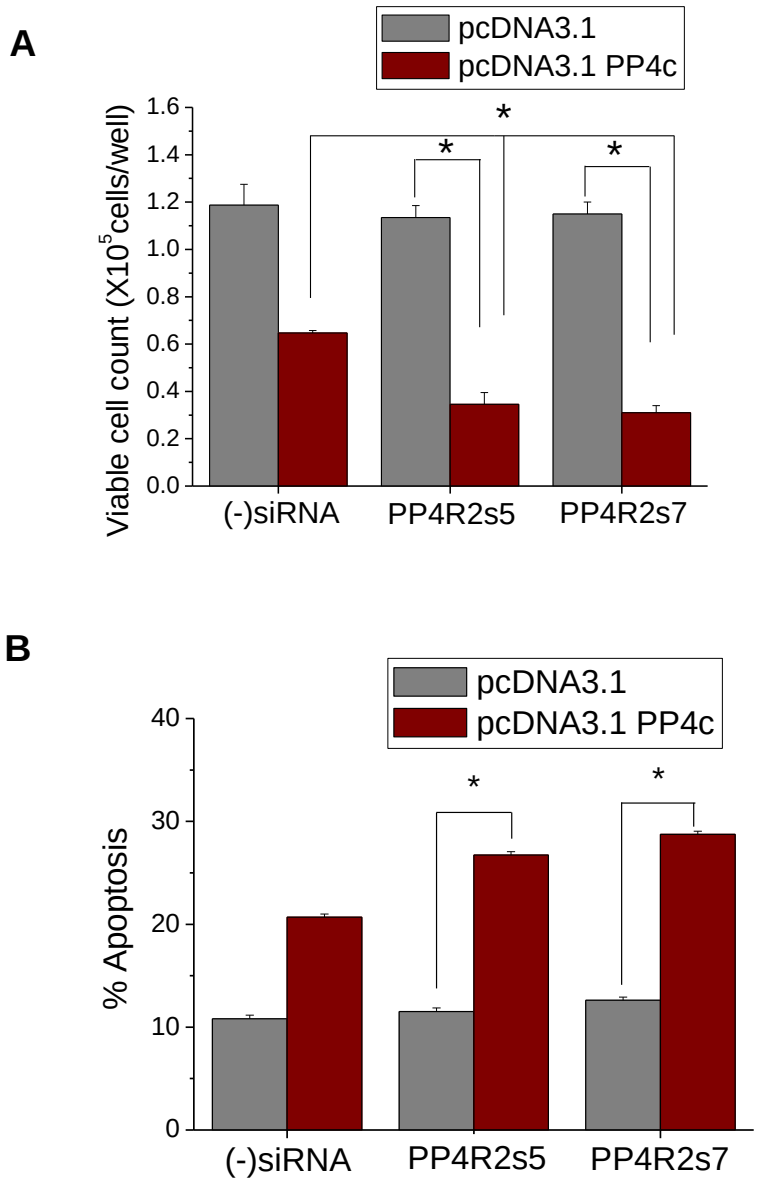


Figure 4.16: PP4R2 down-regulation increases PP4c over-expression-mediated reduction in viable cell count and promotion of basal apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with PP4R2-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared with (-)siRNA or pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

4.3.4.3 SMEK1 and SMEK2 down-regulation has no effect on viable cell count and apoptosis of MCF7 and MDA-MB-231 breast cancer cells

To investigate the effect of SMEK1 and SMEK2 down-regulation on MCF7 and on MDA-MB-231 cells, two SMEK1-targeted siRNAs (SMEK1s2 and SMEK1s5) and SMEK2-targeted siRNAs (SMEK2s1 and SMEK2s5) were used to knockdown endogenous SMEK1 and SMEK2 protein level. Cells were transfected with (-)siRNA as a control or with SMEK1 or SMEK2-specific siRNAs. Cells were harvested and down-regulation of these two PP4c regulatory subunits was confirmed by immunoblotting 72 hours post-transfection. The cells were re-plated for assessment of cell survival after a further 48 hours. In MCF7 cells, SMEK1s2 and SMEK1s5 reduced the expression level of SMEK1 protein by 67% and 75% respectively (Figure 4.17). SMEK2s1 and SMEK2s5 targeted siRNAs reduced the expression of SMEK2 protein by 78% and 88% respectively (Figure 4.19). There was no significant changes in viable cell number or in the percentage of total apoptosis in cultures transfected with SMEK1 (Figure 4.18A, B) or SMEK2 (Figure 20A, B) specific siRNAs compared to cultures transfected with (-)siRNA as assessed by FACS analysis.

In MDA-MB-231 cells SMEK1s2 reduced the endogenous level of SMEK1 protein by 80% and the reduction was 85% for SMEK1s5 (Figure 4.21). For SMEK2 the reduction was also 80% and 85 % for SMEK2s1 and SMEK2s5 respectively (Figure 4.23). The influence of silencing of the above regulatory subunits on short term cell survival and apoptosis was examined by FACS analysis under basal conditions. There was no significant change in viable cell count or in the % of basal apoptosis in the cultures transfected with SMEK1 (Figure 22A, B) or SMEK2 (Figure 24A, B) specific siRNAs compared to the cultures transfected with (-)siRNA. Above data

indicated that SMEK1 or SMEK2 down-regulation has no effect on viable cell number and on the percentage of basal apoptosis in both MCF7 and in MDA-MB-231 cells.

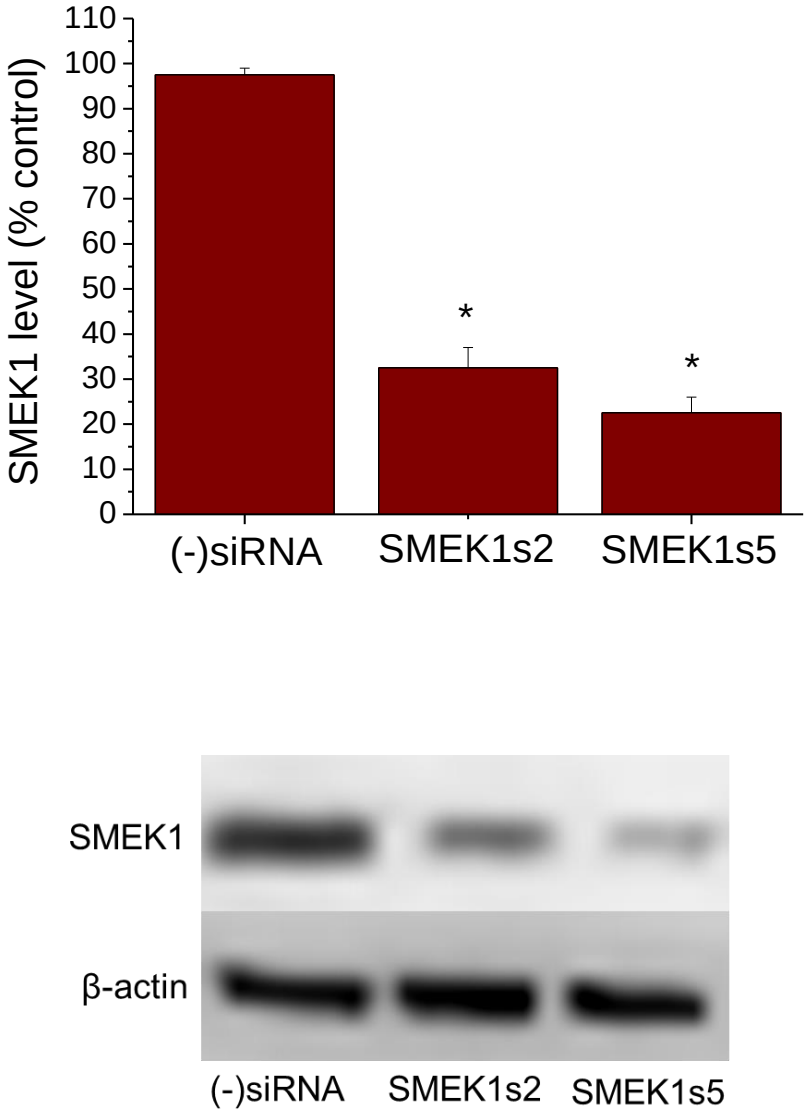


Figure 4.17: Expression level of SMEK1 protein in MCF7 cells with SMEK1 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. SMEK1 protein expression levels were normalised with that of β-actin *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

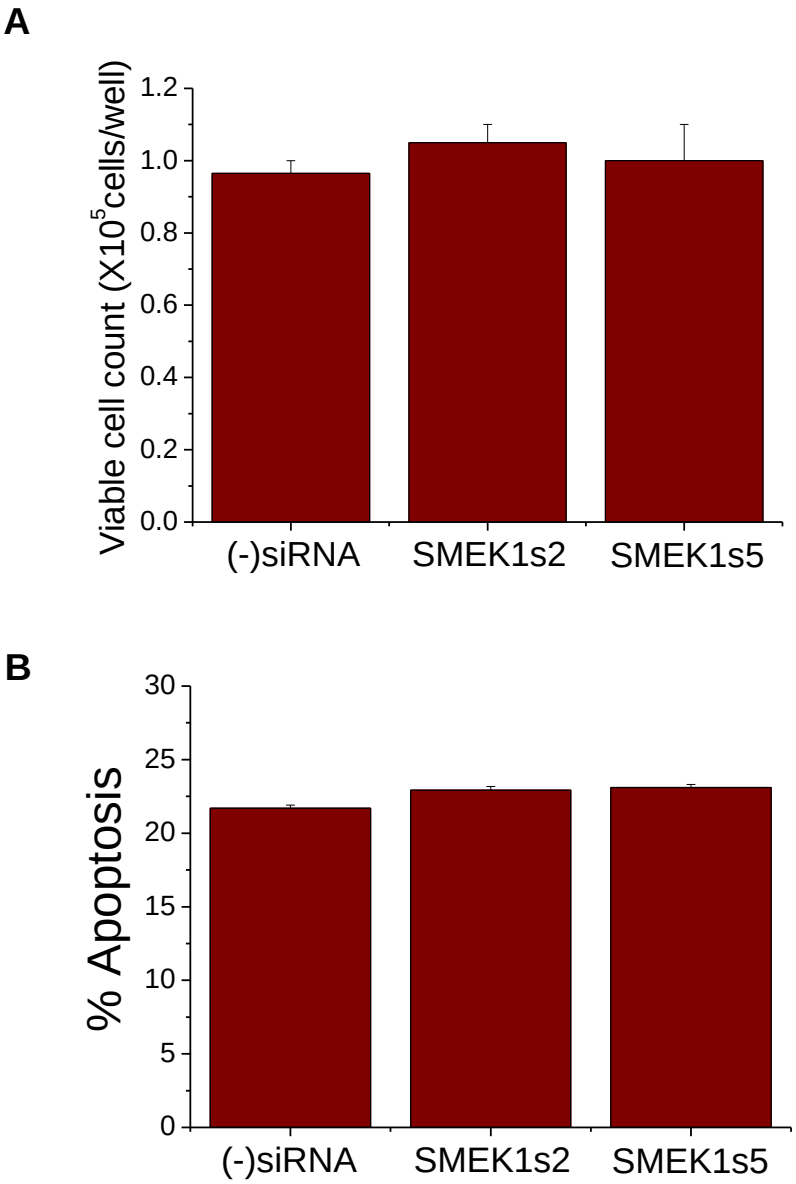


Figure 4.18: SMEK1 down-regulation has no effect on cell viability or on basal apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or with SMEK1-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis under basal conditions. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P > 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

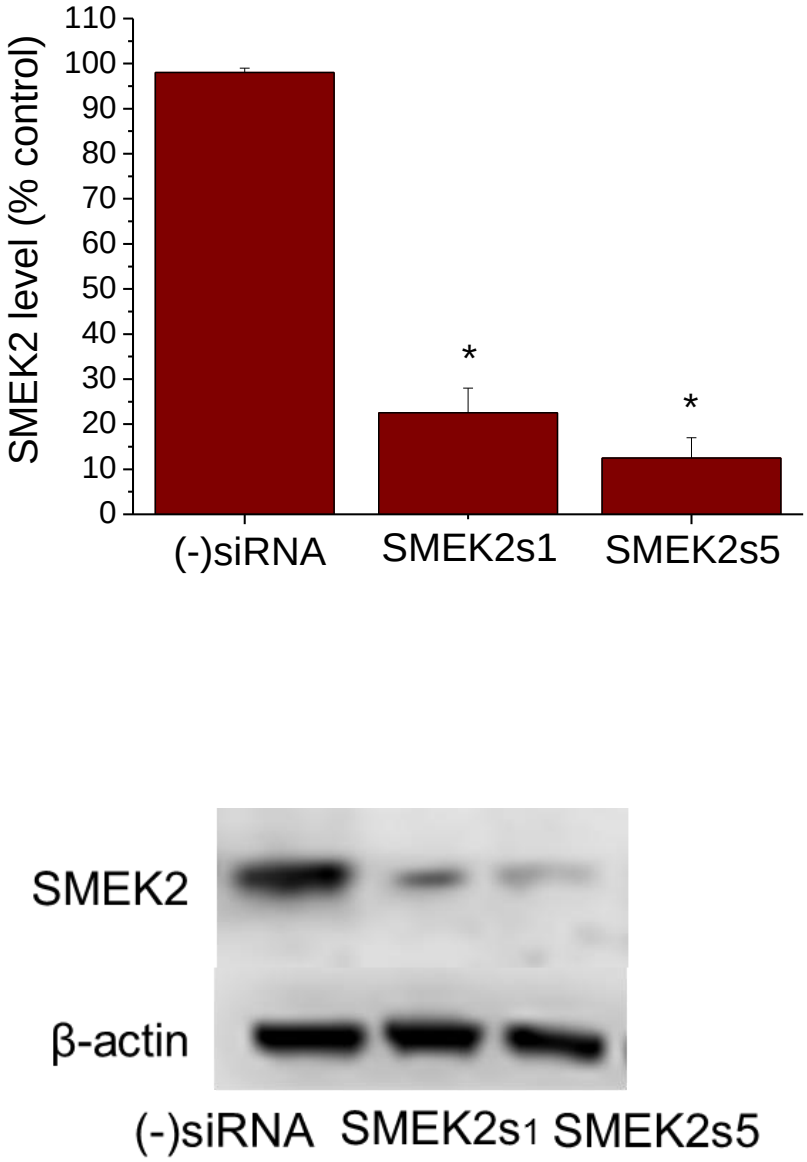


Figure 4.19: Expression level of SMEK2 protein in MCF7 cells with SMEK2 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. SMEK2 protein expression levels were normalised with that of β-actin *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

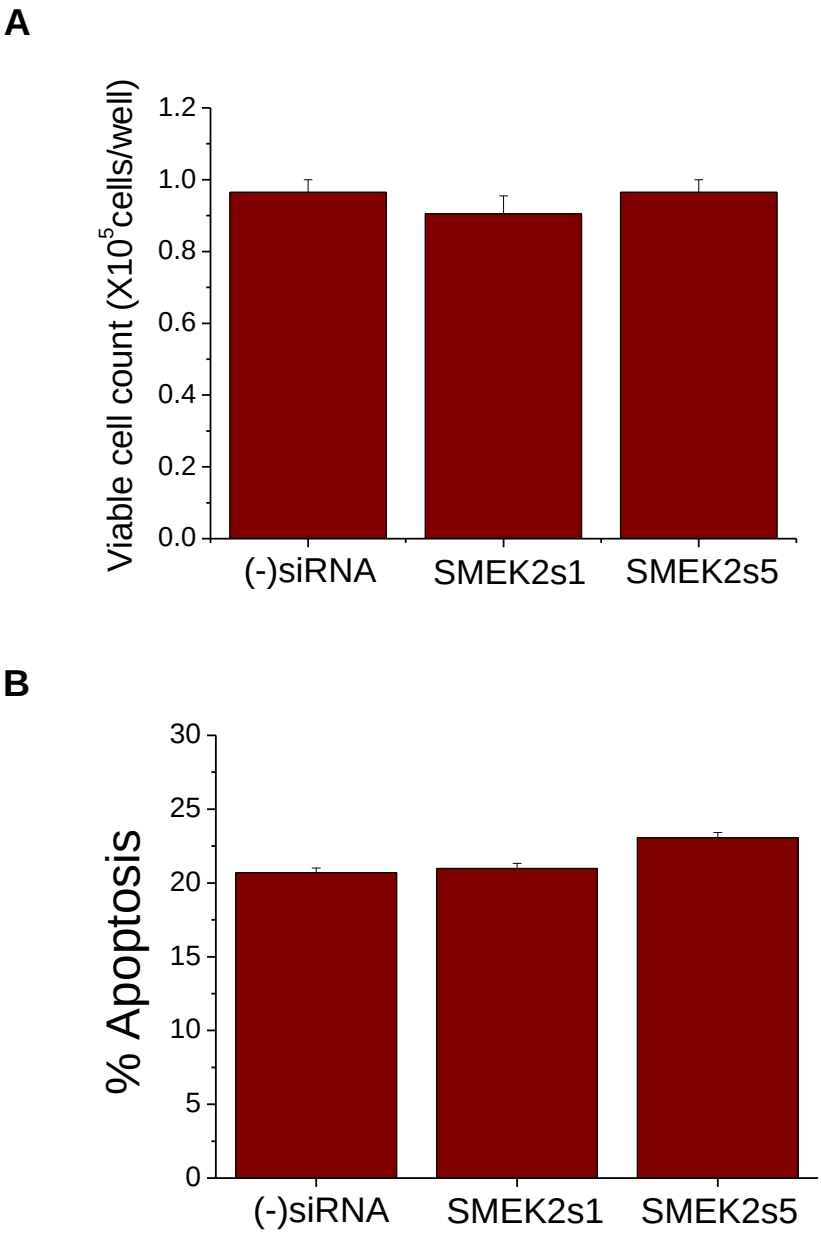


Figure 4.20: SMEK2 down-regulation has no effect on cell viability and basal apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or with SMEK2-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis under basal conditions. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P > 0.05 versus cells transfected with (-)siRNA (one-wayANOVA and Bonferrroni's MCT)

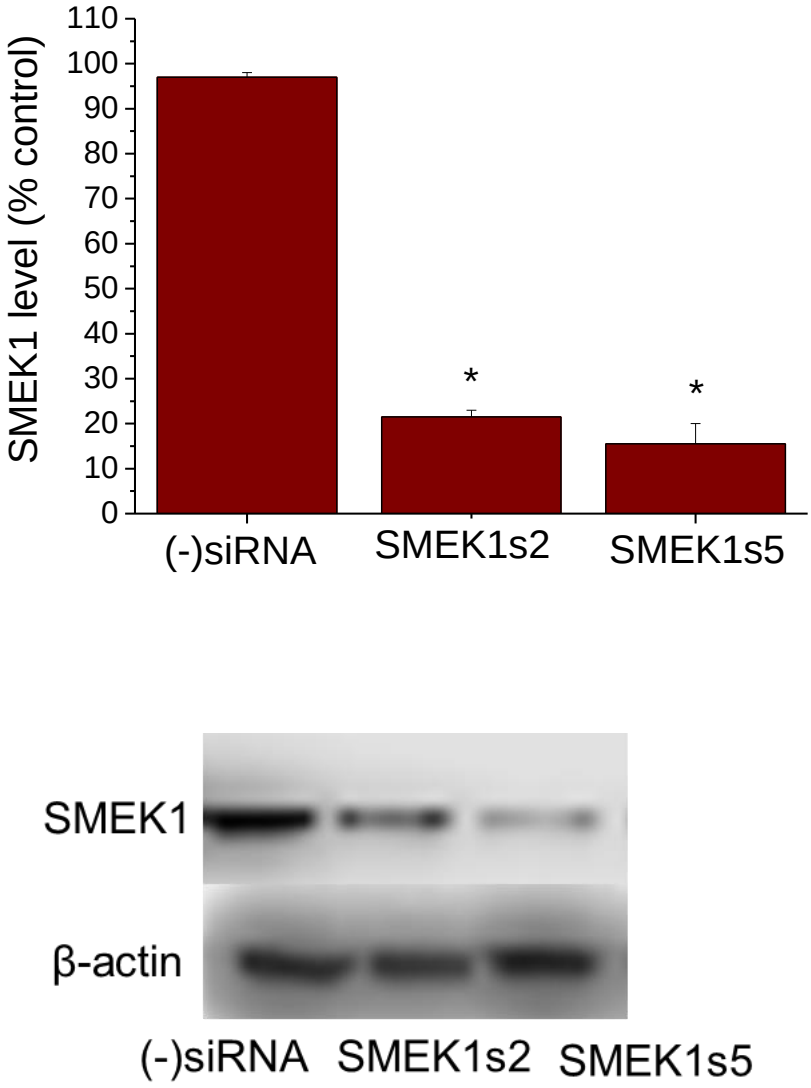


Figure 4.21: Expression level of SMEK1 protein in MDA-MB-231 cells with SMEK1 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. SMEK1 protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

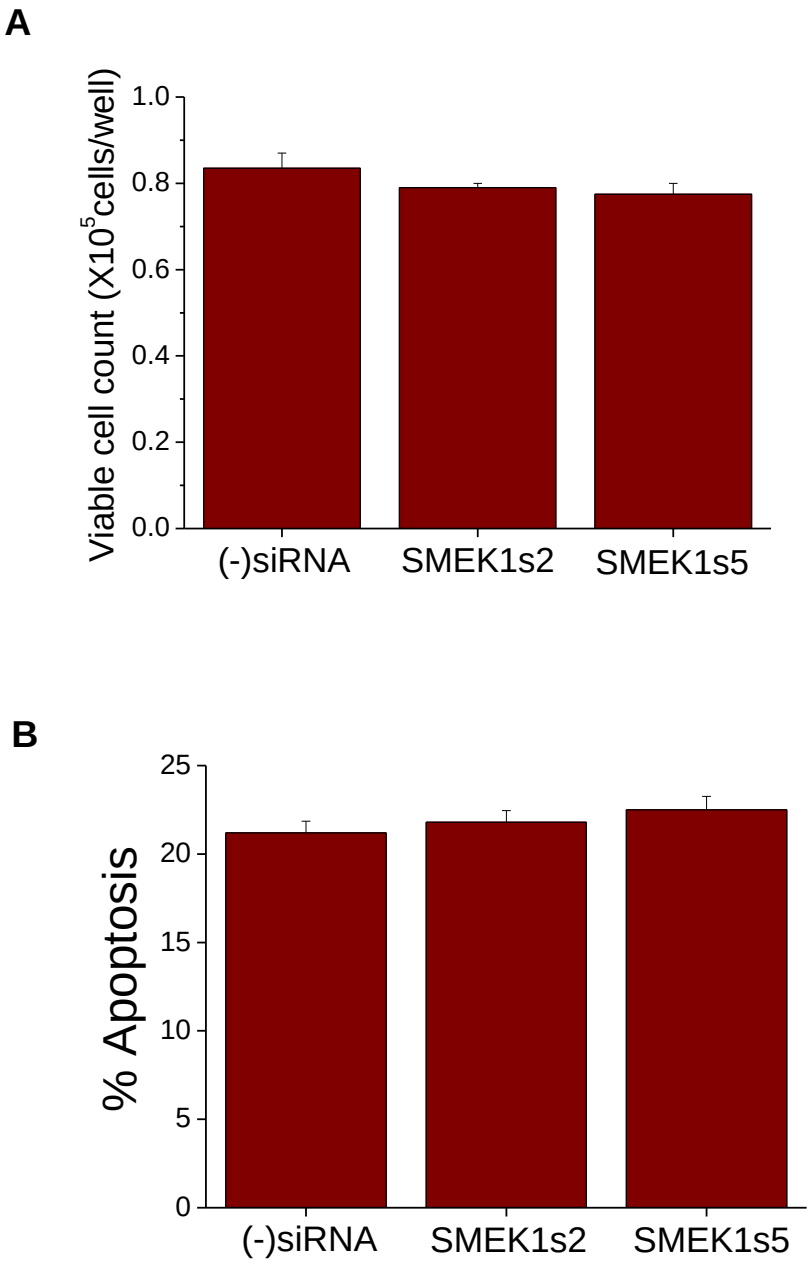


Figure 4.22: SMEK1 down-regulation has no effect on cell viability and on basal apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with SMEK1-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis under basal conditions. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P > 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

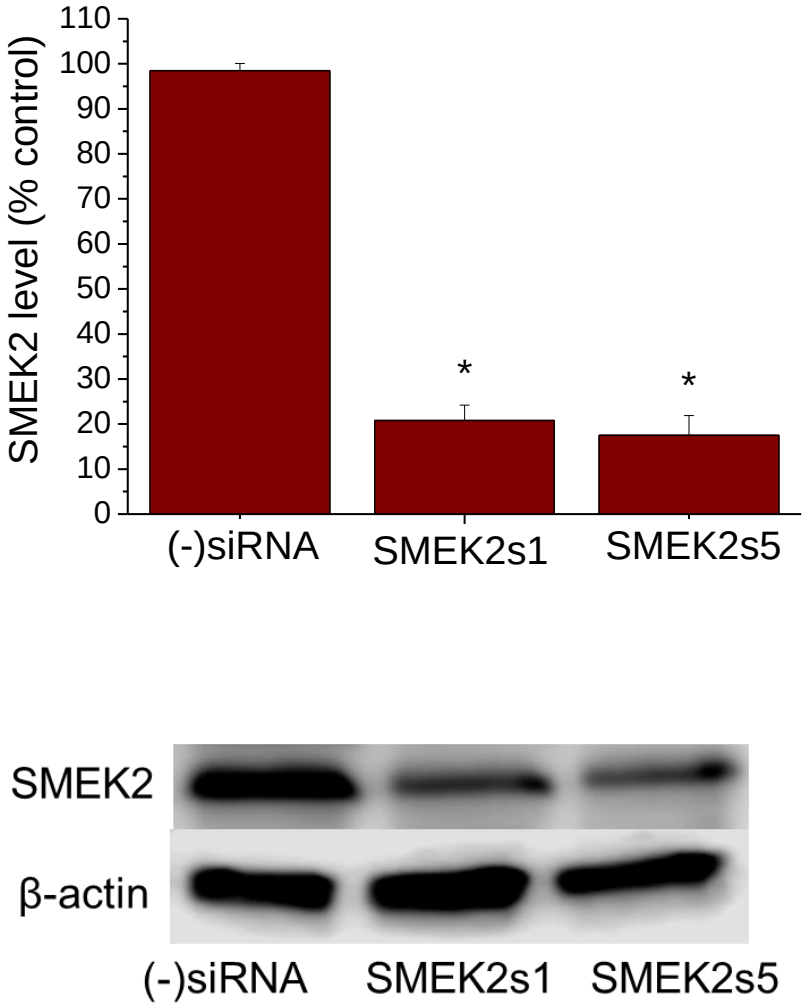


Figure 4.23: Expression level of SMEK2 protein in MDA-MB-231 cells with SMEK2 down-regulation was determined by western blotting 72 hours after transfection and equivalent loading was demonstrated using anti-β-actin antibody. A representative autoradiograph is also presented. The quantified data (in graph form) is the mean +/- the std. error inter-assay and not intra-assay from four independent experiments. SMEK2 protein expression levels were normalised with that of β-actin. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

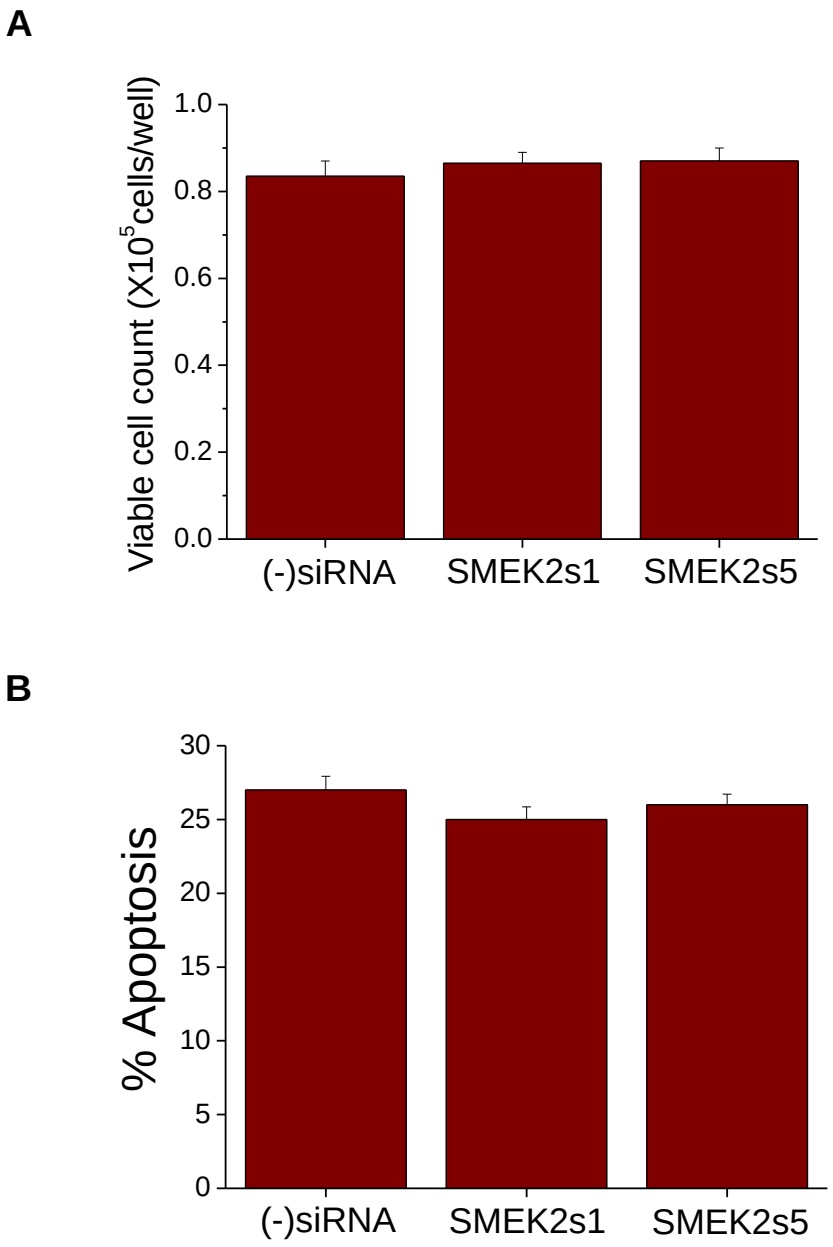


Figure 4.24: SMEK2 down-regulation has no effect on cell viability and on basal apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with SMEK2-specific siRNAs. Cell count and apoptosis were assessed by FACS analysis under basal condition. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as measured by Muse® Annexin V and Dead Cell Assay kit. The bar graphs represent means ± S.E. from four independent experiments. *P > 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

4.3.4.4 SMEK1 or SMEK2 down-regulation does not promote cell death and apoptosis induced by PP4c over-expression in MCF7 and MDA-MB-231 cells

In order to examine the significance of SMEK1 and SMEK2 in regulating the function of PP4c in MCF7 and in MDA-MB-231 cells, cells were transfected with (-)siRNA or with SMEK1 or SMEK2-specific siRNAs. 72 hours post-transfection; SMEK1/SMEK2 down-regulation was confirmed by western blotting analysis. Control cells transfected with (-)siRNA as well as cultures transfected with SMEK1 or SMEK2-specific siRNAs were transiently transfected with pcDNA3.1 empty vector or with pcDNA3.1-PP4c expression vector. Viable cell number and the percentage of total apoptosis were examined by FACS analysis after incubation of the transfected cells for a further 48 hours. PP4c over-expression in MCF7 cells transfected with (-)siRNA resulted in a reduction in the viable cell counts by 50% compared to the cultures transfected with pcDNA3.1, confirming the results obtained in Chapter 3 (Figure 4.25A, Figure 4.26A). Similarly, PP4c over-expression in cultures transfected with SMEK1-targeted siRNAs (SMEK1s2 and SMEK1s5) and those with SMEK2-targeted siRNAs (SMEK2s1 and SMEK2s5) resulted in a reduction in viable cell counts by 50% compared to the cells transfected with pcDNA3.1 empty vector so there was no further effect on viable cell count of MCF7 by combining PP4c over-expression and SMEK1 or SMEK2 down-regulation (Figure 4.25A, Figure 4.26A).

Furthermore, in MCF7 PP4c over-expression in cultures transfected with (-)siRNA increased basal apoptosis by 50% compared to the pcDNA3.1 empty vector transfected cells (Figure 4.25B, Figure 4.26B). Similarly, 50% increase in the percentage apoptosis was observed in the cells having over-expression of PP4c and down-regulation of SMEK1 or SMEK2 compared to the control cells transfected with (-)siRNA and pcDNA3.1 (Figure 4.25B, Figure 4.26B). Indicating that, SMEK1 or

SMEK2 down-regulation has no promoting effect on the function of PP4c in inducing apoptosis in MCF7 cells.

In MDA-MB-231 cells; PP4c over-expression in the cells transfected with (-)siRNA reduced viable cell count by 50% compared to the cultures transfected with pcDNA3.1 confirming the results obtained in Chapter 3 (Figure 4.27A, 4.28A). Similarly, PP4c over-expression in cultures transfected with SMEK1-targeted siRNAs (SMEK1s2 and SMEK1s5) and those with SMEK2-targeted siRNAs (SMEK2s1 and SMEK2s5) resulted in reduction in viable cell count by 50% compared to the cells transfected with (-)siRNA and pcDNA3.1 empty vector so there was no further effect on viable cell count of MDA-MB-231 by combining PP4c over-expression and SMEK1 or SMEK2 down-regulation compared to the results obtained by PP4c over-expression alone (Figure 4.27A, 4.28A).

On the other hand, PP4c over-expression in MDA-MB-231 in cultures transfected with (-)siRNA increased apoptosis by 50% compared to the pcDNA3.1 empty vector transfected cultures (Figure 4.27B, 4.28B). Similarly, 50% increase in the percentage apoptosis was observed in the cells having combined over-expression of PP4c and SMEK1 or SMEK2 down-regulation compared to the cells transfected with (-)siRNA and pcDNA3.1 (Figure 4.27B, 4.28B). Data showed that, SMEK1 or SMEK2 down-regulation has no promoting effect on the function of PP4c in induction of apoptosis in MDA-MB-231 cells. Data indicated that SMEK1 and SMEK2 down-regulation have no additional effect on the regression of cell growth and induction of apoptosis caused by PP4c over-expression in MCF7 and in MDA-MB-231 cells.

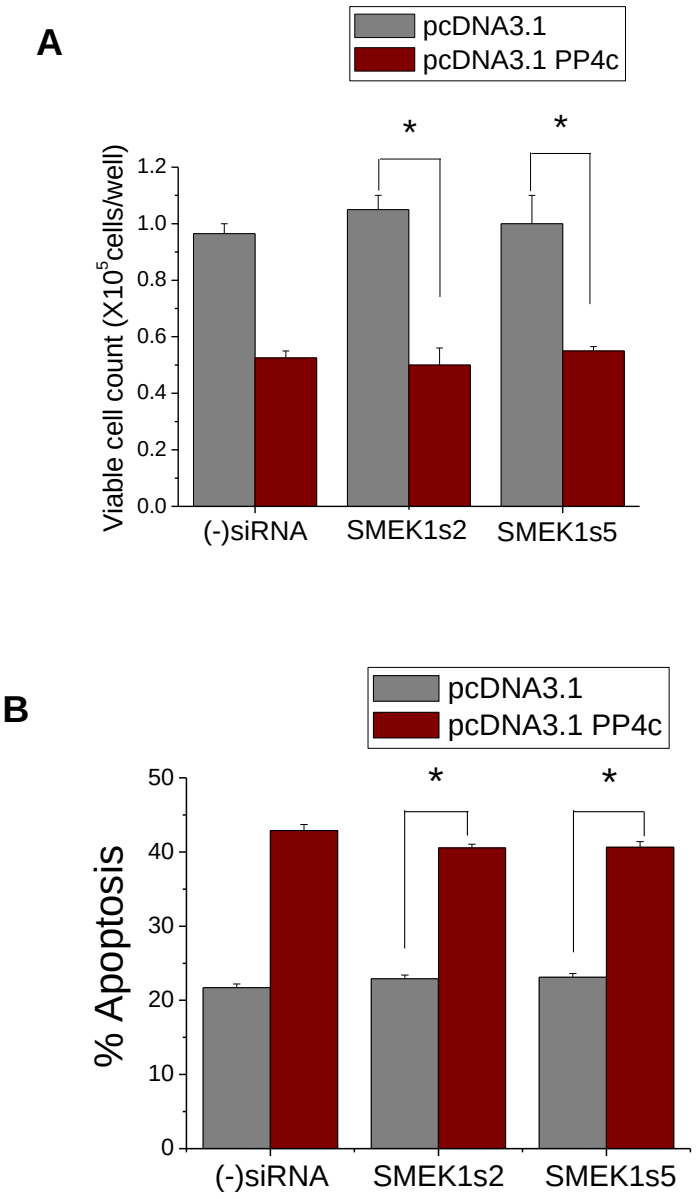


Figure 4.25: SMEK1 down-regulation does not alter PP4c over-expression mediated reduction of cell viability and increase of apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or SMEK1-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as assessed Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared to pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

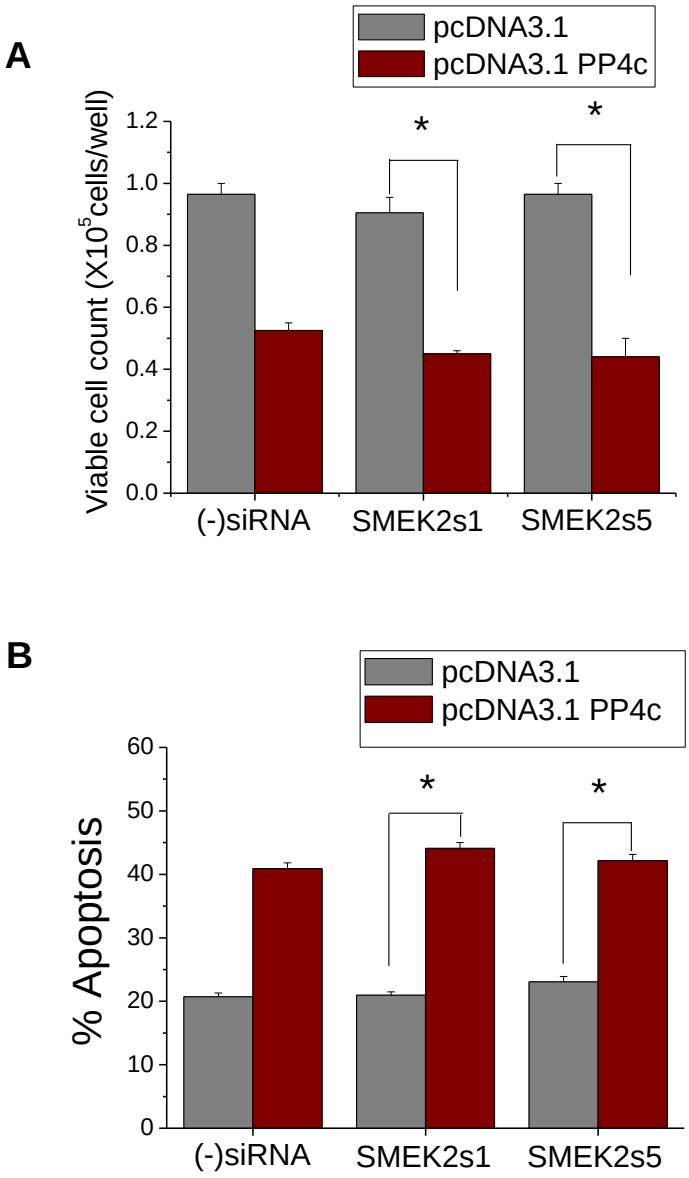


Figure 4.26: SMEK2 down-regulation does not alter PP4c over-expression mediated reduction of cell viability and increase of apoptosis in MCF7 cells. MCF7 cells were transfected with (-)siRNA or SMEK2-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as assessed Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared to pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

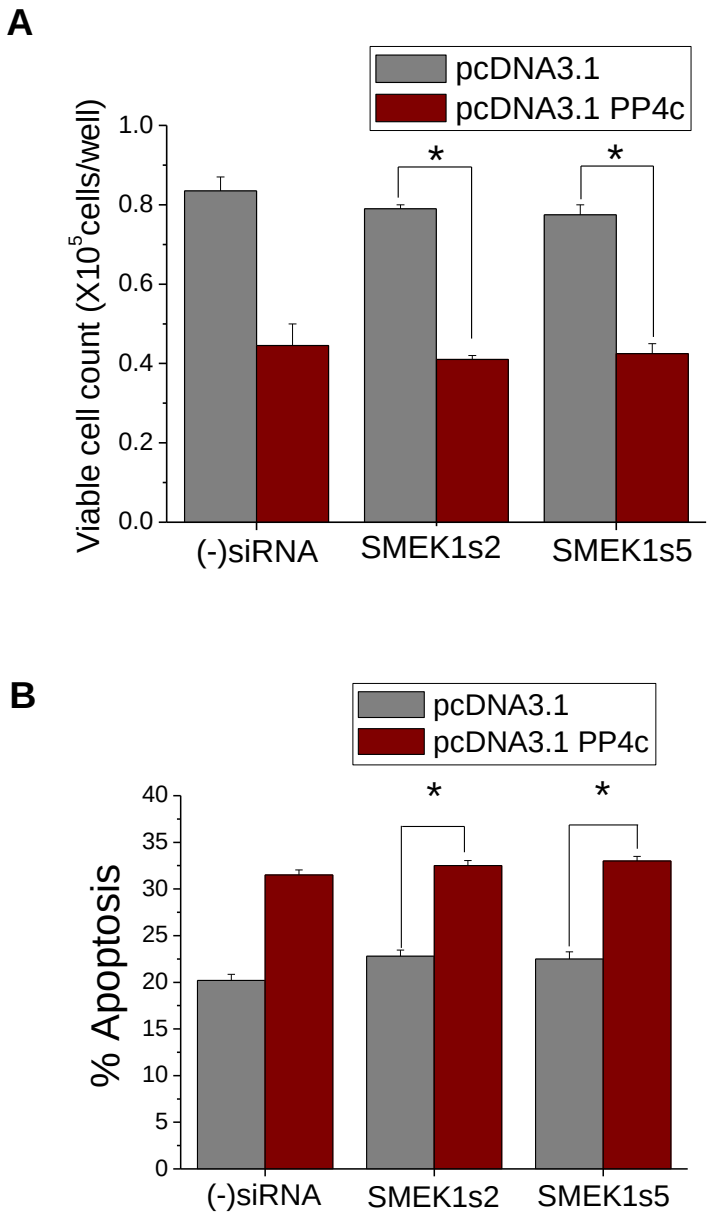


Figure 4.27: SMEK1 down-regulation does not alter PP4c over-expression mediated reduction of cell viability and the increase in apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with SMEK1-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as assessed Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared to pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

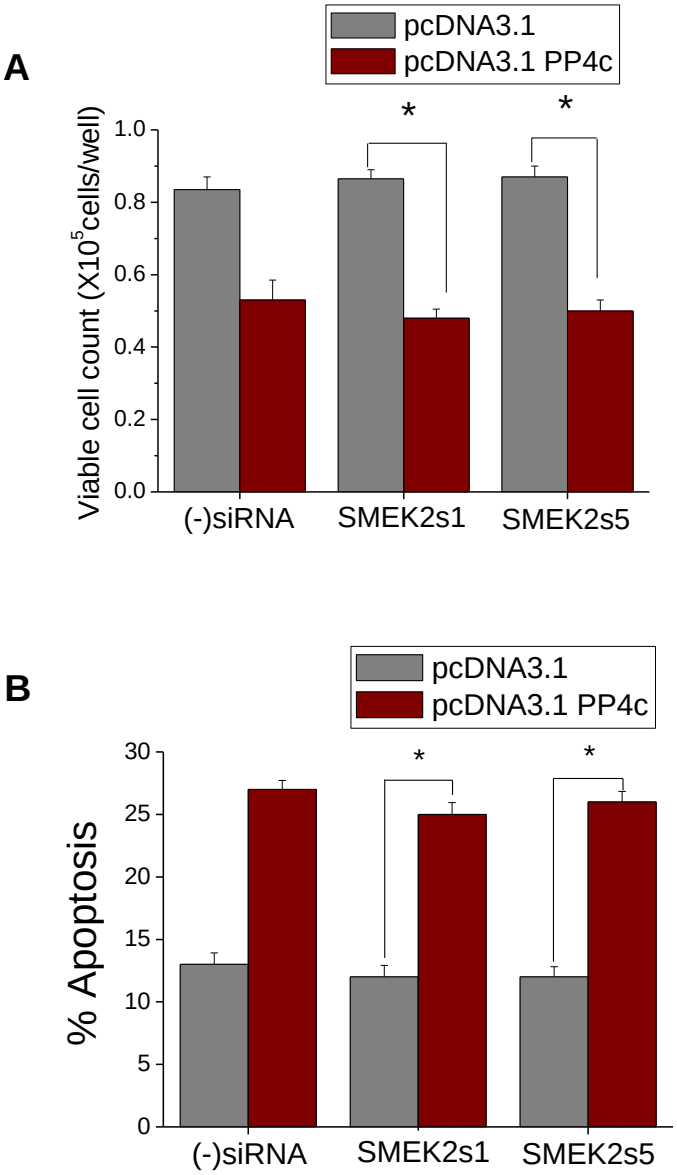


Figure 4.28: SMEK2 down-regulation does not alter PP4c over-expression mediated reduction of cell viability and increase of apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA or with SMEK2-specific siRNAs, 72 hours post-transfection both types of cultures were transiently transfected with pcDNA3.1empty vector or pcDNA3.1-PP4c expression vector. Cell survival was assessed by FACS analysis after incubating the transfected cells for a further 48 hours. **A)** Viable cell count as assessed by FACS analysis using Muse® Count & Viability Assay kit. **B)** % basal apoptosis as assessed Muse® Annexin V and Dead Cell Assay kit. Data are presented as the means ± S.E. from four independent experiments. *P < 0.05 compared to pcDNA3.1 (one-way ANOVA and Bonferrroni's MCT)

4.4 Discussion

The complex structure of PP4 holoenzyme facilitates its diverse functions and locations. Results in the previous chapter revealed the over-expression of PP4c in hormone positive breast cancer cells including MCF7 cells and in the triple negative breast cancer cells including MDA-MB-231 and Hs578T cells as well as in breast cancer tissue samples and also revealed the tumour suppressor activities of PP4c in these types of breast cancer cell lines, in terms of suppressing cell growth and mediation of apoptosis.

In this chapter we have studied other components of PP4 holoenzyme which comprise one or more of PP4c regulatory subunits; PP4R1, PP4R2, PP4R3α (SMEK1), PP4R3β (SMEK2) and PP4R4. We have studied mRNA expression of these regulatory subunits in breast cancer tissue samples and investigated if there is any positive correlation in mRNA expression between PP4c and any of these regulatory subunits and how the modulation of the endogenous expression of PP4c affects the expression of these regulatory subunits in both MCF7 and in MDA-MB-231 cells. We have also studied the effect of specific regulatory subunits down-regulation on MCF7 and MDA-MB-231 cell function and investigated if this has any significant effect on the function of PP4c in these cells.

This study demonstrated that PP4c regulatory subunits show changes in their mRNA expression in all stages of breast cancer. Although the changes in the mRNA expression level of these regulatory subunits were not statistically significant, this could be due to wide range difference between the readings of the samples in a specific stage also the small number of normal samples, i.e. only five compared to 43 breast cancer tissue samples.

PP4R1, PP4R2, PP4R3α, and PP4R3β were found to be over-expressed and easily detected in the human primary mammary epithelial cells (HMEC), the non-tumourigenic epithelial cell lines as well as in tumourigenic cell lines such as oestrogen receptor-positive breast cancer lines-MCF7, T47D, MDA-MB-361, ZR75, and SkBr3 and in the oestrogen receptor-negative lines including MDA-MB-231and MDA-MB-453 (Wang et al., 2008), indicating critical roles for these proteins in breast cancer (Wang et al., 2008). This is consistent with our findings that there was also variation in these regulatory subunits mRNA expression in breast cancer tissue samples which include breast cancer samples of different stages and hormone expression status.

This study revealed that changes in endogenous PP4c mRNA expression level produced changes in PP4c-regulatory subunits mRNA expression in both MCF7 and MDA-MB-231 cells. PP4c over-expression resulted in an increase in mRNA expression of all PP4c-regulatory subunits except that of PP4R4 in both MCF7 and in MDA-MB-231 cells. For instance in MCF7, mRNA expression of PP4R1 and PP4R2 increased by 43.9% and 45.8%, respectively. Similarly, the expression level of SMEK1 and SMEK2 increased by 46.8% and 234.6% respectively. However, PP4R4 mRNA expression level reduced by 98.7% in MCF7 cells after PP4c over-expression.

In MDA-MB-231 cells PP4c over-expression resulted in an increase in the expression level of PP4R1, PP4R2, SMEK1, and SMEK2 mRNA by 331.5%, 352.3%, 40%, and 401.2%, respectively, with a contrasting reduction in the expression level of PP4R4 mRNA by 97%.

In case of PP4c down-regulation, PP4c silencing in MCF7 cells decreased the expression level of PP4R1, PP4R2 and PP4R4 mRNA by 42.7%, 80% and 78.2 respectively. Likewise, the expression level of SMEK1 and SMEK2 mRNA was greatly reduced by 95.7% and 93.2%, respectively. In MDA-MB-231 cells, PP4c down-regulation decreased mRNA expression of PP4R1, PP4R2, SMEK1 and SMEK2 by 98.5%, while the reduction was 96.6% for PP4R4 mRNA. These data indicated the great influence of endogenous level of PP4c on the expression level of its regulatory subunits mRNA in both hormone positive and in triple negative breast cancer cells.

Also we have found that PP4R2 down-regulation reduced cell number and induced apoptosis in MCF7 breast cancer cells and had no effect on MDA-MB-231 cells. Furthermore, PP4R2 down-regulation promoted the apoptotic activity and the suppression of cell growth induced by PP4c in both cell lines. In contrast, SMEK1 and SMEK2 down-regulation had no effect on cell survival in both MCF7 and in MDA-MB-231 cells and had no additional effect on the function of PP4c in these cells.

Initial studies have shown that PP4R1 interacts with PP4c forming a stable complex (Kloeker and Wadzinski, 1999; Wada et al., 2001). Further independent studies using proteomic approaches provided further evidence for a specific interaction between PP4R1 and PP4c (Chen et al., 2008; Gingras et al., 2005). Such specific interactions have been confirmed to occur in human T-cells (Brechmann et al., 2012). It was found that PP4R1 down-regulation reduced short and long term survival and induced apoptosis in the ER- positive breast cancer cell line (ZR-75-30) (Qi et al., 2015). Qi et al (2015) reported that the increased apoptosis in ZR-75-30 cells depleted of PP4R1 could be related to the up-regulation of caspase-3 and

down-regulation of PARP (poly ADP ribose polymerase) which is a protein involved in a number of cellular processes mainly DNA repair and programmed cell death (Maxim et al., 2010).

PP4-R1 and PP4-R2 are reported to act as a core regulatory subunits forming PP4c-R1 and the PP4c-R2 complexes, these complexes may attach to a third variable subunit to form different PP4 complexes with a different functions (Carnegie et al., 2003; Janssens and Goris, 2001). Immunocytological studies revealed that PP4c and PP4R2 is predominantly located in the centrosomes of the human cells suggesting that PP4R2 may be involved in targeting PP4c to this location (Hastie et al., 2000). Association of both PP4R1 and PP4R2 with the PP4c is reported to significantly decrease the activity of PP4c which could be a consequence of the inhibition of the catalytic subunit by one of these regulatory subunits or because they induce narrow substrate specificities (Hastie et al., 2000; Kloeker and Wadzinski, 1999). This may explain results obtained in this chapter in which PP4R2 down-regulation produced an additional effect on the function of PP4c in terms of reducing viable cell number and inducing apoptosis in both MCF7 and in MDA-MB-231 cells. PP4R2 inactivates IKK/NF-κB (inhibitor of nuclear factor kappa-B kinase/ nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathway that is stimulated by cellular stimuli such as growth factors and inflammatory mediators, by enhancing binding of PP4c to phosphorylated IKK. This activity of PP4R2 induced and promoted by the transcription factor PATZ1 (POZ-, AT hook-, and zinc finger-containing protein 1) which provides negative feedback on IKK/NF-κB signalling pathway. PATZ1 and PP4R2 are found to be expressed more in primary lung cancers than lymph node metastases and a reduction in both PATZ1 and PP4R2 is functionally associated with cancer migration/invasion (Ho et al., 2016). The lack of

the effect of PP4R2 down-regulation in triple negative cells-MDA-MB-231 could be due to mutations and / or the metastatic state in these cells. Furthermore, PP4R2 mediates the interaction between DBC1 (Deleted in breast cancer-1) and PP4c. DBC1 is a tumour suppressor that plays an important role in the regulation of cell survival and apoptosis (Habuchi et al., 1997). PP4c dephosphorylates DBC1 at Thr454 *in vitro*. PP4-mediated dephosphorylation of DBC1 is necessary for efficient response to DNA damage and p53 activation in cells; it also increases apoptosis in response to DNA damage (Lee et al., 2015). This action of PP4R2 is consistent with the results of Chapter 3 and it may be involved in the promotion of apoptosis induced by PP4c.

DHX38, a DEAH-box RNA helicase involved in RNA splicing, was found to interact with most of PP4 subunits including PP4c, PP4R2, PP4R3α and PP4R3β but not with PP4R1. This interaction is specific to PP4 because there is no detectable association of DHX38 with other seine/threonine phosphatases. DHX38 interaction with PP4 is regarded as DNA damage-independent interaction because it does not occur as a response to DNA damage. PP4R2 is essential for DNA replication and double-strand DNA break (DSB) repairs (Lee et al., 2015). Research has shown that DHX38 act as an endogenous inhibitor of PP4 by disrupting PP4c/PP4R2-mediated functions. PP4R2 enhances PP4c/PP4R2-mediated dephosphorylation of target proteins such as RPA2 (replication protein A2), which acts as a stabilizer of single-stranded DNA intermediates that are generated during DNA replication or upon DNA stress (Iftode et al., 1999), and KAP1 (KRAB-associated protein-1) which is a critical regulator during normal development and differentiation. It has been reported that mice deficient in KAP1 die prior to gastrulation (Cammas et al., 2000), whereas, KAP1 knock-out mice, which is specifically deleted in the adult forebrain, exhibit

heightened levels of anxiety and stress with alterations in learning and memory (Jakobsson et al., 2008). DHX38 over-expression significantly inhibits PP4R2 function and inhibits dephosphorylation of RPA2 and KAP1 by PP4c so that cells are less efficient in repairing DNA double strand damage and cells have a lower survival rate. On the other hand, DHX38 depletion promotes the process of DSB repair through activation of PP4R2 with subsequent dephosphorylation of these target proteins that are essential in this process (Sueji et al., 2015). This could give a clue to the results obtained in this chapter in which we have found that PP4R2 down-regulation reduced viable cell count and induced apoptosis in MCF7 breast cancer cells, also may partly give an explanation as to why PP4R2 down-regulation promoted the function of PP4c in MCF7 and MDA-MB-231 cells. The explanation of these findings may need further investigation toward the expression level of DHX38 in these breast cancer cell lines. Our results obtained in MDA-MB-231 cells agreed with the results obtained by Mourtada-Maarabouni et al., (2016) that PP4R2 had no effects on myeloid leukemic cell survival.

Other PP4c regulatory subunits are PP4R3α and PP4R3β, also termed as (SMEK1 and SMEK2) respectively (suppressor of MEK; mitogen-activated protein/extracellular signal-regulated kinase) (Gingras et al., 2005; Chowdhury et al., 2008). In yeast and mammalian cells, PP4R3/SMEK forms a complex with PP4c and PP4R2 resulting in a trimer PP4c-PP4R2-PP4R3 that has a conserved role in the resistance to cisplatin and other cytotoxic agents (Hastie et al., 2000; Gingras et al., 2005; Keogh et al., 2006). Whereas, another heterotrimeric PP4 complex PP4c-PP4R2-PP4R3 β has been described to be involved in the DNA double strand break repair (DSB) (Lee et al., 2010).

SMEK1 and SMEK2 are directly involved in the regulation of hepatic glucose metabolism in mice. Up-regulation of hepatic SMEK1/2 was observed during fasting or in mouse models of insulin-resistant conditions. SMEK1/2 over-expression stimulates hepatic gluconeogenesis with resulting elevations in plasma glucose level (Yoon et al., 2010). SMEK1/2 stimulates hepatic gluconeogenesis through dephosphorylation of CRTC2 (CREB regulated transcription coactivator 2) which is the transcriptional coactivator for the transcription factor CREB and regarded as the central regulator of gluconeogenic gene expression in response to cAMP (Alan and Alan, 2006). On the other hand, SMEK1/2 down-regulation reduces hyperglycaemia and enhances CRTC2 phosphorylation; the effect is blunted by S171A CRTC2, which is refractory to salt-inducible kinase (SIK)-dependent inhibition (Yoon et al., 2010).

SMEK1 is a tumour suppressor protein and it also possesses a pro-apoptotic activities. SMEK1 is down-regulated in cancer cells and tissues (Byun et al., 2012). The tumour suppressor activity of SMEK1 is obtained by suppressing vascular endothelial growth factor (VEGF)-induced cell proliferation, migration, and capillary-like tubular structure *in vitro*. In ovarian tumours via binding with vascular endothelial growth factor receptor 2 (VEGFR-2), SMEK1 inhibited the phosphorylation of up and downstream proteins of Akt, such as phospholipase Cγ1 (PLC-γ1), 3-phosphoinositide-dependent protein kinase 1 (PDK1), endothelial nitric oxide synthetase (eNOS), and hypoxia-inducible factor 1 (HIF-1α) (Kim et al., 2015). SMEK1 over-expression increased sensitivity of OVCAR-3 cancer cells to gemcitabine. So SMEK1 increased the cytotoxic activity of gemcitabine in the human ovarian carcinoma system and promotes gemcitabine-inhibited cell migration. In addition SMEK1 affects cell cycle progression and it is claimed to cause cell cycle

arrest in the G_0-G_1 phase. Combination of SMEK1 and gemcitabine suppressed the phosphorylation of many important proteins in the PI3K signalling pathway such as PDK1 and Akt and promoted the activities of p53 and p21 promoter luciferase (Byun et al., 2012). Furthermore, combination of SMEK1 plus paclitaxel increased apoptosis of the ovarian carcinoma cells and this was due to reduction in the phosphorylation of p70S6K and 4E-BP1 (Kim et al., 2014). This may explain our results obtained in this chapter that SMEK1 down-regulation doesn't have additional effect on the PP4c function in reducing cell number and promoting apoptosis in both MCF7 and MDA-MB-231 cells, since this could be due to increased phosphorylation of important proteins in the PI3K signalling pathway such as PDK1 protein. It was found that the tumour suppressor BLU enhances the pro-apoptotic activity of SMEK1 through direct interaction between them. The N-terminal of BLU was observed to interact with the C-terminal of SMEK1. Expression of BLU and SMEK1 was down-regulated in ovarian and cervical cancer patients, and its promoter was found to be hyper-methylated (Dong et al., 2012). BLU was also found to be down-regulated in nasopharyngeal carcinoma (Qiu et al., 2004). SMEK1 is a positive regulator of neuronal differentiation and it suppresses the proliferative capacity of neural progenitor cells (NPCs). During mitosis SMEK1 is excluded from the nucleus and it interacts with cortical/cytoplasmic Par3 (the cell polarity protein), Par3 is SMEK1 substrate and it is a negative regulator of neuronal differentiation, Par3 interaction with SMEK1 mediates its dephosphorylation by the catalytic subunit PP4c and suppresses its activity. These results identify the PP4/SMEK1 complex as a key regulator of neurogenesis (Lyu et al., 2013).

SMEK positively regulates PP4c to induce its functions in developmental progression and chemotaxis; and the expression of genes specifically involved in cellular

responses to stress and leading-edge formation during cell movement. This regulation in PP4c function occurs as a result of SMEK regulation of PP4c localization to the nucleus rather than modulation of the endogenous level of PP4c (Mendoza et al., 2007). Our results obtained in MCF7 and MDAMB-231 cells agreed with the results obtained by Mourtada-Maarabouni et al (2016) that PP4R3 had no effects on myeloid leukemic cell survival.

This chapter highlights the importance of the PP4c-regulatory subunits in particular the importance of PP4R2 which seems to be fundamental in controlling the function of PP4c inside the cell and this could be a consequence of the inhibition of the PP4 catalytic subunit or PP4R2 may induces PP4c narrow substrate specificities.

Chapter 5: An investigation into the impact of PP4c silencing on the response of breast cancer cells to chemotherapeutic agents

5.1 Introduction

As established in Chapter 3, over-expression of PP4c inhibits cell proliferation and stimulates apoptosis, key determinants of cell survival that are frequently disrupted in cancer (Hanahan and Weinberg, 2011). Previous studies have also confirmed the growth inhibitory effects of PP4c on other cells (Mourtada-Maarabouni and Williams, 2008; 2009), consistent with a tumour suppressor role for this gene. On the other hand, reduced expression of PP4c promotes cell survival and decreases apoptosis in breast cancer cells and in other cell types (Chapter 3; Mourtada-Maarabouni and Williams, 2008; 2009). In contrast to these tumour suppressor properties of PP4c, results presented in Chapter 4 revealed that PP4c displays increased expression in mRNA level in breast cancer tissue samples compared to samples from normal individuals. Wang et al (2008) also reported an increase in the expression of PP4c in human primary breast tumours and an increase in cisplatin sensitivity in PP4c-depleted MCF7 cells. Similarly, increased PP4c expression has been observed in many cancers including lung cancer, pancreatic ductal adenocarcinoma and colorectal cancer (CRC) (Weng et al., 2012; Li et al., 2015).

The aberrant expression and apoptosis-promoting activity of PP4c indicate that it may serve as a therapeutic target in breast cancer. Moreover, the apoptosis-promoting activity of PP4c should have important implication in cancer therapy, since the action and the potency of many chemotherapeutic drugs ultimately depend on activating the cellular apoptotic machinery (McKenzie and Kyprianou, 2006; Indran et al., 2011). Since PP4c expression level is altered in breast cancer, it is important to

investigate the consequences of reduced PP4c expression on chemotherapeutic drug action in breast cancer cells. Such studies will allow the evaluation of the potential use of PP4c level as a predictive biomarker for response to chemotherapeutic drugs.

Many of chemotherapeutic agents used in breast cancer treatment cause different types of DNA damage through various mechanisms. Some of these drugs directly damage DNA by causing double-strand breaks (DSBs) (d'Andrea and Haseltine, 1978). Another group, such as alkylating agents causes DNA inter-strand cross-links. These cross-links lead to the arrest of DNA replication resulting in DSBs (McHugh et al., 2001; Tercero and Diffley, 2001). A third group of DNA damaging agents inhibits topoisomerases I and II. These enzymes are involved in introducing temporary breaks in the DNA strands to permit DNA unwinding before DNA replication (Shao et al., 1999). Inhibition of topoisomerases promotes the formation of a stable topoisomerase-DNA complex leading to the arrest of DNA replication forks and DSBs (Shao et al., 1999). A fourth group of DNA damaging agents forms adducts with DNA, such as platinum-based compounds including cisplatin (Zdraveski et al., 2002). DSBs induce a phosphorylation-mediated signalling cascade regulated by signal transducing kinases such ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) kinase and effector kinases such as checkpoint kinase 1 and checkpoint kinase 2 (CHK1 and CHK2) (Bartek and Lukas, 2003) but the role of phosphatases in this pathway remains unclear. Much of the signalling of the DNA damage response (DDR) is transmitted by serine and threonine phosphorylation. Therefore, it is not surprising to find that the serine/threonine phosphatases negatively regulate these phosphorylation events. Indeed, the activities of many proteins in the DDR signalling pathway are negatively regulated by

dephosphorylation by serine\threonine phosphatases (Freeman et al., 2010). Phosphorylation of the histone H2A variant H2AX on Ser 139 (γH2AX) by ATM, ATR is one of the earliest events in the response to DSB (Rogakou et al., 1998). The phosphorylation of H2AX which leads to the formation of γH2AX produces a large domain of DNA damage foci which acts as a signal for the recruitment and efficient retention of DNA repair and signalling proteins (Rogakou et al., 1999; Celeste et al., 2003). The PP4 phosphatase complex consisting of PP4c and PP4R1 is implicated in the dephosphorlyation of γH2AX, regulating its role in the DSB response (Nakada et al., 2008).

The PI3K/Akt/mTOR (phosphatidylinositide 3-kinases/ Protein kinase B (PKB)/ mechanistic target of rapamycin) pathway has been recognised to transmit the signals from cell membrane to the nucleus for activation of multiple oncogenic pathways. This pathway has also been found to regulate the growth and proliferation of breast cancer cells; and it is interconnected with multiple metabolic pathways involving glucose, lipid and amino-acids for maintaining the energy balance and autophagy (Grunt and Mariani, 2013). It is also known to regulate oestrogen receptor (ER) function, and communicates with upstream and downstream regulatory systems (Paplomata and O'Regan, 2014). Therefore, it is known as one of the complicated signalling networks that are found in hyperactive states in 70% of breast cancer cases (Grunt and Mariani, 2013). Consequently, protein kinases present in this pathway are considered attractive targets for treating breast cancer. For these reasons, the community of researchers and pharmaceutical companies are engaged in developing multiple inhibitors of the PI3K/Akt/mTOR pathway (Grunt and Mariani, 2013). A number of chemotherapeutic agents that target kinases in this pathway have been developed; these drugs include agents that inhibit the activity of PI3K and

Akt protein kinase complexes and downstream kinases such mTORC1 and mTORC2 (mammalian target of rapamycin complex 1 and mammalian target of rapamycin complex 2) (MacKeigan et al., 2005). Since much of the signalling of the PI3K/Akt/mTOR pathway is relayed by serine and threonine phosphorylation, it is intuitive that protein serine/threonine phosphatases would negatively regulate these phosphorylation events and ultimately affect the cell response to a variety of these kinase inhibitors.

As discussed above, most of these chemotherapeutic drugs target signalling pathways regulated by the reversible phosphorylation of its protein components. It is therefore important to study the role of PP4c in the cellular response to these chemotherapeutic agents. The consequences of reduced PP4c expression on the response of breast cancer cells to various chemotherapeutic agents have not been examined to-date. Therefore, in this chapter, the aims are to focus on the chemotherapeutic implications of reduced PP4c expression in breast cancer, to investigate the hypothesis that PP4c silencing impairs the responses of breast cancer cells to apoptotic stimuli and to analyse the role of PP4c in the regulation of PI3K/Akt/mTOR pathway.

5.2 Methods

5.2.1 Induction of cell death

Cells were transfected with PP4c-specific siRNAs using HiPerFect transfection reagent according to the protocol as described in section (2.2.14.3). PP4s1 and PP4s2 were used for PP4c silencing in MCF7 cells. Similarly, PP4s2 and PP4s8 were utilised to silence endogenous expression of PP4c in the triple negative breast cancer cells-MDA-MB-231 and Hs578T.

For drug treatment, cells were seeded at a density of 0.8 X 10^5 cell/ml into a 12-well plate or a 96-well plate. Cells were cultured for 20 hours before addition of 5-fluorouracil (175 µM) (Pickard and Williams, 2014), doxorubicin (0.2 µM) (Mourtada-Maarabouni et al., 2009), rapamycin (1 µM) (Pickard and Williams, 2014), everolimus (10 nM) (Pickard and Williams, 2014), cisplatin (5 µg/ml) (Mourtada-Maarabouni et al., 2009), BEZ235 (100 nM) (Pickard and Williams, 2014), AZD8055 (50 nM) (Pickard and Williams, 2014), AZD5363 (10 mM) (Addie et al., 2013), LY294002 (10 µM) (Hermanto et al., 2001) or vehicle (0.25 % dimethyl sulphoxide). Cells were cultured for 72 hours post-treatment, and then adherent cells were trypsinised and combined with the non-adherent cells for analysis of cell survival. Cell viability was determined by FACS analysis using Muse® Count & Viability Assay kit and MTS assay as described in sections (2.2.3.2 and 2.2.3.3). For the latter, sample absorbance was read at 490 nm (A490) using Wallac Victor 1420 Multilabel Counter and the readings were corrected for the appropriate medium plus drug blank values.

5.2.2 Multi-antibody array measurement of phosphoproteins involved in the Akt signalling pathway

For PP4c over-expression; cells were transfected with pcDNA3.1 empty vector or with pcDNA3.1-PP4c expression vector using TransIT®-BrCa transfection reagent for the transfection of MCF7 cells as described in section (2.2.15.3) and Amaxa® Cell Line Nucleofector® Kit V for the transfection of MDA-MB-231 cells as described in section (2.2.15.2). For PP4c silencing MCF7 cells and MDA-MB-231 cells were transfected with (-)siRNA or with PP4c-specific siRNAs using HiPerFect transfection reagent according to the protocol as described in section (2.2.14.3).

The PathScan® Akt Signalling Antibody Array kit (Chemiluminescent Readout) was used to detect phosphorylated proteins in the Akt pathway. This assay simultaneously detects 16 phosphoproteins, involved in the Akt signalling cascade. Cell Lysates were prepared from transiently transfected cells with pcDNA3.1 empty vector or with PcDNA3.1-PP4c expression vector for PP4c over-expression and from cells transfected with (-)siRNA or PP4c-specific siRNAs for PP4c down-regulation as described in section (2.2.16.2). Protein quantification was carried using the Bradford protein assay as described in (2.2.16.2). For the quantification of the phosphoproteins; cell lysates were diluted to 1 mg/ml using array diluent buffer, 75 µl of the diluted lysate was added to each well in the glass slide provided in the PathScan® Akt Signalling Antibody Array kit after blocking the wells with 100 µl of array blocking buffer for 15 minutes. Cell lysates were incubated on the slide for two hours at room temperature on an orbital shaker followed by a treatment with 75 µl of 1 X detection antibody cocktail for one hour at room temperature on an orbital shaker. 75 µl of 1 X DyLight 680®-linked Streptavidin were then applied to each well

to visualize the bound detection antibody using the Odyssey® Imager (Li-Cor). Spot intensity was quantified using Image Studio™ Software (version 3.1) by estimating the % spot intensity of each protein related to the % spot intensity of the positive control in the samples transfected with pcDNA3.1-PP4c or PP4c-specific siRNAs and compared to that of the control samples that are transfected with pcDNA3.1 or (-)siRNA.

5.3 Results

5.3.1 PP4c silencing attenuates the response of hormone positive and triple negative breast cancer cells to different DNA damaging agents

To examine the effect of PP4c silencing on the response of breast cancer cells to different chemotherapeutic agents, different PP4c-specific siRNAs were used to reduce the endogenous PP4c expression in three cell lines oestrogen-progesterone positive-MCF7 and triple negative-MDA-MB-231 and Hs578T cells. The influence of PP4c silencing on cell survival was then examined after cell death induction by a range of DNA damaging agents such as cisplatin (5 µg/ml), doxorubicin (0.2 µM) and 5-fluorouracil (175 µM).

In MCF7 cells, siRNA-mediated silencing of PP4c resulted in a significant increase in the number of viable cells under basal conditions, in agreement with the results reported in Chapter 3 (Figure 5.1 A, C and E). PP4c knockdown attenuated the growth inhibition caused by cisplatin treatment where the growth inhibition induced by cisplatin was reduced by 66.7% for PP4s1 and by 77.8% for PP4s2 (Figure 5.1 A and B). Similarly, PP4c silencing also affected the sensitivity of MCF7 to doxorubicin by decreasing doxorubicin-induced cell death by approximately 75% (Figure 5.1 C and D). Likewise, PP4c silencing reduced the sensitivity of MCF7 to 5-fluorouracil by 35-40% (Figure 5.1 E and F).

The effect of PP4c knock-down on the response of triple negative cell lines -MDA-MB-231 and Hs578T cells to DNA damaging agents was also investigated. siRNA-mediated silencing of PP4c reduced the sensitivity of MDA-MB-231 cells to cisplatin by 50% and 40% for PP4s2 and PP4s8 respectively (Figure 5.2 A and B). The sensitivity of Hs578T cells to cisplatin was also reduced by 34% after PP4c down-

regulation (Figure 5.3 A and B). Similarly, PP4c silencing affected the sensitivity of MDA-MB-231 cells to doxorubicin by reducing doxorubicin-induced cell death by 33.4% in comparison to the cultures with mock transfection and cultures transfected with (-)siRNA (Figure 5.2 C and D). PP4c silencing in Hs578T cells also reduced doxorubicin-induced cell death by 28% compared to the cultures with mock transfection and cultures transfected with (-)siRNA (Figure 5.3 B and C). In addition, reduction in the sensitivity of both MDA-MB-231 and Hs578T cells to 5-fluorouracil was also observed as a result of PP4c down-regulation. The growth inhibition in response to 5-fluorouracil was recorded to be reduced by 96% in MDA-MB-231 cells (Figure 5.2 E and F) and by 82% in Hs578T cells (Figure 5.3 E and F) after PP4c down-regulation

These data suggested that PP4c down-regulation increased resistance of oestrogen/ progesterone positive cells and triple negative cells to DNA damaging agents.

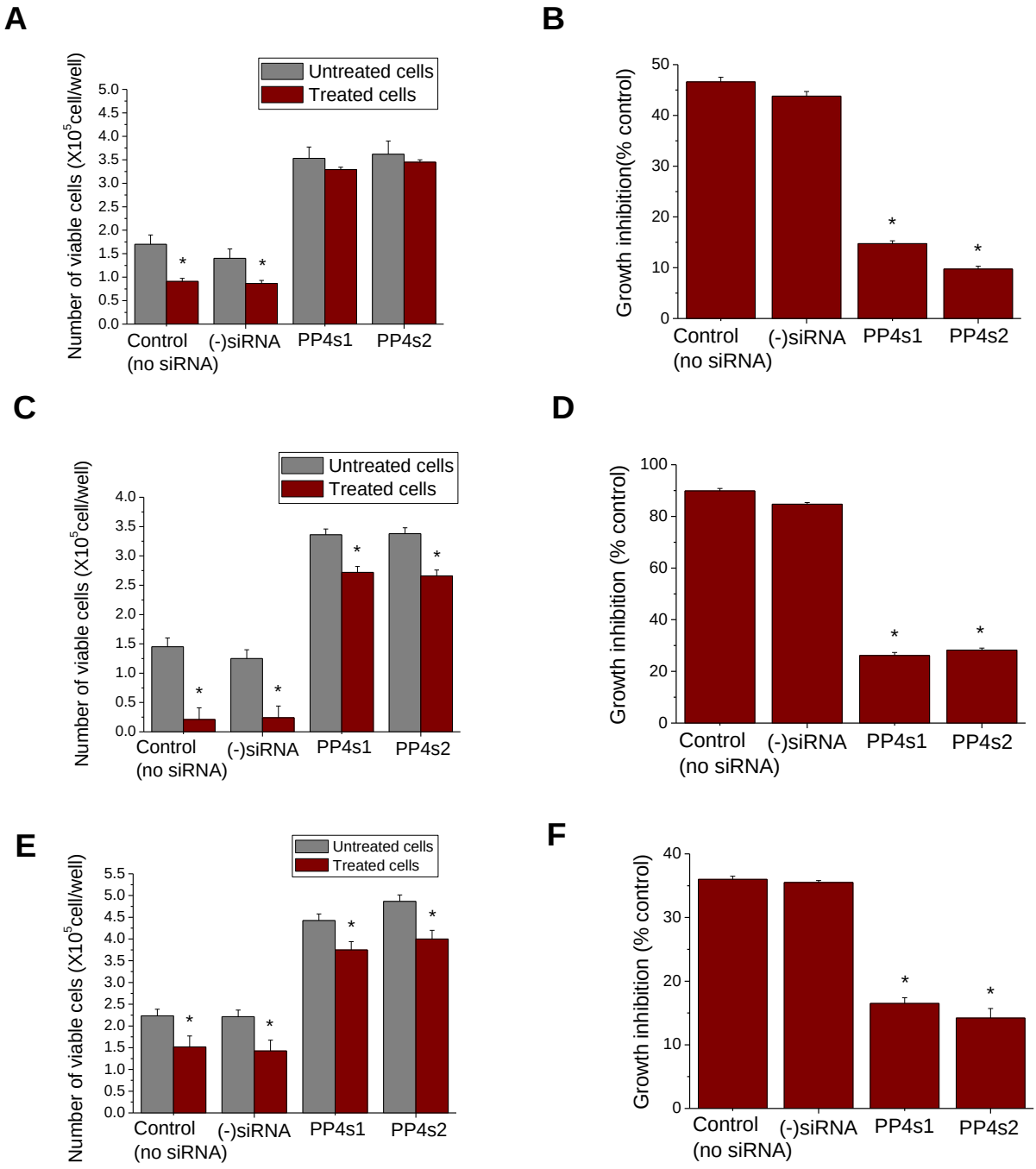


Figure 5.1: Effect of PP4c down-regulation on the response of MCF7 cells to DNA damaging agents. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s1 and PP4s2 reduced expression of PP4c protein by 50-60% in MCF7 cells (Figure 3.23). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by cisplatin (A and B), doxorubicin (C and D) and 5-fluorouracil (E and F). Number of viable cells in the absence and presence of

chemotherapeutic drugs as determined by FACS analysis (A, C and E). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B, D, and F). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A, C, E) and versus mock transfected cells and cells transfected with (-)siRNA control (B, D and F) (one-way ANOVA and Bonferrroni's MCT)

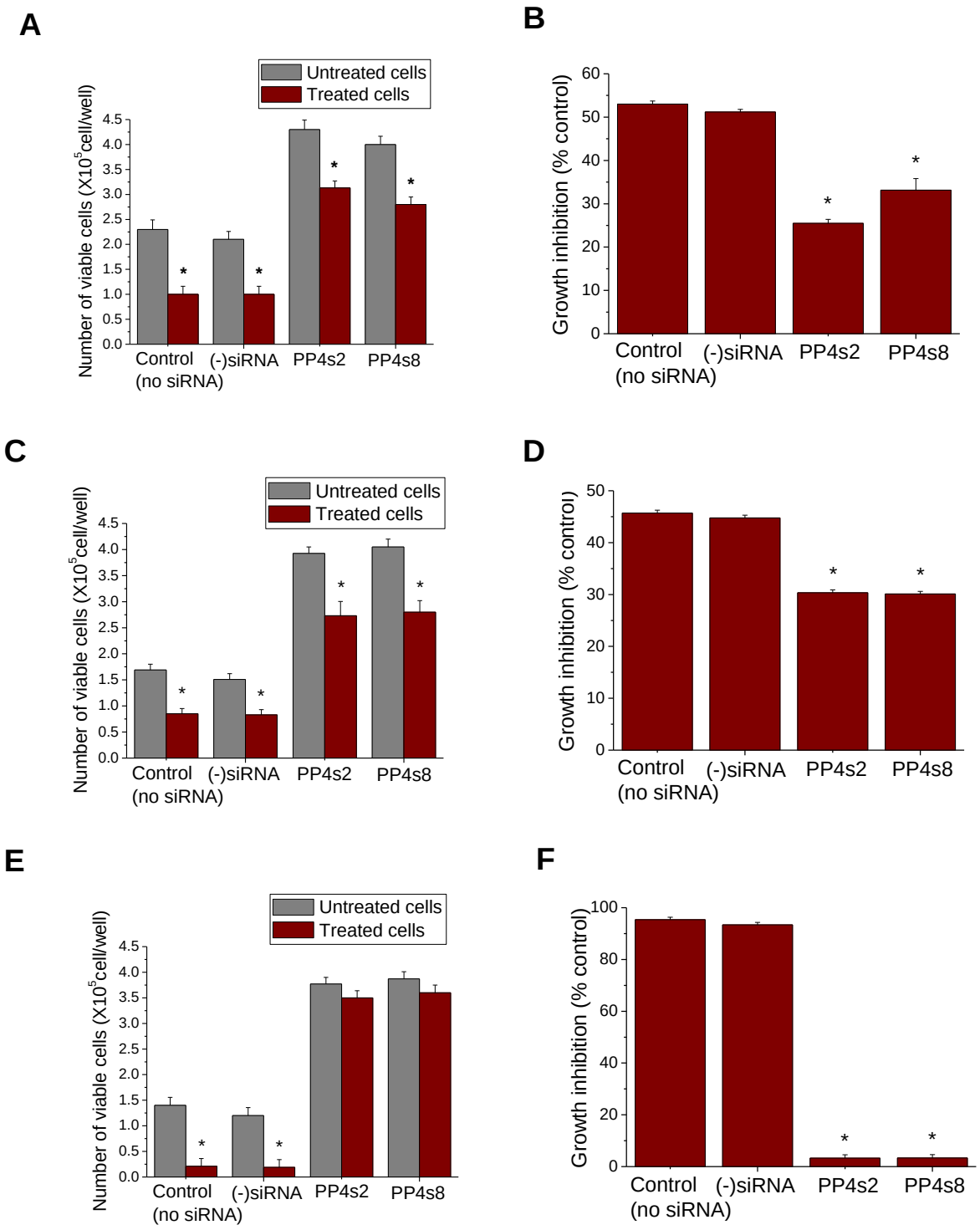


Figure 5.2: Effect of PP4c down-regulation on the response of MDAMB-231 cells to DNA damaging agents. MDAMB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. Both PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 80-90% in MDA-MB-231 cells (Figure 3.27). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by

cisplatin (A and B), doxorubicin (C and D) and 5-fluorouracil (E and F). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A, C and E). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B, D, and F). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A, C, E) and versus cells with mock transfection and cells transfected with (-)siRNA control (B, D and F) (one-way ANOVA and Bonferrroni's MCT)

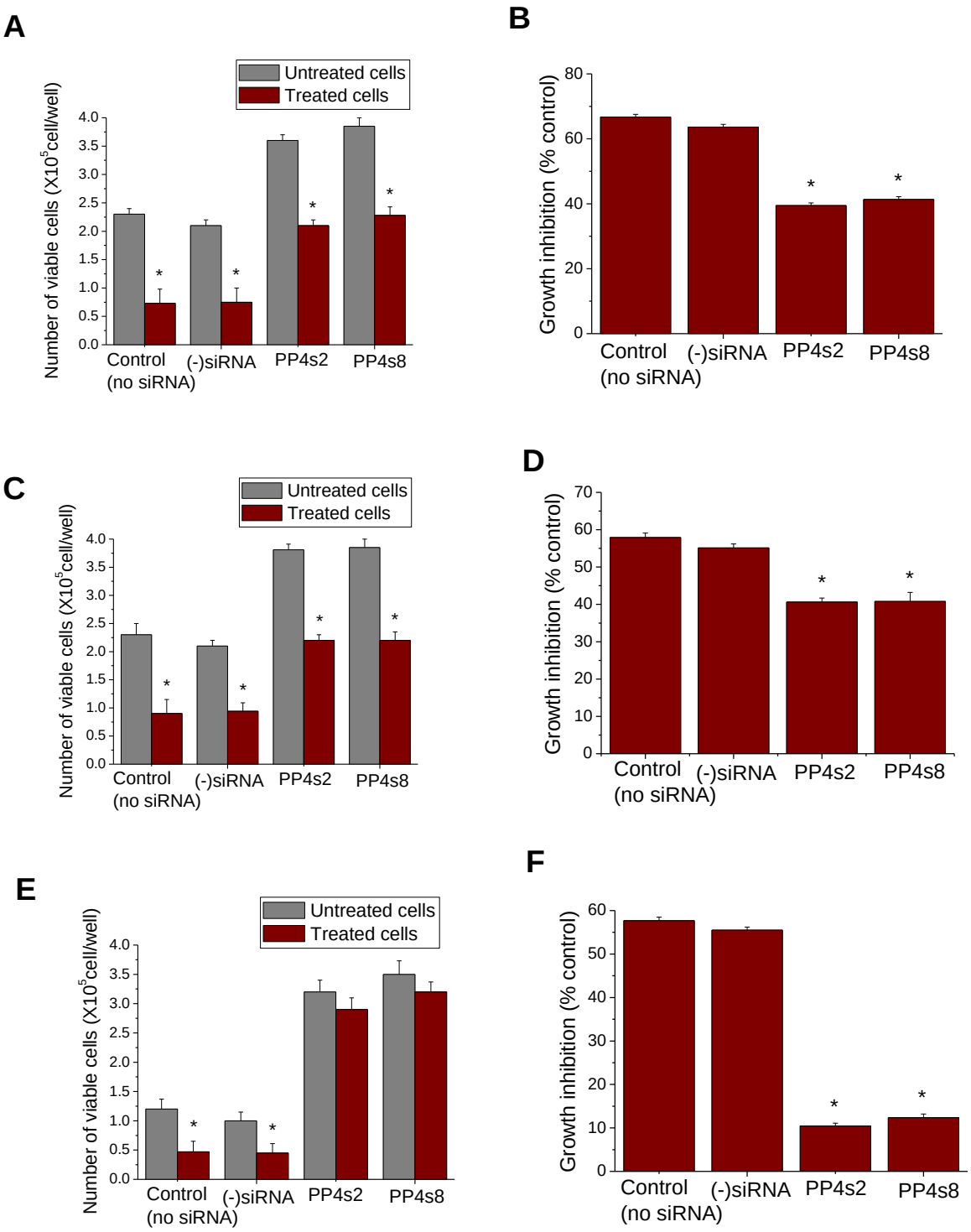


Figure 5.3: Effect of PP4c down-regulation on the response of Hs578T cells to DNA damaging agents. Hs578T cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 50-60% in Hs578T cells (Figure 3.31). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by

cisplatin (A and B), doxorubicin (C and D) and 5-fluorouracil (E and F). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A, C and E). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B, D, and F). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A, C, E) and versus cells with mock transfection and cells transfected with (-)siRNA control (B, D and F) (one-way ANOVA and Bonferrroni's MCT)

5.3.2 Effects of PP4c down-regulation on mTOR inhibitor-induced death of breast cancer cell lines

mTOR inhibitors are a class of drugs that inhibit one of the serine/threonine-specific protein kinase; mTOR (the mechanistic target of rapamycin) that belongs to the family of phosphatidylinositol-3 kinase (PI3K) related kinases (PIKKs). mTOR is a downstream effector of the PI3K/Akt pathway and through its two protein complexes; mTORC1 and mTORC2, it regulates a variety of cellular metabolic activities, cell growth, and cell proliferation (Laplante and Sabatini, 2009). mTOR was found to be deregulated in various human diseases such as cancer and type 2 diabetes (Laplante and Sabatini, 2009). The most established mTOR inhibitors are so-called rapalogs (rapamycin and its analogs), which have shown to be effective in clinical trials against various tumour types (Pópulo, 2012). mTORC1 and mTORC2 each has its specific substrates and characterisation. mTORC1 consists of mTOR and two positive regulatory subunits that are raptor and mammalian LST8 (mLST8) with other two negative regulators that are proline-rich Akt substrate 40 (PRAS40) and DEPTOR (Pópulo, 2012). mTORC1 is considered to be sensitive to rapamycin and is activated by phosphorylated Akt which results in subsequent phosphorylation of S6K1, and 4EBP1 by mTORC1 and finally results in mRNA translation (Pópulo, 2012).

mTORC2 consists of mTOR, mLST8, mSin1, protor, rictor, and DEPTOR (Meric-Bernstam and Gonzalez-Angulo, 2009). mTORC2 is considered to be resistant to rapamycin as well as to nutrient and energy signals. mTORC2 is activated by growth factors to phosphorylate PKCα, AKT and paxillin, and regulate the activity of the small GTPase, Rac, and Rho that are related to cell survival, migration and regulation of the actin cytoskeleton (Meric-Bernstam and Gonzalez-Angulo, 2009).

The impact of PP4c silencing on the response of oestrogen/progesterone positive-MCF7 and triple negative breast cancer cells-MDA-MB-231 and Hs578T to mTORC1 inhibitors and dual mTORC1,2 inhibitors was investigated. Cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-targeted siRNAs to silence PP4c expression. Two important mTORC1 inhibitors including rapamycin (1 μM) and everolimus (10 nM) with one dual mTOR1,2 inhibitor; AZD8055 (50 nM) have been used in this study. Cell survival was assessed by means of FACS analysis and MTS assay 72 hours post-treatment. In MCF7; PP4c-specific siRNAs reduced rapamycin and everolimus-induced cell death by 85% (Figure 5.4 A and B), (Figure 5.4 C and D) respectively and reduced AZD8055-induced cell death by 90% (Figure 5.5 A and B).

In triple negative cells; PP4c-specific siRNAs reduced growth inhibition of MDA-MB-231 cells in response to rapamycin by 50% (Figure 5.6 A and B) and in response to everolimus by 80% (Figure 5.6 C and D) compared to the cultures with mock transfection and the (-)siRNA transfected cultures. The two PP4c-targeted siRNAs decreased rapamycin and everolimus-induced cell death in Hs578T cells by 50% and 66.7% respectively compared to the cultures with mock transfection and the (-)siRNA transfected cultures (Figure 5.8 A and B) and (Figure 5.8 C and D) respectively. On the other hand, silencing of PP4c increased AZD8055- induced cell death of MDA-MB-231 and Hs578T cells by 50% in comparison to the control (cultures with mock transfection and cultures transfected with (-)siRNA) (Figure 5.7 A and B) and (Figure 5.9 A and B) respectively.

These data indicated that PP4c down-regulation increased resistance of oestrogen/ progesterone positive and triple negative breast cancer cells to mTOR1 inhibitors. The above data also showed that PP4c silencing increased resistance of MCF7

while increased the sensitivity of triple negative-MDA-MB-231 and Hs578T cells to dual mTOR1,2 Therefore, PP4c expression plays an important role in cell survival or cell death by interaction directly or indirectly with the downstream proteins in the mTORC1 and mTORC1, 2 signalling pathway.

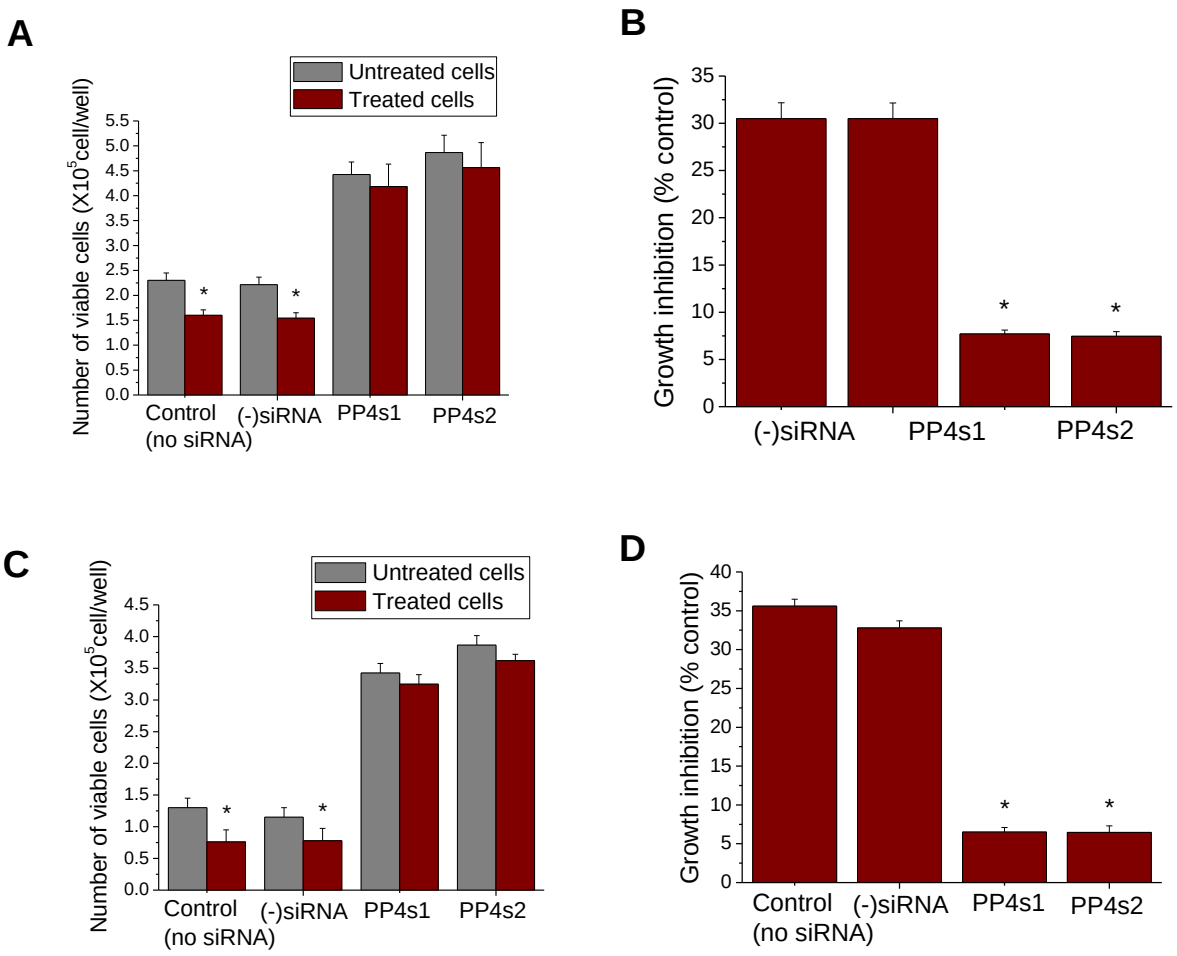


Figure 5.4: Effect of PP4c down-regulation on the response of MCF7 cells to mTORC1 inhibitors. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s1 and PP4s2 reduced the endogenous expression of PP4c protein by 50-60% in MCF7 cells (Figure 3.23). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by rapamycin (A and B), everolimus (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus cells with mock transfection and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

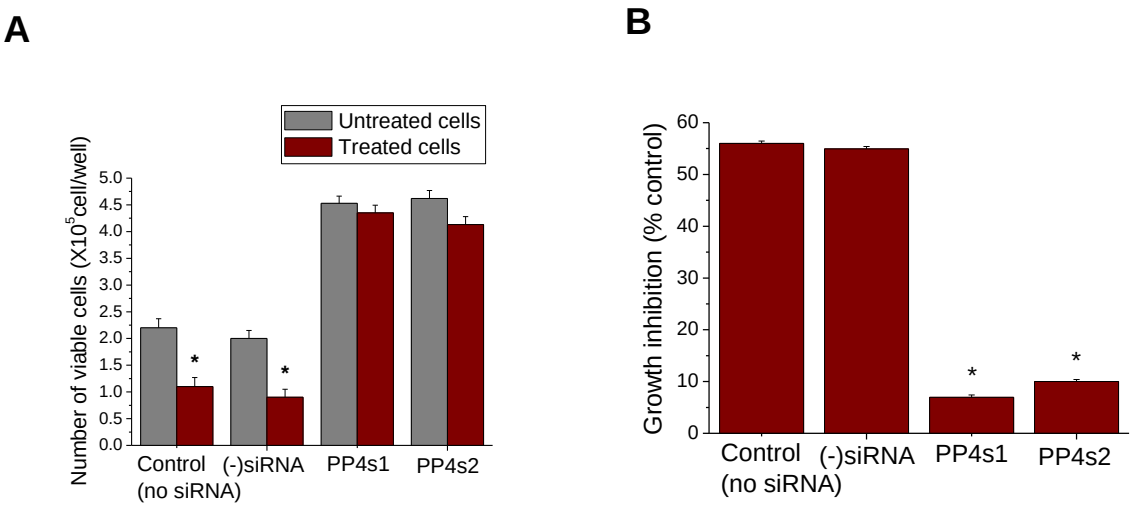


Figure 5.5: Effect of PP4c down-regulation on the response of MCF7 cells to dual mTORC1,2 inhibitors. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s1 and PP4s2 reduced the endogenous expression of PP4c protein by 50-60% in MCF7 cells (Figure 3.23). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD8055 (A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus cells with mock transfection and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

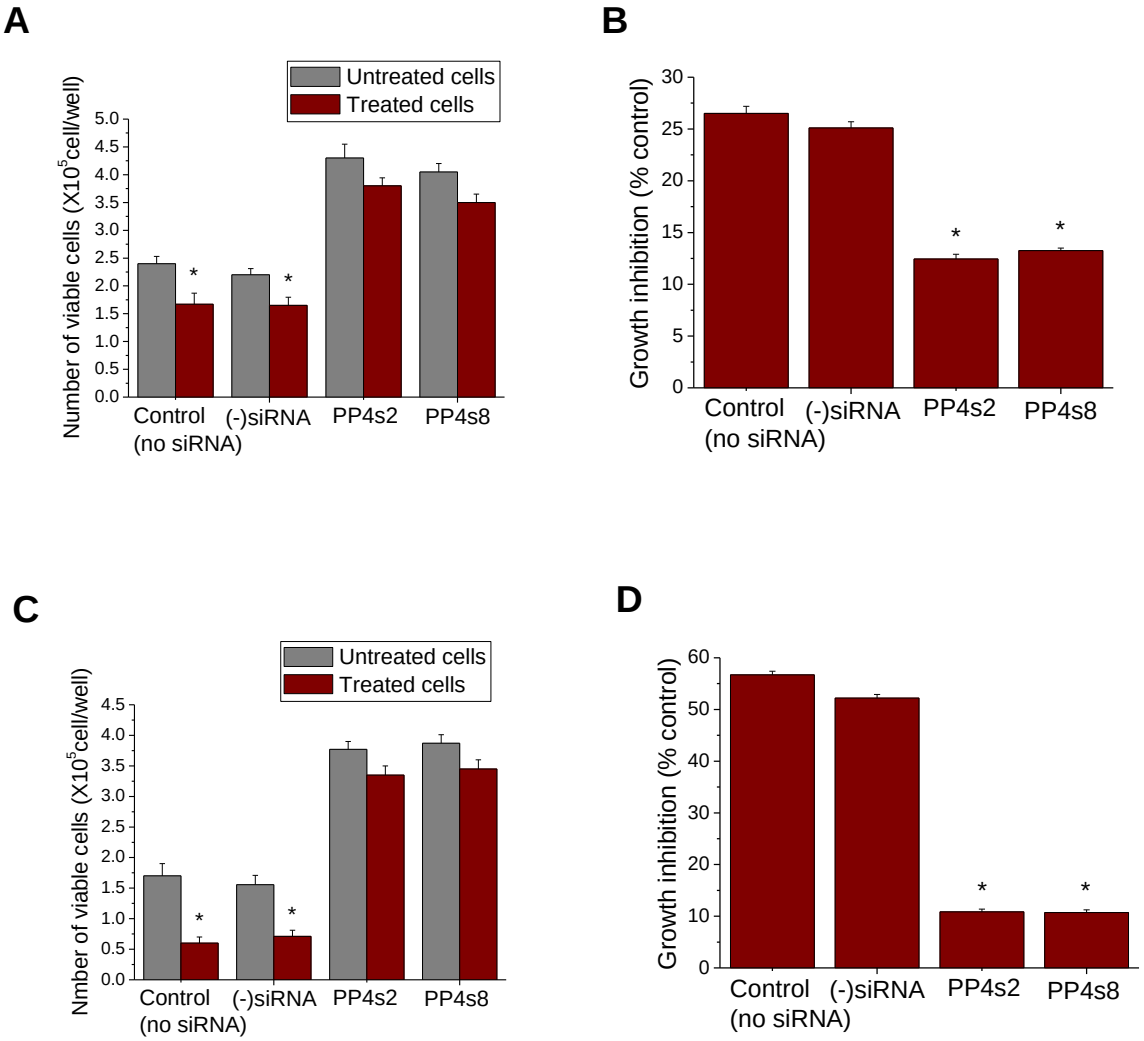


Figure 5.6: Effect of PP4c down-regulation on the response of MDA-MB-231 cells to mTORC1 inhibitors. MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. Both PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 80-85% in MDA-MB-231 cells (Figure 3.27). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by rapamycin (A and B), everolimus (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus cells with mock transfection and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

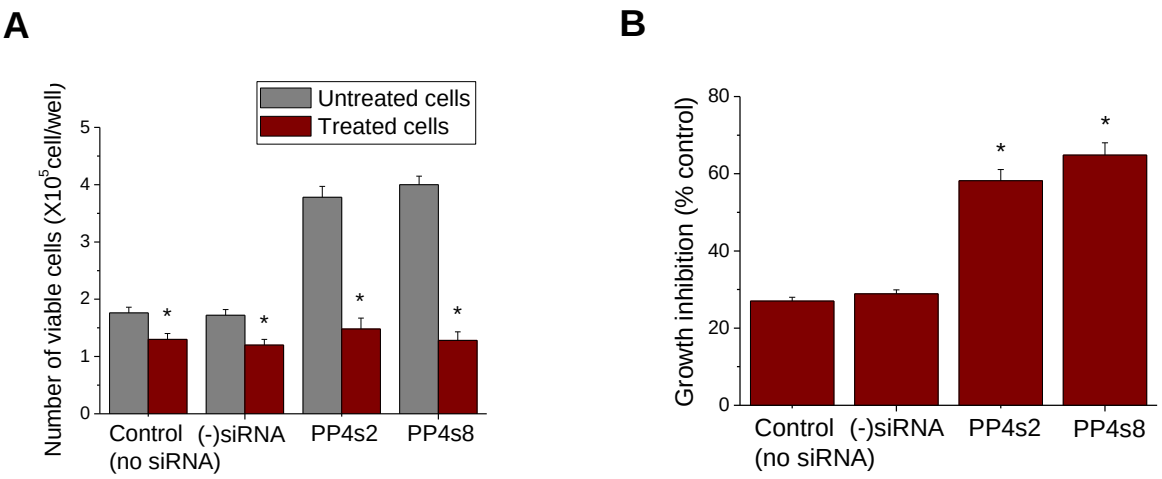


Figure 5.7: Effect of PP4c down-regulation on the response of MDA-MB-231 cells to dual mTORC1,2 inhibitors. MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. Both PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 80-85% in MDA-MB-231 cells (Figure 3.27). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD8055 (A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus cells with mock transfection and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

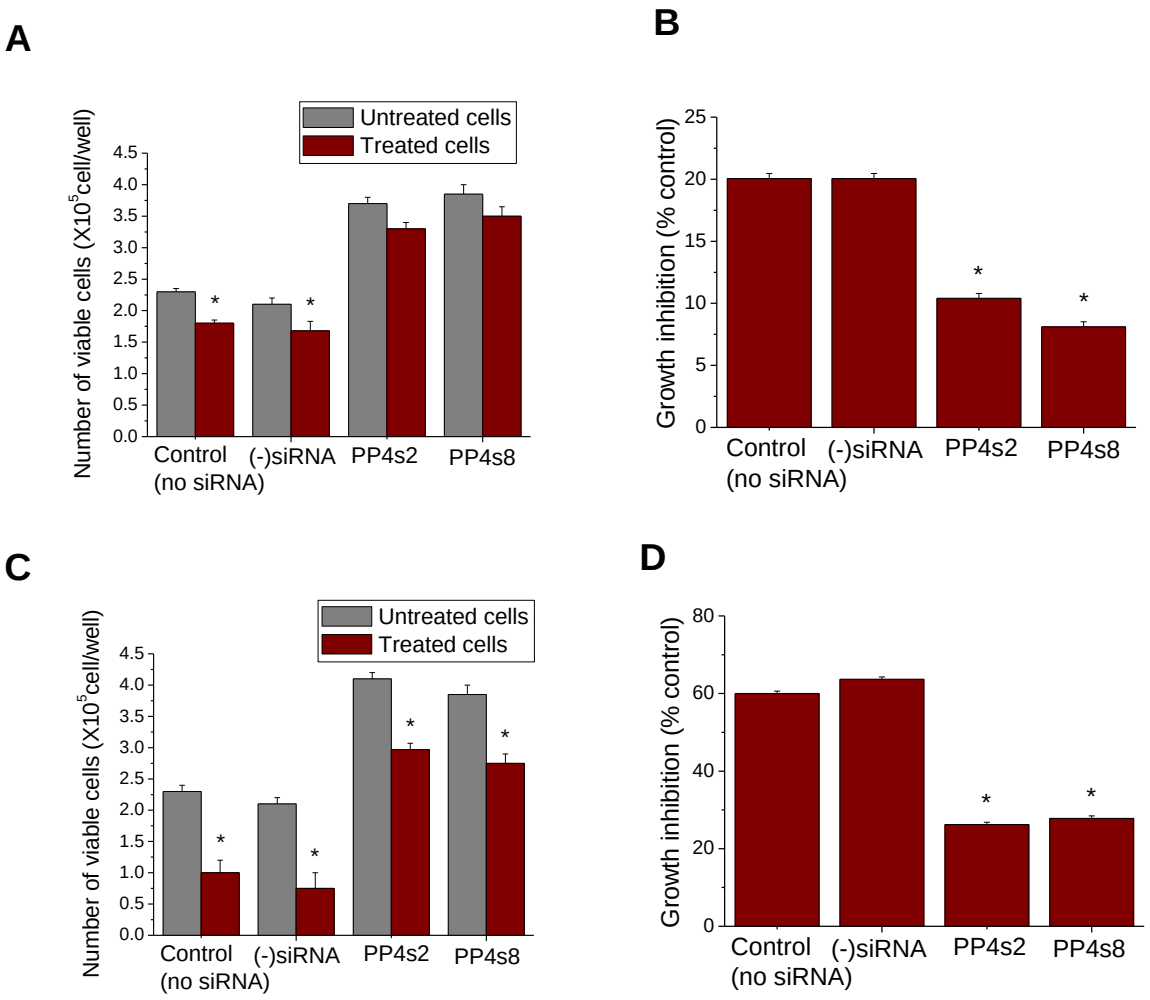


Figure 5.8: Effect of PP4c down-regulation on the response of Hs578T cells to mTORC1 inhibitors. Hs578T cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 50-60% in Hs578T cells (Figure 3.31). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by rapamycin (A and B), everolimus (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus cells with mock transfection and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

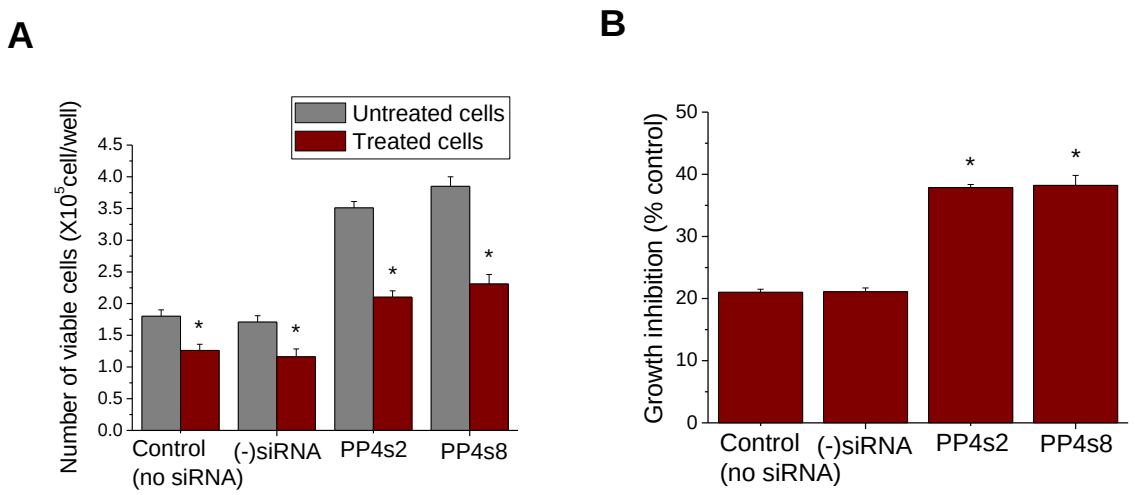


Figure 5.9: Effect of PP4c down-regulation on the response of Hs578T cells to dual mTORC1,2 inhibitors. Hs578T cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 50-60% in Hs578T cells (Figure 3.31). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD8055 (A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus cells with mock transfection and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

5.3.3 PP4c silencing affects the response of breast cancer cells to Akt, PI3K and the dual PI3K/dual mTORC1,2 inhibitors

Akt and PI3K inhibitors interfere with the Akt and the PI3K signalling pathways which control cell survival and cell proliferation in breast cancer. It would be interesting to investigate the impact of PP4c silencing on the response of breast cancer cells to the Akt and PI3K inhibitor chemotherapeutic agents. For this purpose, Cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-targeted siRNAs to silence PP4c expression. AZD5363 (10 mM) has been used as an Akt inhibitor, LY294002 (10 μM) has been used as a PI3K inhibitor and BEZ235 (100 nM) has been used as a PI3K/dual mTORC1,2 inhibitor. Cells were cultured for 72 hours post-treatment before the assessment of cell survival by FACS analysis and MTS assay. PP4c down-regulation reduced the growth inhibition produced by Akt inhibitor by 63% in MCF7 cells (Figure 5.10 A and B). Furthermore, silencing of PP4c in MCF7 cells reduced LY-294002-induced cell death by 45% in comparison to the control cells (Figure 5.10 C and D). Similarly, PP4c down-regulation in MCF7 cells reduced the growth inhibition produced by BEZ235 by 40% (Figure 5.11 A and B).

Results obtained in case of triple negative cells were different from those observed in case of MCF7 cells. In response to Akt inhibitor AZD5363, PP4c-specific siRNAs resulted in 140% increase in the proportion of growth inhibition in MDA-MB-231 cells with 100% increase in the proportion of growth inhibition in Hs578T cells compared to the control cultures (Figure 5.12 A and B), (Figure 5.14 A and B) respectively. On the other hand, silencing of PP4c did not have any effect on the response of MDA-MB-231 and Hs578T cells to the PI3K inhibitor-LY294002 (Figure 5.12 C and D) and (Figure 5.14 C and D) respectively. These data indicated that PP4c down-regulation

increased sensitivity of MDA-MB-231 cells to the Akt inhibitor and has no effect on the response of these cells to the PI3K inhibitor.

The effect of PP4c silencing on the response of triple negative cells to BEZ235 was found to be in contrast to that in MCF7 cells. PP4c silencing increased BEZ235-induced cell death of MDA-MB-231 cells by 50% for PP4s2 and 25% for PP4s8 (Figure 5.13 A and B). Similarly the two siRNA-mediated silencing of PP4c increased the % of growth inhibition of Hs578T cells on response to BEZ235 by 50% compared to the control (Figure 5.15 A and B). Results indicated that PP4c down-regulation decreased the sensitivity of oestrogen/progesterone positive cells to the Akt, PI3K inhibitors and dual PI3K/dual mTOR1,2 inhibitor while it increased sensitivity of triple negative breast cancer cells to Akt and dual PI3K/dual mTOR1,2 inhibitors and had no effect on the response of these cells to the PI3K inhibitor.

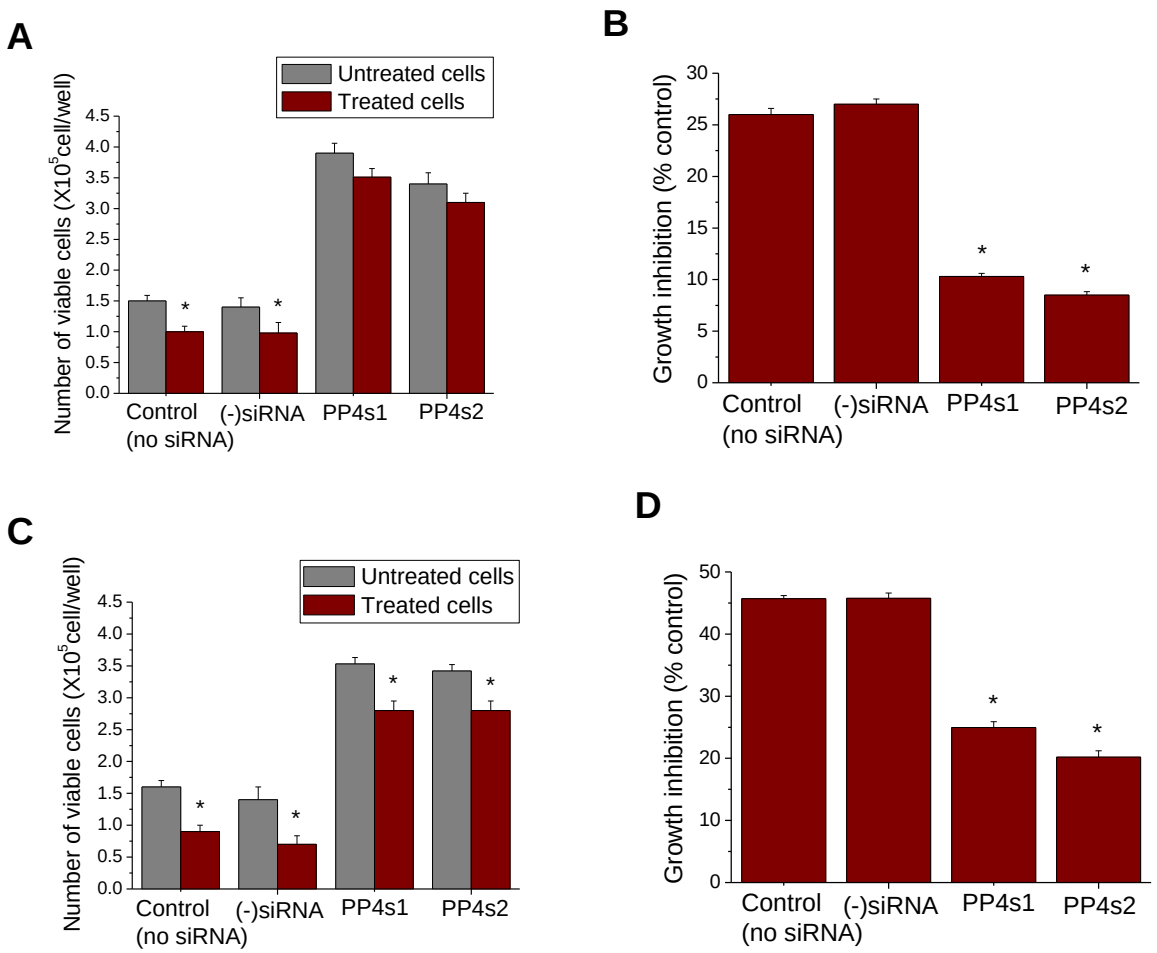


Figure 5.10: Effect of PP4c down-regulation on the response of MCF7 cells to the Akt and the PI3K inhibitors. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s1 and PP4s2 reduced endogenous expression of PP4c protein by 50-60% in MCF7 cells (Figure 3.23). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD5363 (A and B), LY294002 (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus mock transfected cells and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

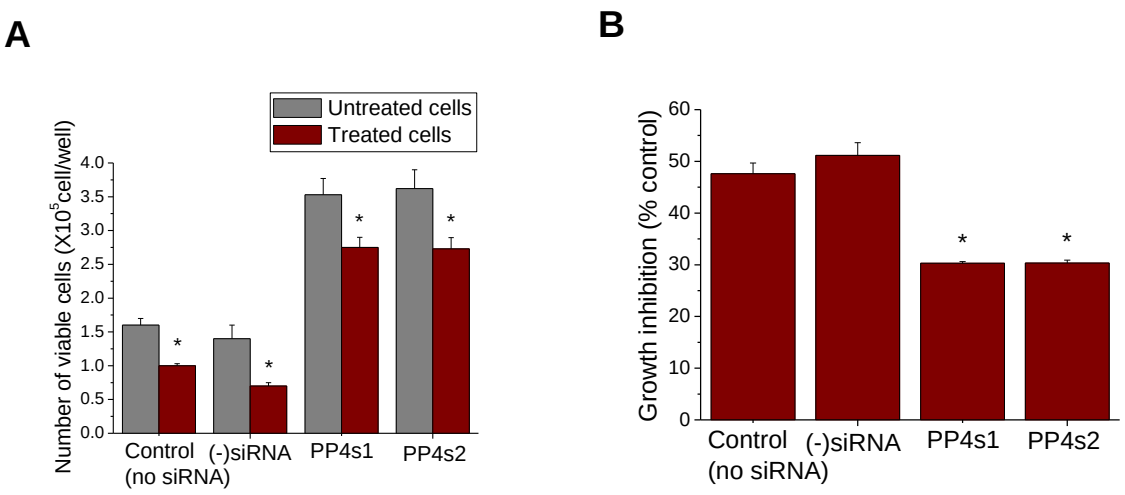


Figure 5.11: Effect of PP4c down-regulation on the response of MCF7 cells to dual PI3K/dual mTOR1C,2 inhibitor. MCF7 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s1 and PP4s2 reduced the endogenous expression of PP4c protein by 50-60% in MCF7 cells (Figure 3.23). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by BEZ235 (A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus cells with mock transfection and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

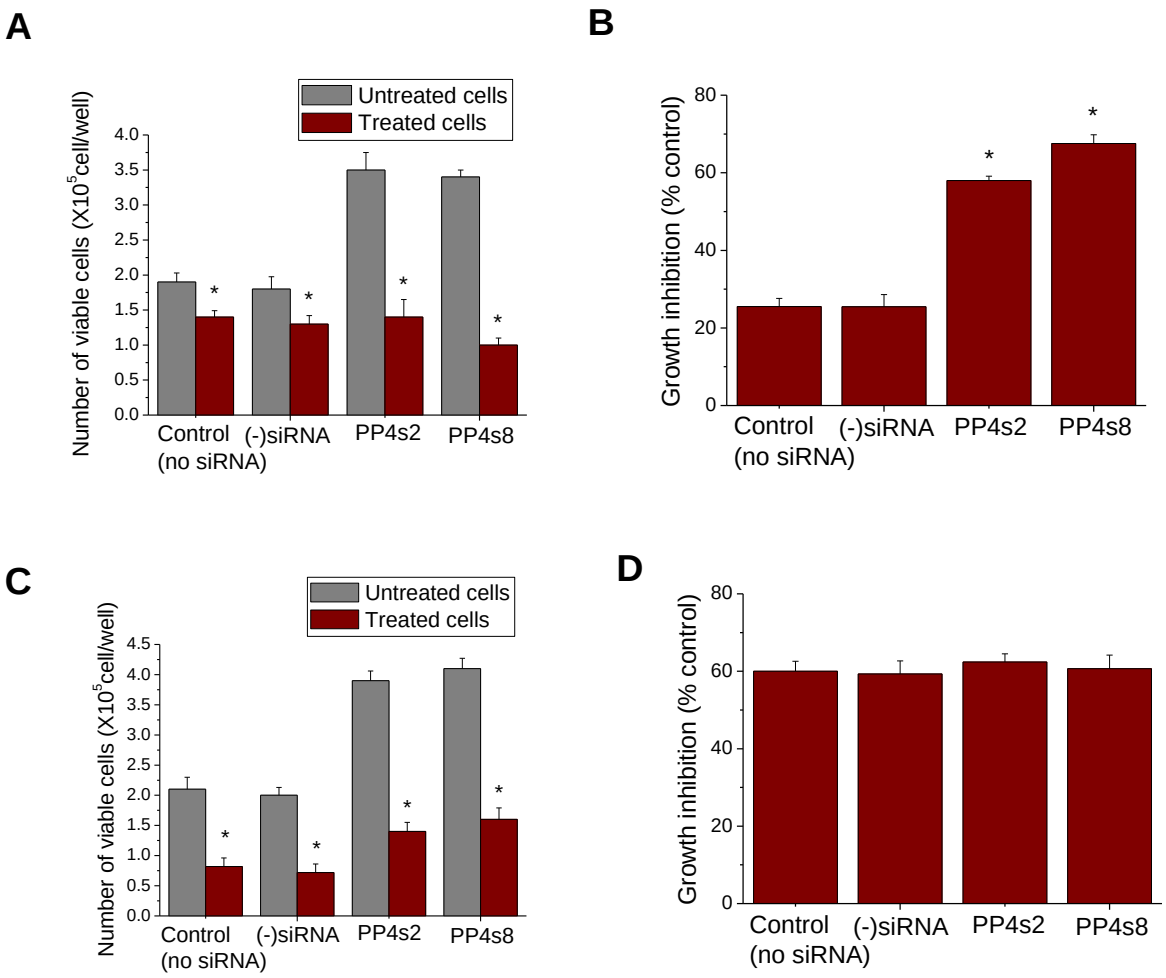


Figure 5.12: Effect of PP4c down-regulation on the response of MDA-MB-231 cells to the Akt and the PI3K inhibitors. MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. Both PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 80-90% in MDA-MB-231 cells (Figure 3.27). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD5363 (A and B), LY294002 (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus cells with mock transfection and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

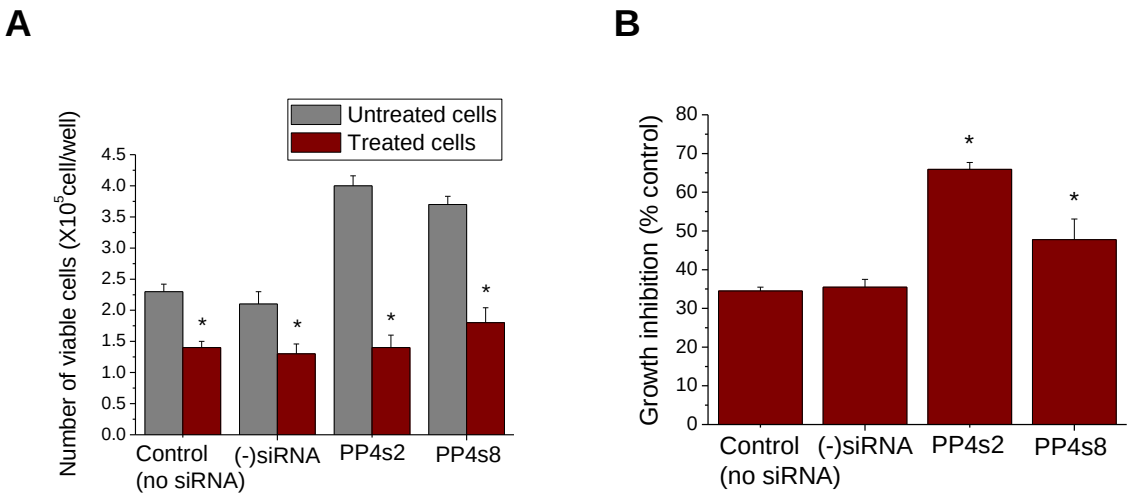


Figure 5.13: Effect of PP4c down-regulation on the response of MDA-MB-231 cells to the dual PI3K/ dual mTORC1,2 inhibitor. MDA-MB-231 cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. Both PP4s2 and PP4s8 reduced the endogenous PP4c protein levels by 80-90% in MDA-MB-231 cells (Figure 3.27). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by BEZ235 (A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus cells with mock transfection and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

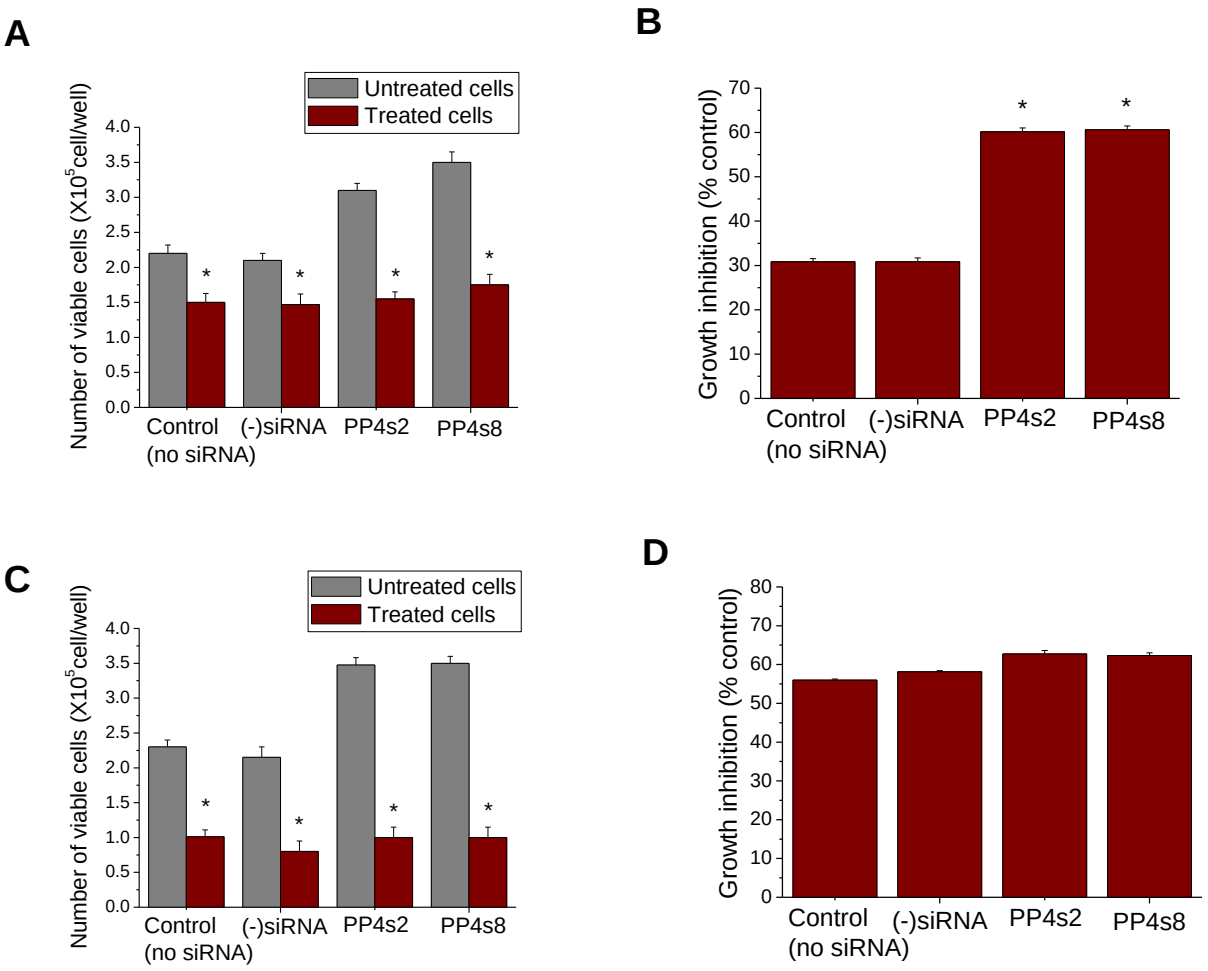


Figure 5.14: Effect of PP4c down-regulation on the response of Hs578T cells to the Akt and the PI3K inhibitors. Hs578T cells were mock transfected or transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 50-60% in Hs578T cells (Figure 3.31). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by AZD5363 (A and B), LY294002 (C and D). Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis (A and C). Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug (B and D). The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A and C) and versus mock transfected cells and cells transfected with (-)siRNA control (B and D) (one-way ANOVA and Bonferrroni's MCT)

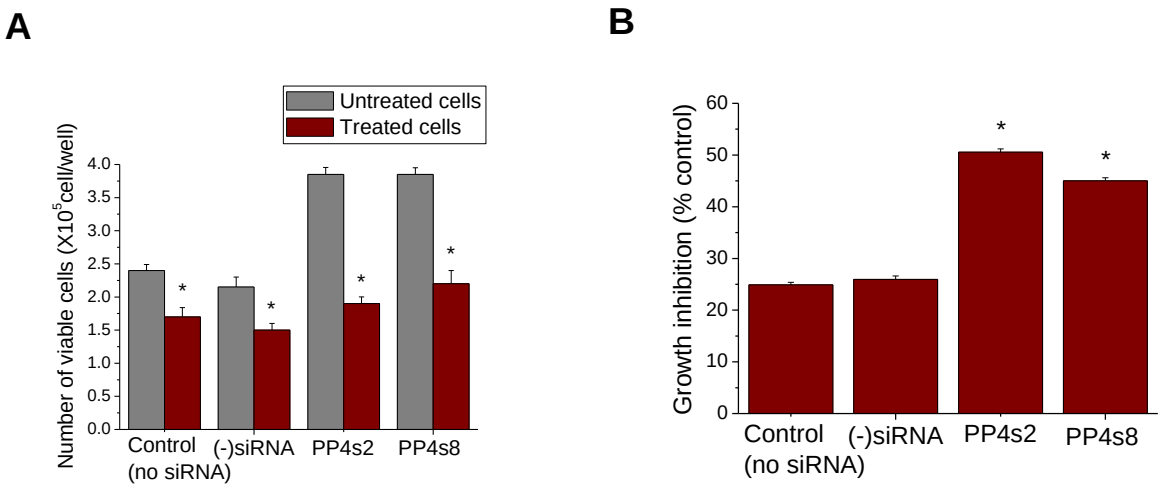


Figure 5.15: Effect of PP4c down-regulation on the response of Hs578T cells to the dual PI3K/dual mTORC1,2 inhibitor. Hs578T cells mock transfected or were transfected with (-)siRNA as a control or with PP4c-specific siRNAs. PP4s2 and PP4s8 reduced the endogenous expression of PP4c protein by 50-60% in Hs578T cells (Figure 3.31). Transfected cells were re-plated at a density of 0.8 X 10^5 cell/ml and incubated for 20 hours before being exposed to chemotherapeutic agents. Cell viability was determined 72 hours post-treatment. Effects of PP4c down-regulation on cell death induced by BEZ235(A and B). **A)** Number of viable cells in the absence and presence of chemotherapeutic drugs as determined by FACS analysis. **B)** Cell proliferation was measured using MTS assay and results are represented as the percentage inhibition of cell growth compared with control in the absence of drug. The bar graphs represent means ± S.E. from four independent experiments. *P < 0.05 versus control cells in the absence of chemotherapeutic drug (A) and versus mock transfected cells and cells transfected with (-)siRNA control (B) (one-way ANOVA and Bonferrroni's MCT)

5.3.4 Modulation of PP4c expression and its impact on the phosphorylation status of proteins in the Akt/mTOR pathway in breast cancer cells

The cellular level of PP4c has an important influence on cell growth and total apoptosis in hormone positive and in triple negative breast cancer cell lines under both basal condition and after induction of cell death by a range of anti-cancer drugs. This suggests that PP4c may interact with the signalling pathways that are targeted by these drugs.

Based on the data gathered from PP4c silencing and its effects on breast cancer cell response to mTOR1, Akt, PI3K inhibitors and the dual mTOR1,2 inhibitor, it was hypothesised that modulation of PP4c affects the phosphorylation status of proteins in the Akt/mTOR pathway which have an established role in breast cancer cell survival and proliferation. Therefore, PP4c expression was up-regulated and down-regulated in the hormone positive cell line (MCF7) and in the triple negative cell line (MDA-MB-231) to identify the impact of modulating PP4c expression on the phosphorylation status of proteins involved in the Akt/mTOR pathway.

5.3.4.1 Modulation of PP4c expression in MCF7 cells affects the phosphorylation status of proteins involved in the Akt/mTOR signalling pathway

For PP4c up-regulation, MCF7 cells were transfected with pcDNA3.1 empty vector as a control or with pcDNA3.1-PP4c expression vector. Cells were harvested and PP4c over-expression was confirmed by immunoblotting 24 hours post-transfection. Cell Lysates were prepared from both types of cultures and protein quantification

was carried out using the Bradford protein assay. Analysis of the expression of the phosphorylated proteins in the Akt/mTOR pathway was carried out using the PathScan® Akt Antibody Array kit (Chemiluminescent Readout). Signals were detected using Odyssey® Imager (Li-Cor) and the spot intensity was quantified using Image Studio™ Software (version 3.1) and was normalized with the control samples transfected with pcDNA3.1. Proteins included; Akt Thr308, Akt Ser473, S6 Ribosomal Protein Ser235/236, AMPKa Thr172, PRAS40 Thr246, mTOR Ser2481, GSK-3a Ser21, GSK-3b Ser9, p70 S6 Kinase Thr389, p70 S6 Kinase Thr421/Ser424, Bad Ser112, RSK1 Thr421/Ser424, PTEN Ser380, PDK 1 Ser241, Erk1/2 Thr202/Tyr204, 4E-BP1 Thr37/46 were tested for their phosphorylation status in response to PP4c up-regulation. In MCF7 cells PP4c over-expression reduced the phosphorylation of 4E-BP1, Bad, GSK3-α, S6 ribosomal protein, PDK1, PRAS40 and PTEN (Figure 5.16). The phosphorylation level of PTEN, GSK3-α and PDK1 was decreased by about 90%. Whereas, the level of P-4E-BP1 was decreased by 60%, the level of P- PRAS40, P-Bad and P-S6 ribosomal protein was decreased by 87.1, 82.1 and 80% respectively (Figure 5.16).

PP4c down-regulation was expected to increase the phosphorylation level of proteins involved in the Akt/mTOR signalling pathway. Hence, we have used the same antibody array to characterize the phosphoproteome of the MCF7 cells that have been transfected with PP4c-specific siRNA. PP4c down-regulation in MCF7 cells increased the phosphorylation level of Bad, 4E-BP1, GSK3-α and PTEN by 250%, 150%, 83% and 55%, respectively (Figure 5.17). Comparatively small changes were observed in the phosphorylation level of PRAS40, S6 ribosomal protein and PDK which showed 36% 33% and 20% increase, respectively (Figure 5.17). These results pointed to the great importance of PP4c in the regulation of the

phosphorylation of key proteins in the Akt/mTOR pathway, these proteins are known to play an important role in the regulation of cell growth, proliferation and apoptosis.

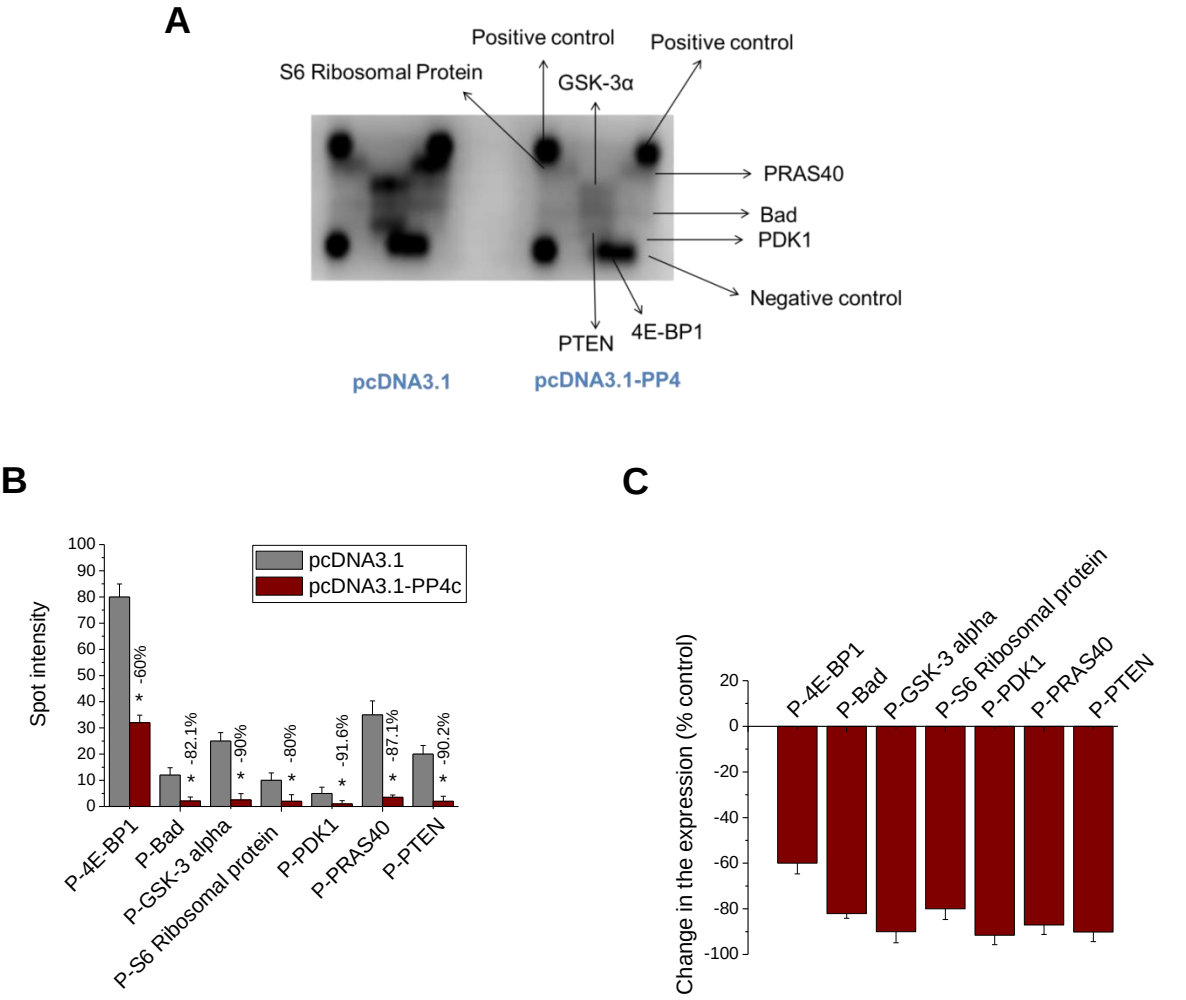


Figure 5.16: PP4c over-expression reduced the phosphorylation level of the Akt/mTOR pathway proteins in MCF7 cells. MCF7 cells were transfected with pcDNA3.1 empty vector as a control or with PcDNA3.1-PP4c. Cell lysates were prepared from the transfected cells and protein quantification was carried out using the Bradford protein assay. 75 µl of 1 mg/ml cell lysate was used. Analysis of the phosphorylation level of the proteins involved in the Akt/mTOR1 signalling pathway was carried out using the PathScan® Akt Antibody Array. Signals were detected using Odyssey® Imager (Li-Cor) and spot intensity was measured using Image Studio™ Software (version 3.1). Normalized spot intensities were used to calculate fold changes in phosphorylation levels. **A)** Representative image of the slides. **B)** Normalised spot intensities of the proteins with marked changes in their phosphorylation levels. **C)** % change in the phosphorylation levels was calculated relative to cells transfected with pcDNA3.1. The bar graphs represent means ± S.E. from three independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 empty vector (one-way ANOVA and Bonferrroni's MCT)

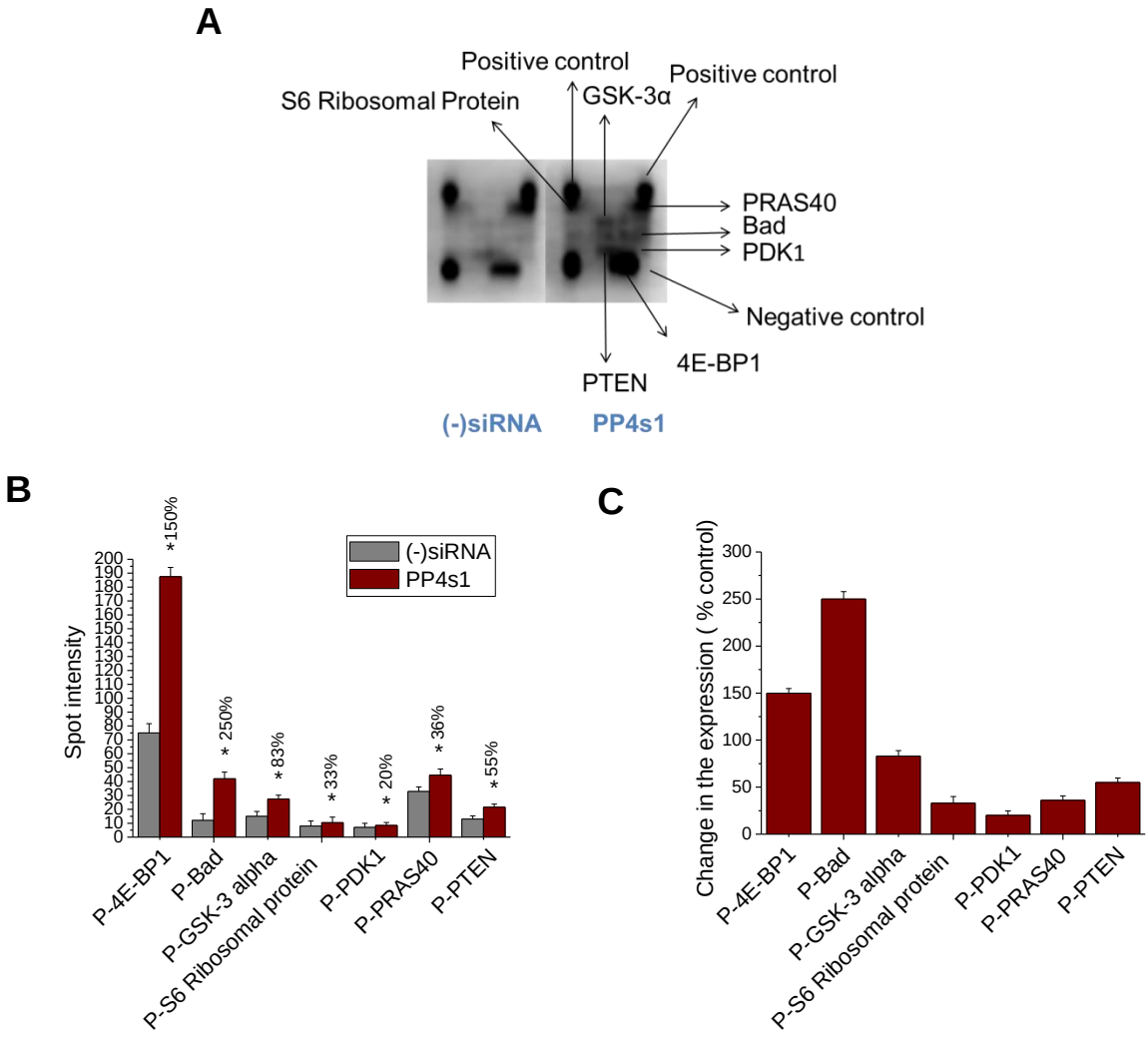


Figure 5.17: PP4c down-regulation increased the phosphorylation level of the Akt/mTOR pathway proteins in MCF7 cells. MCF7 cells were transfected with (-)siRNA or with PP4s1. Cell lysates were prepared from the transfected cells and protein quantification was carried out using the Bradford protein assay. 75 µl of 1 mg/ml cell lysate was used. Analysis of the phosphorylation levels of the proteins involved in the Akt/mTOR1 signalling pathway was carried out using the PathScan® Akt Antibody Array. Signals were detected using Odyssey® Imager (Li-Cor) and spot intensity was measured using Image Studio™ Software (version 3.1). Normalized spot intensities were used to calculate fold changes in phosphorylation levels. **A)** Representative image of the slides. **B)** Normalised spot intensities of the proteins with marked changes in their phosphorylation levels. **C)** % change in the phosphorylation levels was calculated relative to cells transfected with (-)siRNA. The bar graphs represent means ± S.E. from three independent experiments. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

5.3.4.2 Modulation of PP4c expression in the triple negative breast cancer cells changes the phosphorylation status of proteins involved in the Akt/mTOR signalling pathway

The effect of PP4c over-expression on the phosphorylation status of proteins in the Akt/mTOR pathway in the triple negative-MDA-MB-231 cells was investigated. MDA-MB-231 cells were transfected with pcDNA3.1 empty vector as a control or with pcDNA3.1-PP4c expression vector. Cells were harvested and PP4c over-expression was confirmed by immunoblotting 24 hours post-transfection. Cell Lysates were prepared from both types of cultures and protein quantification was carried out using the Bradford protein assay. Analysis of the expression of the phosphorylated proteins in the Akt/mTOR pathway was carried out using the PathScan® Akt Antibody Array kit (Chemiluminescent Readout). Signals were detected using Odyssey® Imager (Li-Cor) and spot intensity was quantified using Image Studio™ Software (version 3.1) and was normalized with that of the control samples transfected with pcDNA3.1. In MDA-MB-231 cells; PP4c up-regulation decreased the phosphorylation level of Bad, PDK1, PRAS40 and PTEN by 20%, 33%, 22% and 30%, respectively (Figure 5.18). On the other hand, PP4c up-regulation in MDA-MB-231 cells increased the phosphorylation level of 4E-BP1, GSK3-α and S6-ribosoml protein by 40%, 47% and 46.3% respectively (Figure 5.18). These data showed that PP4c up-regulation in triple negative cell line involving MDA-MB-231 changed the phosphorylation status of various proteins in the Akt/mTOR1 pathway and these changes are likely to have an important impact on the cell outcome in terms of cell growth, proliferation and apoptosis.

To investigate the effect of PP4c down-regulation on the phosphorylation status of proteins in the Akt/mTOR pathway in triple negative breast cancer cells, MDA-MB-231 cells were transfected with (-)siRNA as a control or with PP4s2. Cells were harvested and PP4c down-regulation was confirmed 72 hours post-transfection. Proteins were extracted from the transfected cells and protein quantification was carried out using the Bradford protein assay. The expression of the phosphorylated proteins in the Akt/mTOR pathway was analysed using the PathScan® Akt Antibody Array kit (Chemuliscence Readout).

In contrast to MCF7, the phosphorylation level of 4E-BP1, GSK3-α, S6 ribosomal protein were decreased by 45.2%, 80% and 95% respectively in MDA-MB-231 in response to the PP4c down-regulation (Figure 5.19). However, the phosphorylation of other proteins including Bad, PDK1, PRAS40 and PTEN was increased by 20%, 95%, 20% and 85%, respectively, in response to the PP4c down-regulation (Figure 5.19). These observations therefore illustrate the complexity of the cellular phosphorylation/dephosphorylation system regulated by PP4c in MDA-MB-231 cell line. The data provided above showed that PP4c induces its effect on oestrogen/progesterone-positive cells and TNBC cells most likely through its effect on the phosphorylation status of critical proteins in the Akt/mTOR pathway.

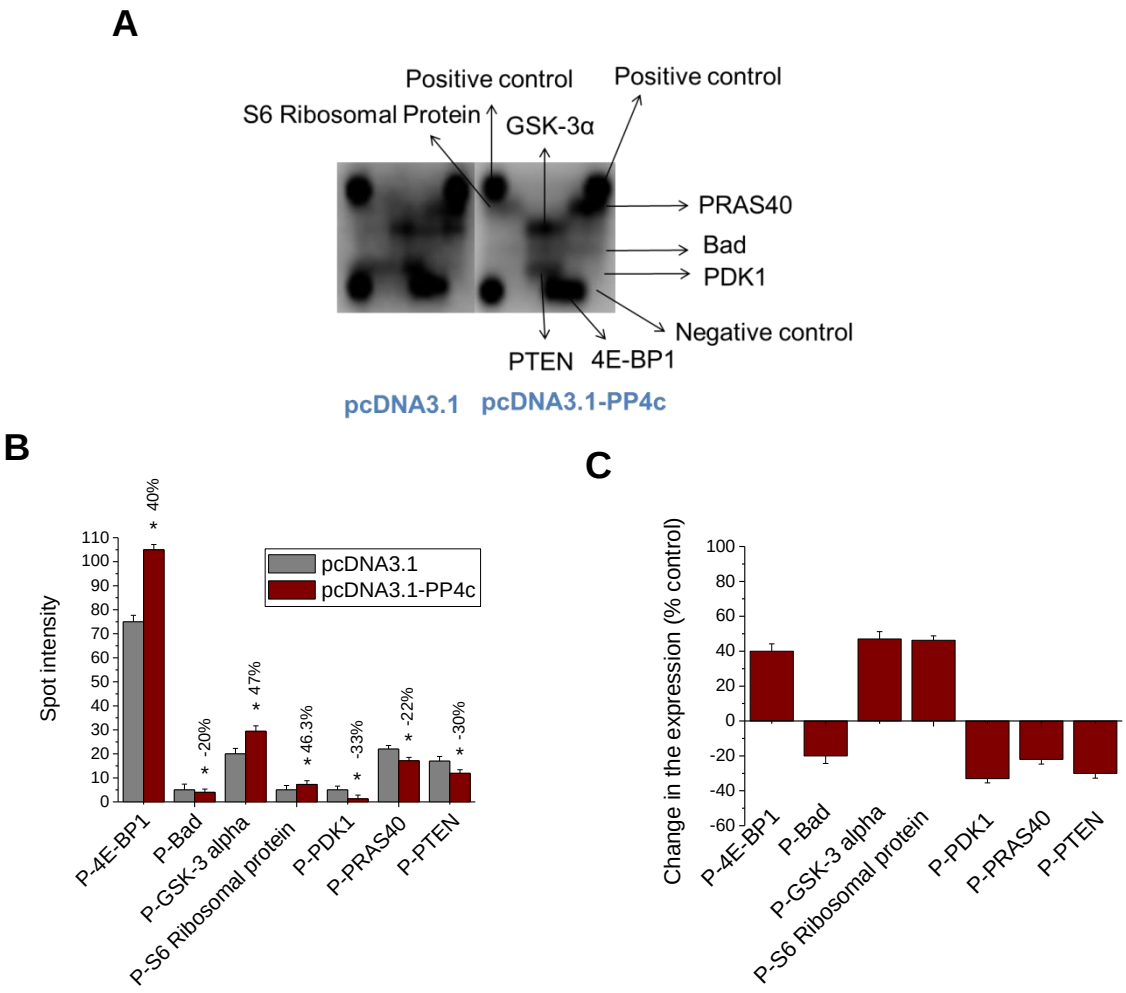


Figure 5.18: PP4c over-expression changes the phosphorylation status of Akt/mTOR pathway proteins in MDA-MB-231 cells. MDA-MB-231 cells were transfected with pcDNA3.1 empty vector as a control or with pcDNA3.1-PP4c. Cell lysates were prepared from the transfected cells and protein quantification was carried out using the Bradford protein assay. 75 µl of 1 mg/ml cell lysate was used. Analysis of the phosphorylation levels of the proteins involved in the Akt/mTOR1 signalling pathway was carried out using the PathScan® Akt Antibody Array. Signals were detected using Odyssey® Imager (Li-Cor) and spot intensity was measured using Image Studio™ Software (version 3.1). Normalized spot intensities were used to calculate fold changes in phosphorylation levels. **A)** Representative image of the slides. **B)** Normalised spot intensities of the proteins with marked changes in their phosphorylation levels. **C)** % change in the phosphorylation levels was calculated relative to cells transfected with pcDNA3.1. The bar graphs represent means ± S.E. from three independent experiments. *P < 0.05 versus cells transfected with pcDNA3.1 empty vector (one-way ANOVA and Bonferrroni's MCT)

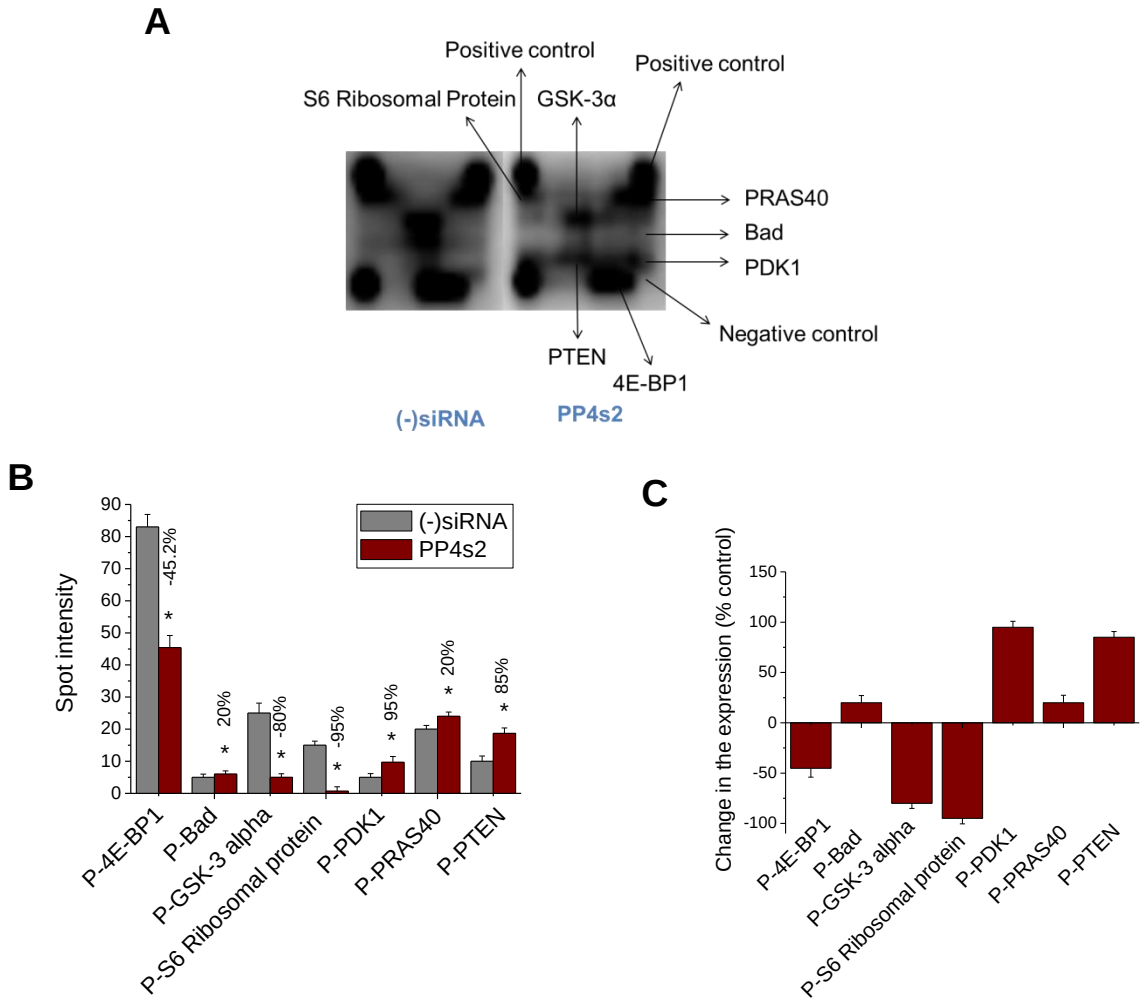


Figure 5.19: PP4c down-regulation changes the phosphorylation level of the Akt/mTOR pathway proteins in MDA-MB-231 cells. MDA-MB-231 cells were transfected with (-)siRNA as a control or with PP4s2. Cell lysates were prepared from the transfected cells and protein quantification was carried out using the Bradford protein assay. 75 µl of 1 mg/ml cell lysate was used. Analysis of the phosphorylation levels of the proteins involved in the Akt/mTOR1 signalling pathway was carried out using the PathScan® Akt Antibody Array. Signals were detected using Odyssey® Imager (Li-Cor) and spot intensity was measured using Image Studio™ Software (version 3.1). Normalized spot intensities were used to calculate fold changes in phosphorylation levels. **A)** Representative image of the slides. **B)** Normalised spot intensities of the proteins with marked changes in their phosphorylation levels. **C)** % change in the phosphorylation levels was calculated relative to cells transfected with (-)siRNA. The bar graphs represent means ± S.E. from three independent experiments. *P < 0.05 versus cells transfected with (-)siRNA (one-way ANOVA and Bonferrroni's MCT)

5.4 Discussion

This study was designed to investigate the effects of PP4c silencing on the response of hormone positive and triple negative breast cancer cells to various chemotherapeutic agents. Breast cancer is the most common cancer in women, and optimising breast tumour therapies is intended to increase the cure rates in the earliest stages of the disease thus improving the life expectancy for patients. In some cases, the targeted therapies perform their function just like the antibodies made naturally by the body's immune system (Burstein, 2011). The advance of strategies combining directed therapies with molecular therapies is the most efficient and logical way ahead to combat the development of drug-resistant tumours. In addition to chemotherapy and hormone therapy, currently, there are effective treatments geared towards attacking particular breast cancer cells without having to harm the normal functioning of the body cells (Burstein, 2011). Targeted cancer therapies are cancer treatments which target certain traits of the tumour cells, for instance, proteins which allow the tumour cells to grow rapidly or abnormally. The targeted methods in most cases are used together with traditional chemotherapy. Nonetheless, targeted drugs have less severe side effects as compared to the standard drugs used in chemotherapy. Breast cancer-targeted therapy makes use of drugs that hinder the growth of breast tumour cells in a particular manner. For instance, targeted therapy may hamper the activity of the abnormally expressed protein HER2 which facilitates the growth of tumour cells in the breast (Barillot et al., 2012).

Therapists have come up with a solution to deal with tumour resistance to targeted-breast cancer therapies. PP4c is of particular interest since it is over-expressed in breast, lung and pancreatic cancers (Chapter 4; Wang et al., 2008; Weng et al.,

2012). Over-expression of PP4c correlated with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma (Weng et al., 2012). Although functional analysis has shown that increased level of PP4c has pro-apoptotic and anti-survival effects on breast cancer cells (Chapter 3) and expression studies have shown increased expression of PP4c in breast cancer samples, findings in this chapter demonstrate for the first time that PP4c silencing changes the response of breast cancer cells to various chemotherapeutic agents. We have demonstrated that silencing of PP4c reduced the sensitivity of MCF7 cells to various chemotherapeutic agents such as DNA damaging agents, mTORC1 inhibitors, mTORC1,2 inhibitor, PI3K inhibitor, dual PI3K/dual mTORC1,2 inhibitor, and Akt inhibitor. Although in triple negative breast cancer cells (MDA-MB-231 and Hs578T), the effect of PP4c silencing on the response of these cells to various chemotherapeutic agents is complicated.

To start with, the study has showed that PP4c silencing attenuated growth inhibition of the oestrogen/progesterone-positive breast cancer cells (MCF7) and triple negative breast cancer cells (MDA-MB-231 and Hs578T) in response to the DNA damaging agents such as cisplatin, doxorubicin and 5- fluorouracil (5FU). These results are in agreement with the results reported by Takahashi et al (2013) which showed that TNBC displayed resistance to 5-fluoracil through the up-regulation of DNA recombination, cell cycle activation and negative regulation of the cell death. In our results, the possible mechanism of TNBC resistance to DNA damaging agents may be related to the negative regulation of cell death which was observed in this study as a result of PP4c down-regulation. This negative regulation of apoptosis may be caused by activating the tumour promoting molecules or inactivating the tumour suppressors involved in the Akt/PI3K/mTORC1,2 pathway through the absence of

PP4c mediated dephosphorylation events. Other mechanisms behind 5-fluoracil resistance may be through control of the cell cycle as PP4c is involved in regulating maturation and growth of centrosome and nucleation during the cell cycle (Helps et al., 1998; Sumiyoshi et al., 2002). Another study carried out by Smith et al (2006) concluded that dephosphorylation or reduced expression of phosphorylated ERK enzyme in the MAPK pathway was a possible mechanism for TNBC resistance to doxorubicin. Several other studies showed the sensitivity to cisplatin is modulated by regulation of mTOR, JNK and NF-kB pathways by the over-expression of PP4c (Mourtada-Maarabouni et al., 2008; Yeh et al., 2004). This strengthens the idea that PP4c is an important cause of fluctuation in the phosphorylation and dephosphorylation status of proteins in the MAPK pathway.

The signalling pathways in mTOR are deemed to accommodate various downstream pathways which regulate the progression of the cell cycle, cell proliferation, translation, cell survival, protein stability and the transcriptional responses (Wagle et al., 2014). mTOR is a downstream effector of PI3K and Akt; it forms mTORC1 and mTORC2 as the major multi-protein complexes (Loewith et al., 2002). Thus, deregulation of the mTOR pathway such as by over-expression of Akt or by mutation of PI3K has been linked to various types of cancer (Burris, 2013). mTORC2 is activated by the growth aspects of a cell such as growth factors whereas mTORC1 is activated by the phosphorylated Akt. mTORC1 is extremely sensitive to rapamycin whereas mTORC2 is insensitive to rapamycin and it is considered to be also insensitive to the nutrient levels. Results of this study showed that PP4c silencing attenuated growth inhibition in MCF7, MDA-MB-231 and Hs578T on response to mTOR1 inhibitors. Everolimus and rapamycin are mTORC1 inhibitors and are used currently in immunosuppressive therapies in patients with organ transplantation

(Wagle et al., 2014). Modelling of rapamycin bounds to mTOR suggests that rapamycin has no effect on inhibiting substrate recruitment (Baselga et al., 2012). However, rapamycin blocks the correct alignment of other substrates to the catalytic cleft (Baselga et al., 2012). This explains why rapamycin is extremely effective in blocking phosphorylation of ribosomal protein S6 kinase. Moreover, the effects of clinical anticancer mTORC1 inhibitors are mainly restricted to the advanced breast carcinomas which are positive for the oestrogen receptor or are negative for HER2 (Nagaraj and Ma 2015). There is a limited success with rapamycin as a cancer therapy since it does not prevent modification of some of the mTORC1 substrates and is ineffective on mTORC2 (Wagle et al., 2014). This drove development of mTOR inhibitor Torin 1 that is an effective inhibitor of both mTORC1 and mTORC2. Current study also demonstrates that PP4c silencing decreased sensitivity of MCF7 cells to dual mTORC1,2 inhibitor (AZD8055) and dual PI3K/dual mTOR inhibitor (BEZ235). Additionally, PP4c silencing increased sensitivity of MDA-MB-231 and Hs578T cells to dual mTOR1,2 inhibitor (AZD8055) and PI3K/dual mTOR1,2 inhibitor (BEZ235).

PI3K and Akt are crucial signalling pathways involved in the resistance of breast cancer cells to chemotherapy (Lien et al., 2016). Akt inhibitor (AZD5363) and PI3K inhibitor (LY 294002) were found to inhibit cell proliferation in MCF7 cells and to induce apoptosis (Burris, 2013). Results of this study showed that PP4c silencing reduced growth inhibition in MCF7 cells in response to Akt inhibitor (AZD5363) and PI3K inhibitor (LY 294002). Moreover, PP4c silencing increased the percentage of growth inhibition in MDA-MB-231 and Hs578T cells in response to Akt inhibitor (AZD5363) and it had no effect on response of MDA-MB-231 and Hs578T to PI3K inhibitor. MDA-MB-231 cells are considered to be a home of various mutation sites

that enable generation of genes expression signatures which are linked to certain metastatic sites (Burris, 2013). The nature of these sites provides an environment that enables PP4c silencing to increase the growth percentage inhibition in MDA-MB-231 in response to Akt inhibitor (Lien et al., 2016).

The exact mechanism behind the changes in the sensitivity of the breast cancer cells to these chemotherapeutic agents is still not clear. From the above data, it can be concluded that PP4c expression is one of the controlling mechanism of many important proteins in the PI3K/Akt/mTOR pathway many of which are targeted by the therapeutic agents, changes in the PP4c expression resulted in changes in the phosphorylation status of these proteins with the resulted changes in the activity of these proteins. In order to investigate the above-mentioned hypothesis, the effect of modulation of PP4c expression on the phosphorylation status of different proteins in the Akt/mTOR pathway was tested. From the results of this study, over-expression of PP4c in MCF7 cells directly or indirectly reduced the phosphorylation of several proteins in the Akt/ mTOR pathway such as 4E-BP1 (4E-binding protein 1), Bad (Bcl-2-associated death promoter), GSK3-α (Glycogen synthase kinase 3-α), S6 ribosomal protein, PDK1 (3-phosphoinositide dependent protein kinase-1), PRAS40 (Proline-rich AKT1 substrate 1 of 40 kDa) and PTEN (Phosphatase and tensin homolog). The phosphorylation level of PTEN, GSK3-alpha and PDK1 was decreased by about 90%. Whereas, the level of P-4E-BP1 was decreased by 60%, and the level of P- PRAS40, P-Bad and P-S6 ribosomal protein was decreased by 87.1, 82.1 and 80% respectively (Table 5.1). This study found out that PP4c up-regulation in the triple negative MDA-MB-231 cells decreased the phosphorylation level of Bad, PDK1, PRAS40 and PTEN by 20%, 33%, 22% and 30%, respectively. On the other hand, up-regulation of PP4c in MDA-MB-231 breast cancer cells

increased the phosphorylation level of 4E-BP1, GSK3-α and S6-ribosoml protein by 40%, 47% and 46.3% respectively (Table 5.1).

Phosphorylated protein	MCF7 cells	MDA-MB-231 cells
4E-BP1 Thr37/46	-60%	+40%
Bad Ser112	-82.1%	-20%
GSK3-α Ser21	-90%	+47%
S6 ribosomal protein Ser235/236	-80%	+46.3%
PDK1 Ser241	-91.6%	-33%
PRAS40 Thr246	-87.1%	-22%
PTEN Ser380	-90.2%	-30%

Table 5.1: Effect of PP4c up-regulation on the phosphorylation level of proteins involved in the Akt/mTOR signalling pathway in both MCF7 and MDA-MB-231 cell lines

Proteins such as Bad, PDK1, PRAS40 and PTEN are linked to the growth and progression of cancer cells in the human body (Nader and Suzanne, 2009). On the other hand, proteins such as of 4E-BP1, GSK3-α and S6-ribosoml are associated with repressing development of cancer cells in the human body (Zhou et al., 2015). PP4c up-regulation in the triple negative-MDA-MB-231 cells reduced phosphorylation of proteins that mediate the growth of cancer cells (Zhao et al., 2015). This agreed with our results which showed that PP4c up-regulation in MDA-MB-231 cells reduced the phosphorylation of Bad, PDK1, PRAS40 and PTEN.

Down-regulation of PP4c increased the survival, proliferation and the anchorage dependent growth of the breast cancer cells (Chapter 3). There is a potential link among the level of PP4c expression, metastasis and tumourigenesis (Mourtada-Maarabouni and Williams 2009). As presented in the results of this study, the phosphorylation level of the protein components of MCF7 and MDA-MB-231 varies greatly in response to PP4c down-regulation. In MCF7 cells PP4c down-regulation increased the phosphorylation level of Bad, 4E-BP1, GSK3-α and PTEN by 250%, 150%, 83% and 55%, respectively. Comparatively small changes were observed in the phosphorylation level of PRAS40, S6 ribosomal protein and PDK which showed 36% 33% and 20% increase, respectively (Table 5.2). In MDA-MB-231 cells the phosphorylation level of 4E-BP1, GSK3-α and S6 ribosomal protein were decreased by 45.2%, 80% and 95% respectively in response to PP4c down-regulation. However, the phosphorylation of other proteins including Bad, PDK1, PRAS40 and PTEN was increased by 20%, 95%, 20% and 85%, respectively, in response to the PP4c down-regulation in MDA-MB-231 cells (Table 5.2).

Phosphorylated protein	MCF7 cells	MDA-MB-231 cells
4E-BP1 Thr37/46	+150%	-45.2%
Bad Ser112	+250%	+20%
GSK3-α Ser21	+83%	-80%
S6 ribosomal protein Ser235/236	+33%	-95%
PDK1 Ser241	+20%	+95%
PRAS40 Thr246	+36%	+20%
PTEN Ser380	+55%	+85%

Table 5.2: Effect of PP4c down-regulation on the phosphorylation level of proteins involved in the Akt/mTOR signalling pathway in both MCF7 and MDA-MB-231 cell lines

PDK1 is a serine/threonine kinase that localizes to the plasma membrane by recognizing the phosphoinositides phosphorylated in position 3 by phosphatidylinositol 3 kinase (PI3K) through its C-terminal pleckstrin homology (PH) domain, and this event results in phosphorylation and activation of Akt (Currie et al., 1999). Other PDK1 substrates such as p70S6K, SGK, RSK, and PKC isoforms lack such PH domains (Toker and Newton, 2000) and these substrates are activated via binding of PDK1 to the hydrophobic motif (HM) on these substrates through its PDK1-interacting fragment (PIF)-binding pocket, leading to their phosphorylation and full activation (Biondi et al., 2001). PDK1 expression resulted in reduced cell growth and promoting apoptosis of breast cancer cells (Gagliardi et al., 2012). PDK1 was found to be over-expressed at the protein as well as at the mRNA level in most cases of breast cancer with frequent genomic amplifications. PDK1-Ser241 phosphorylated form was found to be over-expressed in human breast carcinoma (Xie et al., 2006). Another study conducted by Liang et al (2006) demonstrated that PDK1 is superior target for cancer therapy compared to Akt1 in the PI3K pathway. Over-expression of PDK1 increased the resistance of breast cancer cells to many chemotherapeutic agents including paclitaxel, doxorubicin and gemcitabine compared to Akt1 over-expressing cells (Liang et al., 2006). In addition, Du et al (2016) reported similar outcomes showing that reduced level of phosphorylated PDK1 impeded the survival of breast cancer cells; and it can consequently be a potential therapeutic target for breast cancer. These data suggest that regulation of phosphorylated PDK1 is important for the control of cellular proliferation and apoptosis in breast cancer cells. In this study we have found that PP4c down regulation increased the phosphorylation level PDK1 at Ser241 in both MCF7 and MDA-MB-231 cells and this may explain increased resistance of these cells to some

of the chemotherapeutic agents such as DNA damaging agents and mTOR1 inhibitors.

GSK3-α is a serine/threonine kinase which is a key regulator of differentiation, metabolism, apoptosis, autophagy and tumourigenesis. GSK3-α is also implicated in the regulation of glycogen synthesis. Akt has been shown to phosphorylate and inactivate GSK3 (Rayasam et al., 2009). Phosphorylation of GSK3-α makes it inactive and this results in activation of glycogen synthase (Embi et al., 1980). The aberrant cellular level of GSK3-α is involved in many pathological conditions such as Parkinson's disease, Alzheimer's disease, cancer and non-insulin dependent diabetes mellitus. Consequently, targeting GSK3-α has therapeutic implications for these disorders. Furthermore, GSK3-α is implicated in causing cells to become resistant to targeted chemotherapy (Shimura, 2011) and GSK3-α has been targeted to overcome the resistance to chemotherapeutic agents (Kawazoe et al., 2012). The effect of modulation of the endogenous level of PP4c on the phosphorylation of GSK3-α in MCF7 was found to be opposite to that in MDA-MB-231. PP4c over-expression in the MCF7 breast cancer cell line reduced the phosphorylated GSK3-α. This means the presence of more active form of GSK3-α and this negatively regulates glycogen synthase, which is as expected as MCF7 cells are oestrogen positive cells and require a regulator to control the glycogen biosynthesis for metabolic purposes. Thus it has direct role in controlling cell growth possibly through regulating the activity of a transcription factor such as eIF2B (eukaryotic initiation factor 2B) (Mishra 2010). On the other hand PP4c over-expression in MDA-MB-231 breast cancer cell line unexpectedly increased the cellular level of phosphorylated GSK3-α, which means that presence of inactive form of GSK3-α with increased activity of glycogen synthase which results in more fatty acid synthesis as expected

in MDA-MB-231 cell line in which glucose and insulin pathway is active. So it might be possible that PP4c activates some protein component involved in insulin pathway by dephosphorylating them, which mediates the activation of kinases responsible for phosphorylation of GSK3-α. However, the exact mechanism behind the increased level of phosphorylated GSK3-α is not clear and it needs to be further investigated.

Another protein which was found to be affected due to modulating the endogenous level of PP4c in breast cancer cells is phosphatase and tensin homolog (PTEN) which has been identified as a tumour suppressor gene and the mutation of which results in various types of cancer. PTEN is similar to the dual specificity protein tyrosine phosphatases in having a tensin-like domain and a catalytic domain, it dephosphorylates phosphoinositide substrates and negatively regulates intracellular level of phosphatidylinositol-3,4,5-trisphosphate. By this mean it acts as a tumour suppressor and it is the major 3-phosphatase in the PI3K/Akt pro-apoptotic pathway (Li and Sun 1997). Phosphorylation of PTEN in its C-terminus is fundamental to the stabilization and functionality of PTEN (Okahara et al., 2004). The protein interacting with C-terminus (PICT-1) plays an important role in regulating the phosphorylation status through creating conformational changes in the C-terminus domain. This shows that the phosphorylated state is very important for controlling tumour suppressor activity. The reduced level of phosphorylated PTEN mediated by PP4c over-expression in MCF7 and MDA-MB-231 cells increased its tumour suppressor activity by mediating its recruitment to the plasma membrane. It was demonstrated that PTEN-negative breast cancer cells were more sensitive to the growth inhibitory impact of PI3K and mTOR inhibitors. The reduced level of phosphorylated PTEN was found to cause more sensitivity of breast cancer cells to the anti-proliferative effect induced by rapamycin (deGraffenried et al., 2004). These results strengthen

and interpret why PP4c down-regulation reduced sensitivity of MCF7 and MDA-MB-231 cells to mTOR1 inhibitors, and also to the PI3K inhibitor in the case of MCF7 cells. In the absence of dephosphorylated PTEN, the PI3K/Akt signalling pathway becomes major pathway promoting cell proliferation and cell survival. This suggests that PP4c over-expression is an important factor in the control of the PI3K/Akt pathway by increasing the tumour suppressor activity of PTEN.

Bad (Bcl-2-associated death promoter) is a pro-apoptotic member of the Bcl-2 gene family. The pro-apoptotic activity of Bad is regulated by the process of reversible protein phosphorylation. Only non-phosphorylated Bad aggregates with BCL-xl or Bcl-2 and induces apoptosis by stimulating cytochrome-c release from the mitochondria with subsequent activation of the caspase cascade and apoptosis (Bergmann, 2002). Five phosphorylation sites have been identified on Bad and all are of serine type and are phosphorylated by a variety of kinases: Ser112, Ser128, Ser136, Ser155 and Ser170 (Burlacu, 2003). The pro-apoptotic molecule Bad is regulated by phosphorylation of two sites; serine-112 (Ser112) and serine-136 (Ser136) (Zha et al., 1996). Cell survival signals such as activation of insulin-like growth factor receptor (IGF-1R) and epidermal growth factor receptor (EGFR) result in activation of the phosphatidylinositol 3-kinase (PI3K) signalling cascade with subsequent activation of Akt and 70-kDa ribosomal protein S6 kinases. Both Akt and P70 S6 kinases phosphorylate Bad at Ser136 thereby reducing the level of apoptosis (Harada et al., 2001). Another study showed that treatment of MCF7 cells with estradiol inhibited apoptosis through Bad inactivation (Fernando and Wimalasena, 2004). Bad over-expression inhibits the oestrogen mediated cyclin D1 synthesis and cell proliferation while its inhibition in MCF7 cells resulted in higher proliferative activity, increased the activated state of Cdks and enhanced the cyclin D1 protein

synthesis (Fenando et al., 2004). Bad phosphorylation inhibits apoptosis in a murine model (Fernando and Wimalasena, 2004). PP4c has been shown to regulate the level of phosphorylated Bad (Mourtada-Maarabouni and Williams, 2009). In this study, PP4c over-expression reduced the level of phosphorylated Bad at Ser112 in both MCF7 and MDA-MB-231 cells. Regulation of Bad phosphorylation at Ser112 is usually done by the Ras-mitogen-activated protein kinase pathway (Fang et al., 1999). Increased expression of phosphorylated Bad in MCF7 and MDA-MB-231 cells as a result of PP4c down-regulation may explain increased resistance of these cells to some of the chemotherapeutic agents. On the other hand, reduced expression of phosphorylated Bad as a result of PP4c over-expression may explain the increase in proportion of basal apoptosis in both MCF7 and MDA-MB-231 cells as a result of PP4c over-expression (Chapter 3). The effects of PP4c over-expression on the cell survival and apoptosis observed in Chapter 3 could be due to changes in the phosphorylation level of many proteins in the PI3K/Akt /mTOR pathway, start with reduced phosphorylation of PTEN which act as a tumour suppressor gene in its dephosphorylated form and it inactivates PI3K which lead to inactivation of Akt with subsequent reduction in the phosphorylation of Bad with the resulted increase in its pro-apoptotic activity.

Other proteins that are affected by modulating the endogenous level of PP4c are PRAS40 (The proline-rich Akt substrate of 40 kDa) and 4E-BP1 (Eukaryotic translation initiation factor 4E-binding protein 1). Both of these proteins are substrates of mTOR1 and activation of both promotes translation. PP4 over-expression reduced the phosphorylation level of these proteins in MCF7 cells. On the other hand, PP4c over-expression in MDA-MB-231 cells reduced the level of

phosphorylated PRAS40 and increased the level of phosphorylated 4E-BP1 and S6 ribosomal protein.

S6 ribosomal protein (rpS6) is involved in the regulation of cell size, cell proliferation, and glucose homeostasis (Magnuson et al., 2012). There is a strong correlation between mitogenic stimulation of cells and the phosphorylation of rpS6 on its serines (Bandi et al., 1993). *In vitro* rpS6 was found to be phosphorylated by two classes of protein kinases, the S6K1/2 and the p90 ribosomal S6 kinase (RSK) family of serine/threonine kinases (Roux et al., 2007). Subsequent studies indicated that S6K1/2 were the main physiological rpS6 kinases and found that rpS6 phosphorylation was largely sensitive to the mTOR inhibitor-rapamycin (Chung et al., 1992). The p70S6 is a serine/threonine kinase which mediates phosphorylation of rpS6 protein at serine235 and 236 sites and many other sites. Growth factor receptors and oncogenic signals activate the PI3K pathway, which activates Akt. Activation of Akt phosphorylates mTOR which in turn activates p70S6 kinase through its phosphorylation. p70S6 phosphorylation activates translation apparatus to synthesise proteins required for cell proliferation and growth (Dufner and Thomas, 1999). In the present study reduced level of the rpS6 protein phosphorylated at Ser 235/236 as a result of PP4c over-expression indicated that MCF7 cells are subject to the growth arrest due to defect in translation mechanism. The opposite effect was observed in MDA-MB-231 cells in which PP4c over-expression increased the phosphorylation level of rpS6 protein at Ser235/236 with subsequent activation and induction of translation. This indicates that PP4c over-expression controls the activation of S6 ribosomal protein indirectly through likely activation of some protein kinases in the mTOR pathway. This indicates that PP4c acts through different

pathways to produce or exert its effect as a tumour suppressor or oncogene in these two cell lines.

Another protein which shows different responses to PP4c over-expression between MCF7 and MDA-MB-231 cells is 4E-BP1 which is a translation repressor. Binding of the 4E-BPs to eIF4E (Eukaryotic translation initiation factor 4E) is regulated by phosphorylation; hypo-phosphorylated 4E-BP isoforms interact strongly with eIF4E, whereas hyper-phosphorylated isoforms do not (Gingras et al., 1999). Therefore, 4E-BP1 is a key component in the mTOR/Akt pathway, which is inhibited by the Akt/mTOR pathway and activates downstream kinases required for transition of G_1 to S phase during the cell cycle (Gingras et al., 1999). Thus the reduced level of phosphorylated 4E-BP1 as a result of PP4 over-expression induces growth arrest and apoptosis in MCF7 as the dephosphorylated form of 4E-BP1 binds to the eIF4E and inhibits its function as translation stimulator. On the other hand, the elevated level of phosphorylated 4E-BP1 in the MBA-MD-231 cell line after PP4c up-regulation mediates cell proliferation due to dissociation of phosphorylated 4E-BP1 from eIF4E and this activates the translation suppressor function of 4E-BP1. It has previously been found that MDA-MB-231 cells are sensitive to rapamycin induced 4E-BP1 phosphorylation while MCF7 cells are not (Yellen et al., 2011).

Another striking finding in this study was the effect of PP4c over-expression on PRAS40 phosphorylation. The cellular level of phosphorylated PRAS40 was decreased as a result of PP4c over-expression in MCF7 and in MDA-MB-231 cells. PRAS40 is phosphorylated at threonine 246 by Akt/PKB and the mTORC1 complex. As a result of phosphorylation, PRAS40 becomes separated from the mTORC1 complex and it relieves the inhibitory effect on the function of mTORC1. The phosphorylation of PRAS40 is mediated through external cellular stimuli such as

glucose, insulin, amino acids, nerve growth factor, factors like platelet derived growth factor (Kovacina et al., 2003; Saito et al., 2004). Nevertheless, the Akt/PKB/PI3K pathway inhibitors decrease the phosphorylation of PRAS40 at threonine 246 position. This may explain increased resistance of MCF7 cells to Akt inhibitor and PI3K inhibitor as a result of PP4c down-regulation. This study supports the role of PP4c as an inhibitor of the phosphorylation of PRAS40, and may be serve as an important biomarker to measure PRAS40 phosphorylation status and the pro-apoptotic signals which in turn measure the PI3K and the Akt inhibitors sensitivity. Moreover, PP4c expression status determines cell survival and apoptosis of the cancer cells indirectly through determination of PRAS40 activity because the latter is associated with the regulation of apoptosis and cell survival (Saito et al., 2004; Yu et al., 2008).

The results of this chapter highlight the importance of PP4c protein as a key player in regulating the phosphorylation of many important proteins in the PI3K/Akt pathway that are responsible for regulating cell growth, differentiation, translation and apoptosis; these proteins are usually targeted by various chemotherapeutic agents (this discussed thoroughly in Chapter 6). Modulating the intracellular level of PP4c in both hormone positive and triple negative breast cancer cells may produce a synergistic effect with some of the targeted therapies through increasing or decreasing the activity of some of these proteins via changing the phosphorylation status of these proteins and this increased sensitivity of breast cancer cells to various chemotherapeutic agents.

Chapter 6: Discussion

There is increasing evidence that breast cancer and other cancers develop as a result of multi-step genetic and epigenetic changes of the host cells, as well as the surrounding tissue cells that interact with these malignant cells, such as immune, vascular and stromal cells (Balmain et al., 2003). Genomic instability may result in gene loss, gene mutation, amplification or chromosomal translocation. Most of these changes will end in cell death but sometimes these changes may occur in genes that play a crucial role in cell proliferation, differentiation and the cell cycle and may also control migration, invasion and metastasis of tumour cells (Osborne et al., 2004). So understanding genes, proteins and the biological pathways that are involved in the development of cancer may lead to new genetic therapies of cancer. These therapies could be more targeted than the usual therapies, with fewer side effects and lower recurrence rates (Osborne et al., 2004). According to estimates, one out of eight women is in danger of developing breast malignancies during her life-time. Despite the efforts to develop targeted therapies and cytotoxic chemotherapies that have resulted in increasing survival rates of patients with breast cancer, 40,000 women with breast cancer still die annually in the USA (Siegel et al., 2013).

The PI3K/Akt/mTOR pathway has been recognized to transmit signals from the cell membrane to the nucleus for activation of multiple oncogenic pathways. This pathway is also found to regulate growth and proliferation of breast cancer cells; and it is interconnected with multiple metabolic pathways including glucose, lipid and amino-acid metabolism for maintaining energy balance and autophagy (Grunt and Mariani, 2013). This pathway is also known to regulate ER function, and to communicate with upstream and downstream regulatory systems. The

PI3K/Akt/mTOR pathway is found in a hyperactive state in 70% of breast cancer cases and, consequently, the protein kinases present in this pathway are considered attractive targets for treating breast cancer (Grunt and Mariani, 2013). Chemotherapeutic agents are being developed, which block the function of different upstream protein kinases, whereas other drugs directly inhibit the function of mTORC1 or mTORC2 or mTORC1+mTORC2 or PI3K+mTORC1+mTORC2 (MacKeigan et al., 2005).

The PI3K/Akt/mTOR pathway has been implicated in inducing resistance to chemotherapy, HER2-directed therapy and endocrine therapy (Paplomata and O'Regan, 2013; Nahta, 2012). Consequently, the community of researchers and pharmaceutical companies are engaged in developing multiple inhibitors of the PI3K/Akt/mTOR pathway. It has been reported that HER2 over-expressing breast cancer is resistant to trastuzumab due to the involvement of the PI3K/Akt/mTOR pathway. Pre-clinical studies have indicated that in resistant cells, PI3K/Akt/mTOR pathway inhibitors act synergistically with trastuzumab to overcome this resistance (Nahta, 2012). Andre et al (2010) showed that combinations of chemotherapeutic agents such as everolimus or paclitaxel with trastuzumab improved the response rate up to 44% in patients with trastuzumab-resistance. Resistance or sensitivity of breast cancer cells to a particular chemotherapeutic agent depends on the phosphorylation status of different components located along the PI3K/Akt/mTOR pathway (Bonavida, 2008).

Protein phosphorylation is regarded as a key process in cellular signal transduction pathways; any disturbance in this mechanism contributes into many disease states including cancer. The process of reversible phosphorylation regulates the function of about one third of proteins in the cell including proteins that are identified to play a

critical role in breast cancer. The process of reversible protein phosphorylation is specifically controlled by two groups of enzymes that counteract each other, the protein kinases and protein phosphatases (Bennett et al., 2006). Protein kinases include many oncogenes and growth factor receptors, many of which have been linked to the pathogenesis and the progression of breast cancer (Nunes-Xavier et al., 2013). The involvement of oncogenic kinases in breast cancer highlights the importance of identifying functional phosphatases that may counteract their activities (Nunes-Xavier et al., 2013). PP4 is a highly conserved serine/threonine phosphatase belonging to the PPP family of phosphatases which has been implicated in a variety of cellular functions independently of other protein phosphatases in the PPP family (Cohen et al., 2005). In this study we have found that PP4c is over-expressed in many types of breast cancer cell lines including hormone positive-MCF7, T47D and MDA-MB-361 cells and triple negative breast cancer cell lines-MDA-MB-231 and Hs578T cells (Figure 3.10), it was also found to be over-expressed in all stages of breast cancer in breast cancer tissue samples (Figure 3.11). PP4c was found to be over-expressed in many types of human cancer such as in colorectal carcinoma (Li et al., 2015) and in pancreatic ductal adenocarcinoma (Weng et al., 2012). PP4c over-expression in colorectal carcinoma was found to have a negative impact on patients, where it is associated with promotion of cell growth and invasion through indirect induction of Akt phosphorylation and up-regulation of MMP-2/9 (matrix metallopeptidase 2/9) expression (Li et al., 2015). PP4c over-expression correlates with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma through mediated activation of both JNK and NF-κB pathways (Weng et al., 2012). In the present study, effects of modulating PP4c expression in various types of breast cancer cell lines have been studied. Cell lines used were the MCF7 breast cancer

cells that are ER+, PR+ and HER2- and the triple negative (ER-, PR-, HER2-) breast cancer cells-MDA-MB-231 and Hs578T. The data presented revealed that over-expression of PP4c in MCF7, MDA-MB-231 and Hs578T breast cancer cells reduced their total and viable cell counts and increased the level of apoptosis in the absence of any extracellular apoptotic stimuli (Chapter 3). The effect of PP4c in increasing cell death was also confirmed in the cell cycle analysis where it caused an increase in the percentage of cells in Sub-G_0 (Chapter 3). These observations are consistent with previous work which showed that over-expression of PP4c in HEK293T cells and in human T-leukemic cell lines (CEM-C7 and Jurkat cells) resulted in suppression of cell proliferation, induction of apoptosis and promotion of cell cycle arrest (Mourtada-Maarabouni and Williams, 2008; Mourtada-Maarabouni and Williams, 2009). On the other hand, the present study revealed that PP4c down-regulation in these breast cancer cell lines increased cell proliferation and decreased basal apoptosis, which is also in agreement with the results presented by Mourtada-Maarabouni and Williams, 2008 and Mourtada-Maarabouni and Williams, 2009. However, the present results contrast with previous work which was reported by Lifeng et al (2006) which showed that PP4c suppression in MCF7 cells resulted in an increase in cell death. This may be explained by the important role that PP4c plays in various vital biological cellular functions, so that a minimum level of the enzyme is essential to maintain cell survival. Such an apparent discrepancy could therefore be the result of reducing PP4c to a level below the minimum required level for cell survival in the work performed by Lifeng et al (2006). In this regard, deletion of PP4c in mouse thymocytes resulted in an inhibition of thymocyte development and impairment of calcium mobilisation (Shui et al., 2007). In addition, it has been

reported that PP4c knockout in mouse neural progenitor cells led to defects in spindle orientation and in the development of the neocortex (Xie et al., 2013).

A key finding in this study is that PP4c influenced the phosphorylation status of PEA15, and this finding was consistent with previous proteomic analysis that has shown that changes in PP4c expression affect the phosphorylation status of many proteins involved in apoptosis and cell proliferation, including PEA15 (Mourtada-Maarabouni and Williams, 2009). PEA15 is a cytoplasmic protein that is involved in the regulation of a variety of cellular functions and its activity depends on its phosphorylation status. PEA15 prevents nuclear translocation of MAPK and the extra-cellular signal regulated kinase 1/2 (ERK1/2) and regulates many cellular ERK1/2 dependent processes including cell proliferation (Greig and Nixon, 2014). PEA15 inhibits apoptosis through its death effector domain (DED) which interacts with the DED of other proteins such as apoptotic adaptor protein (FADD) and inhibits apoptosis via inhibiting assembly of apoptotic signalling complexes (Greig and Nixon, 2014). PEA15 function is tightly regulated by phosphorylation, since the anti-apoptotic effect of PEA15 is stabilised by Akt (Protein Kinase B) which promotes its phosphorylation (Toker and Newton, 2000). Chapter 3 points the importance of PEA15 as a crucial PP4c substrate in both hormone positive and triple negative breast cancer cell lines. We have reported an increase in the phosphorylation state of PEA15 after PP4c down-regulation and this is consistent with other studies that have shown that the phosphorylation level of PEA15 was increased dramatically after PP4c down-regulation in HEK293T (Mourtada-Maarabouni and Williams, 2009). Mourtada-Maarabouni and Williams (2009) have also reported that the pro-apoptotic effect of PP4c in leukemic cells was partly mediated by the dephosphorylation of PEA15, providing evidence that the interaction between PEA15 and PP4c may be

critical in leukaemogenesis and/or leukaemia progression (Mourtada-Maarabouni and Williams, 2009). In this study we have reported that PEA15 was over-expressed in about one third of the breast cancer tissue samples (Figure 4.8), and this agreed with previous investigations that have reported over-expression of PEA15 in human breast cancer and other human cancers (Hwang et al., 1997). Such PEA15 over-expression was found to be associated with resistance to a broad range of anti-cancer drugs (Stassi et al., 2005). On the other hand, it was found that biphosphorylated PEA15 at Ser104 and Ser116 sensitized ovarian cancer cells to paclitaxel. Biphosphorylated PEA15 induces this effect via reducing expression and possibly by inducing posttranslational modification of SCLIP (SCG10-like protein), which resulted in impairment of SCLIP-mediated MT destabilization (microtubule-destabilizing protein) which is involved in pPEA15-mediated chemosensitisation, this will enhance mitotic arrest and apoptotic cell death after paclitaxel treatment due to MT polymerization (Xie et al., 2013). Akt, which phosphorylates and stabilises the anti-apoptotic action of PEA15, is also up-regulated in breast cancer and the hyper activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is implicated in the tumourigenesis of ER+ breast cancer and resistance to endocrine therapy, suggesting that Akt and PEA15 might function cooperatively in breast tumourigenesis (Ciruelos, 2014). PTEN was reported to modulate phosphorylation of PEA15 at Ser116 through modulating Akt activity, but not through direct dephosphorylation of PEA15 (Hayashi et al., 2012). PTEN mutation is commonly seen in many types of tumours and this resulted in an increase in the phosphorylation of PEA15 at Ser116 with the inhibition of Fas-induced apoptosis (Hayashi et al., 2012). These findings support results obtained in this study where we observed an increase in the expression of phosphorylated

PTEN upon PP4c down-regulation in both hormone positive and triple negative breast cancer cell lines (Figure 5.17 and Figure 5.19). Therefore, PP4c down-regulation may indirectly increase phosphorylation of PEA15 through activation of Akt and this resulted in an increase in cell proliferation and a reduction in basal apoptosis in case of PP4c down-regulation in these cell lines.

Accumulation of genetic alterations is the most common cause of cell transformation from the benign to the malignant state. In early stages these malignant cells are not invasive or metastatic but later when new genetic alterations accumulate the cells become highly malignant with metastatic characteristics (Yokota, 2000). Activation of the ERK/MAP kinase pathway has also been described to elicit changes in intracellular architecture and adhesion (Weinberg, 2006). Transfection of PP4c-specific siRNAs into MCF7 and MDA-MB-231 breast cancer cells resulted in an increase in both anchorage-dependent growth of the cells and in the metastatic ability of these cells (Chapter 3). This effect of PP4c could be as a result of changes in the phosphorylation status of important proteins in the PI3K/Akt/mTOR pathway such as PTEN and PDK1. Dephosphorylated PTEN was found to suppress migration of a variety of cell types, including primary human fibroblasts, non-transformed mouse fibroblasts, and tumour cells (Liliental et al., 2000). PTEN produces its effect on cell migration via several mechanisms, one of these mechanisms is through its effects on phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)-P3) (Yamada and Araki, 2001). PDK1 was found to be over-expressed in gastric cancer and promotes gastric cancer cell proliferation, migration, and invasion by activation of the NF-kB signalling pathway (Wu et al., 2017). These findings support results obtained in this study where it was observed that PP4c down-regulation increased expression of phosphorylated PTEN and PDK1 in both MCF7 and in MDA-MB-231 cells, and this

may have a great impact on cell migration in these cells after PP4c down-regulation. Our results contrast with a study done by Martin-Granados et al (2008) which showed that depletion of PP4c in HEK293 cells resulted in severe reduction in cell migration. This study suggested that PP4c complexes may coordinate centrosome maturation and cell migration via regulation of Rho GTPases (Martin-Granados et al., 2008). Another study showed that PP4c over-expression significantly promoted cell proliferation and increased cell invasion by increasing the expression levels of MMP-2 and MMP-9 (matrix metallopeptidase 2/9) (Li et al., 2015). The PI3K/Akt axis was suggested to be a potential oncogenic mechanism in which PP4c contributes to the up-regulation of MMP-2 and MMP-9 and consequently to cell invasion (Li et al., 2015). Overall, the results presented provide strong evidence that PP4c plays a crucial role in the regulation of cell proliferation, apoptosis, cell cycle, cell migration and metastasis.

The present study went on to investigate the effects of PP4c expression on the regulation of different molecules in the PI3K/Akt/mTOR signalling pathway since modulation of PP4c expression has a great impact on the response of breast cancer cells to various chemotherapeutic agents. We observed that PP4c down-regulation reduced the sensitivity of MCF7, MDA-MB-231 and Hs578T cells to DNA damaging agents such as cisplatin, doxorubicin and 5FU and also reduced the sensitivity of these cells to mTORC1 inhibitors such as rapamycin and everolimus (Chapter 5). PP4c down-regulation reduced the sensitivity of MCF7 to the Akt inhibitor (AZD5363), dual mTOR1,2 inhibitor (AZD8055) and PI3K inhibitor (LY294002). In contrast, PP4c down-regulation increased sensitivity of MD-MB-231 and Hs578T cells to the Akt inhibitor, dual mTOR1,2 inhibitor and had no effect on the response of these cells to the PI3K inhibitor (Chapter 5).

There is accumulating evidence that changes in the phosphorylation status of various components of the PI3K/Akt and PI3K/mTOR pathways can result in an increase in the resistance of cancer cells to various chemotherapeutic agents, thereby affecting the outcome of treatment (Liang et al., 2006). Multidrug resistance (MDR) of cancer cells can be sometimes overcome by silencing breast cancer resistant protein (BCRP) using specific siRNA and by PI3K/Akt inhibition using LY294002, and this approach was used to re-sensitise MCF7 cells to mitoxantrone (MTX) chemotherapy (Komeili-Movahhed et al., 2015). By studying the effect of PP4c expression on the phosphorylation status of different proteins in the PI3K/Akt/mTOR pathway we have found that PP4c down-regulation in MCF7 cells increased the phosphorylation of 4EBP1 at Thr 37/46, GSK3-α at Ser21, PDK1 at Ser241, Bad at Ser112, S6 ribosomal protein at Ser235/236, PTEN at Ser380 and PRAS40 at Thr246 (Figure 5.17) with the corresponding opposite effects obtained when PP4c was over-expressed (Figure 5.16). Changes in the phosphorylation state of these proteins could be a direct or an indirect effect of PP4c. Increased phosphorylation of all of these proteins upon PP4c down-regulation would be expected to result in induction of translation and cell growth stimulation; furthermore, increased phosphorylation of Bad would be expected to result in inhibition of apoptosis. On the other hand, when PP4c is over-expressed reduced phosphorylation of these proteins is consistent with inhibition of translation, reduction of cell growth and stimulation of apoptosis. This may therefore explain results obtained in case of PP4c down-regulation and PP4c up-regulation in MCF7 breast cancer cells, respectively. Each of these substrates is considered individually below in figure 6.1 and figure 6.2.

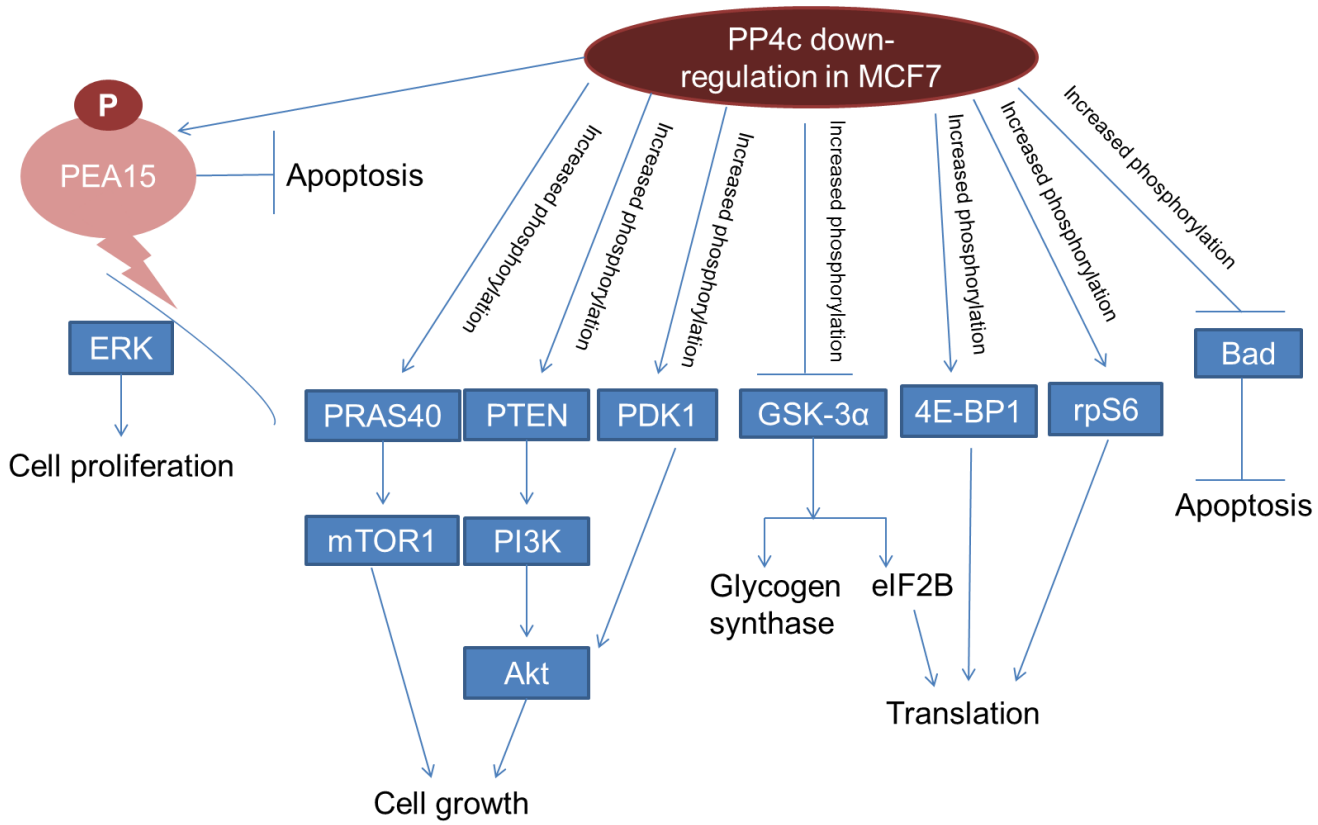


Figure 6.1: PP4c substrates in case of PP4c down-regulation in MCF7 breast cancer cells. The increase in phosphorylation is an indirect effect of PP4c down-regulation

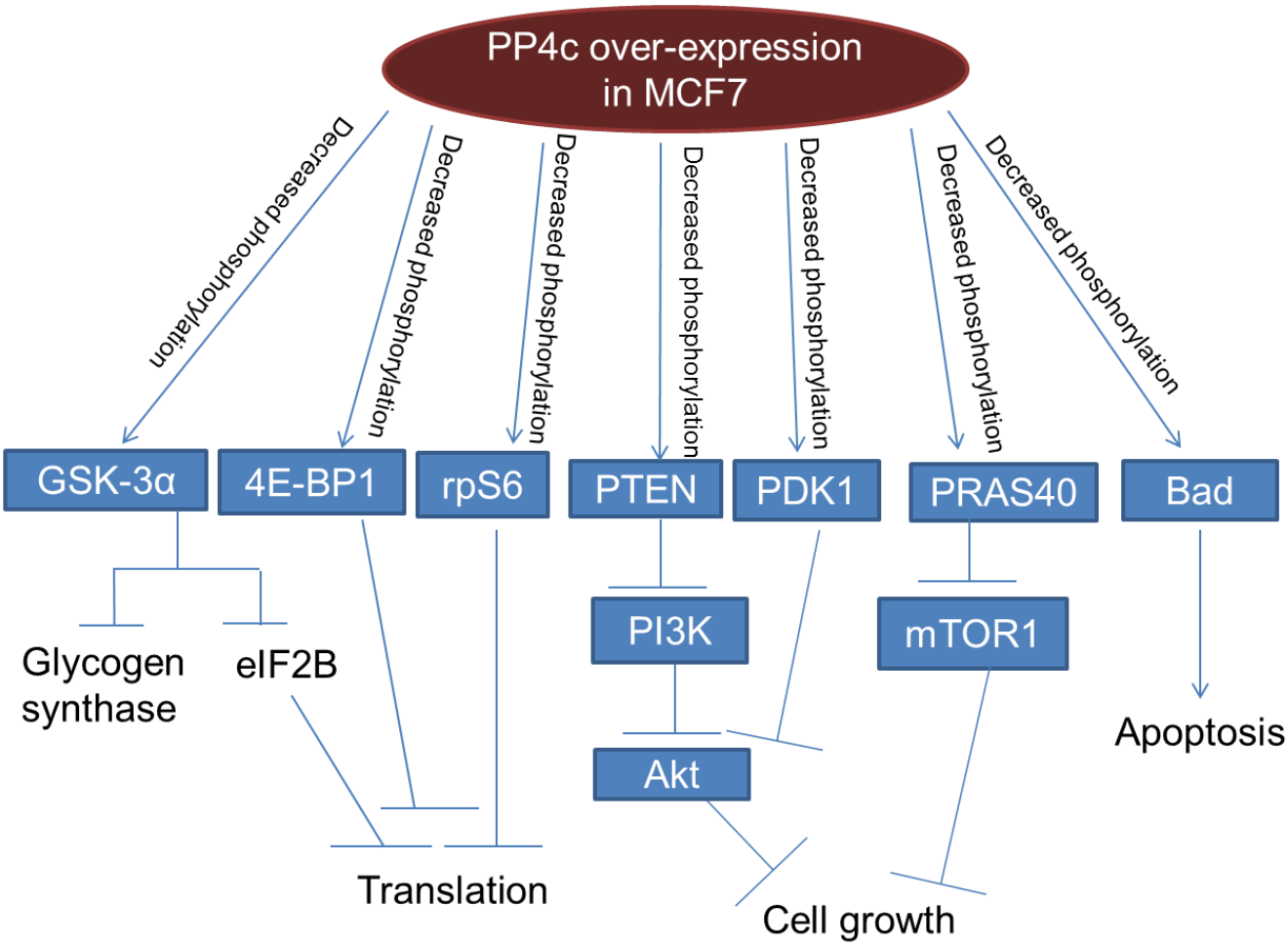


Figure 6.2: PP4c substrates in case of PP4c over-expression in MCF7 breast cancer cells. The decrease in phosphorylation could be a direct or an indirect effect of PP4c over-expression

Phosphorylation status of 4E-BP1 determines its translation suppresser activity through its binding to the eIF4E (Eukaryotic translation initiation factor 4E). Hypo phosphorylated 4E-BP1 interacts strongly with eIF4E, whereas hyper phosphorylated 4E-BP1 does not (Kleijn et al., 1998). 4E-BP1 phosphorylation is stimulated after cellular exposure to a wide variety of extracellular stimuli such as hormones, mitogens, growth factors, cytokines, and G-protein-coupled receptor agonists; this phosphorylated 4E-BP1 therefore permits translation (Kleijn et al., 1998). This may explain increased MCF7 cell number and cell proliferation after PP4c down-regulation as a result of increased phosphorylation of 4E-BP1. It was reported that treatment of cells with LY294002 caused reduction in 4E-BP1 phosphorylation due to inhibition of PI3K signalling pathway, whereas activated PI3K increased expression of phosphorylated 4E-BP1 (von Manteuffel et al., 1997). Activated Akt also induces 4E-BP1 phosphorylation (Gingras et al., 1998). The present study showed that PP4c down-regulation reduced sensitivity of MCF7 breast cancer cells to the Akt inhibitor and to the PI3K inhibitor which could be explained by the increase in the phosphorylation of 4E-BP1.

It was found that both FRAP/mTOR (FKBP12-rapamycin associated protein/mammalian target of rapamycin) and a baculovirus-expressed FRAP/mTOR protein induced phosphorylation of 4E-BP1 at Thr37 and Thr46. FRAP is one of rapamycin's targets besides mTOR, inhibition of FRAP by rapamycin causes cell cycle arrest at G_1 phase (Dennis et al., 1999). It was reported that the most rapamycin and LY294002-resistant phosphopeptides are those containing phosphorylated Thr37 and Thr46 (von Manteuffel et al., 1997) and this may explain increased MCF7 resistance to rapamycin and LY294002 after PP4c down-regulation.

GSK-3α (Glycogen synthase kinase 3) is a serine/threonine kinase known to regulate glycogen synthesis in response to insulin (Hughes et al., 1993). GSK-3α phosphorylates a broad range of substrates, including the translation factor eIF2B (Eukaryotic Initiation Factor 2B) (Welsh et al., 1996). In mammalian cells inactivation of the GSK-3α occurs upon its phosphorylation at serine21 as a result of stimulation with insulin or other growth factors (Sutherland et al., 1993). Protein kinase B (PKB/Akt), a serine/threonine kinase has also been implicated to induce phosphorylation of GSK-3α at serine21 (Cross et al., 1994). GSK-3α is implicated in causing cancer cells to become resistant to targeted chemotherapy (Shimura, 2011); therefore, GSK-3α has been targeted to overcome resistance to chemotherapeutic agents (Fu et al., 2011; Kawazoe et al., 2012). In our study we have demonstrated that PP4c down-regulation increased the expression of phosphorylated GSK-3α at serine21 in MCF7 cells and this may help to explain increased cell proliferation as a result of PP4c down-regulation in these cells. This effect may also explain reduced sensitivity of MCF7 cells to the Akt inhibitor after PP4c down-regulation.

Ribosomal protein S6 (rpS6) is known to have five phosphorylation sites at its C terminus, these are; Ser235, Ser236, Ser240, Ser244, and Ser247 (Krieg et al., 1988). These five phosphorylation sites are phosphorylated by mTOR upon growth factor stimulation (Ruvinsky and Meyuhas, 2006) and determine the potential role of S6 phosphorylation in regulating cell growth capacity (Zoncu et al., 2011). It was found that seleno-compound 2,4-dihydroselenoquinazoline (3a) reduced cell viability and induced cell cycle arrest at G_2/M in MCF7 cells due to inhibition of the PI3K/Akt/mTOR/S6 ribosomal protein signalling pathway. This effect of 3a was found to be dose-and time-dependent effect (Moreno et al., 2014). It was reported that everolimus and BEZ235 synergistically decrease proliferation of the ER+ MCF7 sub-

lines (TamC3 and TamR3). Everolimus alone acts by inhibiting p70S6K pathway downstream of mTORC1 (Leung et al., 2015), in this study PP4c down-regulation increased the phosphorylation of rpS6 in MCF7 cells; this may help to explain reduced sensitivity of these cells to everolimus after PP4c down-regulation.

Our results revealed that PP4c down-regulation in MCF7 cells increased the phosphorylation of Bad at Ser112. Bad (Bcl-2-associated death promoter) is a pro-apoptotic member of the Bcl-2 gene family. Non-phosphorylated Bad interacts with BCL-xl or Bcl-2 and induces apoptosis (Bergmann, 2002). Bad phosphorylation inhibits apoptosis in a murine model (Fernando and Wimalasena, 2004). PP4c has been shown to regulate the levels of phosphorylated Bad (Mourtada-Maarabouni and Williams, 2009). Oestrogens exert anti-apoptotic effects in the ER-positive MCF7 breast cancer cell line through induction of transcription of the BCL2 gene and increase expression of BCL2 protein which exerts an anti-apoptotic action (Huang et al., 1997). It was reported that E2 induces Bad phosphorylation and inactivation through Ras/PI3K/Akt pathway as well as Ras/ERK/p90RSK1 pathway (Fernando and Wimalasena, 2004). It was also reported that 17β-Estradiol (E2)-dependent phosphorylation of ERα on S118 is counteracted by PP5 (another member of PPP family). This may help to explain reduced cell number and promotion of apoptosis as a result of PP4c up-regulation in MCF7 cells which could be due inhibition of E2 activity. MCF7 human breast cancer cells are relatively resistant to cisplatin treatment compared to other breast cancer cell lines which could be due to high expression of anti-apoptotic Bcl-2 protein relative to more cisplatin sensitive breast cancer cells (Yde and Issinger, 2006; Lukyanova et al., 2009). It was reported that down-regulation of both Bcl-2 and cyclin D1 resulted in sensitisation of MCF7 cells to cisplatin (Eytan, 2005). Fenando et al (2007) also reported that Bad over-expression

inhibited oestrogen mediated cyclin D1 synthesis and cell proliferation, and its inhibition in MCF7 cells resulted in higher proliferative activity in these breast cancer cells as a result of increased activated state of Cdks and enhanced cyclin D1 protein level. In our study, PP4c over-expression reduced level of phosphorylated Bad which resulted in potentiation of apoptosis and reduction of cell number in the MCF7 and in MDA-MB-231 breast cancer cell lines.

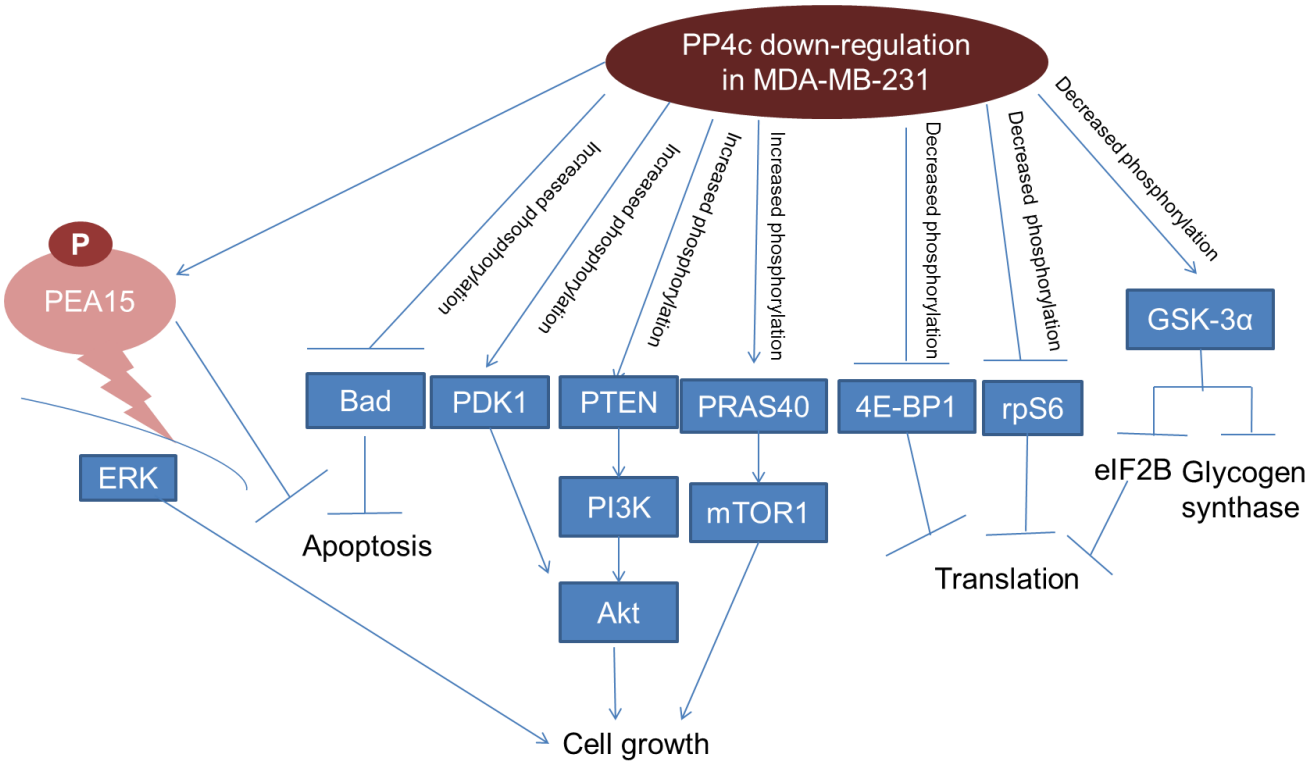
PTEN (Phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor gene the abnormality of which has been recorded in different types of human cancers (Myers and Tonks, 1997). PTEN regulates the PI3K/Akt signal transduction pathway (Cantley and Neel, 1999) and is composed of an N-terminal dual specificity phosphatase-like enzyme domain and a C-terminal regulatory domain (Lee et al., 1999). Phosphorylation is the most important mechanism of post-translational modification of PTEN (Vazquez et al., 2000). Phosphorylation of PTEN at Ser380, Thr382, and Thr383 results in loss of its tumour suppressor activity (Vazquez et al., 2000). PTEN phosphorylation at Ser380 was found to be increased in gastric cancer (Yang et al., 2013). DeGraffenried et al (2004) demonstrated that PTEN negative breast cancer cell lines were more sensitive to the growth inhibitory impact of PI3K and mTOR inhibitors. PTEN knockdown in MCF7 breast cancer cell line was found to increase MCF7 cell growth by modulating CKIs, CDKs, cyclin A, and Cdc25C expressions with the resulted increase in M phase cells and decrease in G_0/G_1 phase cells (Chiang et al., 2015). Our data showed increased expression of p-PTEN at Ser380 in both MCF7 and in MDA-MB-231 cells after PP4c down-regulation and this may mediate in part the effect of PP4c down-regulation in these cells through increased activity of the PI3K/Akt pathway.

PDK1 (3-Phosphoinositide-dependent kinase 1) is regarded as a master kinase in the PI3K pathway that activates and transduces signals from phosphoinositides to several other kinases including Akt, ribosomal protein S6 kinases and mTOR (Kannan et al., 2007). Abnormalities in the PI3K pathway, which could be at several levels, play an important role in breast cancer tumourigenesis and in breast cancer resistance to various chemotherapeutic agents (Fry, 2001). It has been reported that MCF7 and MDA-MB-231 breast cancer cell lines expressed higher level of PDK1 than other breast cancer cell lines with MCF7 cells containing a high level of phosphorylated PDK1 at Ser241 and a very low level of phosphorylated Akt1 at Thr308 (Liang et al., 2006). Du et al (2016) reported that reduced level of phosphorylated PDK1 impeded the survival of breast cancer cells; and it can consequently be a potential therapeutic target for breast cancer. Nevertheless, increased level of phosphorylated PDK1 is essential for promoting tumour growth and metastasis in spontaneous mouse tumour model (Du et al., 2016). These data suggest regulation of phosphorylated PDK1 is important for the control of cell proliferation and apoptosis in breast cancer. The data further support the empirical results obtained from our study, and corroborate the pivotal role of PP4c in dephosphorylation of PDK1 and in the regulation of apoptosis and cell proliferation. Over-expression of PP4c decreased the cellular level of phosphorylated PDK1 in MCF7 and in MDA-MB-231 cells with reduced level of phosphorylated GSK-3α in MCF7 cells which is consistent with the results obtained by Liang et al (2006) which showed that PDK1 knock-down in MCF7 was found to be accompanied by reduced level of phosphorylated GSK3 particularly GSK-3α (Liang et al., 2006). PDK1 knock-down in MCF7 moderately increased sensitivity of these cells to doxorubicin (Liang et al., 2006). These data may therefore help to explain the reduced sensitivity of

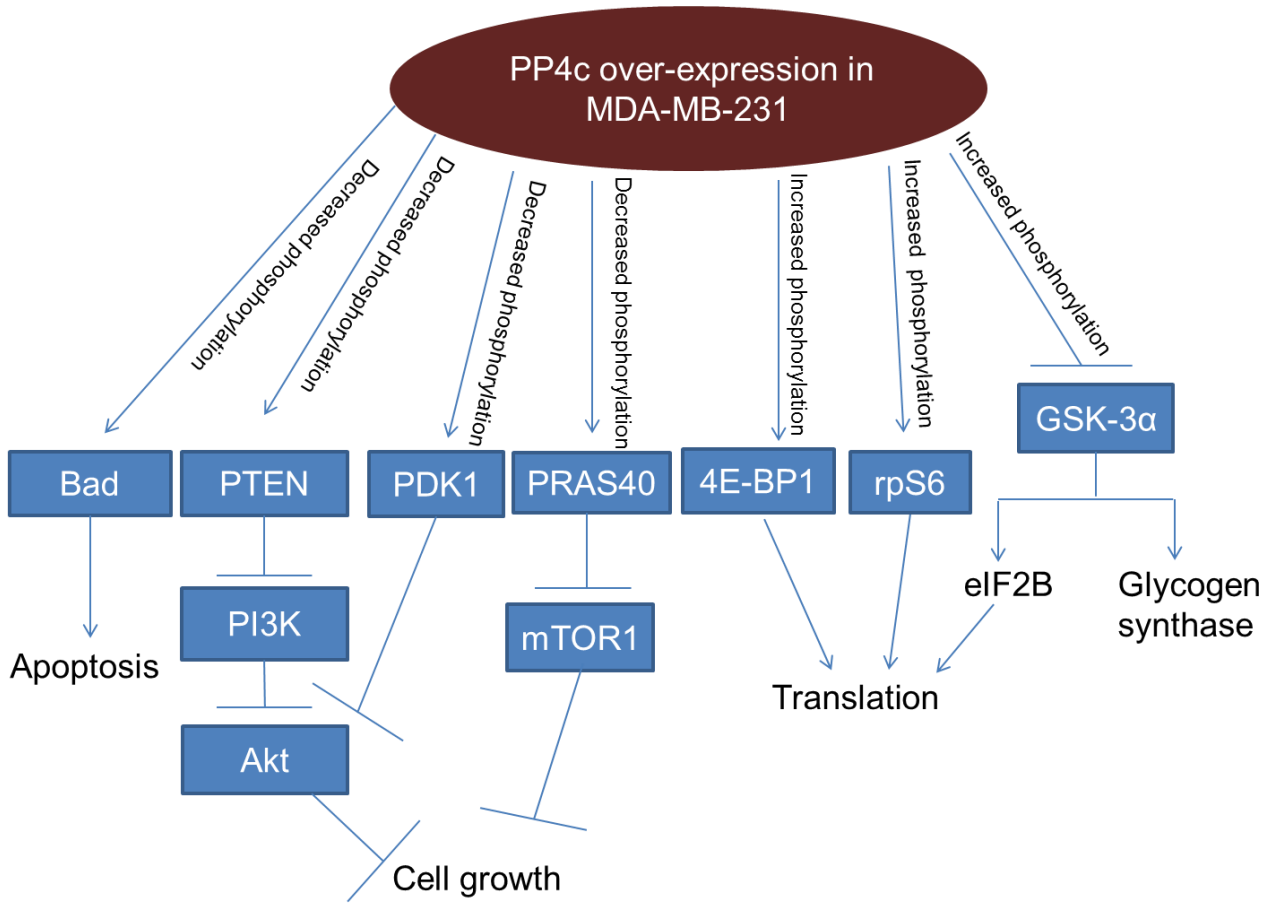
MCF7 to doxorubicin after PP4c down-regulation which could be due to increased level of phosphorylated PDK1.

PRAS40 (the proline-rich Akt substrate of 40 kDa) acts as an intermediate between Akt and mammalian target of rapamycin (mTOR) (Fonseca et al., 2007). Akt is regarded to be the major kinase that phosphorylates PRAS40 at Thr246 (Taniguchi et al., 2006). PRAS40 phosphorylation at Thr246 is inhibited by PI3K inhibitors (Kovacina et al., 2003). Phosphorylation of PRAS40 by Akt and by mTORC1 itself results in dissociation of PRAS40 from mTORC1 and may relieve the inhibitory effect on mTORC1 activity resulting in phosphorylation of mTORC1 substrates including ribosomal protein S6 kinase 1 (S6K1) and 4E-BP (Fonseca et al., 2007). Phosphorylation of PRAS40 was increased in many types of human cancers such as prostate cancer (Shipitsin et al., 2014) and breast cancer (Fu et al., 2012). Phosphorylated PRAS40 at Thr246 is regarded as a biomarker in breast cancer, over-expression of phosphorylated PRAS40 suggests that those patients may get better response to PI3K inhibitors than other patients (Fu et al., 2012). Phosphorylated PRAS40 was also increased in patients with melanoma especially those with elevated Akt activity, and targeting PRAS40 increased apoptosis and reduced the anchorage-independent growth of melanoma cells (Madhunapantula et al., 2007). The level of phosphorylated PRAS40 at Thr246 and phosphorylated Akt at Thr308 is also increased in human non-small cell lung cancer (NSCLC) and it is regarded to be a good biomarker to assess Akt activity in NSCLC (Vincent et al., 2011). This study supports the role of PP4c as an inhibitor of the phosphorylation of PRAS40 in both MCF7 and in MDA-MB-231 cell lines, and PP4c may serve as an important biomarker to measure PRAS40 phosphorylation status and the pro-apoptotic signals which will in turn measures PI3K and Akt inhibitors sensitivity.

In triple negative-MDA-MB-231 breast cancer cells modulating the endogenous level of PP4c expression has changed the phosphorylation status of various proteins in the PI3K/Akt/mTOR1 pathway and these changes have an important impact on the cell outcome including cell growth, proliferation, apoptosis and cell response to various chemotherapeutic agents. PP4c down-regulation in MDA-MB-231 breast cancer cells reduced the phosphorylation level of 4E-BP1 at Thr 37/46, GSK-3α at Ser21 and S6-ribosomal protein at Ser235/236 with an increase in the phosphorylation of PDK1 at Ser241, Bad at Ser112, PTEN at Ser380 and PRAS40 at Thr246 (Figure 5.19). Corresponding opposite effect was obtained in case of PP4c up-regulation in these cells (Figure 5.18). Each of these substrates is considered individually below in figure 6.3 and figure 6.4. These observations therefore illustrate the complexity of the cellular phosphorylation/dephosphorylation systems regulated by PP4c in triple negative breast cancer cells.



6.3: PP4c substrates in case of PP4c down-regulation in MDA-MB-231 breast cancer cells. Changes in the phosphorylation state of these substrates could be a direct or an indirect effect of PP4c down-regulation



6.4: PP4c substrates in case of PP4c over-expression in MDA-MB-231 breast cancer cells. Changes in the phosphorylation state of these substrates could be a direct or an indirect effect of PP4c over-expression

Increased phosphorylation of PDK1 at Ser241, Bad at Ser112, PTEN at Ser380 and PRAS40 at Thr246 in MDA-MB-231 upon PP4c down-regulation may explain increased cell number, cell proliferation and the reduction in apoptosis in these cells after PP4c down-regulation. Consistent with this, reduced phosphorylation of the above proteins after PP4c up-regulation may explain reduced cell number and increased apoptosis. Changes in the phosphorylation status of the proteins in the PI3K/Akt/mTOR pathway as a result of modulation in the expression of PP4c in triple negative cells may also explain changes in the response of these cells to various chemotherapeutic agents upon PP4c down-regulation. In triple-negative breast cancer cells, cell proliferation is regulated independently of ER or HER2 signalling, triple-negative breast cancer cells also show high level of p-Akt compared to other types of breast cancer cells (Umemura et al., 2007) which may be the cause of the increased Bad phosphorylation observed in these cells (Umemura et al., 2007). Increased expression of phosphorylated Bad and Erk2 has been identified as a result of PP4c down-regulation in HEK293T cells (Mourtada-Maarabouni and Williams, 2008) and this is consistent with the results obtained in this study. Furthermore, phosphorylated PRAS40 at Thr246 was reported to be positively correlated with the activation of the PI3K pathway and this resulted in increased sensitivity of lung and breast cancer cell lines to Akt inhibitors (Andersen et al, 2010) and this support our results of increased sensitivity of MDA-MB-231 cells to the Akt inhibitor after PP4c down-regulation which could be due to increased level of p-PRAS40.

It is the association between PP4c with its regulatory subunits that determine the function of the final holoenzyme. The results in Chapter 4 showed a positive correlation in between PP4c and PP4R2 and between PP4c and PP4R3 in the term

of mRNA expression in breast cancer tissue samples. Our results agree with the results reported by Wang et al (2008) which showed over-expression of PP4R2 in human primary breast tumours but not in the breast cancer cell lines. This PP4R2 over-expression reduced the activity of PP4c, since it has been reported that association of PP4R1 and PP4R2 with PP4c significantly reduces the activity of PP4c (Hastie et al., 2000; Kloeker and Wadzinski, 1999). This could be as a result of general inhibition of the catalytic subunit by one of these regulatory subunits or may be due to induction of narrower substrate specificities by these regulatory subunits (Hastie et al., 2000; Kloeker and Wadzinski, 1999). PP4R2 and PP4R3 interact with PP4c to form a multimeric complex called PP4cs (protein phosphatase 4, cisplatin-sensitive complex) which is involved in cisplatin sensitivity (Gingras et al., 2005). PP4c down-regulation was associated with reduced expression of both PP4R2 and PP4R3 in both MCF7 and MDA-MB-231 cells (Chapter 4) which may explain increased resistance of these cells to cisplatin after PP4c down-regulation. PP4R3α (SMEK1) was found to be over-expressed in human primary breast tumours but not in the breast cancer cell lines (Wang et al., 2005). Whereas, it was reported that the expression of SMEK1 is found to be reduced in patients with ovarian tumour and cervical tumour as well as in the tumour cell lines (Dong et al., 2012). Our results showed a significant positive correlation in mRNA expression in between PP4c and SMEK1 in breast cancer tissue samples (Figure 4.9). A positive correlation between PP4c and SMEK1 plays a major role in promoting the function of PP4c in the regulation of cell proliferation, cell cycle and apoptosis (Dong et al., 2012). Dong et al (2012) reported that SMEK1 plays a role in increasing the sensitivity of the ovarian cancer cells (OVCAR-3) to gemcitabine. SMEK1 reduced cell proliferation via induction of cell cycle arrest at the G_1–G_0 phase, increased expression of p53

and p21 and reduced expression of the anti-apoptotic Bcl-2 and Bcl-xL proteins (Dong et al., 2012). Furthermore, SMEK1 sensitizes OVCAR-3 to paclitaxel by inducing cell cycle arrest at G_1 or G_2 phase and promoting paclitaxel-induced apoptosis by enhancing caspase-3 activation and PARP (poly ADP ribose polymerase) cleavage (Kim et al., 2014).

Consequently, this study suggests that PP4c plays a critical role in the delicate balance between cell survival and cell death in breast cancer through direct or indirect changes in the phosphorylation state of important proteins in the PI3K/Akt/mTOR signalling pathway and it highlights the importance of targeting PP4c expression and the high potential therapeutic value of targeting PEA15 in particular in the treatment of breast cancer. These effects of PP4c are very likely to be modulated by its corresponding regulatory subunits. Further studies are required around PEA15 and its associated interactions which could provide novel avenues for treatment strategies in breast cancer. Further work is also required to investigate the substrate specificity changes brought about by changing the expression of each PP4c-regulatory subunit and to further investigate whether the different effects of PP4c are mediated via direct or indirect dephosphorylation of its substrates. These questions must be addressed by *in vitro* combination of PP4c with each substrate. Our knowledge of the role of PP4 in the control of cell fate will also be significantly advanced by determining which one of these substrates has a significant impact on breast cancer cell survival and proliferation.

References

Adams, J.M. and Cory, S., 2007. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene,* 26(9), pp.1324-1337.

Addie, M., Ballard, P., Buttar, D., Crafter, C., Currie, G., Davies, B.R., Debreczeni, J., Dry, H., Dudley, P., Greenwood, R., Johnson, P.D., Kettle, J.G., Lane, C., Lamont, G., Leach, A., Luke, R.W., Morris, J., Ogilvie, D., Page, K., Pass, M., Pearson, S., Ruston, L., 2013. Discovery of 4-amino-N-[(1S)-1-(4-chlorophenyl)-3-hydroxypropyl]-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamide (AZD5363), an orally bioavailable, potent inhibitor of Akt kinases. *Journal of Medicinal Chemistry*, 56(5), pp.2059-73.

Ahmed, A.A., Etemadmoghadam, D., Temple, J., Lynch, A.G., Riad, M., Sharma, R., Stewart, C., Fereday, S., Caldas, C., Defazio, A. and Bowtell, D., 2010. Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. *The Journal of Pathology*, 221(1), pp.49-56.

Ahn, N.G., Weiel, J.E., Chan, C.P. and Krebs, E.G., 1990. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *Journal of Biological Chemistry*, 265(20), pp.11487-11494.

Alan, C. and Alan, R. S., 2006. More TORC for the gluconeogenic engine. *BioEssays*, 28(3), pp.231–4.

Allred, D.C., Mohsin, S.K. and Fuqua, S.A., 2001. Histological and biological evolution of human premalignant breast disease. *Endocrine-Related Cancer*, 8(1), pp.47-61.

Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J. and Mustelin, T., 2004. Protein tyrosine phosphatases in the human genome. *Cell*, 117(6), pp.699-711.

American Joint Committee on Cancer, 2002. Cancer Staging Manual. Sixth Edition, pp.227-228.

Andersen, J.N., Sathyanarayanan, S., Di Bacco, A., Chi, A., Zhang, T., Chen, A.H., Dolinski, B., Kraus, M., Roberts, B., Arthur, W., Klinghoffer, R.A., Gargano,

D., Li, L., Feldman, I., Lynch, B., Rush, J., Hendrickson, R.C., Blume-Jensen, P., Paweletz, C.P., 2010. Pathway-based identification of biomarkers for targeted therapeutics: personalized oncology with PI3K pathway inhibitors. *Science Translational Medicine,* 2(43), pp.43ra55.

Andrade, M.A. and Bork, P., 1995. HEAT repeats in the Huntington's disease protein. *Nature Genetics*, 11(2), pp.115-116.

Andre, F., Campone, M., O'Regan, R., Manlius, C., Massacesi, C., Tarek, S., Mukhopadhyay, P., Soria, J.C., Naughton, M. and Hurvitz, S.A., 2010. Phase I study of everolimus plus weekly paclitaxel and trastuzumab in patients with metastatic breast cancer pretreated with trastuzumab. *Journal of Clinical Oncology*, 28(34), pp.5110-5115.

Antoniou, A., Pharoah, P.D.P., Narod, S., Risch, H.A., Eyfjord, J.E., Hopper, J.L., Loman, N., Olsson, H., Johannsson, O., Borg, Å. and Pasini, B., 2003. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: a combined analysis of 22 studies. *The American Journal of Human Genetics*, 72(5), pp.1117-1130.

Apostolou, P. and Fostira, F., 2013. Hereditary breast cancer: the era of new susceptibility genes. *BioMed Research International*, 2013. 747318.

Araujo, H., Danziger, N., Cordier, J., Glowinski, J. and Chneiweiss, H., 1993. Characterization of PEA-15, a major substrate for protein kinase C in astrocytes. *Journal of Biological Chemistry*, 268(8), pp.5911-5920.

Aylon, Y. and Oren, M., 2011. New plays in the p53 theater. *Current Opinion in Genetics & Development*, 21(1), pp.86-92.

Baehrecke, E.H., 2002. How death shapes life during development. *Nature Reviews Molecular Cell Biology,* 3(10), pp.779-787.

Balmain, A., Gray, J. and Ponder, B., 2003. The genetics and genomics of cancer. *Nature Genetics*, 33, pp.238-244.

Bandi, H.R., Ferrari, S., Krieg, J., Meyer, H.E. and Thomas, G., 1993. Identification of 40 S ribosomal protein S6 phosphorylation sites in Swiss mouse 3T3 fibroblasts stimulated with serum. *Journal of Biological Chemistry*, 268(6), pp.4530-4533.

Barford, D., 1996. Molecular mechanisms of theprotein serine/threonine phosphatases. *Trends in Biochemical Sciences*, 21(11), pp.407-412.

Barillot, E., Calzone, L., Hupe, P., Vert, J.P. and Zinovyev, A., 2012. Computational Systems Biology of Cancer. *Chapman & Hall/CRC Mathematical & Computational Biology*, pp. 461.

Bartek, J. and Lukas, J., 2003. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell*, 3(5), pp.421-429.

Bartholomeusz, C., Rosen, D., Wei, C., Kazansky, A., Yamasaki, F., Takahashi, T., Itamochi, H., Kondo, S., Liu, J. and Ueno, N.T., 2008. PEA-15 induces autophagy in human ovarian cancer cells and is associated with prolonged overall survival. *Cancer Research*, 68(22), pp.9302-9310.

Bartholomeusz, C., Rosen, D., Wei, C., Kazansky, A., Yamasaki, F., Takahashi, T., Itamochi, H., Kondo, S., Liu, J. and Ueno, N.T., 2008. PEA-15 induces autophagy in human ovarian cancer cells and is associated with prolonged overall survival. *Cancer Research,* 68(22), pp.9302-9310.

Baselga, J., Campone, M., Piccart, M., Burris III, H.A., Rugo, H.S., Sahmoud, T., Noguchi, S., Gnant, M., Pritchard, K.I., Lebrun, F. and Beck, J.T., 2012. Everolimus in postmenopausal hormone-receptor–positive advanced breast cancer. *New England Journal of Medicine*, 366(6), pp.520-529.

Bastians, H., Krebber, H., Hoheisel, J., Ohl, S., Lichter, P., Ponstingl, H. and Joos, S., 1997. Assignment of the Human Serine/Threonine Protein Phosphatase 4 Gene (PPP4C) to Chromosome 16p11–p12 by Fluorescencein SituHybridization. *Genomics*, 42(1), pp.181-182.

Beeram, M., Tan, Q.T., Tekmal, R.R., Russell, D. and Middleton, A., 2007. Akt-induced endocrine therapy resistance is reversed by inhibition of mTOR signalling. *Annals of Oncology*, 18(8), pp.1323-1328.

Bennett, D., Matthews, R.J. and Sathish, J.G., 2006. The whys and wherefores of phosphate removal. *European Molecular Biology Organization Reports*, 7(3), pp.263-268.

Bergmann, A., 2002. Survival signalling goes BAD. *Developmental Cell*, 3(5), pp.607-608.

Bertram, P.G., Choi, J.H., Carvalho, J., Ai, W., Zeng, C., Chan, T.F. and Zheng, X.S., 2000. Tripartite regulation of Gln3p by TOR, Ure2p, and phosphatases. *Journal of Biological Chemistry*, 275(46), pp.35727-35733.

Bhardwaj, A., Singh, S., Srivastava, S.K., Honkanen, R.E., Reed, E. and Singh, A.P., 2011. Modulation of protein phosphatase 2A activity alters androgen-independent growth of prostate cancer cells: therapeutic implications. *Molecular Cancer Therapeutics,* 10(5), pp.720-731.

Bines, J., Dienstmann, R., Obadia, R.M., Branco, L.G.P., Quintella, D.C., Castro, T.M., Camacho, P.G., Soares, F.A. and Costa, M.E.F., 2014. Activity of megestrol acetate in postmenopausal women with advanced breast cancer after nonsteroidal aromatase inhibitor failure: a phase II trial. *Annals of Oncology,* 25(4), pp.831-836.

Blows, F.M., Driver, K.E., Schmidt, M.K., Broeks, A., Van Leeuwen, F.E., Wesseling, J., Cheang, M.C., Gelmon, K., Nielsen, T.O., Blomqvist, C. and Heikkilä, P., 2010. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLOS Medicine,* 7(5),e1000279.

Bocchinfuso, W.P. and Korach, K.S., 1997. Mammary gland development and tumourigenesis in estrogen receptor knockout mice. *Journal of Mammary Gland Biology and Neoplasia*, 2(4), pp.323-334.

Bonavida, B., 2008. Sensitization of cancer cells for chemo/ immuno/ radiotherapy. *Springer*, pp.419.

Bosio, Y., Berto, G., Camera, P., Bianchi, F., Ambrogio, C., Claus, P. and Di Cunto, F., 2012. PPP4R2 regulates neuronal cell differentiation and survival,

functionally cooperating with SMN. *European Journal of Cell Biology*, 91(8), pp.662-674.

Brechmann, M., Mock, T., Nickles, D., Kiessling, M., Weit, N., Breuer, R., Müller, W., Wabnitz, G., Frey, F., Nicolay, J.P. and Booken, N., 2012. A PP4 holoenzyme balances physiological and oncogenic nuclear factor-kappa B signalling in T lymphocytes. *Immunity*, 37(4), pp.697-708.

Brewis, N.D. and Cohen, P.T., 1992. Protein phosphatase X has been highly conserved during mammalian evolution. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1171(2), pp.231-233.

Brewis, N.D., Street, A.J., Prescott, A.R. and Cohen, P.T., 1993. PPX, a novel protein serine/threonine phosphatase localized to centrosomes. *The European Molecular Biology Organization journal*, 12(3), p.987.

Brinkley, B.R., Beall, P.T., Wible, L.J., Mace, M.L., Turner, D.S. and Cailleau, R.M., 1980. Variations in cell form and cytoskeleton in human breast carcinoma cells *in vitro*. *Cancer Research*, 40(9), pp.3118-3129.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. and Schreiber, S.L., 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, 369(6483), p.756.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell,* 96(6), pp.857-868.

Burkhart, D.L. and Sage, J., 2008. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature Reviews Cancer*, 8(9), pp.671-682.

Burlacu, A., 2003. Regulation of apoptosis by Bcl-2 family proteins. *Journal of Cellular and Molecular Medicine*, 7(3), pp.249-257.

Burris, H.A., 2013. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. *Cancer Chemotherapy and Pharmacology*, 71(4), pp.829-842.

Burstein, H., 2011. Targeted Therapies in Breast Cancer. *Oxford University Press*.

Buxton, R.S. and Magee, A.I., 1992. Structure and interactions of desmosomal and other cadherins. *Seminars in Cell Biology*, 3(3), pp. 157-167.

Byun, H.J., Kim, B.R., Yoo, R., Park, S.Y. and Rho, S.B., 2012. sMEK1 enhances gemcitabine anti-cancer activity through inhibition of phosphorylation of Akt/mTOR. *Apoptosis*, 17(10), pp.1095-1103.

Byun, H.J., Kim, B.R., Yoo, R., Park, S.Y. and Rho, S.B., 2012. sMEK1 enhances gemcitabine anti-cancer activity through inhibition of phosphorylation of Akt/mTOR. *Apoptosis*, 17(10), pp.1095-1103.

Cailleau, R., Young, R., Olive, M. and Reeves, W.J., 1974. Breast tumour cell lines from pleural effusions. *Journal of the National Cancer Institute*, 53(3), pp.661-674.

Cammas, F., Mark, M., Dollé, P., Dierich, A., Chambon, P. and Losson, R., 2000. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. *Development,* 127(13), pp.2955-2963.

Campbell, R.A., Bhat-Nakshatri, P., Patel, N.M., Constantinidou, D., Ali, S. and Nakshatri, H., 2001. Phosphatidylinositol 3-Kinase/AKT-mediated activation of estrogen receptor α A new model for anti-estrogen resistance. *Journal of Biological Chemistry*, 276(13), pp.9817-9824.

Campeau, P.M., Foulkes, W.D. and Tischkowitz, M.D., 2008. Hereditary breast cancer: new genetic developments, new therapeutic avenues. *Human genetics*, 124(1), pp.31-42.

Campisi, J. and di Fagagna, F.D.A., 2007. Cellular senescence: when bad things happen to good cells. *Nature Reviews Molecular Cell Biology*, 8(9), pp.729-740.

Cantley, L.C. and Neel, B.G., 1999. New insights into tumour suppression: PTEN suppresses tumour formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings of the National Academy of Sciences*, 96(8), pp.4240-4245.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C., 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science*, 282(5392), pp.1318-1321.

Carnegie, G.K., Sleeman, J.E., Morrice, N., Hastie, C.J., Peggie, M.W., Philp, A., Lamond, A.I. and Cohen, P.T., 2003. Protein phosphatase 4 interacts with the Survival of Motor Neurons complex and enhances the temporal localisation of snRNPs. *Journal of Cell Science*, 116(10), pp.1905-1913.

Carracedo, A., Alimonti, A. and Pandolfi, P.P., 2011. PTEN level in tumour suppression: how much is too little? *Cancer Research,* 71(3), pp.629-633.

Celeste, A., Difilippantonio, S., Difilippantonio, M.J., Fernandez-Capetillo, O., Pilch, D.R., Sedelnikova, O.A., Eckhaus, M., Ried, T., Bonner, W.M. and Nussenzweig, A., 2003. H2AX haploinsufficiency modifies genomic stability and tumour susceptibility. *Cell*, 114(3), pp.371-383.

Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., Franklin, R. A. and Mccubrey, J.A., 2003. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia,* 17(3), pp.590-603.

Chen, G. and Goeddel, D.V., 2002. TNF-R1 signalling: a beautiful pathway. *Science*, 296(5573), pp.1634-1635.

Chen, G.I., Tisayakorn, S., Jorgensen, C., D'Ambrosio, L.M., Goudreault, M. and Gingras, A.C., 2008. PP4R4/KIAA1622 forms a novel stable cytosolic complex with phosphoprotein phosphatase 4. *Journal of Biological Chemistry*, 283(43), pp.29273-29284.

Chen, H.Y., Yang, Y.M., Stevens, B.M. and Noble, M., 2013. Inhibition of redox/Fyn/c-Cbl pathway function by Cdc42 controls tumour initiation capacity and tamoxifen sensitivity in basal-like breast cancer cells. *European Molecular Biology Organization Molecular Medicine*, 5(5), pp.723-736.

Chen, J., Peterson, R.T. and Schreiber, S.L., 1998. α4 associates with protein phosphatases 2A, 4, and 6. *Biochemical and Biophysical Research Communications*, 247(3), pp.827-832.

Chen, X., 1999. The p53 family: same response, different signals? *Molecular Medicine Today*, 5(9), pp.387-392.

Chen, X., Ko, L.J., Jayaraman, L. and Prives, C., 1996. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumour cells. *Genes & Development,* 10(19), pp.2438-2451.

Cheng, N., Chytil, A., Shyr, Y., Joly, A. and Moses, H.L., 2008. Transforming growth factor-β signalling–deficient fibroblasts enhance hepatocyte growth factor signalling in mammary carcinoma cells to promote scattering and invasion. *Molecular Cancer Research*, 6(10), pp.1521-1533.

Chiang, C.W., Harris, G., Ellig, C., Masters, S.C., Subramanian, R., Shenolikar, S., Wadzinski, B.E. and Yang, E., 2001. Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin-3–dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. *Blood*, 97(5), pp.1289-1297.

Chiang, K.C., Chen, H.Y., Hsu,S.Y., Pang, J.H., Wang, S.Y., Hsu, J.T., Yeh, T.S., Chen, L.W., Kuo, S.F., Sun, C.C., Lee, J.M., Yeh, C.N., Juang, H.H., 2015. PTEN insufficiency modulates ER+ breast cancer cell cycle progression and increases cell growth *in vitro* and *in vivo*. *Drug Design, Development and Therapy,* 13(9), pp.4631-8.

Chowdhury, D., Keogh, M.C., Ishii, H., Peterson, C.L., Buratowski, S. and Lieberman, J., 2005. γ-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Molecular Cell*, 20(5), pp.801-809.

Chowdhury, D., Xu, X., Zhong, X., Ahmed, F., Zhong, J., Liao, J., Dykxhoorn, D.M., Weinstock, D.M., Pfeifer, G.P. and Lieberman, J., 2008. A PP4-phosphatase complex dephosphorylates γ-H2AX generated during DNA replication. *Molecular Cell*, 31(1), pp.33-46.

Chu, Q. D., King, T. and Hurd, T., 2012. Triple-negative breast cancer. *International Journal of Breast Cancer,* 2012 (12), pp.671684.

Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J., 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signalling by the 70 kd S6 protein kinases. *Cell*, 69(7), pp.1227-1236.

Clegg, L.X., Li, F.P., Hankey, B.F., Chu, K. and Edwards, B.K., 2002. Cancer survival among US whites and minorities: a SEER (Surveillance, Epidemiology, and End Results) Program population-based study. *Archives of Internal Medicine*, 162(17), pp.1985-1993.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. *Biochemical Journal*, 326(1), pp.1-16.

Cohen, P.T., Philp, A. and Vázquez-Martin, C., 2005. Protein phosphatase 4–from obscurity to vital functions. *Federation of European Biochemical Societies letters,* 579(15), pp.3278-3286.

Condorelli, G., Vigliotta, G., Cafieri, A., Trencia, A., Andalo, P., Oriente, F., Miele, C., Caruso, M., Formisano, P. and Beguinot, F., 1999. PED/PEA-15: an anti-apoptotic molecule that regulates FAS/TNFR1-induced apoptosis. *Oncogene*, 18(31), pp.4409-4415.

Condorelli, G., Vigliotta, G., Trencia, A., Maitan, M.A., Caruso, M., Miele, C., Santopietro, S., Formisano, P. and Beguinot, F., 2001. Protein kinase C (PKC)-α activation inhibits PKC-ζ and mediates the action of PED/PEA-15 on glucose transport in the L6 skeletal muscle cells. *Diabetes*, 50(6), pp.1244-1252.

Cory, S. and Adams, J.M., 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer*, 2(9), pp.647-656.

Croce, C. M. 2008. Oncogenes and cancer. *New England Journal of Medicine*, 358(5), pp.502-11.

Cross, D.A., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S. and Cohen, P., 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochemical Journal*, 303(1), pp.21-26.

Currie R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R. and Lucocq, J., 1999. Role of phosphatidylinositol 3, 4, 5-

trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochemical Journal*, 337(3), pp.575-583.

D'Andrea, A.D. and Haseltine, W.A., 1978. Sequence specific cleavage of DNA by the antitumour antibiotics neocarzinostatin and bleomycin. *Proceedings of the National Academy of Sciences*, 75(8), pp.3608-3612.

Davis, A.J. and Chen, D.J., 2012. A role for protein phosphatase 4 in regulating non-homologous end-joining. *Cell Cycle*, 11(19), pp.3535-3535.

DeGraffenried LA, Friedrichs WE, Russell DH, Donzis EJ, Middleton AK, Silva JM, Roth, R.A. and Hidalgo, M., 2004. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. *Clinical Cancer Research*, 10(23), pp.8059-8067.

DeGraffenried, L.A., Fulcher, L., Friedrichs, W.E., Grünwald, V., Ray, R.B., Hidalgo, M., 2004. Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. *Annals of Oncology,* 15(10), pp.1510-6.

Dejean, L.M., Martinez-Caballero, S. and Kinnally, K.W., 2006. Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis? *Cell Death & Differentiation*, 13(8), pp.1387-1395.

den Hollander, P., Savage, M.I. and Brown, P.H., 2013. Targeted therapy for breast cancer prevention. *Frontiers in Oncology*, 3, p.250.

Deng, X., Ito, T., Carr, B., Mumby, M. and May, W.S., 1998. Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. *Journal of Biological Chemistry*, 273(51), pp.34157-34163.

Dennis, P.B., Fumagalli, S. and Thomas, G., 1999. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Current Opinion in Genetics & Development,* 9(1), pp.49-54.

Denu, J.M., Stuckey, J.A., Saper, M.A. and Dixon, J.E., 1996. Form and function in protein dephosphorylation. *Cell*, 87(3), pp.361-364.

Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B. and Martinou, J.C., 2001. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Molecular Cell*, 8(3), pp.601-611.

Donegan, W.L., 1997. Tumour-related prognostic factors for breast cancer. *CA: a Cancer Journal for Clinicians,* 47(1), pp.28-51.

Dong, S.M., Byun, H.J., Kim, B.R., Lee, S.H., Trink, B. and Rho, S.B., 2012. Tumour suppressor BLU enhances pro-apoptotic activity of sMEK1 through physical interaction. *Cellular Signalling*, 24(6), pp.1208-1214.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J. and Wicha, M.S., 2003. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & Development*, 17(10), pp.1253-1270.

Dowsett, M., Cuzick, J., Wale, C., Howell, T., Houghton, J. and Baum, M., 2005. Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: an hypothesis-generating study. *Journal of Clinical Oncology*, 23(30), pp.7512-7517.

Du, L., Chen, X., Cao, Y., Lu, L., Zhang, F., Bornstein, S., Li, Y., Owens, P., Malkoski, S., Said, S. and Jin, F., 2016. Overexpression of PIK3CA in murine head and neck epithelium drives tumour invasion and metastasis through PDK1 and enhanced TGFβ signalling. *Oncogene*, 35(35), pp.4641-52.

Duffy, M.J., 2013. The war on cancer: are we winning?. *Tumour Biology*, 34(3), pp.1275-1284.

Dufner, A. and Thomas, G., 1999. Ribosomal S6 kinase signalling and the control of translation. *Experimental Cell Research*, 253(1), pp.100-109.

Dupuy, F., Griss, T., Blagih, J., Bridon, G., Avizonis, D., Ling, C., Dong, Z., Siwak, D.R., Annis, M.G., Mills, G.B. and Muller, W.J., 2013. LKB1 is a central regulator of tumour initiation and pro-growth metabolism in ErbB2-mediated breast cancer. *Cancer & Metabolism*, 1(1), p.18.

Easton, D.F., 1999. How many more breast cancer predisposition genes are there? *Breast Cancer Research*, 1(1), p.14.

Eckert, A., Böck, B.C., Tagscherer, K.E., Haas, T.L., Grund, K., Sykora, J., Herold-Mende, C., Ehemann, V., Hollstein, M., Chneiweiss, H. and Wiestler, O.D., 2008. The PEA-15/PED protein protects glioblastoma cells from glucose deprivation-induced apoptosis via the ERK/MAP kinase pathway. *Oncogene*, 27(8), pp.1155-1166.

Edinger, A.L. and Thompson, C.B., 2004. Death by design: apoptosis, necrosis and autophagy. *Current Opinion in Cell Biology*, 16(6), pp.663-669.

Eichhorn, P.J., Creyghton, M.P. and Bernards, R., 2009. Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 1795(1), pp.1-15.

Embi, N., Rylatt, D.B. and Cohen, P., 1980. Glycogen Synthase Kinase-3 from Rabbit Skeletal Muscle. *European Journal of biochemistry*, 107(2), pp.519-527.

Eshleman, J.R., Lang, E.Z., Bowerfind, G.K., Parsons, R., Vogelstein, B., Willson, J.K., Veigl, M.L., Sedwick, W.D. and Markowitz, S.D., 1995. Increased mutation rate at the hprt locus accompanies microsatellite instability in colon cancer. *Oncogene,* 10(1), pp.33-37.

Estep, A.L., Palmer, C., McCormick, F. and Rauen, K.A., 2007. Mutation analysis of BRAF, MEK1 and MEK2 in 15 ovarian cancer cell lines: implications for therapy. *PLOS One*, 2(12), p.e1279.

Eytan, G.D., 2005. Mechanism of multidrug resistance in relation to passive membrane permeation. *Biomedicine & Pharmacotherapy*, 59(3), pp.90-97.

Fang, X., Yu, S., Eder, A., Mao, M., Bast, R.C., Boyd, D. and Mills, G.B., 1999. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene*, 18(48), pp.6635-6640.

Favaloro, B., Allocati, N., Graziano, V., Di Ilio, C. and De Laurenzi, V., 2012. Role of apoptosis in disease. *Aging (Albany NY)*, 4(5), pp.330-349.

Fedele, C.G., Ooms, L.M., Ho, M., Vieusseux, J., O'Toole, S.A., Millar, E.K., Lopez-Knowles, E., Sriratana, A., Gurung, R., Baglietto, L. and Giles, G.G., 2010. Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signalling and is lost in human basal-like breast cancers. *Proceedings of the National Academy of Sciences*, 107(51), pp.22231-22236.

Ferlay, J., Parkin, D.M. and Steliarova-Foucher, E., 2010. Estimates of cancer incidence and mortality in Europe in 2008. *European Journal of Cancer*, 46(4), pp.765-781.

Fernando, R., Foster, J.S., Bible, A., Ström, A., Pestell, R.G., Rao, M., Saxton, A., Baek ,S.J., Yamaguchi, K., Donnell, R., Cekanova, M., Wimalasena, J., 2007. Breast cancer cell proliferation is inhibited by BAD: regulation of cyclin D1. *The Journal of Biological Chemistry*, 282(39), pp.28864-73.

Fernando, R.I. and Wimalasena, J., 2004. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signalling through ERK and Akt. *Molecular Biology of the Cell*, 15(7), pp.3266-3284.

Ferrara, N. 2009. Vascular endothelial growth factor. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29 (6), pp.789-91.

Fingar, D.C., Salama, S., Tsou, C., Harlow, E.D. and Blenis, J., 2002. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes & Development*, 16(12), pp.1472-1487.

Fiory, F., Formisano, P., Perruolo, G. and Beguinot, F., 2009. Frontiers: PED/PEA-15, a multifunctional protein controlling cell survival and glucose metabolism. *American Journal of Physiology-Endocrinology and Metabolism*, 297(3), pp.E592-E601.

Fonseca, B.D., Smith, E.M., Lee, V.H.Y., MacKintosh, C. and Proud, C.G., 2007. PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signalling downstream of this complex. *Journal of Biological Chemistry*, 282(34), pp.24514-24524.

Formstecher, E., Ramos, J.W., Fauquet, M., Calderwood, D.A., Hsieh, J.C., Canton, B., Nguyen, X.T., Barnier, J.V., Camonis, J., Ginsberg, M.H. and Chneiweiss, H., 2001. PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. *Developmental Cell*, 1(2), pp.239-250.

Fouladi, B., Sabatier, L., Miller, D., Pottier, G. and Murnane, J.P., 2000. The relationship between spontaneous telomere loss and chromosome instability in a human tumour cell line. *Neoplasia*, 2(6), pp.540-554.

Fowler, J., Cohen, L., 1990. Practical Statistics for Field Biology. *Open University Press.* Milton, Keynes, UK.

Freeman, A.K., Dapic, V. and Monteiro, A.N., 2010. Negative regulation of CHK2 activity by protein phosphatase 2A is modulated by DNA damage. *Cell Cycle*, 9(4), pp.736-747.

Fry, M.J., 2001. Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play? *Breast Cancer Research*, 3(5), p.304.

Fu, R., 2012. Clinicopathological and prognostic significance of phosphorylated PRAS40 (Thr246) in breast cancer. *Asian Biomedicine,* 6(4), pp.573–577.

Fyffe, C. and Falasca, M., 2013. 3-Phosphoinositide-dependent protein kinase-1 as an emerging target in the management of breast cancer. *Cancer Management and Research*, 5(1), pp.271-280.

Gagliardi, P.A., di Blasio, L., Orso, F., Seano, G., Sessa, R., Taverna, D., Bussolino, F. and Primo, L., 2012. 3-phosphoinositide-dependent kinase 1 controls breast tumour growth in a kinase-dependent but Akt-independent manner. *Neoplasia*, 14(8), pp.719IN14-731IN19.

Gajria, D. and Chandarlapaty, S., 2011. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Review of Anticancer Therapy*, 11(2), pp.263-275.

García-Martínez, J.M. and Alessi, D.R., 2008. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum-and

glucocorticoid-induced protein kinase 1 (SGK1). *Biochemical Journal*, 416(3), pp.375-385.

Gawecka, J.E., Geerts, D., Koster, J., Caliva, M.J., Sulzmaier, F.J., Opoku-Ansah, J., Wada, R.K., Bachmann, A.S. and Ramos, J.W., 2012. PEA15 impairs cell migration and correlates with clinical features predicting good prognosis in neuroblastoma. *International Journal of Cancer*, 131(7), pp.1556-1568.

Giaccia, A.J. and Kastan, M.B., 1998. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes & Development*, 12(19), pp.2973-2983.

Gil, E.M.C., 2014. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. *Cancer Treatment Reviews*, 40(7), pp.862-871.

Gingras, A.C., Caballero, M., Zarske, M., Sanchez, A., Hazbun, T.R., Fields, S., Sonenberg, N., Hafen, E., Raught, B. and Aebersold, R., 2005. A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity. *Molecular & Cellular Proteomics*, 4(11), pp.1725-1740.

Gingras, A.C., Gygi, S.P., Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R. and Sonenberg, N., 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes & Development, 13(11), pp.1422-1437.

Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. and Hay, N., 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt (PKB) signalling pathway. *Genes & Development*, 12(4), pp.502-513.

Gioeli, D., Ficarro, S.B., Kwiek, J.J., Aaronson, D., Hancock, M., Catling, A.D., White, F.M., Christian, R.E., Settlage, R.E., Shabanowitz, J., Hunt, D.F., Weber, M.J., 2002. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *Journal of Biological Chemistry*, 277(32), pp.29304-14.

Glading, A., Koziol, J.A., Krueger, J. and Ginsberg, M.H., 2007. PEA-15 inhibits tumour cell invasion by binding to extracellular signal-regulated kinase 1/2. *Cancer Research*, 67(4), pp.1536-1544.

Goh, A.M., Coffill, C.R. and Lane, D.P., 2011. The role of mutant p53 in human cancer. *The Journal of pathology*, 223(2), pp.116-126.

González, D., Bejarano, I., Barriga, C., Rodriguez, A.B. and Pariente, J.A., 2010. Oxidative stress-induced caspases are regulated in human myeloid HL-60 cells by calcium signal. *Current Signal Transduction Therapy*, 5(2), pp.181-186.

Green, D.R. and Evan, G.I., 2002. A matter of life and death. *Cancer Cell*, 1(1), pp.19-30.

Green, D.R., 2005. Apoptotic pathways: ten minutes to dead. *Cell*, 121(5), pp.671-674.

Greig, F.H. and Nixon, G.F., 2014. Phosphoprotein enriched in astrocytes (PEA)-15: A potential therapeutic target in multiple disease states. *Pharmacology & Therapeutics*, 143(3), pp.265-274.

Grunt, T.W. and Mariani, G.L., 2013. Novel approaches for molecular targeted therapy of breast cancer: interfering with PI3K/AKT/mTOR signalling. *Current Cancer Drug Targets*, 13(2), pp.188-204.

Habuchi, T., Yoshida, O. and Knowles, M.A., 1997. A novel candidate tumour suppressor locus at 9q32–33 in bladder cancer: localization of the candidate region within a single 840 kb YAC. *Human Molecular Genetics*, 6(6), pp.913-919.

Hackett, A.J., Smith, H.S., Springer, E.L., Owens, R.B., Nelson-Rees, W.A., Riggs, J.L. and Gardner, M.B., 1977. Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. *Journal of the National Cancer Institute*, 58(6), pp.1795-1806.

Hadweh, P., Habelhah, H., Kieff, E., Mosialos, G. and Hatzivassiliou, E., 2014. The PP4R1 subunit of protein phosphatase PP4 targets TRAF2 and TRAF6 to mediate inhibition of NF-κB activation. *Cellular Signalling*, 26(12), pp.2730-2737.

Halperin, E.C., Brady, L.W., Wazer, D.E. and Perez, C.A., 2013. Perez & Brady's principles and practice of radiation oncology. 6th. Lippincott Williams & Wilkins; Baltimore, MD.

Hanahan, D. and Weinberg, R.A., 2000. The hallmarks of cancer. *Cell*, 100(1), pp.57-70.

Hanahan, D. and Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646-674.

Harada, H., Andersen, J.S., Mann, M., Terada, N. and Korsmeyer, S.J., 2001. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proceedings of the National Academy of Sciences*, 98(17), pp.9666-9670.

Harris, H.A., 2007. Estrogen receptor-β: recent lessons from *in vivo* studies. *Molecular Endocrinology*, 21(1), pp.1-13.

Hastie, C.J., Graeme, K., Morrice, N. and Cohen, P.T., 2000. A novel 50 kDa protein forms complexes with protein phosphatase 4 and is located at centrosomal microtubule organizing centres. *Biochemical Journal,* 347(3), pp.845-855.

Hayashi, N., Peacock, J.W., Beraldi, E., Zoubeidi, A., Gleave, M.E. and Ong, C.J., 2012. Hsp27 silencing coordinately inhibits proliferation and promotes Fas-induced apoptosis by regulating the PEA-15 molecular switch. *Cell Death & Differentiation,* 19(6), pp.990-1002.

Hayashi, N., Peacock, J.W., Beraldi, E., Zoubeidi, A., Gleave, M.E., Ong, C.J., 2012. Hsp27 silencing coordinately inhibits proliferation and promotes Fas-induced apoptosis by regulating the PEA-15 molecular switch. *Cell Death & Differentiation,* 19(6), pp.990-1002.

Hayden, M.S. and Ghosh, S., 2008. Shared principles in NF-κB signalling. *Cell*, 132(3), pp.344-362.

Helps, N.R., Brewis, N.D., Lineruth, K., Davis, T., Kaiser, K. and Cohen, P.T., 1998. Protein phosphatase 4 is an essential enzyme required for organisation of

microtubules at centrosomes in Drosophila embryos. *Journal of Cell Science*, 111(10), pp.1331-1340.

Hermanto, U., Zong, C.S., Wang, L.H., 2001. ErbB2-overexpressing human mammary carcinoma cells display an increased requirement for the phosphatidylinositol 3-kinase signalling pathway in anchorage-independent growth. *Oncogene*, 20(51), pp.7551-62.

Hernandez-Aya, L.F. and Gonzalez-Angulo, A.M., 2011. Targeting the phosphatidylinositol 3-kinase signalling pathway in breast cancer. *The Oncologist*, 16(4), pp.404-414.

Hinds, T.D. and Sánchez, E.R., 2008. Protein phosphatase 5. *The International Journal of Biochemistry & Cell Biology,* 40(11), pp.2358-2362.

Hindupur, S.K., Balaji, S.A., Saxena, M., Pandey, S., Sravan, G.S., Heda, N., Kumar, M.V., Mukherjee, G., Dey, D. and Rangarajan, A., 2014. Identification of a novel AMPK-PEA15 axis in the anoikis-resistant growth of mammary cells. *Breast Cancer Research*, 16(4), p.420.

Ho, M.Y., Liang, C.M. and Liang, S.M., 2016. PATZ1 induces PP4R2 to form a negative feedback loop on IKK/NF-κB signalling in lung cancer. *Oncotarget*, 7(32), p.52255.

Hu, M.C.T., Shui, J.W., Mihindukulasuriya, K.A. and Tan, T.H., 2001. Genomic structure of the mouse PP4 gene: a developmentally regulated protein phosphatase. *Gene,* 278(1), pp.89-99.

Hu, M.C.T., Tang-Oxley, Q., Qiu, W.R., Wang, Y.P., Mihindukulasuriya, K.A., Afshar, R. and Tan, T.H., 1998. Protein phosphatase X interacts with c-Rel and stimulates c-Rel/nuclear factor κB activity. *Journal of Biological Chemistry,* 273(50), pp.33561-33565.

Huang, Y., Ray, S., Reed, J.C., Ibrado, A.M., Tang, C., Nawabi, A. and Bhalla, K., 1997. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. *Breast Cancer Research and Treatment,* 42(1), pp.73-81.

Hughes, K., Nikolakaki, E., Plyte, S.E., Totty, N.F. and Woodgett, J.R., 1993. Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *The European Molecular Biology Organization journal*, 12(2), p.803.

Humphreys, R.C., Lydon, J.P., O'malley, B.W. and Rosen, J.M., 1997. Use of PRKO mice to study the role of progesterone in mammary gland development. *Journal of Mammary Gland Biology and Neoplasia*, 2(4), pp.343-354.

Humphries, J.D., Byron, A. and Humphries, M.J., 2006. Integrin ligands at a glance. *Journal of Cell Science*, 119(19), pp.3901-3903.

Hutchinson, L., 2010. Breast cancer: Challenges, controversies, breakthroughs. *Nature Reviews Clinical Oncology*, 7(12), pp.669–670.

Hwang, S.I., Kuo, W.L., Cochran, J.F., Guzman, R.C., Tsukamoto, T., Bandyopadhyay, G., Myambo, K. and Collins, C.C., 1997. Assignment of HMAT1, the human homolog of the murine mammary transforming gene (MAT1) associated with tumourigenesis, to 1q21. 1, a region frequently gained in human breast cancers. *Genomics,* 42(3), pp.540-542.

Hynes, N.E., 2000. Tyrosine kinase signalling in breast cancer. *Breast Cancer Research,* 2 (3), pp.154–157.

Iftode, C., Daniely, Y. and Borowiec, J.A., 1999. Replication protein A (RPA): the eukaryotic SSB. *Critical Reviews in Biochemistry and Molecular Biology*, 34(3), pp.141-180.

Igney, F.H. and Krammer, P.H., 2002. Death and anti-death: tumour resistance to apoptosis. *Nature Reviews Cancer*, 2(4), pp.277-288.

Indran, I.R., Tufo, G., Pervaiz, S. and Brenner, C., 2011. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1807(6), pp.735-745.

Inostroza, J., SÁENZ, L., CALAF, G., CABELLO, G. and PARRA, E., 2005. Role of the phosphatase PP4 in the activation of JNK-1 in prostate carcinoma cell

lines PC-3 and LNCaP resulting in increased AP-1 and EGR-1 activity. *Biological Research*, 38(2-3), pp.163-178.

Jacobson, M.D., Weil, M. and Raff, M.C., 1997. Programmed cell death in animal development. *Cell*, 88(3), pp.347-354.

Jakobsson, J., Cordero, M.I., Bisaz, R., Groner, A.C., Busskamp, V., Bensadoun, J.C., Cammas, F., Losson, R., Mansuy, I.M., Sandi, C. and Trono, D., 2008. KAP1-mediated epigenetic repression in the forebrain modulates behavioral vulnerability to stress. *Neuron*, 60(5), pp.818-831.

Janssens, V. and Goris, J., 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochemical Journal,* 353(3), pp.417-439.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J. and Thun, M.J., 2007. Cancer statistics, 2007. *CA: A Cancer Journal for Clinicians*, 57(1), pp.43-66.

Jones, R.G. and Thompson, C.B., 2009. Tumour suppressors and cell metabolism: a recipe for cancer growth. *Genes & Development*, 23(5), pp.537-548.

Junttila, M.R. and Evan, G.I., 2009. p53—a Jack of all trades but master of none. *Nature Reviews Cancer*, 9(11), pp.821-829..

Kannan, N., Haste, N., Taylor, S.S. and Neuwald, A.F., 2007. The hallmark of AGC kinase functional divergence is its C-terminal tail, a cis-acting regulatory module. *Proceedings of the National Academy of Sciences*, 104(4), pp.1272-1277.

Kato, S., Endoh, H., Masuhiro, Y. and Kitamoto, T., 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*, 270(5241), p.1491.

Kawazoe, H., Bilim, V.N., Ugolkov, A.V., Yuuki, K., Naito, S., Nagaoka, A., Kato, T. and Tomita, Y., 2012. GSK-3 inhibition *in vitro* and *in vivo* enhances antitumour effect of sorafenib in renal cell carcinoma (RCC). *Biochemical and Biophysical Research Communications*, 423(3), pp.490-495.

Keogh, M.C., Kim, J.A., Downey, M., Fillingham, J., Chowdhury, D., Harrison, J.C., Onishi, M., Datta, N., Galicia, S., Emili, A. and Lieberman, J., 2006. A phosphatase complex that dephosphorylates γH2AX regulates DNA damage checkpoint recovery. *Nature*, 439(7075), pp.497-501.

Kerr, J.F., Wyllie, A.H. and Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 26(4), p.239.

Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J., Radu, M., Chaitcik, S. and Brenner, R.H., 1979. Establishment and characterization of a cell line of human breast carcinoma origin. *European Journal of Cancer*, 15(5), pp.659-670.

Kim, B.R., Seo, S.H., Park, M.S., Lee, S.H., Kwon, Y. and Rho, S.B., 2015. sMEK1 inhibits endothelial cell proliferation by attenuating VEGFR-2-dependent-Akt/eNOS/HIF-1α signalling pathways. *Oncotarget,* 6(31), p.31830.

Kim, B.R., Yoon, K., Byun, H.J., Seo, S.H., Lee, S.H. and Rho, S.B., 2014. The anti-tumour activator sMEK1 and paclitaxel additively decrease expression of HIF-1α and VEGF via mTORC1-S6K/4E-BP-dependent signalling pathways. *Oncotarget,* 5(15), pp.6540-6551.

Kim, B.R., Yoon, K., Byun, H.J., Seo, S.H., Lee, S.H. and Rho, S.B., 2014. The anti-tumour activator sMEK1 and paclitaxel additively decrease expression of HIF-1α and VEGF via mTORC1-S6K/4E-BP-dependent signalling pathways. *Oncotarget*, 5(15), pp.6540-6551.

Kim, M.Y., Oskarsson, T., Acharyya, S., Nguyen, D.X., Zhang, X.H.F., Norton, L. and Massagué, J., 2009. Tumour self-seeding by circulating cancer cells. *Cell*, 139(7), pp.1315-1326.

Kim, Y.N., Koo, K.H., Sung, J.Y., Yun, U.J. and Kim, H., 2012. Anoikis resistance: an essential prerequisite for tumour metastasis. *International Journal of Cell Biology*, 2012, pp.306879.

Kleijn, M., Scheper, G.C., Voorma, H.O. and Thomas, A.A., 1998. Regulation of translation initiation factors by signal transduction. *European Journal of Biochemistry,* 253(3), pp.531-544.

Kloeker, S. and Wadzinski, B.E., 1999. Purification and identification of a novel subunit of protein serine/threonine phosphatase 4. *Journal of Biological Chemistry*, 274(9), pp.5339-5347.

Kloeker, S., Reed, R., McConnell, J.L., Chang, D., Tran, K., Westphal, R.S., Law, B.K., Colbran, R.J., Kamoun, M., Campbell, K.S. and Wadzinski, B.E., 2003. Parallel purification of three catalytic subunits of the protein serine/threonine phosphatase 2A family (PP2A C, PP4 C, and PP6 C) and analysis of the interaction of PP2A C with alpha4 protein. *Protein Expression and Purification*, 31(1), pp.19-33.

Knutson, S.K., Wigle, T.J., Warholic, N.M., Sneeringer, C.J., Allain, C.J., Klaus, C.R., Sacks, J.D., Raimondi, A., Majer, C.R., Song, J. and Scott, M.P., 2012. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nature Chemical Biology*, 8(11), pp.890-896.

Komeili-Movahhed, T., Fouladdel, S., Barzegar, E., Atashpour, S., Hossein Ghahremani, M., Nasser, O. S., Madjd, Z., Azizi, E., 2015. PI3K/Akt inhibition and down-regulation of BCRP re-sensitize MCF7 breast cancer cell line to mitoxantrone chemotherapy. *Iranian Journal of Basic Medical Sciences*, 18(5), pp.472-7.

Kong, M., Ditsworth, D., Lindsten, T. and Thompson, C.B., 2009. α4 is an essential regulator of PP2A phosphatase activity. *Molecular Cell*, 36(1), pp.51-60.

Kovacina, K.S., Park, G.Y., Bae, S.S., Guzzetta, A.W., Schaefer, E., Birnbaum, M.J. and Roth, R.A., 2003. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. *Journal of Biological Chemistry*, 278(12), pp.10189-10194.

Krieg, J., Hofsteenge, J. and Thomas, G., 1988. Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. *Journal of Biological Chemistry*, 263(23), pp.11473-11477.

Krueger, J., Chou, F.L., Glading, A., Schaefer, E. and Ginsberg, M.H., 2005. Phosphorylation of phosphoprotein enriched in astrocytes (PEA-15) regulates extracellular signal-regulated kinase-dependent transcription and cell proliferation. *Molecular Biology of the Cell*, 16(8), pp.3552-3561.

Kubes, Miroslav, Jocelyne Cordier, Jacques Glowinski, Jean-Antoine Girault, and Hervé Chneiweiss. 1998. "Endothelin Induces a Calcium-Dependent Phosphorylation of PEA-15 in Intact Astrocytes: Identification of Ser104 and Ser116 Phosphorylated, Respectively, by Protein Kinase C and Calcium/Calmodulin Kinase II *In Vitro*." *Journal of Neurochemistry*, 71(3), pp.1307-1314.

Le Romancer, M., Poulard, C., Cohen, P., Sentis, S., Renoir, J.M. and Corbo, L., 2011. Cracking the estrogen receptor's posttranslational code in breast tumours. *Endocrine Reviews*, 32(5), pp.597-622.

Lee, D.H. and Chowdhury, D., 2011. What goes on must come off: phosphatases gate-crash the DNA damage response. *Trends in Biochemical Sciences*, 36(11), pp.569-577.

Lee, D.H., Pan, Y., Kanner, S., Sung, P., Borowiec, J.A. and Chowdhury, D., 2010. A PP4 phosphatase complex dephosphorylates RPA2 to facilitate DNA repair via homologous recombination. *Nature Structural & Molecular Biology*, 17(3), pp.365-372.

Lee, J., Adelmant, G., Marto, J.A. and Lee, D.H., 2015. Dephosphorylation of DBC1 by protein phosphatase 4 is important for p53-mediated cellular functions. *Molecules and Cells*, 38(8), p.697.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P. and Pavletich, N.P., 1999. Crystal structure of the PTEN tumour suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell,* 99(3), pp.323-334.

Leung, E.Y., Askarian-Amiri, M., Finlay, G.J., Rewcastle, G.W., Baguley, B.C., 2015. Potentiation of Growth Inhibitory Responses of the mTOR Inhibitor

Everolimus by Dual mTORC1/2 Inhibitors in Cultured Breast Cancer Cell Lines. *PLOS One,* 10(7). e0131400

Li, D.M. and Sun, H., 1997. TEP1, encoded by a candidate tumour suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. *Cancer Research*, 57(11), pp.2124-2129.

Li, X., Liang, L., Huang, L., Ma, X., Li, D. and Cai, S., 2015. High expression of protein phosphatase 4 is associated with the aggressive malignant behavior of colorectal carcinoma. *Molecular Cancer*, 14(1), p.95.

Liang, K., Lu, Y., Li, X., Zeng, X., Glazer, R.I., Mills, G.B. and Fan, Z., 2006. Differential roles of phosphoinositide-dependent protein kinase-1 and akt1 expression and phosphorylation in breast cancer cell resistance to Paclitaxel, Doxorubicin, and gemcitabine. *Molecular Pharmacology*, 70(3), pp.1045-1052.

Lien, E.C., Lyssiotis, C.A., Juvekar, A., Hu, H., Asara, J.M., Cantley, L.C. and Toker, A., 2016. Glutathione biosynthesis is a metabolic vulnerability in PI (3) K/Akt-driven breast cancer. *Nature Cell Biology*, 18(5), pp.572-578.

Lifeng, N., Long, Z., Huang, X., Sun, L. and Sang, J., 2006. Effects of PP4 suppression on the proliferation of MCF7 cells. *Chinese Science Bulletin*, 51(19), pp.2335-2341.

Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H. and Wu, H., 2000. Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Current Biology,* 10, pp.401-404.

Lukyanova, N.Y., Rusetskya, N.V., Tregubova, N.A. and Chekhun, V.F., 2009. Molecular profile and cell cycle in MCF-7 cells resistant to cisplatin and doxorubicin. *Experimental Oncology,* 31(2), pp.87-91.

Lumachi, F., Luisetto, G., Basso, S. M., Basso, U., Brunello, A. & Camozzi, V. 2011. Endocrine therapy of breast cancer. *Current Medicinal Chemistry,* 18(4), pp.513-22.

Lyu, J., Kim, H.R., Yamamoto, V., Choi, S.H., Wei, Z., Joo, C.K. and Lu, W., 2013. Protein phosphatase 4 and Smek complex negatively regulate Par3 and promote neuronal differentiation of neural stem/progenitor cells. *Cell Reports,* 5(3), pp.593-600.

MacKeigan, J.P., Murphy, L.O. and Blenis, J., 2005. Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nature Cell Biology*, 7(6), pp.591-600.

Madhunapantula, S.V., Sharma, A., Robertson, G.P., 2007. PRAS40 deregulates apoptosis in malignant melanoma. *Cancer Research*,67(8), pp.3626-36.

Magnuson, B., Ekim, B. and Fingar, D.C., 2012. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochemical Journal*, 441(1), pp.1-21.

Manning, J.R., Jacobs, J., Fried, I. and Kahana, M.J., 2009. Broadband shifts in local field potential power spectra are correlated with single-neuron spiking in humans. *Journal of Neuroscience*, 29(43), pp.13613-13620.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. *Nature,* 454(7203), pp.436-44.

Mao, Z., Jiang, Y., Liu, X., Seluanov, A., Gorbunova, V., 2009. DNA repair by homologous recombination, but not by nonhomologous end joining, is elevated in breast cancer cells. *Neoplasia,* 11(7), pp.683-91.

Marks, J.L., Gong, Y., Chitale, D., Golas, B., McLellan, M.D., Kasai, Y., Ding, L., Mardis, E.R., Wilson, R.K., Solit, D. and Levine, R., 2008. Novel MEK1 mutation identified by mutational analysis of epidermal growth factor receptor signalling pathway genes in lung adenocarcinoma. *Cancer Research*, 68(14), pp.5524-5528.

Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L., Zheng, Z., Liaw, D., Caron, S., Duboué, B., Lin, A.Y. and Richardson, A.L., 1998. Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. *Human Molecular Genetics*, 7(3), pp.507-515.

Martin-Granados, C., Philp, A., Oxenham, S.K., Prescott, A.R. and Cohen, P.T., 2008. Depletion of protein phosphatase 4 in human cells reveals essential roles in centrosome maturation, cell migration and the regulation of Rho GTPases. *The International Journal of Biochemistry & Cell Biology*, 40(10), pp.2315-2332.

Mathews, S.T., Plaisance, E.P. and Kim, T., 2009. Imaging systems for westerns: chemiluminescence vs. infrared detection. *Methods in Molecular Biology*, 536, pp.499-513.

Maxim, I., Moreel, X., Gagné, J.P., Rouleau, M., Ethier, C., Gagné, P., Hendzel, M.J. and Poirier, G.G., 2010. Investigation of PARP-1, PARP-2, and PARG interactomes by affinity-purification mass spectrometry. *Proteome Science*, 8(1), p.22.

McConnell, J.L., Watkins, G.R., Soss, S.E., Franz, H.S., McCorvey, L.R., Spiller, B.W., Chazin, W.J. and Wadzinski, B.E., 2010. Alpha4 is a ubiquitin-binding protein that regulates protein serine/threonine phosphatase 2A ubiquitination. *Biochemistry*, 49(8), pp.1713-1718.

McHugh, P.J., Spanswick, V.J. and Hartley, J.A., 2001. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *The lancet Oncology*, 2(8), pp.483-490.

McKenzie, S. and Kyprianou, N., 2006. Apoptosis evasion: the role of survival pathways in prostate cancer progression and therapeutic resistance. *Journal of Cellular Biochemistry*, 97(1), pp.18-32.

Meerasahib, M.F. and Asseri, A.H., 2011. Evidence of a positive correlation between accelerated oestrogen receptor phosphorylation and breast cancer progression. *Journal of Cancer Research and Experimental Oncology*, 3(9), pp.105-114.

Meijers-Heijboer, H., Wasielewski, M., Wagner, A., Hollestelle, A., Elstrodt, F., van den Bos, R., de Snoo, A., Fat, G.T.A., Brekelmans, C., Jagmohan, S. and Franken, P., 2003. The CHEK2 1100delC mutation identifies families with a

hereditary breast and colorectal cancer phenotype. *The American Journal of Human Genetics*, 72(5), pp.1308-1314.

Mendoza, M.C., Booth, E.O., Shaulsky, G. and Firtel, R.A., 2007. MEK1 and protein phosphatase 4 coordinate Dictyostelium development and chemotaxis. *Molecular and Cellular Biology*, 27(10), pp.3817-3827.

Mendoza, M.C., Du, F., Iranfar, N., Tang, N., Ma, H., Loomis, W.F. and Firtel, R.A., 2005. Loss of SMEK, a novel, conserved protein, suppresses MEK1 null cell polarity, chemotaxis, and gene expression defects. *Molecular and Cellular Biology*, 25(17), pp.7839-7853.

Meric-Bernstam, F. and Esteva, F.J., 2005. Potential role of mammalian target of rapamycin inhibitors in breast cancer therapy. *Clinical Breast Cancer*, 6(4), pp.357-360.

Mester, J. and Redeuilh, G., 2008. Proliferation of breast cancer cells: regulation, mediators, targets for therapy. *Anti-Cancer Agents in Medicinal Chemistry,* 8(8), pp.872-885.

Mihindukulasuriya, K.A., Zhou, G., Qin, J. and Tan, T.H., 2004. Protein phosphatase 4 interacts with and down-regulates insulin receptor substrate 4 following tumour necrosis factor-α stimulation. *Journal of Biological Chemistry*, 279(45), pp.46588-46594.

Mishra, R., 2010. Glycogen synthase kinase 3 beta: can it be a target for oral cancer. *Molecular Cancer*, 9(1), p.144.

Moorhead, G.B., De Wever, V., Templeton, G. and Kerk, D., 2009. Evolution of protein phosphatases in plants and animals. *Biochemical Journal*, 417(2), pp.401-409.

Moreno, E., Doughty-Shenton, D., Plano, D., Font, M., Encío, I., Palop, J.A., Sanmartín, C., 2014. A dihydroselenoquinazoline inhibits S6 ribosomal protein signalling, induces apoptosis and inhibits autophagy in MCF-7 cells. *European Journal of Pharmaceutical Sciences*,15(63), pp.87-95.

Mosselman, S., Polman, J. and Dijkema, R., 1996. ERβ: identification and characterization of a novel human estrogen receptor. *Federation of European Biochemical Societies letters*, 392(1), pp.49-53.

Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R. and Johansson, C.C., 2010. Regulatory T cells in cancer. *Advances in Cancer Research*, 107, pp.57-117.

Mourtada-Maarabouni M, Hanson, R., Sivanandan, C. and Ladna, A., 2016. Role of Protein Phosphatase 4 Regulatory Subunits in Myeloid Leukaemia. Poster sessions. Cancer website (http://abstracts. ncri. org. uk/year_published/2016/? s= pp4).

Mourtada-Maarabouni, M., Pickard, M.R., Hedge, V.L., Farzaneh, F., Williams, G.T., 2009. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. *Oncogene*, 28(2), pp.195-208.

Mourtada-Maarabouni, M. and Williams, G.T., 2008. Protein phosphatase 4 regulates apoptosis, proliferation and mutation rate of human cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1783(8), pp.1490-1502.

Mourtada-Maarabouni, M. and Williams, G.T., 2009. Protein phosphatase 4 regulates apoptosis in leukemic and primary human T-cells. *Leukemia Research*, 33(11), pp.1539-1551.

Mourtada-Maarabouni, M., Kirkham, L., Jenkins, B., Rayner, J., Gonda, T.J., Starr, R., Trayner, I., Farzaneh, F. and Williams, G.T., 2003. Functional expression cloning reveals proapoptotic role for protein phosphatase 4. *Cell Death & Differentiation*, 10(9), pp.1016-1024.

Mumby, M.C. and Walter, G., 1993. Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. *Physiological Reviews*, 73(4), pp.673-699.

Murata, K., Wu, J. and Brautigan, D.L., 1997. B cell receptor-associated protein α4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein

phosphatase 2A. *Proceedings of The National Academy of Sciences*, 94(20), pp.10624-10629.

Murphy, L.C., Seekallu, S.V. and Watson, P.H., 2011. Clinical significance of estrogen receptor phosphorylation. *Endocrine-Related Cancer*, 18(1), pp.R1-R14.

Myers, M.P. and Tonks, N.K., 1997. PTEN: sometimes taking it off can be better than putting it on. *American Journal of Human Genetics,* 61(6), p.1234.

Nader Chalhoub, Suzanne J. Baker, 2009. PTEN and the PI3-kinase pathway in cancer. *Annual Review of Pathological Mechanical Disease*, 4, pp.127-150.

Nagaraj, G. and Ma, C., 2015. Revisiting the estrogen receptor pathway and its role in endocrine therapy for postmenopausal women with estrogen receptor-positive metastatic breast cancer. *Breast Cancer Research and Treatment*, 150(2), pp.231-242.

Nahta, R., 2012. Pharmacological strategies to overcome HER2 cross-talk and Trastuzumab resistance. *Current Medicinal Chemistry*, 19(7), pp.1065-1075.

Nakada, S., Chen, G.I., Gingras, A.C. and Durocher, D., 2008. PP4 is a γH2AX phosphatase required for recovery from the DNA damage checkpoint. *European Molecular Biology Organization Reports*, 9(10), pp.1019-1026.

Negrini, S., Gorgoulis, V.G., Halazonetis, T.D., 2010. Genomic instability--an evolving hallmark of cancer. *Nature Reviews Molecular Cell Biology*, 11(3), pp.220-8.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F. and Speed, T., 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, 10(6), pp.515-527.

Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B.W., Liu, S., Mao, H., Chang, J.S., Galietta, A., Uttam, A. and Roy, D.C., 2005. The tumour suppressor PP2A is functionally inactivated in blast crisis CML through the

inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*, 8(5), pp.355-368.

Nuciforo, P.G., Aura, C., Holmes, E., Prudkin, L., Jimenez, J., Martinez, P., Ameels, H., de la Peña, L., Ellis, C., Eidtmann, H. and Piccart-Gebhart, M.J., 2015. Benefit to neoadjuvant anti-human epidermal growth factor receptor 2 (HER2)-targeted therapies in HER2-positive primary breast cancer is independent of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) status. *Annals of Oncology*, 26(7), pp.1494-1500.

Nunes-Xavier, C.E., Martín-Pérez, J., Elson, A. and Pulido, R., 2013. Protein tyrosine phosphatases as novel targets in breast cancer therapy. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 1836(2), pp.211-226.

Oakman, C., Viale, G. and Di Leo, A., 2010. Management of triple negative breast cancer. *The Breast,* 19(5), pp.312-321.

Okada, H. and Mak, T.W., 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nature Reviews Cancer,* 4(8), pp.592-603.

Okahara, F., Ikawa, H., Kanaho, Y. and Maehama, T., 2004. Regulation of PTEN phosphorylation and stability by a tumour suppressor candidate protein. *Journal of Biological Chemistry*, 279(44), pp.45300-45303.

Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P. and Mann, M., 2006. Global, *in vivo*, and site-specific phosphorylation dynamics in signalling networks. *Cell*, 127(3), pp.635-648.

O'Regan, R. and Hawk, N.N., 2011. mTOR inhibition in breast cancer: unraveling the complex mechanisms of mTOR signal transduction and its clinical implications in therapy. *Expert Opinion on Therapeutic Targets*, 15(7), pp.859-872.

O'Reilly, K.E., Warycha, M., Davies, M.A., Rodrik, V., Zhou, X.K., Yee, H., Polsky, D., Pavlick, A.C., Rosen, N., Bhardwaj, N., Mills, G., Osman, I., 2009. Phosphorylated 4E-BP1 is associated with poor survival in melanoma. *Clinical Cancer Research,* 15(8), pp.2872-8.

Osborne, C., Wilson, P. and Tripathy, D., 2004. Oncogenes and tumour suppressor genes in breast cancer: potential diagnostic and therapeutic applications. *The Oncologist,* 9(4), pp.361-377.

Osborne, C.K., Zhao, H. and Fuqua, S.A., 2000. Selective estrogen receptor modulators: structure, function, and clinical use. *Journal of Clinical Oncology*, 18(17), pp.3172-3186.

Paik, S., Hazan, R., Fisher, E.R., Sass, R.E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M.E. and King, C.R., 1990. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. *Journal of Clinical Oncology*, 8(1), pp.103-112.

Pandey, P., Seshacharyulu, P., Das, S., Rachagani, S., Ponnusamy, M.P., Yan, Y., Johansson, S.L., Datta, K., Lin, M.F. and Batra, S.K., 2013. Impaired expression of protein phosphatase 2A subunits enhances metastatic potential of human prostate cancer cells through activation of AKT pathway. *British Journal of Cancer*, 108(12), pp.2590-2600.

Pankaj, T., Maglic, D., Kai, F., Zhu, S., Kendig, R. D., Fry, E. A. and InouE, K., 2010. Classical and Novel Prognostic Markers for Breast Cancer and their Clinical Significance. *Clinical Medicine Insights*, 4, pp.15–34.

Paplomata, E. and O'Regan, R., 2013. New and emerging treatments for estrogen receptor-positive breast cancer: focus on everolimus. *Therapeutics and Clinical Risk Management,* 9, pp.27-36.

Paplomata, E. and O'Regan, R., 2014. The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. *Therapeutic Advances in Medical Oncology*, 6(4), pp.154-166.

Parker, M.G., 1993. Steroid and related receptors. *Current Opinion in Cell Biology*, 5(3), pp.499-504.

Partridge, A.H., Rumble, R.B., Carey, L.A., Come, S.E., Davidson, N.E., Di Leo, A., Gralow, J., Hortobagyi, G.N., Moy, B., Yee, D. and Brundage, S.B., 2014. Chemotherapy and targeted therapy for women with human epidermal

growth factor receptor 2–negative (or unknown) advanced breast cancer: American Society of Clinical Oncology clinical practice guideline. *Journal of Clinical Oncology*, 32(29), pp.3307-3329.

Payne, S.J.L., Bowen, R.L., Jones, J.L. and Wells, C.A., 2008. Predictive markers in breast cancer–the present. *Histopathology*, 52(1), pp.82-90.

Peller, S. and Rotter, V., 2003. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Human Mutation*, 21(3), pp.277-284.

Pettersson, K., Delaunay, F. and Gustafsson, J.Å., 2000. Estrogen receptor [beta] acts as a dominant regulator of estrogen signalling. *Oncogene*, 19(43), pp.4970.

Pickard, M.R., Williams, G.T., 2014. Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy. *Breast Cancer Research and Treatment*, 145(2), pp.359-70.

Power, R.F. and Mani, S.K., 1991. Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science*, 254(5038), pp.1636.

Pulido R. and Hooft van Huijsduijnen R., 2008. Protein tyrosine phosphatases: dual-specificity phosphatases in health and disease. *Federation of European Biochemical Societies Journal.* 275(5), pp.848–866.

Pusztai, L., Mazouni, C., Anderson, K., Wu, Y. and Symmans, W.F., 2006. Molecular classification of breast cancer: limitations and potential. *The Oncologist*, 11(8), pp.868-877.

Qi, Y., Hu, T., Li, K., Ye, R. and Ye, Z., 2015. Lentivirus-mediated short-hairpin RNA targeting protein phosphatase 4 regulatory subunit 1 inhibits growth in breast cancer. *Journal of Breast Cancer*, 18(3), pp.218-224.

Qiu, G.H., Tan, L.K., Loh, K.S., Lim, C.Y., Srivastava, G., Tsai, S.T., Tsao, S.W. and Tao, Q., 2004. The candidate tumour suppressor gene BLU, located at the commonly deleted region 3p21. 3, is an E2F-regulated, stress-responsive

gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. *Oncogene*, 23(27), pp.4793-4806.

Raimondi, C. and Falasca, M., 2011. Targeting PDK1 in cancer. *Current Medicinal Chemistry*, 18(18), pp.2763-2769.

Rakha, E.A., El-Sayed, M.E., Green, A.R., Lee, A.H., Robertson, J.F. and Ellis, I.O., 2007. Prognostic markers in triple-negative breast cancer. *Cancer*, 109(1), pp.25-32.

Ramos, J.W., Kojima, T.K., Hughes, P.E., Fenczik, C.A. and Ginsberg, M.H., 1998. The death effector domain of PEA-15 is involved in its regulation of integrin activation. *Journal of Biological Chemistry*, 273(51), pp.33897-33900.

Rayasam, G.V., Tulasi, V.K., Sodhi, R., Davis, J.A. and Ray, A., 2009. Glycogen synthase kinase 3: more than a namesake. *British Journal of Pharmacology*, 156(6), pp.885-898.

Reisner, A.H., Nemes, P. and Bucholtz, C., 1975. The use of Coomassie Brilliant Blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Analytical Biochemistry*, *64*(2), pp.509-516.

Reitman, Z.J. and Yan, H., 2010. Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. *Journal of the National Cancer Institute*, 102(13), pp.932-41.

Renganathan, H., Vaidyanathan, H., Knapinska, A. and Ramos, J.W., 2005. Phosphorylation of PEA-15 switches its binding specificity from ERK/MAPK to FADD. *Biochemical Journal*, 390(3), pp.729-735.

Rice, S. and Whitehead, S.A., 2014. Targets for the Action of Phytoestrogens in Breast Cancer—Focus on Isoflavones and Resveratrol. *Current Breast Cancer Reports*, 6(2), pp.88-95.

Rogakou, E.P., Boon, C., Redon, C. and Bonner, W.M., 1999. Megabase chromatin domains involved in DNA double-strand breaks *in vivo. The Journal of Cell Biology*, 146(5), pp.905-916.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M., 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *Journal of Biological Chemistry*, 273(10), pp.5858-5868.

Rose, B.A., Force, T. and Wang, Y., 2010. Mitogen-activated protein kinase signalling in the heart: angels versus demons in a heart-breaking tale. *Physiological Reviews*, 90(4), pp.1507-1546.

Ross, J. S. and Harbeck, N., 2005. Prognostic and Predictive Factors Overview. *In: Ross JS, Hortobagyi GN, editors. Molecular Oncology of Breast Cancer. Sudbury, MA: Jones and Bartlett Publishers,* pp.128–141.

Roux, P.P., Shahbazian, D., Vu, H., Holz, M.K., Cohen, M.S., Taunton, J., Sonenberg, N. and Blenis, J., 2007. RAS/ERK signalling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *Journal of Biological Chemistry*, 282(19), pp.14056-14064.

Rubin, I. and Yarden, Y., 2001. The basic biology of HER2. *Annals of Oncology*, 12(1), pp.S3-S8.

Ruediger, R., Hood, J.V.W., Mumby, M. and Walter, G., 1991. Constant expression and activity of protein phosphatase 2A in synchronized cells. *Molecular and Cellular Biology*, 11(8), pp.4282-4285.

Ruvinsky, I. and Meyuhas, O., 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends in Biochemical Sciences,* 31(6), pp.342-348.

Saito, A., Narasimhan, P., Hayashi, T., Okuno, S., Ferrand-Drake, M. and Chan, P.H., 2004. Neuroprotective role of a proline-rich Akt substrate in apoptotic neuronal cell death after stroke: relationships with nerve growth factor. *Journal of Neuroscience*, 24(7), pp.1584-1593.

Salmena, L., Carracedo, A. and Pandolfi, P.P., 2008. Tenets of PTEN tumour suppression. *Cell*, 133(3), pp.403-414.

Sant, M., Capocaccia, R., Coleman, M.P., Berrino, F., Gatta, G., Micheli, A., Verdecchia, A., Faivre, J., Hakulinen, T., Coebergh, J.W.W. and Martinez-Garcia, C., 2001. Cancer survival increases in Europe, but international differences remain wide. *European Journal of Cancer*, 37(13), pp.1659-1667.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. and Sabatini, D.M., 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular Cell*, 22(2), pp.159-168.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. and Sabatini, D.M., 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*, 307(5712), pp.1098-1101.

Schiff, R. and Fuqua, S., 2002. The importance of the estrogen receptor in breast cancer. *Breast cancer: Prognosis, Treatment, and Prevention*, pp.149-186.

Schiff, R. and Osborne, C.K., 2005. Endocrinology and hormone therapy in breast cancer: New insight into estrogen receptor-α function and its implication for endocrine therapy resistance in breast cancer. *Breast Cancer Research,* 7(5), p.205.

Schmadeka, R., Harmon, B.E. and Singh, M., 2014. Triple-negative breast carcinoma. *American Journal of Clinical Pathology*, 141(4), pp.462-477.

Schneider, M.R., Werner, S., Paus, R. and Wolf, E., 2008. Beyond wavy hairs: the epidermal growth factor receptor and its ligands in skin biology and pathology. *The American Journal of Pathology*, 173(1), pp.14-24.

Scholzen, T. and Gerdes, J., 2000. The Ki-67 protein: from the known and the unknown. *Journal of Cellular Physiology*, 182(3), pp.311-322.

Seshacharyulu, P., Pandey, P., Datta, K. and Batra, S.K., 2013. Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Letters*, 335(1), pp.9-18.

Shao, R.G., Cao, C.X., Zhang, H., Kohn, K.W., Wold, M.S. and Pommier, Y., 1999. Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA: DNA-PK

complexes. *The European Molecular Biology Organization journal*, 18(5), pp.1397-1406.

Shen Z., 2011. Genomic instability and cancer: an introduction. Journal of Molecular Cell Biology, 3, pp.1–3.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A. and Greene, G.L., 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell,* 95(7), pp.927-937.

Shimada, M. and Nakanishi, M., 2013. Response to DNA damage: why do we need to focus on protein phosphatases? *Frontiers in Oncology*, 3, pp.8.

Shimura, T., Kakuda, S., Ochiai, Y., Kuwahara, Y., Takai, Y. and Fukumoto, M., 2011. Targeting the AKT/GSK3β/cyclin D1/Cdk4 survival signalling pathway for eradication of tumour radioresistance acquired by fractionated radiotherapy. *International Journal of Radiation Oncology* Biology* Physics*, 80(2), pp.540-548.

Shipitsin, M., Small, C., Giladi, E., Siddiqui, S., Choudhury ,S., Hussain, S., Huang, Y.E., Chang, H., Rimm, D.L., Berman, D.M., Nifong, T.P., Blume-Jensen, P., 2014. Automated quantitative multiplex immunofluorescence in situ imaging identifies phospho-S6 and phospho-PRAS40 as predictive protein biomarkers for prostate cancer lethality. *Proteome Science,* 12.pp.12:40

Shrivastav, A., Bruce, M.C., Jaksic, D., Bader, T., Seekallu, S., Penner, C., Nugent, Z., Watson, P. and Murphy, L., 2014. The mechanistic target for rapamycin pathway is related to the phosphorylation score for estrogen receptor-α in human breast tumours *in vivo. Breast Cancer Research,* 16(3), p.3407.

Shui, J.W., Hu, M.C.T. and Tan, T.H., 2007. Conditional knockout mice reveal an essential role of protein phosphatase 4 in thymocyte development and pre-T-cell receptor signalling. *Molecular and Cellular Biology*, 27(1), pp.79-91.

Siegel, R., Naishadham, D. and Jemal, A., 2013. Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians,* 63(1), pp.11-30.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., McGuire, W.L., 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/Neu oncogene. *Science*, 235(4785), pp.177–182.

Sledge, G.W., Mamounas, E.P., Hortobagyi, G.N., Burstein, H.J., Goodwin, P.J. and Wolff, A.C., 2014. Past, present, and future challenges in breast cancer treatment. *Journal of Clinical Oncology*, 32(19), pp.1979-1986.

Smith, L., Watson, M.B., O'Kane, S.L., Drew, P.J., Lind, M.J. and Cawkwell, L., 2006. The analysis of doxorubicin resistance in human breast cancer cells using antibody microarrays. *Molecular Cancer Therapeutics*, 5(8), pp.2115-2120.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F. and Brooks, S.C., 1990. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Research*, 50(18), pp.6075-6086.

Soule, H.D., Vazquez, J., Long, A., Albert, S. and Brennan, M., 1973. A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute*, 51(5), pp.1409-1416.

Stanbridge, E.J., 1976. Suppression of malignancy in human cells. *Nature,* 260(5546), pp.17.

Stange, D.E., Radlwimmer, B., Schubert, F., Traub, F., Pich, A., Toedt, G., Mendrzyk, F., Lehmann, U., Eils, R., Kreipe, H. and Lichter, P., 2006. High-resolution genomic profiling reveals association of chromosomal aberrations on 1q and 16p with histologic and genetic subgroups of invasive breast cancer. *Clinical Cancer Research*, 12(2), pp.345-352.

Stassi, G., Garofalo, M., Zerilli, M., Ricci-Vitiani, L., Zanca, C., Todaro, M., Aragona, F., Limite, G., Petrella, G. and Condorelli, G., 2005. PED mediates AKT-dependent chemoresistance in human breast cancer cells. *Cancer Research*, 65(15), pp.6668-6675.

Stern, D.F. and Kamps, M.P., 1988. EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. *The European Molecular Biology Organization Journal*, 7(4), pp.995.

Stracker, T.H., Usui, T. and Petrini, J.H., 2009. Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. *DNA Repair*, 8(9), pp.1047-1054.

Su, Y.W., Chen, Y.P., Chen, M.Y., Reth, M. and Tan, T.H., 2013. The serine/threonine phosphatase PP4 is required for pro-B cell development through its promotion of immunoglobulin VDJ recombination. *PLOS One*, 8(7), pp.e68804.

Sueji Han, Jaehong Park and Dong-Hyun Lee, 2015. Protein DHX38 is a novel inhibitor of protein phosphatase 4. *Animal Cells and Systems*, 19(4), pp.236-244.

Sulzmaier, F.J., Opoku-Ansah, J. and Ramos, J.W., 2012. Phosphorylation is the switch that turns PEA-15 from tumour suppressor to tumour promoter. *Small GTPases*, 3(3), pp.173-177.

Sumiyoshi, E., Sugimoto, A. and Yamamoto, M., 2002. Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in C. elegans. *Journal of Cell Science*, 115(7), pp.1403-1410.

Sun, H. and Wang, Y., 2012. Novel Ser/Thr protein phosphatases in cell death regulation. *Physiology,* 27(1), pp.43-52.

Sutherland, C., Leighton, I.A. and Cohen, P., 1993. Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochemical Journal,* 296(1), pp.15-19.

Takahashi, K., Tanaka, M., Inagaki, A., Wanibuchi, H., Izumi, Y., Miura, K., Nagayama, K., Shiota, M. and Iwao, H., 2013. Establishment of a 5-fluorouracil-resistant triple-negative breast cancer cell line. *International Journal of Oncology*, 43(6), pp.1985-1991.

Taniguchi, C.M., Emanuelli, B. and Kahn, C.R., 2006. Critical nodes in signalling pathways: insights into insulin action. *Nature Reviews Molecular Cell Biology*, 7(2), pp.85-96.

Tercero, J.A. and Diffley, J.F., 2001. Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, 412(6846), pp.553-557.

Theobald, B., Bonness, K., Musiyenko, A., Andrews, J.F., Urban, G., Huang, X., Dean, N.M. and Honkanen, R.E., 2013. Suppression of ser/thr phosphatase 4 (PP4C/PPP4C) mimics a novel post-mitotic action of fostriecin, producing mitotic slippage followed by tetraploid cell death. *Molecular Cancer Research*, 11(8), pp.845-855.

Thomas, G. and Hall, M.N., 1997. TOR signalling and control of cell growth. *Current Opinion in Cell Biology*, 9(6), pp.782-787.

Toker, A. and Newton, A.C., 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *Journal of Biological Chemistry*, 275(12), pp.8271-8274.

Toker, A. and Newton, A.C., 2000. Cellular signalling: pivoting around PDK-1. *Cell*, 103(2), pp.185-188.

Toyooka, Y., Shimosato, D., Murakami, K., Takahashi, K. and Niwa, H., 2008. Identification and characterization of subpopulations in undifferentiated ES cell culture. *Development,* 135(5), pp.909-918.

Trotman, L.C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N.P., Carver, B.S., Cordon-Cardo, C., Erdjument-Bromage, H. and Tempst, P., 2007. Ubiquitination regulates PTEN nuclear import and tumour suppression. *Cell*, 128(1), pp.141-156.

Ubersax, J.A. and Ferrell Jr, J.E., 2007. Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology*, 8(7), pp.530-541.

Umemura, S., Yoshida, S., Ohta, Y., Naito, K., Osamura, R.Y. and Tokuda, Y., 2007. Increased phosphorylation of Akt in triple-negative breast cancers. *Cancer Science*, 98(12), pp.1889-1892.

Vahteristo, P., Bartkova, J., Eerola, H., Syrjäkoski, K., Ojala, S., Kilpivaara, O., Tamminen, A., Kononen, J., Aittomäki, K., Heikkilä, P. and Holli, K., 2002. A CHEK2 genetic variant contributing to a substantial fraction of familial breast cancer. *The American Journal of Human Genetics*, 71(2), pp.432-438.

Van Hoof, C. and Goris, J., 2003. Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1640(2), pp.97-104.

Vazquez, F., Ramaswamy, S., Nakamura, N. and Sellers, W.R., 2000. Phosphorylation of the PTEN tail regulates protein stability and function. *Molecular and Cellular Biology*, 20(14), pp.5010-5018.

Veitch, N.C., 2004. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry*, 65(3), pp.249-59.

Verma, S., Zhao, L.J. and Chinnadurai, G., 2001. Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. *Journal of Biological Chemistry*, 276(7), pp.4671-4676.

Vignon, F., Bardon, S., Chalbos, D. and Rochefort, H., 1983. Antiestrogenic effect of R5020, a synthetic progestin in human breast cancer cells in culture. *The Journal of Clinical Endocrinology & Metabolism,* 56(6), pp.1124-1130.

Vincent, E.E., Elder, D.J., Thomas, E.C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M.T., Hetzel ,M.R., Tavare, J.M., 2011. Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *British Journal of Cancer,* 104, pp.1755–1761.

Virshup, D.M. and Shenolikar, S., 2009. From promiscuity to precision: protein phosphatases get a makeover. *Molecular Cell*, 33(5), pp.537-545.

Von Manteuffel, S.R., Dennis, P.B., Pullen, N., Gingras, A.C., Sonenberg, N. and Thomas, G., 1997. The insulin-induced signalling pathway leading to S6 and

initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k. *Molecular and Cellular Biology*, 17(9), pp.5426-5436.

Wada, T., Miyata, T., Inagi, R., Nangaku, M., Wagatsuma, M., Suzuki, D., Wadzinski, B.E., Okubo, K. and Kurokawa, K., 2001. Cloning and characterization of a novel subunit of protein serine/threonine phosphatase 4 from mesangial cells. *Journal of the American Society of Nephrology,* 12(12), pp.2601-2608.

Wagle, N., Grabiner, B.C., Van Allen, E.M., Hodis, E., Jacobus, S., Supko, J.G., Stewart, M., Choueiri, T.K., Gandhi, L., Cleary, J.M. and Elfiky, A.A., 2014. Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. *Cancer Discovery*, 4(5), pp.546-553.

Wajant, H., 2002. The Fas signalling pathway: more than a paradigm. *Science*, 296(5573), pp.1635-1636.

Walsh, T., Casadei, S., Coats, K.H., Swisher, E., Stray, S.M., Higgins, J., Roach, K.C., Mandell, J., Lee, M.K., Ciernikova, S. and Foretova, L., 2006. Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *The Journal of the American Medical Association,* 295(12), pp.1379-1388.

Wang, B., Zhao, A., Sun, L., Zhong, X., Zhong, J., Wang, H., Cai, M., Li, J., Xu, Y., Liao, J. and Sang, J., 2008. Protein phosphatase PP4 is overexpressed in human breast and lung tumours. *Cell Research*, 18(9), p.974.

Wang, D. and Lippard, S.J., 2005. Cellular processing of platinum anticancer drugs. *Nature Reviews Drug Discovery*, 4(4), pp.307-320.

Wang, H.Y., Cheng, Z. and Malbon, C.C., 2003. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. *Cancer letters*, 191(2), pp.229-237.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J. and Qin, J., 2000. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes & Development,* 14(8), pp.927-939.

Wang, Y., Klijn, J.G., Zhang, Y., Sieuwerts, A.M., Look, M.P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M.E., Yu, J. and Jatkoe, T., 2005. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *The Lancet*, 365(9460), pp.671-679.

Wei, Y., 2015. On the Quest of Cellular Functions of PEA-15 and the Therapeutic Opportunities. *Pharmaceuticals*, 8(3), pp.455-473.

Weinberg, R.A., 2006. The biology of cancer. *The Yale Journal of Biology and Medicine,* 80(2), pp.91.

Welsh, G.I., Wilson, C., Proud, C.G., 1996. GSK3: a SHAGGY frog story. *Trends Cell Biology,* 6(7), pp.274-9.

Weng, S., Wang, H., Chen, W., Katz, M.H., Chatterjee, D., Lee, J.E., Pisters, P.W., Gomez, H.F., Abbruzzese, J.L., Fleming, J.B. and Wang, H., 2012. Overexpression of protein phosphatase 4 correlates with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma. *Cancer Epidemiology, Biomarkers & Prevention*, 21(8), pp.1336-43.

Wu, G., Ma, Z., Qian, J. and Liu, B., 2015. PP4R1 accelerates cell growth and proliferation in HepG2 hepatocellular carcinoma. *OncoTargets and Therapy*, 8, pp.2067.

Wu, H.I., Brown, J.A., Dorie, M.J., Lazzeroni, L. and Brown, J.M., 2004. Genome-wide identification of genes conferring resistance to the anticancer agents cisplatin, oxaliplatin, and mitomycin C. *Cancer Research,* 64(11), pp.3940-3948.

Wu, N., He, C., Zhu, B., Jiang, J., Chen, Y., Ma, T.M 2017. T.3-Phosphoinositide Dependent Protein Kinase-1 (PDK-1) Promotes Migration and Invasion in Gastric Cancer Cells through Activating the NF-κB Pathway. *Oncology Research*, 25(7), pp.1153-1159.

Xie, X., Bartholomeusz, C., Ahmed, A.A., Kazansky, A., Diao, L., Baggerly, K.A., Hortobagyi, G.N., Ueno, N.T. 2013. Bisphosphorylated PEA-15 sensitizes ovarian cancer cells to paclitaxel by impairing the microtubule-destabilizing effect of SCLIP. *Molecular Cancer Therapeutics,* 12(6), pp.1099-111.

Xie, X., Tang, H., Liu, P., Kong, Y., Wu, M., Xiao, X., Yang, L., Gao, J., Wei, W., Lee, J. and Bartholomeusz, C., 2015. Development of PEA-15 using a potent non-viral vector for therapeutic application in breast cancer. *Cancer Letters*, 356(2), pp.374-381.

Xie, Y., Jüschke, C., Esk, C., Hirotsune, S. and Knoblich, J.A., 2013. The phosphatase PP4c controls spindle orientation to maintain proliferative symmetric divisions in the developing neocortex. *Neuron*, 79(2), pp.254-265.

Xie, Z., Yuan, H., Yin, Y., Zeng, X., Bai, R. and Glazer, R.I., 2006. 3-phosphoinositide-dependent protein kinase-1 (PDK1) promotes invasion and activation of matrix metalloproteinases. *BMC cancer*, 6(1), p.77.

Yamada, K.M., Araki, M.J, 2001. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *Cell Science,* 114(13), pp.2375-82.

Yamnik, R.L. and Holz, M.K., 2010. mTOR/S6K1 and MAPK/RSK signalling pathways coordinately regulate estrogen receptor α serine 167 phosphorylation. *Federation of European Biochemical Societies Letters*, 584(1), pp.124-128.

Yang, L., Pang, Y. and Moses, H.L., 2010. TGF-β and immune cells: an important regulatory axis in the tumour microenvironment and progression. *Trends in Immunology*, 31(6), pp.220-227.

Yang, Z., Yuan, X.G., Chen, J., Luo, S.W., Luo, Z.J. and Lu, N.H., 2013. Reduced expression of PTEN and increased PTEN phosphorylation at residue Ser380 in gastric cancer tissues: a novel mechanism of PTEN inactivation. *Clinics and Research in Hepatology and Gastroenterology,* 37(1), pp.72-79.

Yarden, Y. and Sliwkowski, M.X., 2001. Untangling the ErbB signalling network. *Nature Reviews Molecular Cell Biology*, 2(2), pp.127-137.

Yde, C.W. and Issinger, O., 2006. Enhancing cisplatin sensitivity in MCF-7 human breast cancer cells by down-regulation of Bcl-2 and cyclin D1. *International Journal of Oncology,* 29(6), pp.1397-1404.

Yeh, P.Y., Yeh, K.H., Chuang, S.E., Song, Y.C. and Cheng, A.L., 2004. Suppression of MEK/ERK signalling pathway enhances cisplatin-induced NF-κB activation by protein phosphatase 4-mediated NF-κB p65 Thr dephosphorylation. *Journal of Biological Chemistry,* 279(25), pp.26143-26148.

Yellen, P., Saqcena, M., Salloum, D., Feng, J., Preda, A., Xu, L., Rodrik-Outmezguine, V. and Foster, D.A., 2011. High-dose rapamycin induces apoptosis in human cancer cells by dissociating mTOR complex 1 and suppressing phosphorylation of 4E-BP1. *Cell Cycle*, 10(22), pp.3948-3956.

Yokota, J., 2000. Tumour progression and metastasis. *Carcinogenesis,* 21(3), pp.497-503.

Yoon, Y.S., Lee, M.W., Ryu, D., Kim, J.H., Ma, H., Seo, W.Y., Kim, Y.N., Kim, S.S., Lee, C.H., Hunter, T. and Choi, C.S., 2010. Suppressor of MEK null (SMEK)/protein phosphatase 4 catalytic subunit (PP4C) is a key regulator of hepatic gluconeogenesis. *Proceedings of the National Academy of Sciences*, 107(41), pp.17704-17709.

Yu, F., Narasimhan, P., Saito, A., Liu, J. and Chan, P.H., 2008. Increased expression of a proline-rich Akt substrate (PRAS40) in human copper/zinc-superoxide dismutase transgenic rats protects motor neurons from death after spinal cord injury. *Journal of Cerebral Blood Flow & Metabolism*, 28(1), pp.44-52.

Zdraveski, Z.Z., Mello, J.A., Farinelli, C.K., Essigmann, J.M. and Marinus, M.G., 2002. MutS preferentially recognizes cisplatin-over oxaliplatin-modified DNA. *Journal of Biological Chemistry*, 277(2), pp.1255-1260.

Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S.J., 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X L. *Cell*, 87(4), pp.619-628.

Zhang, Q. and Claret, F.X., 2011. Phosphatases: the new brakes for cancer development? *Enzyme Research*, 2012(2012), p.659649.

Zhang, X., Ozawa, Y., Lee, H., Wen, Y.D., Tan, T.H., Wadzinski, B.E. and Seto, E., 2005. Histone deacetylase 3 (HDAC3) activity is regulated by interaction with

protein serine/threonine phosphatase 4. *Genes & Development*, 19(7), pp.827-839.

Zhou, G., Mihindukulasuriya, K.A., MacCorkle-Chosnek, R.A., Van Hooser, A., Hu, M.C.T., Brinkley, B.R. and Tan, T.H., 2002. Protein phosphatase 4 is involved in tumour necrosis factor-α-induced activation of c-Jun N-terminal kinase. *Journal of Biological Chemistry*, 277(8), pp.6391-6398.

Zhou, T., Du, Y. and Wei, T., 2015. Transcriptomic analysis of human breast cancer cells reveals differentially expressed genes and related cellular functions and pathways in response to gold nanorods. *Biophysics Reports*, 1(2), pp.106-114.

Zhu, J., Nozell, S., Wang, J., Jiang, J., Zhou, W. and Chen, X., 2001. p73 cooperates with DNA damage agents to induce apoptosis in MCF7 cells in a p53-dependent manner. *Oncogene*, 20(30), p.4050.

Zoncu, R., Efeyan, A. and Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature Reviews Molecular cell biology*, 12(1), pp.21-35.

Appendix

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The protein phosphatase 4 - PEA15 axis regulates the survival of breast cancer cells

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ABSTRACT

Background: The control of breast cell survival is of critical importance for preventing breast cancer initiation and progression. The activity of many proteins which regulate cell survival is controlled by reversible phosphorylation, so that the relevant kinases and phosphatases play crucial roles in determining cell fate. Several protein kinases act as oncoproteins in breast cancer and changes in their activities contribute to the process of transformation. Through counteracting the activity of oncogenic kinases, the protein phosphatases are also likely to be important players in breast cancer development, but this class of molecules is relatively poorly understood. Here we have investigated the role of the serine/threonine protein phosphatase 4 in the control of cell survival of breast cancer cells.

Methods: The breast cancer cell lines, MCF7 and MDA-MB-231, were transfected with expression vectors encoding the catalytic subunit of protein phosphatase 4 (PP4c) or with PP4c siRNAs. Culture viability, apoptosis, cell migration and cell cycle were assessed. The involvement of phosphoprotein enriched in astrocytes 15 kDa (PEA15) in PP4c action was investigated by immunoblotting approaches and by siRNA-mediated silencing of PEA15.

Results: In this study we showed that PP4c over-expression inhibited cell proliferation, enhanced spontaneous apoptosis and decreased the migratory and colony forming abilities of breast cancer cells. Moreover, PP4c down-regulation produced complementary effects. PP4c is demonstrated to regulate the phosphorylation of PEA15, and PEA15 itself regulates the apoptosis of breast cancer cells. The inhibitory effects of PP4c on breast cancer cell survival and growth were lost in PEA15 knockdown cells, confirming that PP4c action is mediated, at least in part, through the de-phosphorylation of apoptosis regulator PEA15.

Conclusion: Our work shows that PP4 regulates breast cancer cell survival and identifies a novel PP4c-PEA15 signalling axis in the control of breast cancer cell survival. The dysfunction of this axis may be important in the development and progression of breast cancer.

1. Introduction

Breast cancer is a highly complex and heterogeneous disease, and can be classified into different molecular subtypes according to the expression status of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptors (HERs), HER2/Neu and HER1/EGFR [1,2]. Phosphorylation of these receptors on tyrosine (Tyr) residues constitutes a major regulatory mechanism of their activities. Aberrant activation of these receptors dysregulates multiple signalling cascades and plays a vital role in the initiation, development and progression of breast cancer, highlighting the importance of protein phosphorylation in the context of breast oncogenesis [3]. While the protein tyrosine kinase (PTK) families comprising the HERs and the non-receptor Src-family kinases (SFKs) have been directly implicated in the development and progression of breast cancer [3], their downstream signalling is mainly mediated via the activation of effector pathways that involve serine/threonine (Ser/Thr) kinases, including MAP kinase (MAPK), the phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the JAK/STAT signalling pathways [4]. The role of Ser/Thr kinases in breast cancer as crucial effectors in oncogenic PTK signalling has been well studied and multiple compounds that target their activity are being evaluated in clinical trials [5].

In contrast, the role of protein phosphatases, the enzymes that reverse the action of protein kinases, is relatively under-studied. This is despite the tumour suppressor function of protein phosphatase 2A (PP2A), a major Ser/Thr phosphatase, having been clearly demonstrated in breast, gastric and ovarian cancer cells [6]. Notably, the Ser/Thr

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