Investigating the Production and Function of Oxidative Stress-Induced PtdIns5*P*

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Medical and Human Sciences

2014

Rebecca Ann Foulger

School of Medicine

Contents

Abbreviations

^{32}P	^{32} Phosphorus
4E-BP1	Eukaryotic Translation Initiation Factor 4E-Binding Protein 1
ABG	Knock down of PIP4K2A, PIP4K2B and PIP4K2C in combination
AML	Acute Myeloid Leukaemia
ATM	Ataxia Telangiectasia Mutated
AtMTM1	Arabidopsis Thaliana Myotubularin homologue 1
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3 related
Atx1	Ataxin 1
BAD	Bcl2-Associated Death Promoter
BRCA	Breast Cancer Susceptibility Protein
Cdc25	Cell Division Cycle 25
cDNA	complementary DNA
$CHCl_3$	Chloroform
CH_2OH	Methanol
CHO T cells	Chinese Hamster Ovary T cells
CO_2	Carbon Dioxide
CMV	Cytomegalovirus
Cul3	Cullin 3
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA- Protein Kinase
Dok	Downstream of Tyrosine Kinase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

Eph	Ephrin Receptors
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence Activated Cell Sorter
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FGF1	Fibroblast Growth Factor 1
FGFR	Fibroblast Growth Factor Receptor
G	G-force
GFP	Green Fluorescent Protein
GLUT4	Glucose Transporter Type 4
GPCR	G-protein Coupled Receptor
GSK3	Glycogen Synthase Kinase 3
GST	Glutathione *S*-Transferases
H_2O	Water
H_2O_2	Hydrogen Peroxide
H3PO4	Phosphoric Acid
HCl	Hydrochloric Acid
HEK	Human Embryonic Kidney
His-tag	Polyhistidine Tag
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
IGF1R	Insulin-like Growth Factor Receptor 1
ING2	Inhibitor of Growth Family Member 2
InsR	Insulin Receptor
IP	Immunoprecipitation

IpgD	Inositol Phosphate Phosphatase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS1	Insulin Receptor Substrate 1
KCl	Potassium Chloride
kDa	kiloDalton
LB	Luria Broth
LDS	Lithium Dodecyl Sulphate
MAPK	Mitogen Activated Protein Kinase
Mdm2	Mouse Double Minute 2 Homologue
MEFs	Mouse Embryonic Fibroblasts
mRNA	messenger RNA
MS	Mass Spectrometry
MTM	Myotubularin
mTOR	Mammalian Target of Rapamycin
mTORC	Mammalian Target of Rapamycin Complex
MW	Molecular Weight
NAD^+/NADH	Nicotinamide Adenine Dinucleotide
NaIO4	Sodium Periodate
NEDD4-1	Neural precursor cell Expressed Developmentally Down-Regulated Protein 4-1
NEM	*N*-ethylmaleimide
NF-κB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
SCLC	Non Small Cell Lung Cancer
p38	p38 Mitogen-Activated Protein Kinase
P70S6K	p70-S6 Kinase

PARP1	Poly [ADP-ribose] Polymerase 1
PBS	Phosphate Buffered Saline
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	Phosphoinositide-Dependent Kinase-1
PEI	Polyethylenimine
PGP	Phosphatidylglycerophosphate
PH	Pleckstrin Homology
PHD	Plant Homeodomain
PI3K	Phosphatidylinositol-3-kinase
PIKfyve	Phosphoinositide Kinase, FYVE Finger Containing
PIP4K	Phosphatidylinositol 5-Phosphate 4-Kinase
PIP5K	Phosphatidylinositol 4-Phosphate 5-Kinase
PKB	Protein Kinase B
PLIP	PTEN-Like Phosphatase
PP2A	Protein Phosphatase 2A
PRAS40	Proline-Rich Akt Substrate of 40kDa
PtdIns	Phosphatidylinositol
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5) Trisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol (4,5) Bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol (3,5) Bisphosphate
PtdIns(3,4)P_2	Phosphatidylinositol (3,4) Bisphosphate
PtdIns3*P*	Phosphatidylinositol 3 Phosphate
PtdIns4*P*	Phosphatidylinositol 4 Phosphate
PtdIns5*P*	Phosphatidylinositol 5 Phosphate
PTPMT1	Protein Tyrosine Phosphatase Localised to Mitochondrion 1

PS	Phosphatidylserine
PTEN	Phosphatase and Tensin Homologue
PTP1B	Protein Tyrosine Phosphatase 1B
qRT-PCR	Quantitative Reverse Transcription- Polymerase Chain Reaction
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
RFP	Ret Finger Protein
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
Ser473	Serine 473
shRNA	short hairpin RNA
SHX	non-targeting shRNA control construct
siRNA	short interfering RNA
SIRT1	Silent Mating Type Information Regulation 2 Homologue 1
SPOP	Speckle-Type POZ Domain Protein
TB	Terrific Broth
TBHq	*tert*-Butylhydroquinone
TC45	T-Cell Protein Tyrosine Phosphatase of 45kDa
Thr308	Threonine 308
TLC	Thin Layer Chromatography
TSC2	Tuberous Sclerosis Complex 2
TUP	Theoretical Upper Phase
UV	Ultraviolet
VEGFR	Vascular Endothelial Growth Factor Receptor
VO-OH	VO-OHpic Trihydrate

VSV-G	Vesicular Stomatitis Virus-Glycoprotein
WWP2	WW Domain-Containing Protein 2
XLMTM	X-Linked Recessive Myotubularmyopathy

Abstract

Phosphoinositides regulate a wide range of essential cellular processes in almost every cellular compartment. Research conducted to better understand the diverse and widespread influence of this small family of phospholipids has uncovered unique signalling functions for each phosphoinositide however the most recently discovered phosphoinositide, PtdIns5*P*, remains poorly understood. Since its discovery there have been numerous enzymes implicated in the metabolism of PtdIns5*P* but exactly where and how PtdIns5*P* is made is not completely understood. The importance of PtdIns5*P* has been highlighted in publications that have revealed its involvement in regulating signal transduction pathways and cellular processes in various cellular compartments. The initial aim of the project was to uncover novel regulators of oxidative stress-induced PtdIns5*P* using small molecular weight inhibitors targeted against pathways activated in response to oxidative stress and a library of shRNA constructs targeted against phosphoinositide-related enzymes and co-enzymes. Using these two approaches several PtdIns5*P* regulators were identified including PTEN, the PI3K pathway and the NAD^+/NADH pathway. Similarly to previously published work, PIP4K was shown to negatively regulate H_2O_2-induced PtdIns5*P* and in particular PIP4K2A was shown to be important.

Numerous groups have described a role for PtdIns5*P* in the regulation of PKB signalling. Activated PKB is responsible for phosphorylating many downstream targets in order to initiate cell signalling pathways involved in cell survival, growth and proliferation and consequently the regulation of PKB is frequently altered in many types of cancers. The role of PtdIns5*P* in the regulation of PKB activation was examined using differential expression of PIP4K2A to manipulate the cellular level of PtdIns5*P*. It was shown that increasing the level of PtdIns5*P* causes enhanced PKB activation and decreasing the level of PtdIns5*P* suppresses PKB activation. Furthermore this is likely to be occurring through the regulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, two lipids that can mediate PKB activation at the plasma membrane. Interestingly, this effect seems to be specific to oxidative stress as insulin stimulation led to increased PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 however this did not translate to an increase in PKB activation and importantly, insulin was unable to induce an increase in PtdIns5*P* suggestive of a vital role for PtdIns5*P* in the activation of PKB. Further work suggested PtdIns5*P* may regulate the ubiquitination of PTEN. Finally, analysis of multiple signalling molecules that impinge on the PKB pathway showed that PtdIns5*P* may be able to maintain several signalling molecules in a phosphorylated and therefore active state in order to enhance PKB activation.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Acknowledgements

There are many people who have helped me on my tumultuous journey over the past four years. I'd like to thank my supervisor Nal, whose passion for science energised my often flagging enthusiasm. Thanks to everyone in the Inositide Lab but in particular David, who was endlessly generous sharing his expertise, time and efforts teaching me how to do the experiments that make up this thesis. Also special thanks go to Yvette who is truly one of the most organised people I've ever met. I am so grateful for all of her help and all of our chats!

My PhD experience would not have been the same without our neighbours the Leukaemia Biology group and their boss Tim. I'm grateful to the entire group for all the scientific advice and discussions but without their friendship I'm not sure if I would have made it to the end. To the socialites, Bill, James, Xu, Gary, Filippo, Little Tim, Dan and Emma, thanks for all the fun. Christmas parties, K2B, Student Conferences (we really do love culture) and endless lovely lunches. The Bar Rally 2012 will always be one of my favourite ever nights.
Championes!

I was so lucky to have a bunch of amazing girls to share all the ups and downs of academia with; Hadir, Eva, Elli, Sara and of course my favourite little itch, Marija. We've come from far and wide but the last four years has brought us together and I'm very grateful for that.

My family has been incredibly supportive throughout my entire education from repetitive rounds of 'the... cat... sat... on... the... mat...' to maintaining genuine interest in a conversation about the cellular metabolism of phosphoinositides. I really wouldn't be where I am today without you all. Mama, Ped, Amoire, Smish, Posie and all the Newman clan, thank you for always looking after me.

Neil, you've been there for me in the good times and the bad. From the train journey up to Manchester for my interview to writing up this thesis, you have helped me all the way through my PhD. Thank you for everything you've done.

Finally, the biggest thanks go to my best little girls Rosie and Daisy and all the babies at Greeba Zoo.

1. Introduction

1.1. Phosphoinositides

Lipids are important molecules in cells. They make up the bilayers in membranes which define not only the cell itself but also the intracellular organelles. Phospholipids are the major lipid species found in cell membranes and it is their amphipathic characteristics that allow proper formation of the bilayer. Phosphoinositides make up only a small portion of the total phospholipids found in cellular membranes [1]. Despite their scarcity they influence a range of essential cellular processes including proliferation, migration, differentiation and apoptosis. The key building block of the phosphoinositide family is phosphatidylinositol (PtdIns) which contains a *myo*-inositol head group that can be phosphorylated on three free hydroxyl groups at positions 3, 4 and 5 on the inositol ring. There are seven distinct phosphoinositide derivatives of PtdIns that each have a unique phosphorylation state: three monophosphates [PtdIns3*P*, PtdIns4*P* and PtdIns5*P*], three bisphosphates [PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2] and one trisphosphate [PtdIns(3,4,5)P_3]. Dynamic interconversion between these phosphorylation states by lipid kinases and phosphatases and metabolism by phospholipases controls the availability and localisation of each phosphoinositide (Figure 1.1) [2].

Due to their specific cellular localisation and rapid turnover, phosphoinositides make very good signalling molecules. Signalling is achieved through the recruitment of proteins with binding domains that are able to specifically interact with differentially phosphorylated myoinositol head groups, thereby allowing each phosphoinositide to recruit a specific subset of signalling molecules. There are many different phosphoinositide binding domains that bind to their target lipids in different ways due to their varied structures. There are about 20 PH domains that recognise PtdIns(3,4,5)P_3 and due to the rapid and transient accumulation of PtdIns(3,4,5)P_3 many of these domains need to interact with high specificity and high affinity however many PH domains that bind PtdIns(3,4,5)P_3 are also able to bind to other phosphoinositides [3]. Not only are binding domains responsible for mediating interaction between a protein and its phosphoinositide target, they can also affect the physical properties of the target membrane. For example the protein epsin-1 binds to PtdIns(4,5)P_2 during clathrin-mediated endocytosis and upon PtdIns(4,5)P_2 binding induces membrane curvature [4]. This demonstrates the importance of phosphoinositides at multiple levels of cell signalling.

The presence of a specific phosphoinositide profile can define sub-cellular compartments. For example PtdIns(4,5)P_2 is strongly localised to the plasma membrane, PtdIns3*P* predominantly

appears to mark the early endosome, PtdIns(3,5)P_2 is thought to be present in lysosomes and PtdIns4P appears to mark the endoplasmic reticulum and specific vesicles derived from those organelles (Figure 1.2). The picture is unlikely to be quite as simple as shown as for example PtdIns3P is known to be generated and to be functional at the plasma membrane [5] and also PtdIns4P is the substrate for a plasma membrane localised kinase that generates PtdIns(4,5)P_2. Generation of specific phosphoinositides is thought to not only mark a certain membrane but also to contribute to its functionality. The endocytic system is a network of vesicles that transport plasma membrane components to be recycled back to the plasma membrane or degraded. Endocytic vesicles are known to mature from early endosomes to late endosomes and finally to lysosomes where degradation takes place. PtdIns3P is produced on early endosomes by PI3-Kinases. Many proteins required for early endosome function contain a PtdIns3P binding domain such as the FYVE [6] or PX domain [7] which allows their recruitment specifically to the early endosome. The association of one such protein with endosomal vesicles, early endosomal antigen 1 is lost when cells are treated with wortmannin to block PtdIns3P production [8]. Furthermore, the ability of EEA1 to induce early endosome fusion is PI3K-depenedent [9] [10] showing that the presence of PtdIns3P directly impacts upon the function of early endosomes. This epitomises the important roles phosphoinositides play in defining cellular membranes through the recruitment of proteins that then provide specific functionality to the membrane.

The signalling roles of each phosphoinositide have been studied to varying degrees. Individual members of the phosphoinositide family of phospholipids have been shown to regulate a diverse array of signalling pathways, many of which become de-regulated during cancer initiation and progression. Certain phosphoinositides have been extensively studied including PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 which are both known to be produced at the plasma membrane in response to receptor activation and are able to recruit the survival protein kinase B (PKB), a protein frequently involved in cancer. One phosphoinositide however remains elusive. PtdIns5P is the most recently discovered member of the phosphoinositide family [12]. It is relatively poorly understood, but it is becoming clear that PtdIns5P is a key regulator in many signalling pathways particularly those that arise from perturbations in the external cell environment. Often such external signals are cellular stressors that affect the PtdIns5P level which then engenders a cellular response [11].

1.2. An Introduction to PtdIns5P

PtdIns5*P* was originally isolated as a novel substrate for PtdIns(4,5)P_2 synthesis [12]. Previously it was believed that PtdIns(4,5)P_2 was made exclusively from the phosphorylation of PtdIns4*P* by PIP5Ks. Originally two forms of PIP5Ks, types I and II, were thought to be enzymatically active towards different membranes but both were thought to target PtdIns4*P* [176]. Further investigation into the specificities of type I and II PIP5Ks confirmed that type I phosphorylates PtdIns4*P* at the 5-position but contrary to previous research it was proven that type II or PIP4K actually phosphorylates PtdIns5*P* at the 4- position [12]. This work highlighted that PtdIns4*P* is far more abundant in cells than PtdIns5*P* and that it is difficult to separate PtdIns4*P* from PtdIns5*P* using high performance liquid chromatography due to their similar elution properties. This initially hampered research into the generation and regulation of PtdIns5*P.* Since then alternative methods have been developed to overcome technical difficulties associated with separating PtdIns4*P* from PtdIns5*P* including optimised HPLC methodology [38] and enzymatic assays based on the specificity of PIP4K [13] (Figure 2.1).

Despite considerable research efforts relatively little is known about this elusive member of the phosphoinositide family but its potential importance has been noted in a number of different cell types. The level of PtdIns5*P* increases after thrombin stimulation of platelets [14], in response to hyperosmotic stress in plant cells [15], during the G1 phase of the cell cycle [13], in response to oncogene over-expression [16] [17] and in response to insulin [18] [19] and fibroblast growth factor [20] [21]. PtdIns5*P* is also dramatically increased in cells during bacterial infection and this is thought to regulate the activation of PKB and the closure of the hemi-desmosome channels during infection to prevent inflammatory responses [22]. More recently it was shown that the level of PtdIns5*P* also increases when cells are oxidatively stressed [11]. Thus far, there has been no conclusive study that has convincingly proved which enzymes are responsible for the metabolism of PtdIns5*P* *in vivo*. Although numerous enzymes have been implicated in the regulation of PtdIns5*P* production (Figure 1.3), some reactions have only been observed *in vitro* [24] [115] and others may not represent the major route of synthesis *in vivo* [30]. Studies have shown the involvement of different enzymes in both the synthesis and loss of PtdIns5*P* suggesting that the *in vivo* situation is likely to be complex and dependent on the dynamic balance between production and depletion of PtdIns5*P*.

1.3. Are kinases responsible for raising the level of PtdIns5P?

1.3.1. PtdIns4P 5-kinases (PIP5Ks)

PIP5K is a 5-kinase that phosphorylates PtdIns*4*P to generate PtdIns(4,5)P_2 although to a lesser extent PIP5K are also able to phosphorylate PtdIns, PtdIns3*P* and PtdIns(3,4)P_2 [23] [24] [25]. There are three isoforms of PIP5K; PIP5K1A, PIP5K1B and PIP5K1C. Distinct cellular localisations and non-redundant functions observed in knockout mice [26] [27] [28] strongly imply that each isoform performs different functions and are likely to regulate separate pools of PtdIns(4,5)P_2. Whether they use PtdIns as a substrate to directly generate PtdIns5*P in vivo* is still unclear. One study found that PIP5K1A and PIP5K1B were able to phosphorylate PtdIns to produce PtdIns5*P in vitro* [24]. Another study failed to see any effect of over-expression of PIP5K1A on the level of PtdIns5*P* in HeLa cells [174]. The ability of PIP5Ks to generate PtdIns5*P in vivo* therefore remains unclear.

1.3.2. PIKfyve

The FYVE domain containing PtdIns5-kinase, PIKfyve, has been proposed to generate PtdIns5*P* from PtdIns. When it was originally discovered, sequence homology studies of PIKfyve revealed a zinc finger FYVE domain and a PIP5K homology domain [29] and initial experiments indicated a kinase specificity towards PtdIns and PtdIns3*P* to produce PtdIns5*P* and PtdIns(3,5)P_2 respectively [30]. Importantly it has been shown that the catalytic activity of PIKfyve may be higher towards PtdIns3*P* than towards PtdIns [31] [32]. The presence of a FYVE domain within PIKfyve may also point towards a preference for catalysing the phosphorylation of PtdIns3*P* over PtdIns as there is evidence that FYVE domains mediate specific binding to PtdIns3*P* [6]. Homozygous deletion of mouse PIKfyve is embryonic lethal at the pre-implantation stage however heterozygous deletion of the PIKfyve gene reduces the PIKfyve protein level by 50% causing a 35% reduction in the level of PtdIns5*P* [175]. Numerous studies have shown that PIKfyve indirectly regulates the cellular level of PtdIns5*P* through the production of PtdIns(3,5)P_2 which can then be de-phosphorylated to PtdIns5*P* by myotubularins (described in Chapter 1.4.1.). Strong evidence implicates PIKfyve in PtdIns5*P* synthesis and further research will determine in which cell type and under what conditions PIKfyve enzymatic activity is significant in the production of PtdIns5*P*.

1.4. Are phosphatases responsible for raising the level of PtdIns5P?

The metabolism of PtdIns5*P* is unlikely to be regulated only by kinase activity. Another proposed route of synthesis is via phosphatase activity towards higher phosphorylated phosphoinositides. One research group tested numerous kinases and phosphatases associated with PtdIns5*P* metabolism and although the contribution of kinase activity was not discredited the group concluded that phosphatase activity may actually have a more significant effect on the level of this lipid [174].

1.4.1. Myotubularins

One family of phosphatases that has been strongly linked to PtdIns5*P* metabolism is the myotubularin (MTM) family. MTM1 was originally isolated as the causative gene in X-linked recessive myotubular myopathy (XLMTM) [33]. It was shown later that MTM1 specifically targeted PtdIns3*P* to produce PtdIns [123]. After the initial link to phosphoinositides was made, further analysis revealed that MTM1 could target not only PtdIns3*P* but also had 3phosphatase activity towards PtdIns(3,5)P_2 and was therefore able to produce PtdIns5*P* [173]. At this time little was known of the functions of PtdIns5*P* but it was speculated to have an important role in muscle cells as the loss of MTM1 activity and subsequent reduced level of PtdIns5*P* could be causative of XLMTM. One group investigating the enzymatic properties of different myotubularins towards both PtdIns3*P* and PtdIns(3,5)P_2 found that PtdIns5*P* may act in a positive feedback loop by triggering oligomerisation of single myotubularins and therefore allosterically activating them [127]. A mechanism was recently described where PIKfyve and MTMR3 are able to produce PtdIns5*P* in response to FGFR1 signalling and the increased PtdIns5*P* stimulated cell migration [20] [34]. Further work showed that Rac1 is involved in PIKfyve/MTMR3-mediated regulation of migration and it was hypothesised that PtdIns5*P* recruits effectors of Rac to co-ordinate its activation [35]. Myotubularins clearly have the ability to produce functionally important PtdIns5*P* from PtdIns(3,5)P_2 in certain conditions but what contribution their enzymatic activity has to the level of PtdIns5*P* in response to alternative external signals is not clear.

1.4.2. 4-phosphatases

Polyphosphate 5-phosphatases have long been known to control the levels of PtdIns(4,5)P_2 by removing the 5-phosphate to produce PtdIns4*P.* Accordingly it has been suggested that there may be a 4-phosphatase that targets PtdIns(4,5)P_2 and subsequently generates PtdIns5*P*. The first phosphatase of this nature to be identified was the bacterial IpgD from *Shigella flexneri.* Upon

bacterial invasion, this phosphatase is injected into target host cells where it de-phosphorylates PtdIns(4,5)P_2 to generate PtdIns5*P* at the plasma membrane. This change in the phosphoinositide profile disturbs plasma membrane contact with the actin cytoskeleton and allows *S. flexneri* to enter the cell [186]. Further research established that different bacterial species contained similar enzymes and this allowed the identification of two human PtdIns(4,5)P_2 4-phosphatases using sequence homology analysis [203]. This study described how type I and II 4-phosphatases are both ubiquitously expressed and localise on late endosomal and lysosomal membranes. It was suggested that the presence of PtdIns5*P* in these membranes could be vital for their function, including regulating receptor degradation such as epidermal growth factor receptors (EGFRs). This is a particularly interesting observation as over-expression or mutated forms of EGFRs or the lack of EGFR degradation may contribute to continued aberrant signalling in some cases of cancer. Interestingly, PtdIns5*P* has been implicated in the regulation of EGFR recycling in early endosomes [36] (described in Chapter 1.7.9.). Furthermore both comparative genomics and PtdIns5*P* measurements using HPLC analysis have indicated that PtdIns5*P* is likely to be involved in vesicular trafficking [37] [38] suggesting that PtdIns5*P* and 4phosphatases reside in the same sub-cellular compartment, making 4-phosphatases good candidates for PtdIns5*P* regulators.

1.5. Are kinases responsible for decreasing the level of PtdIns5P?

1.5.1. PIP4-Kinases (PIP4Ks)

The importance of negative regulation of PtdIns5*P* has been highlighted in numerous publications with particular emphasis on the role of PIP4K. PtdIns5*P* can be removed by phosphorylation to PtdIns(4,5)P_2 by PIP4Ks. Three isoforms of PIP4K exist, namely α, β and γ isoforms (PIP4K2A, PIP4K2B and PIP4K2C) [39] [40] [41]. Each isoform is thought to reside in a different cellular compartment which has led to the suggestion that each PIP4K isoform may regulate PtdIns5*P* in a specific sub-cellular compartment. PIP4K2A is thought to reside predominantly at the plasma membrane and in the cytosol. Pervanadate-provoked elevation of protein tyrosine phosphorylation leads to an increase in PtdIns5*P* in HeLa S3 cells, an increase which is subdued when PIP4K2A is over-expressed but augmented when PIP4K2A is silenced using siRNA [42]. Similarly, over-expression of PIP4K2A suppresses H_2O_2-induced production of PtdIns5*P* in U2OS cells [11] and knock down of PIP4K2A in the AML cell line Thp1, enhances both basal and H_2O_2-induced PtdIns5*P* levels [43]. PIP4K2B has been implicated in the regulation of a nuclear pool of PtdIns5*P*. UV light-mediated activation of p38 leads to inhibition of PIP4K2B and consequently an increase in nuclear PtdIns5*P* [148]. It has been suggested that PIP4K2B may function mainly to direct the localisation of

PIP4K2A as the two isoforms can heterodimerise and PIP4K2B actually has 2000-fold lower enzymatic activity than PIP4K2A [44]. PIP4K2C was the last PIP4K isoform to be discovered. It is particularly highly expressed in the kidneys and has been shown to reside in the endoplasmic reticulum (ER) and the Golgi suggestive of a specialised role [41, 45]. Recently PtdIns5*P* was shown to be enriched in the ER and Golgi networks and interestingly when each PIP4K isoform was knocked down, loss of PIP4K2C had the greatest effect on the level of PtdIns5*P* [38]. Each PIP4K isoform might be important in the regulation of specific sub-cellular pools of PtdIns5*P*.

1.6. Are phosphatases responsible for decreasing the level of PtdIns5P?

1.6.1. PTEN like phosphatase

PTEN like phosphatase (PLIP) was identified in the *Dictyostelium* genome due its highly conserved active site motif. *In vitro* it was shown to be highly selective towards PtdIns5*P* [46] [115]. Northern blotting revealed that PLIP is present in a wide range of tissue types but is particularly enriched in the testis, a common feature of other PTEN-like phosphatases. Interestingly these studies showed that PLIP resides in the Golgi which coincides with the predicted localisation of PtdIns5*P* based on HPLC data [38]. The ability of PLIP to specifically target PtdIns5*P* *in vivo* was not demonstrated. PLIP was later renamed protein tyrosine phosphatase localised to mitochondrion 1 (PTPMT1) and was found to be localised on the inner mitochondrial membrane [115]. Further research showed that PTPMT1 de-phosphorylates phosphatidylglycerophosphate (PGP), an important precursor in cardiolipin biosynthesis [47], which along with the mitochondrial localisation of PTPMT1 strongly suggests that PtdIns5*P* is not likely to be a significant substrate for PTPMT1 *in vivo*.

1.7. Cell Signalling Functions of PtdIns5P

As more and more studies have been conducted in an effort to establish the origin of PtdIns5*P* and how it is metabolised there has been increased interest in its role as a signalling molecule in its own right. These studies highlight the importance of PtdIns5*P* in the regulation of critical cell signalling pathways and further emphasise the need to understand this phosphoinositide in greater detail.

1.7.1. Transcription

PtdIns5*P* has been implicated in a number of different cellular processes in various parts of the cell and it has been suggested that there may be pools of PtdIns5*P* that are each responsible for a different process. As mentioned previously, when cells are irradiated with UV light, nuclear PtdIns5*P* accumulates as PIP4K2B is inhibited and therefore PtdIns5*P* is not removed by phosphorylation to PtdIns(4,5)P_2. For PtdIns5*P* to function as a signalling phosphoinositide it is likely that there would be nuclear proteins that specifically interact with it. Indeed, nuclear PtdIns5*P* was shown to interact with inhibitor of growth protein-2 (ING2) via its plant homeodomain (PHD). Interaction of ING2 with PtdIns5*P* appeared to stimulate the association of ING2 with chromatin and thereby increase p53 acetylation and enhance its transcription [48] [148]. This suggests that nuclear PtdIns5*P* acts as a signalling molecule by relaying stress activation signals from p38 to the nuclear transcription machinery to initiate a cell cycle arrest when the cell is stressed and damaged.

Another study revealed that stress caused by DNA damaging agents such as etoposide also led to an increased level of nuclear PtdIns5*P* due to translocation of a 4-phosphatase into the nucleus which de-phosphorylated PtdIns(4,5)P_2 to generate PtdIns5*P*. This was shown to increase p53 acetylation and stabilisation via ING2 and subsequently p53 dependent apoptosis was increased [197]. Both of these studies emphasise the regulatory role of nuclear PtdIns5*P* in the cellular responses to stress and its ability to regulate gene expression (Figure 1.4). Recent studies show that a subset of genes that are repressed by ING2 in response to etoposide treatment are also dependent on the ability of ING2 to interact with PtdIns5*P* as shown by the disruption of etoposide-induced nuclear programs by a mutant ING2 that cannont bind PtdIns5*P*. In this instance PtdIns5*P* appears to stimulate the recruitment of ING2 to the promoter of these PtdIns5*P* regulated genes. Gene repression is a consequence of ING2 mediated histone deacetylase (HDAC) recruitment, a protein which decreases the acetylation of histones at the gene promoter [49].

Cytosolic PtdIns5*P* can also cross talk directly with the regulation of gene transcription. Plant trithorax factor is a functional homolog of the mammalian trithorax protein. A common feature in all trithorax proteins is the SET domain which possesses histone methylation activity, in particular histone 3 lysine 4 trimethylase activity [50]. Histone 3 lysine 4 trimethylation (H3K4me3) has been associated with active gene promoters [51]. All trithorax members including the mammalian ones contain at least one PHD finger although the exact function of the domain in these proteins is not well understood [52]. PHD fingers have been shown to mediate interaction with H3K4me3, although the PHD finger of Arabidopsis thaliana trithorax-like protein (Atx1) does not interact with histone. However the Atx1 PHD finger motif does interact specifically with PtdIns5*P*. Studies showed that in response to drought,

stress increased cellular PtdIns5*P* leads to the cytoplasmic translocation and retention of Atx1 and decreased trimethylation at a drought stress regulated gene. Decreased trimethylation of the target gene was associated with decreased transcription [198]. Further work demonstrated that the drought stress mediated increase in PtdIns5*P* was dependent on the plant myotubularin homolog AtMTM1 [185]. Importantly, this study was the first to show a link between PtdIns5*P* external cellular stress and Atx1 mediated epigenetic regulation.

How exactly PtdIns5*P* regulates proteins is not clear. It might, as in the case of Atx1, modulate its localisation or impact on protein conformation. Recent studies on the UHRF1 protein support this latter possibility. UHRF1 links histone methylation with the methylation of DNA. UHRF1 interacts with H3K4me3 through its Tudor domains and with unmethylated H3 via its PHD finger. PtdIns5*P* appears to interact with a polybasic region that bridges these domains and thereby impacts on the interaction of UHRF1 with methylated or nonmethylated histone [53]. These data suggest that nuclear PtdIns5*P* has a pleiotropic role in regulating aspects of transcription. Interestingly recent proteomics studies have identified many nuclear proteins that apparently are able to interact with phosphoinositides suggesting that these described studies represent just the tip of the iceberg [54] [55].

1.7.2. Ubiquitination

A yeast two hybrid screen identified an interaction between PIP4K2B and speckle-type POZ domain protein, a substrate adaptor for the ubiquitin ligase Cul3 [149]. The interaction between Cul3-SPOP and PIP4K2B leads to Cul3-mediated ubiquitination of PIP4K2B, which the authors suggested might be dependent on PtdIns5*P* and p38 MAPK activity. This was the first demonstration of PtdIns5*P* and PIP4K2B regulating nuclear protein ubiquitination. The exact role PtdIns5*P* plays in nuclear speckles and in ubiquitination is not clear, nor is the role of PIP4K2B ubiquitination as it does not appear to induce degradation of PIP4K2B and it has not been demonstrated that the endogenous PIP4K2B is ubiquitinated by this system.

1.7.3. Exocytosis

The localisation of PtdIns5*P* metabolising enzymes suggests that PtdIns5*P* plays a role in membrane trafficking. Comparative genomics have been used to determine the conserved pathways of PtdIns5*P* metabolism and this highlighted a link with PtdIns3*P,* PtdIns(3,5)P_2 and PtdIns(4,5)P_2 [37]. By considering the localisation and known functions of these precursors, it was suggested that PtdIns5*P* may have a role in exocytosis. PtdIns3*P* is present on early endosomes and is phosphorylated to PtdIns(3,5)P_2 on late endosomes. PtdIns(4,5)P_2 is present in the plasma membrane. Enzymes

capable of de-phosphorylating PtdIns(3,5)P_2 to PtdIns5P and those capable of phosphorylating PtdIns5P to PtdIns(4,5)P_2 are conserved across different phyla, leading to the hypothesis that PtdIns5P regulates trafficking between the endosomal network and the plasma membrane via exocytosis. The discovery that PIP4K2C resides in the ER and Golgi network [41] [45] along with evidence that PIP4K2C may regulate PtdIns5P in these cellular compartments [38] supports this theory although there is no strong mechanistic data to validate the hypothesis.

1.7.4. Insulin signalling

PtdIns5P has been implicated as a regulatory factor in insulin signalling. In two insulin sensitive cell lines (Chinese Hamster Ovary T cells (CHO) and 3T3-L1 adipocytes), insulin treatment transiently increased the level of PtdIns5P after 10 minutes which was then reduced to its basal level after 20-30 minutes [18]. Two known effects of acute insulin stimulation were studied, namely disassembly of F-actin stress fibres and translocation of GLUT4 to the plasma membrane, to determine the precise role of PtdIns5P in this signalling cascade. Microinjection of PtdIns5P led to reduced numbers and lengths of F-actin stress fibres in CHO T cells [18]. Interestingly, ectopic expression of PIKfyve had the same effect as addition of exogenous PtdIns5P highlighting its involvement in PtdIns5P production. In 3T3-L1 adipocytes, microinjected PtdIns5P caused GLUT4 translocation to the plasma membrane. Both effects were phosphoinositide specific and could be inhibited using a 3xPHD fusion protein (PHD motif from ING2) that has been suggested to specifically bind to PtdIns5P. Another group demonstrated that PtdIns5P regulates insulin-stimulated glucose uptake through the regulation of PtdIns(3,4,5)P_3 production [19]. Overall the specific action of PtdIns5P in a critical signalling cascade has been clearly demonstrated and these observations add another layer of complexity to the range of functions associated with this poorly understood phosphoinositide. In addition, PIP4K2B knockout mice have an unusual phenotype in that they remain lean when fed on a high fat diet and have better glucose handling. They also show increased insulin induced PKB activation in muscle. These data further highlight the potential of PIP4K2B and PtdIns5P in insulin signalling [102].

1.7.5. Migration

Phosphoinositides are known to regulate cell migration and PtdIns5P has now also been described as a regulator of this important cellular process. Using an siRNA library targeting PtdIns3P-binding proteins, MTMR3 and PIKfyve were uncovered as regulators of FGF1induced migration and further work demonstrated that depletion of MTMR3 or PIKfyve decreased the level of PtdIns5P [20]. Furthermore addition of exogenous PtdIns5P or use of IpgD, a phosphatase that hydrolyses PtdIns(4,5)P_2 to induce PtdIns5P production increased the rate of migration in MTMR3 or PIKfyve

knock down cells consistent with the hypothesis that PtdIns5*P* produced by the concerted actions of MTMR3 and PIKfyve regulates cell migration. Experiments utilising knock down of PIP4K2A to reduce the level of IpgD-induced PtdIns5*P* confirmed that PtdIns5*P* is an important signalling molecule in migration rather than simply acting as an intermediate for PtdIns(4,5)P_2 [34]. More recently MTMR3 and PIKfyve were shown to regulate the activation of Rac1 which led to a mechanism of action being described where PtdIns5*P* produced by MTMR3 and PIKfyve recruits effector proteins to induce activation of Rac1 and consequently stimulate cell migration [35]. Recent studies also show that PtdIns5*P* interacts with a guanine nucleotide exchange factor for Rac1 and in doing so stimulates the exchange of GDP for GTP on Rac. This might underlie how PtdIns5*P* impacts on migration [21].

1.7.6. T-Cell signalling

Numerous groups have postulated a role for PtdIns5*P* in T-cell signalling. Two adaptor proteins that negatively regulate T-cell signalling, Dok-1 and Dok-2, contain a PH domain that preferencially binds to PtdIns5*P* (although the PH domains from both Dok-1 and Dok-2 can bind to numerous phosphoinositides) and this domain is required for their phosphorylation and function [56]. Later, Dok-5 was also shown to bind to PtdIns5*P* via its PH domain and sequestration of PtdIns5*P* using the PH domain of Dok-5 blocked some T-cell signalling events including activation of Src and PKB [57]. Interestingly, these observations suggest that upon activation T-cells employ PtdIns5*P* to induce T-cell signalling but may also initiate a negative feedback loop to limit T-cell receptor-induced signalling. Finally, IpgD expression (which causes dephosphorylation of PtdIns(4,5)P_2 to produce PtdIns5*P*) inhibits T-cell migration and this was shown to be dependent on the enzymatic activity of IpgD [58]. This contradicts other work that implicates PtdIns5*P* as a positive regulator of cell migration however the authors describe the loss of plasma membrane PtdIns(4,5)P_2 rather than increased PtdIns5*P* as the likely cause of blocked migration. Furthermore, the intracellular signalling networks in T-cells could be different to those in other cells so PtdIns5*P* could have a different function in this specialised cell type.

1.7.7. EGFR signalling

PtdIns5*P* has been implicated in the regulation of EGFR recycling in early endosomes [36]. This study utilised the bacterial phosphatase IpgD to increase the level of PtdIns5*P* which caused accumulation of activated EGFR on early endosomes and this was required to maintain PKB signalling. Use of binding probes demonstrated that PtdIns5*P* is enriched in early endosomes as is phosphorylated EGFR and phosphorylated PKB. Furthermore PtdIns5*P* blocked vesicular transport

from early to late endosomes but did not block recycling back to the plasma membrane or retrograde transport to the Trans-Golgi Network (TGN), suggesting that PtdIns5*P* specifically prevents degradation of EGFR. It should be noted, however that IpgD increases the levels of PtdIns5*P* to approximately 500 fold higher than the endogenous level and this has never been observed with any other agonist. Also surprisingly over-expression of PIP4K2B suppressed IpgD mediated effects on EGF recycling but only suppressed the PtdIns5*P* levels to about 250 fold above the endogenous level. Therefore the ability to modulate EGF recycling may reflect the pathological levels of PtdIns5*P* attained from the transfection of IpgD or infection with *S. flexneri*. Whether endogenous levels of PtdIns5*P* can also impact on EGF recycling remain to be determined.

1.8. Oxidative stress signalling

Oxidative stress has recently been described as a potent stimulator of PtdIns5*P* synthesis, specifically H_2O_2-induced oxidative stress [11]. Reactive oxygen species (ROS) have long been recognised as biologically significant molecules in the immune system as immune cells that phagocytose foreign particles undergo a respiratory burst and consequently produce various microbicidal ROS species [59]. This led to the general consensus that ROS were harmful metabolic waste products and the discovery of numerous enzymatic and nonenzymatic antioxidant systems to remove ROS backed up this theory. However it is becoming clear that the cell produces ROS which act as signalling molecules to regulate essential cellular processes such as gene transcription, cell proliferation and cellular senescence. The extent of oxidative stress will affect the cellular consequences. High levels of H_2O_2 can induce apoptosis [60] and even higher levels can induce necrosis [61]. H_2O_2 is produced at relatively low levels in response to activated growth factor receptors such as the platelet-derived growth factor receptor (PDGFR) [62] and EGFR [63] and importantly the production of H_2O_2 is required for receptor-mediated signal transduction. Downstream of plasma membrane receptors, H_2O_2 has been shown to activate numerous transcription factors such as NF-κB [64], to activate several enzymes including protein kinase C [65], and to induce oxidation of phosphatases that become inactivated due to the formation of a reversible protein modification, such as PTEN [66].

Pathological ROS accumulation is thought to be involved in the development of numerous diseases, including inflammatory diseases such as arthritis, neurological conditions such as Parkinson's disease and cancer. The accumulation of high levels of ROS can induce DNA damage, inflammation and apoptosis leading to pathological conditions which underlies the importance of tightly regulating the level of ROS in cells. Another facet of ROS signalling is that it acts hermetically in the cell. That is to say that a first exposure to ROS enables the cell to respond better to a second exposure. This is

thought to occur in part through the activation of genes that can detoxify ROS that are induced by transcription factors such as NRF2 or FOXOs. Whether PtdIns5*P* might play a role in this process is not clear. In one instance cells that appear to produce more PtdIns5*P* in response to oxidative challenge showed lower accumulation of cellular ROS [67], while another study showed that removal of PtdIns5*P* reduced the negative effects of ROS on long term cell growth. None the less it would appear that there is a strong link between the oxidative landscape of the cell and the level of PtdIns5*P* [11].

1.9. PtdIns5P and Tumourigenesis

Several publications have implicated PtdIns5*P* in the process of tumourigenesis. In breast tumours either low or high expression of PIP4K2B correlates with reduced patient survival compared to intermediate expression and specifically low expression of PIP4K2B is associated with increased tumour size and increased metastases [67]. Further investigation revealed that genetic knock down of PIP4K2B in a breast cancer cell line caused enhanced TGF-β mediated epithelial-to-mesenchymal transition (EMT) which was suggested to be the cause of increased metastasis in low PIP4K2B expressing tumours. Another group analysed the expression of PIP4K2A and PIP4K2B in breast tumours and found that a subset of breast cancers displayed elevated protein levels of PIP4K2A and/or PIP4K2B [68]. In a cell line over-expressing both isoforms, knock down of PIP4K2A and PIP4K2B led to enhanced phosphorylation of PKB, increased intracellular ROS and induction of senescence. PIP4K was shown to be required for growth when p53 is absent as $PIP4K2A^{-/-}$ $PIP4K2B^{+/-}$ $TP53^{-/-}$ mice have fewer tumours compared to $TP53^{-/-}$ mice with wild type PIP4K. A role for PtdIns5*P* in the development of leukaemia has also been reported. An RNAi knock down screen evaluating the effect of genetically knocking down over 100 phosphoinositide-related enzymes showed that PIP4K2A is required for the clonogenic potential of acute myeloid leukaemia (AML) cell lines and primary AML cells [43]. Knock down of PIP4K2A led to cell cycle arrest, apoptosis and increased cellular PtdIns5*P*. This was suggested to be dependent on mTOR signalling, a downstream target of PKB. Importantly knock down of PIP4K2A did not affect normal haematopoietic stem cells suggesting that dis-regulation of PIP4K2A could be selectively targeted to treat AML. Overall this data demonstrates that PIP4K is an important enzyme in tumourigenesis and leads to the possibility that PtdIns5*P* could be mediating the effects of differential PIP4K expression during tumourigenesis.

1.10. The Role of PtdIns5P in PKB Regulation

1.10.1. An Introduction to PKB

One function of PtdIns5*P* that has sparked the interest of several research groups is the role of PtdIns5*P* in regulating the PI3K/PKB pathway. PKB was first isolated from the genome of the AKT8 transforming retrovirus and was shown to be an acquired oncogene [69]. There are three highly homologous isoforms of PKB;; PKBα, PKBβ and PKBγ each of which have distinct cellular functions. Despite each isoform being activated to a similar extent and showing similar activity towards target substrates PKBα has been the most thoroughly researched as it has been shown to account for almost all of the insulin-mediated increase in cellular PKB activity in some cell types [70]. The focus of this work was PKBα which herein will be referred to as PKB.

In response to various external signals, PI3K is activated which leads to the phosphorylation of PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3. Consequently PKB translocates to the plasma membrane due to recruitment by PtdIns(3,4,5)P_3 [71] via the PH domain of PKB. Similarly, PKB can be recruited to the plasma membrane by PtdIns(3,4)P_2 [72]. Once PKB has been recruited to the plasma membrane it is in a favourable position to allow phosphorylation. Two residues need to be phosphorylated for full activation of PKB; Threonine 308 (Thr308) in the activation loop which is phosphorylated by Phosphoinositide Dependent Kinase-1 (PDK1) [73] and Serine 473 (Ser473) in the C-terminal hydrophobic region which is predominantly phosphorylated by mammalian Target of Rapamycin (mTORC2) [74]. Fully activated PKB is able to phosphorylate a plethora of downstream targets in order to initiate cell signalling pathways involved in cell survival, growth and proliferation (Figure 1.5).

A good example of a PKB target is the mTOR pathway. mTOR is an evolutionarily conserved regulator of cell growth, cell cycle progression and cellular metabolism. In response to extracellular signals such as insulin, mTOR initiates protein translation by phosphorylating p70S6K and 4E-BP1, two important signalling molecules that regulate ribosomal translation of mRNA to protein [75]. PKB phosphorylates a negative regulator of mTOR, TSC2 and consequently releases the inhibition of mTOR and allows protein translation [76-79]. By regulating numerous cell signalling molecules such as mTOR, PKB is able to control many different signalling pathways that all converge on cell survival, growth and proliferation. Other PKB targets include the anti-apoptotic protein BAD [80], glycogen synthase kinase 3 [81], the proline-rich Akt substrate of 40-kDa (PRAS40), a component of mTORC1 [157] and the negative regulator of p53, Mdm2 [82]. Tumour cells are often found in stressful environments due to factors such as hypoxia and glucose deprivation. As PKB is a key regulator of

cell survival in response to cellular stress, PKB signalling is particularly important in tumour cells. The regulation of PKB is frequently altered in many types of cancers and this can occur upstream of PKB through expression of a constitutively activated PI3K or loss of PTEN activity, a 3-phosphatase that abrogates PI3K activity (see 1.10.3), both of which cause increased PtdIns(3,4,5)P_3 and therefore allows enhanced recruitment and activation of PKB. Much research effort has been focused on understanding the mechanisms involved in PKB signalling in an attempt to develop inhibitors that could potentially be used clinically to treat cancer.

1.10.2. Regulation of PKB by PI3K

PI3Ks are a family of kinases that phosphorylate the 3-OH group of the myo-inositol head group of phosphoinositides. There are three classes of PI3Ks each of which are responsible for the production of a different 3-phosphorylated product. Class I PI3Ks are the most relevant in terms of PKB activation as these enzymes are responsible for the phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 at the plasma membrane in response to extracellular signals [83]. Class IA PI3Ks are made up of two individual subunits, one catalytic and one regulatory. In response to receptor tyrosine kinase (RTK) activation, the cytoplasmic facing part of the receptor becomes phosphorylated which allows recruitment of PI3K to the plasma membrane as the regulatory subunit can bind to the phosphorylated receptor. This results in allosteric activation of the catalytic subunit in the proximity of its substrate, PtdIns(4,5)P_2. Specifically, Class IA PI3Ks, PIK3CA, PIK3CB and PIK3CD (also known as p110α, p110β and p110δ) as described, are recruited to activated RTKs [84] [85] and the single Class IB PI3K, PIK3CG (also known as p110γ) is activated by G-Protein Coupled Receptors (GPCRs) [86]. Subsequent production of PtdIns(3,4,5)P_3 at the plasma membrane leads to recruitment of signalling proteins such as PKB and initiation of downstream signal transduction. Both PIK3CA and PIK3CB are ubiquitously expressed in a wide range of cell types and may have functional redundancy [87]. PIK3CD and PIK3CG are found predominantly in leukocyte cells suggestive of a specialised role for these enzymes.

PI3K signalling is often dis-regulated in tumours. Gene amplification, deletions and somatic mutations of the PIK3CA gene have been found in a wide range of cancers including 32% of colorectal cancers [88], 40% of breast cancers and 30.5% of ovarian cancers [89]. It has been estimated that around 15% of human cancers harbour a mutation in the PIK3CA gene [90] and one-third of all human cancers display altered PI3K signalling. The deregulation of PI3K leads to enhanced production of PtdIns(3,4,5)P_3 and consequently elevated PKB signalling resulting in resistance to apoptosis, increased cell growth and proliferation. Furthermore, aberrant PI3K signalling has been implicated in the development of tumour cell resistance to many different therapies [91] which is why numerous

PI3K inhibitors are being used in clinical trials to treat a range of cancers including; breast, non-small cell lung cancer (NSCLC), prostate and bladder cancer [92].

1.10.3. Regulation of PKB by PTEN

PTEN was originally identified as the tumour suppressor gene present in the frequently mutated chromosome region 10q23-24 [96] and is now known to be one of the most commonly deleted and mutated tumour suppressor genes in cancer. Germ line PTEN mutations cause Cowden's disease which predisposes sufferers to breast, thyroid, and endometrial cancers. PTEN is a lipid 3-phosphatase with activity towards PtdIns(3,4,5)P_3 [184] which is conserved from yeast to humans and the PtdIns(3,4,5)P_3 phosphatase activity of PTEN is required for its tumour suppression function [195]. PTEN also has protein phosphatase activity [96] however the lipid phosphatase activity of PTEN has been most intensly studied due to its well established role in the regulation of the PI3K/PKB pathway. PTEN negatively regulates PKB dependent cell survival [113] and tumours in PTEN$^{+/-}$ mice show loss of heterozygosity of the wild type allele and increased phosphorylated PKB [183]. PTEN is also able to dephosphorylate the 3- position of PtdIns(3,4)P_2 [114] and importantly PtdIns(3,4)P_2 can regulate PKB activation [202].

Due to its critical activity PTEN is tightly regulated in a number of different ways. Post translational modifications play an important role in controlling the activity and localisation of PTEN. Phosphorylation of Ser380, Thr382 and Thr383, which cluster in the C-terminal tail, inhibits the activity of PTEN while maintaining the protein in a stable state [181]. This cluster of phosphorylations has been shown to inhibit the localisation of PTEN at the plasma membrane preventing the degradation of PtdIns(3,4,5)P_3 [182] by favouring a closed conformation due to the formation of intra-molecular bonds [180]. PTEN was hypothesised to function primarily at the plasma membrane, the discovery of a short conserved sequence in the N-terminus that localises PTEN to cytoplasmic membranes and allows PTEN to inhibit PKB activation emphasised this [179] however importantly it has also been shown to function in the nucleus. Ubiquitination is also an important regulatory mechanism. *In vitro* membrane targeting of PTEN enhances its ubiquitination and subsequently inhibits its phosphatase activity towards PtdIns(3,4,5)P_3 [168]. Polyubiquitination of PTEN by NEDD4-1 leads to its proteasomal degradation [178] whereas monoubiquitination controls the nuclear localisation of PTEN [177]. Several E3 ubiquitin ligases have been identified that can ubiquitinate PTEN including WWP2 [93] and RFP [94]. Recently ubiquitination of PTEN was shown to be an important factor in the regulation of PKB activation. NEDD4-mediated PTEN ubiquitination prevents PTEN from dephosphorylating the insulin receptor substrate (IRS1) in response to IGF1/ insulin

which consequently allows activation of PKB [95]. Interestingly this highlights the importance of the protein phosphatase activity of PTEN in the negative regulation of PKB.

Perturbations of PTEN signalling are seen in virtually all tumour types and PTEN itself is mutated or down-regulated in many different types of cancer [96]. Genetic changes in the PTEN gene can range from point mutations, frameshift mutations, loss of heterozygosity and homozygous deletions [97] to promoter methylation [98]. Interestingly, in gliomas PTEN mutations were found in high-grade glioblastoma but not in low-grade or childhood gliomas [99] suggesting that PTEN has an important role in tumour progression rather than initiation. Further to this, a study utilising hypomorphic mouse models showed that the dose of PTEN mediates the progression and biology of prostate tumours, for example reducing the level of PTEN directly correlated with increased phosphorylated PKB [100]. This implies that gradual loss of PTEN contributes to the progression of tumourigenesis at the molecular level. Interestingly, a recent study demonstrated that PTEN can homodimerise and when one allele is mutated the catalytically inactive PTEN can heterodimerise with the wild-type PTEN and control its phosphatase activity in a dominant negative manner leading to reduced PtdIns(3,4,5)P_3 de-phosphorylation and enhanced tumourigenesis [101]. Overall it is clear that PTEN plays an important role in cancer progression but the various genetic alterations make the biological consequences of PTEN de-regulation complicated.

1.10.4. Proposed Roles of PtdIns5P in the Regulation of PKB

Despite the fact that PtdIns5*P* has been implicated in the regulation of PKB signalling, there is still no consensus on the exact role PtdIns5*P* plays. The first observation that suggested a link between PtdIns5*P* and PKB was insulin hypersensitivity and enhanced PKB phosphorylation in PIP4K2B$^{-/-}$ mice [102]. It was hypothesised that by decreasing the level of PtdIns5*P*, PIP4K2B could negatively regulate the activation of PKB. Knock down of PIP4K2A and PIP4K2B in the BT474 cell line also leads to enhanced PKB activation. Surprisingly these cells undergo senescence rather than hyperproliferation as might be expected from increased PKB activation. The authors suggest that increased PKB activation might be a consequence of reduced glucose metabolism observed in the cells. Interestingly PIP4K knockdown cells also have increased ROS levels which might be a cause of the induced senescence [68]. Conversely, PIP4K over-expression in cells decreases the level of insulin-induced PtdIns(3,4,5)P_3 and reduces PKB activation. The authors hypothesised that PIP4K, through PtdIns5*P*, may regulate a PtdIns(3,4,5)P_3-phosphatase [155].

More direct evidence came from studies utilising IpgD, a bacterial 4-phosphatase that dephosphorylates PtdIns(4,5)P_2 to produce PtdIns5*P* at the plasma membrane during invasion.

Expression of IpgD leads to an increase in plasma membrane PtdIns5*P* which can be colocalised with phosphorylated PKB [164]. The authors suggest that increased PtdIns5*P* might attenuate PKB de-phosphorylation by inducing phosphorylation of protein phosphatase 2 (PP2A), a phosphatase that de-phosphorylates PKB, on a negative regulatory residue which therefore prevents de-phosphorylation of PKB [103]. They also show that PtdIns5*P* induces EGF receptor (EGFR) activation in the absence of a ligand which can induce accumulation of active EGFR in early endosomes allowing continued receptor signalling and PKB activation [36]. These varied proposed roles imply that PtdIns5*P* can influence PKB activation at various stages, possibly depending on the cell type or the upstream extracellular signal.

1.11. Conclusions

It is evident that PtdIns5*P* remains poorly understood. It has been shown to influence a range of cellular functions and is clearly an important signalling molecule in its own right. The synthesis and regulation of PtdIns5*P* is still a controversial topic and it is likely that PtdIns5*P* can be regulated by a number of different enzymes in discrete cellular compartments. The ability of numerous signalling systems to rapidly and transiently increase the level of PtdIns5*P* suggests that PtdIns5*P* acts to transduce extracellular signals and mediates acute intracellular signal transduction. However, until the metabolism of PtdIns5*P* has been well characterised it will be difficult to fully appreciate the functions of PtdIns5*P*. The aim of this project is to identify the enzymes and signalling pathways responsible for the generation of PtdIns5*P* in response to oxidative stress. This will be achieved using small molecular weight inhibitors targeted against molecules involved in oxidative stress signalling and by using a small focused library of short hairpin RNA (shRNA) constructs targeting enzymes involved in phosphoinositide metabolism. I aim to understand the localisation and timing of H_2O_2-induced PtdIns5*P* generation. Identification of regulatory pathways will aid in the discovery of novel functions of PtdIns5*P*.

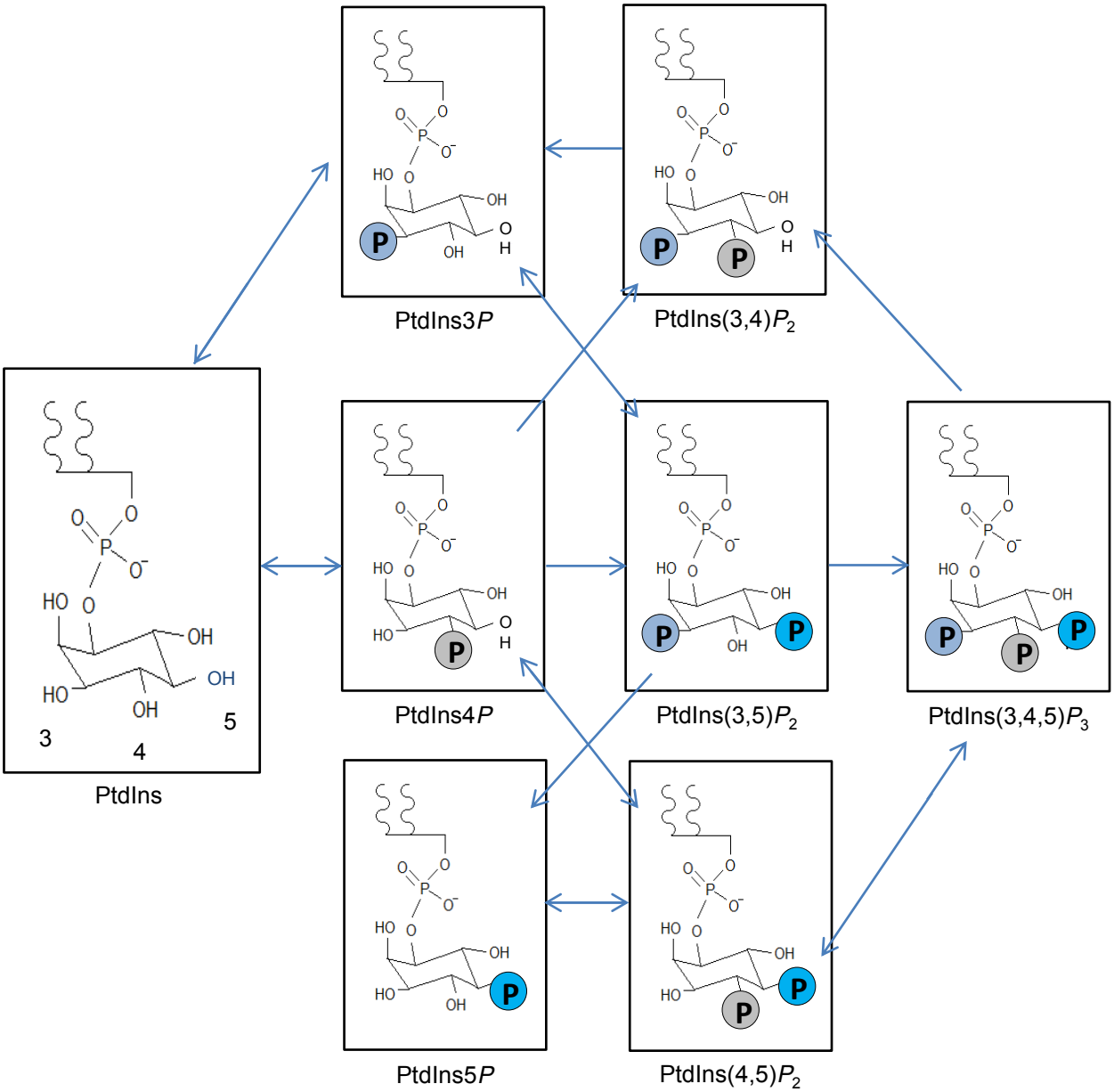


Figure 1.1. Diagram of the eight phosphoinositides and the inter-conversion by kinases (arrows pointing right) and phosphatases (arrows pointing left).

The precursor lipid, phosphatidylinositol (PtdIns) has three hydroxyls that can be phosphorylated (3, 4 and 5 positions on the myo-inositol ring). Differential phosphorylation of these hydroxyls by the action of kinases and phosphatases allows production of seven downstream phosphoinositides, three monophosphates (PtdIns3*P*, PtdIns4*P* and PtdIns5*P*), three bisphosphates (PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2) and one trisphosphate (PtdIns(3,4,5)P_3).

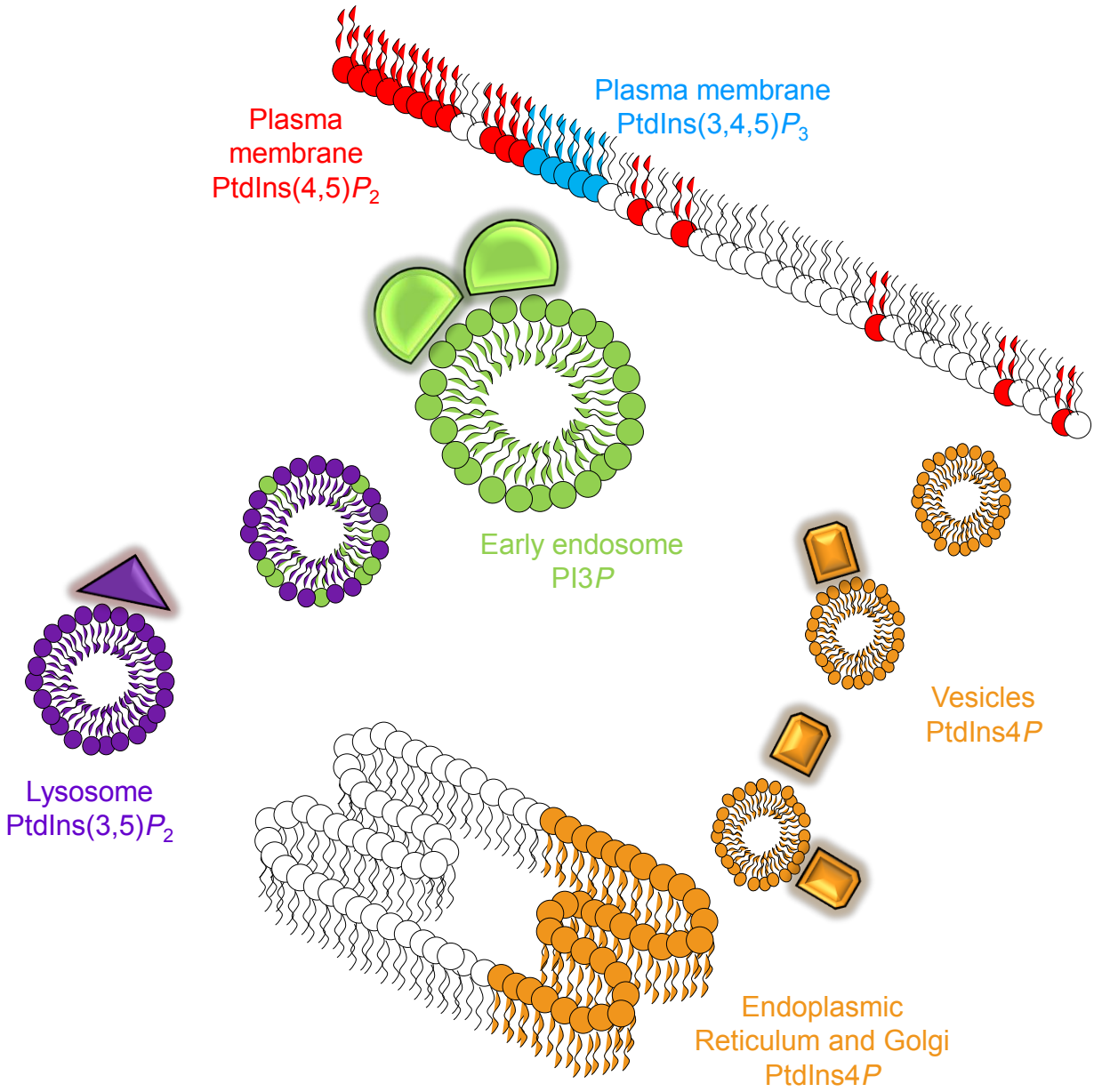


Figure 1.2. Diagram demonstrating that phosphoinositides can define cellular membranes.
PtdIns(4,5)P_2 (red) is largely found in the plasma membrane but upon activation of growth factor receptors it can be phosphorylated to produce PtdIns(3,4,5)P_3 (blue). PtdIns3P (green) is associated with early endosomes and recruits a specific set of proteins (green shapes). Upon maturation of early endosomes to late endosomes and then lysosomes the phosphoinositide profile changes to PtdIns(3,5)P_2 (purple) which causes recruitment of a different set of signalling proteins (purple shapes). Transport vesicles, the endoplasmic reticulum and the golgi all have PtdIns4P (orange) present in their membranes which also recruits specific signalling proteins (orange shapes) required for the functionality of the organelle to which they are recruited.

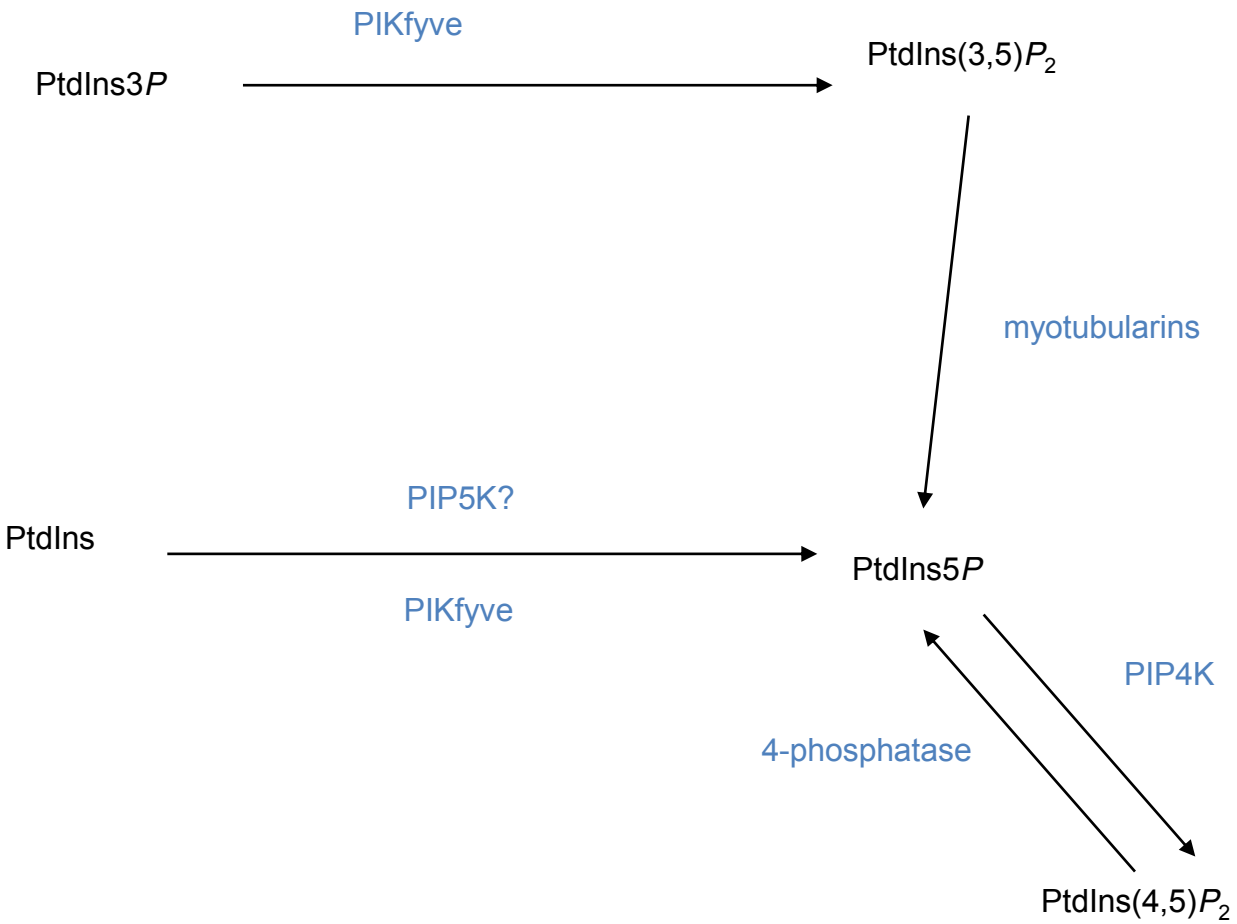


Figure 1.3. Possible routes of PtdIns5*P* generation.

Numerous enzymes have been implicated in the generation of PtdIns5*P* by direct and indirect routes. PIKfyve has been shown to directly phosphorylate PtdIns to produce PtdIns5*P* and can also phosphorylate PtdIns3*P* producing PtdIns(3,5)P_2 which can be de-phosphorylated by myotubularins to make PtdIns5*P*. PLIP, a 4-phosphatase, may be able to de-phosphorylate PtdIns(4,5)P_2 to produce PtdIns5*P*, the opposite reaction to PIP4K which negatively regulates the level of PtdIns5*P*. Alternatively, a 5-kinase such as PIP5K may be able to phosphorylate PtdIns to produce PtdIns5*P*.

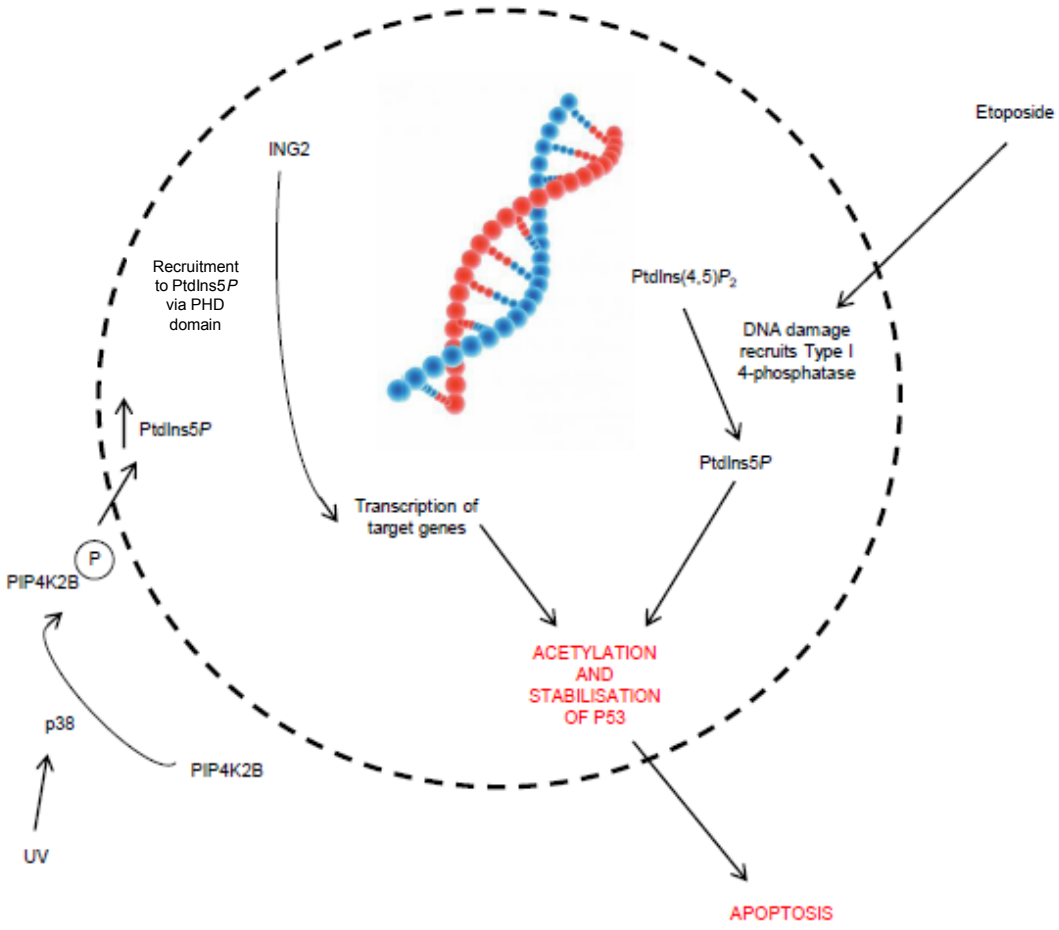


Figure 1.4. PtdIns5*P* induces the activation of p53-mediated apoptosis in two ways.

UV irradiation activates p38 which leads to the phosphorylation of PIP4K2B. This allows the accumulation of nuclear PtdIns5*P* leading to the recruitment of transcription factor ING2 via its PHD domain. Recruitment of ING2 allows transcription of target genes and subsequent activation of p53-mediated apoptosis. In response to etoposide, the DNA damage response recruits a 4-phosphatase to de-phosphorylate PtdIns(4,5)P_2 to PtdIns5*P* which leads to acetylation and stabilisation of p53.

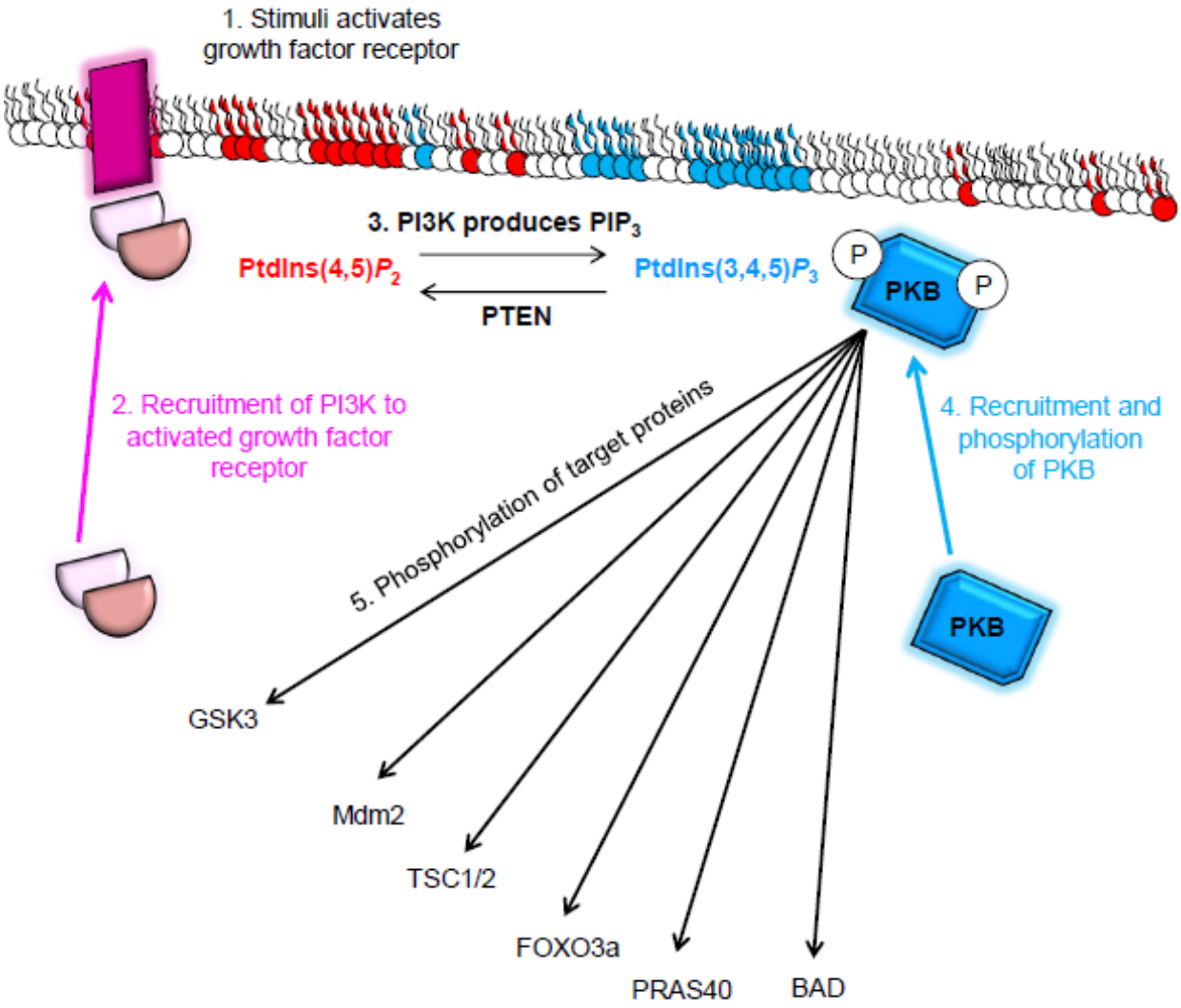


Figure 1.5. Activation of PKB activation.

In response to external stimuli, activated growth factor receptors recruit PI3K to the plasma membrane where it is in close proximity to its substrate PtdIns(4,5)P_2 and can therefore produce PtdIns(3,4,5)P_3. This leads to recruitment of PKB via its PH domain to the plasma membrane and it is subsequently phosphorylated and activated. PKB can then phosphorylate a plethora of downstream targets to initiate a range of signalling pathways resulting in induction of cell cycle progression, protein translation and glucose metabolism as well as inhibition of apoptosis.

2. Materials and Methods

2.1. Cells and Culture Conditions

2.1.1. Cell Culture Media

All cell cultures were maintained in an incubator (Leec) at 5% CO_2 and at 37°C. Cell culture media were made up as follows:

Complete Medium: 500ml Dulbecco's Modified Eagle Medium (D-MEM) (Invitrogen), 50ml foetal bovine serum (FBS) (Sigma Aldrich), 5ml penicillin-streptomycin solution (final concentration of 100 units of penicillin and 100µg streptomycin) (Invitrogen) and 10ml of 200mM L-glutamine (Sigma-Aldrich).

Serum Free Medium: 500ml D-MEM medium and 10ml L-glutamine.

D10 Medium: 500ml D-MEM medium, 50ml FBS and 5ml L-glutamine.

Freezing Medium: 18ml complete medium and 2ml (10%) dimethylsulphoxide (DMSO) (Sigma Aldrich).

2.1.2. 293FT Cells

The 293FT cell line is a human embryonic kidney (HEK) line that combines the fast-growing ability of the 293-F cell line with the high level of lentiviral production of the 239-T cell line. High lentiviral production is controlled by the constitutive expression of SV40 large T antigen from the human cytomegalovirus (CMV) promoter within the pCMVSPORT6TAg.neo plasmid (Invitrogen). Generally cells were maintained in 75cm^2 tissue culture flasks in 15ml D10 medium. Cultures were split 1:10 by trypsinisation every 2-3 days or when the monolayer of these adherent cells neared 100% confluence. Cells were gently washed with sterile PBS and 2ml of trypsin-EDTA (1x) solution (Sigma Aldrich) was added. Flasks were incubated at 37°C for 3 minutes to allow cell detachment and cells were re-suspended in D10 medium for cell counting.

2.1.3. U2OS Cells

The human osteocarcinoma cell line U2OS was chosen due to its general robustness and ease of lentiviral transduction. The cells were maintained in 75cm^2 tissue culture flasks in 15ml complete medium. Cells were split regularly in a similar manner to that described above except 5-10 minutes of incubation at 37°C was required for cell detachment.

2.1.4. Other Cell Lines

MDA-MB-468 cells, HT1080 cells and murine embryonic fibroblasts (MEFs) were all cultured in the same way as U2OS cells.

2.1.5. Cell Counting

After trypsinisation cells were re-suspended in 5-15ml of complete medium depending on cell confluency. From this cell suspension, 100µl was added to 10ml of Casyton (Roche) and cells were counted using a Casy® Model TT counter.

2.1.6. Cryopreservation of Cells

Cell lines were regularly frozen down to maintain stocks. Cells were trypsinised and resuspended in freezing medium. Aliquots of 1ml containing 1x10^6 cells were frozen using a Nalgene Mr Frosty freezing container which was left at -80° for 24 hours after which the vials were transferred to liquid nitrogen for long-term storage.

2.1.7. Thawing of Cells

Cryovials were thawed in a 37°C water bath. Cells were re-suspended in 9ml of appropriate cell culture medium, transferred to a 75cm^2 culture flask and incubated at 5% CO_2 at 37°C for several hours until the cells had adhered. The medium was then changed to remove the DMSO and cells were maintained as described in 2.1.2 and 2.1.3.

2.1.8. Cell Treatments

In a 6-well plate $2.5x10^5$ U2OS cells were plated per well. Cells were incubated overnight at 5% CO_2 at 37°C. The following morning cell culture medium was aspirated and cells were washed once with 2ml PBS and once with 2ml serum free medium. Cells were incubated in 1ml of serum free medium for 2 hours. Inhibitors were added to the serum free medium when required. A stock solution of 100mM hydrogen peroxide (H_2O_2) (Sigma-Aldrich) was prepared of which an appropriate volume was added to each well depending of the desired final concentration. Plates were left in the incubator for the desired amount of time after which, the medium was aspirated, cells were washed once with 2ml PBS and plates were placed on dry ice for 15 minutes to rapidly terminate cell signalling events. Plates were stored at -20ºC for processing at a later time. Other treatments were performed using serum free medium containing 10µg/ml anisomysin, 1mM sodium orthovanadate, 100µM sodium arsenite, 1µg/ml insulin and 1µg/ml EGF.

2.2. RNAi Methods

2.2.1. Polyethylenimine (PEI) Transfection into 293FT Cells

293FT cells were trypsinised and counted as described (2.1.2. and 2.1.5.). In 6-well plates, $1x10^6$ 293FT cells were plated per well. To produce lentiviral particles, each well of 293FT cells was transfected with 2µg of total DNA in a mixture consisting of:

0.28µg VSV-G

0.57µg CMV

1.1µg pLKO.1 containing shRNA targeting sequence 100µl serum free medium

6µl PEI

This mixture was incubated at room temperature for 20-30 minutes then added drop-wise to a well of 293FT cells. After mixing to ensure even distribution the cells were incubated overnight. The medium was aspirated the following morning and 2ml of D10 medium was gently added to each well. The next day, the supernatant containing lentiviral particles was collected and a further 2ml of D10 medium

was added to the wells. This was then collected the following morning. Separate aliquots were stored at -80°C.

2.2.2. Lentiviral Transduction of U2OS Cells

$1x10^5$ U2OS cells were plated into 6-well plates and cells were incubated for 2-4 hours. Lentiviral supernatants were thawed in a 37°C water bath. When the U2OS cells had adhered, the medium was aspirated and 300µl of lentiviral supernatant was applied to the cells, this was made up with a volume of complete medium depending on the number of different constructs being pooled and used in each well. To each well Polybrene® (hexadimethrine bromide at a final concentration of 8µg/ml) was added. After thorough mixing to ensure even distribution the cells were incubated overnight. After 24 hours, the lentiviral containing medium was aspirated and replaced with 2ml complete medium. After a further 24 hours, transduced cells were selected in complete medium with 2µg puromycin/ml (Sigma-Aldrich). The following day puromycin-resistant cells remained attached RNAi screen experiments were set up as shown in Figure 2.2.

2.2.3. siRNA Transfection

$2.5x10^5$ U2OS cells were plated into 6-well plates and cells were incubated overnight. The following morning two mixes were made for each well:

Mix 1

3µl of 20µM siRNA

20µl siRNA buffer

77µl water

Mix 2

2µl Dharmafect

200µl serum free medium

The mixes were left for 5 minutes, mix 1 was added to mix 2 and this was left for 30 minutes after which time it was added to the cells, mixed thoroughly and cell were incubated overnight. The following morning cells were trypsinised, split 1 in 3, plated into fresh cell culture plates and incubated

for a further 24 hours after which time cells could be used. Splitting the cells allows production of a useable number of transfected cells from minimal siRNA.

2.2.4. Analysis of Gene Expression

RNA was extracted from cells using an RNeasy Plus Micro Kit (Qiagen) and mRNA conversion to cDNA was achieved using a TaqMan Reverse Transcription Kit (Applied Biosystems). Oligonucleotides for specific amplification were from Sigma and probes from the Universal Short Hydrolysis Probe Library were also from Applied Biosystems. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was run using the Applied Biosystems 7900 analyser and the data were analysed using SDS software.

2.3. Bacterial Methods

2.3.1. Bacterial Culture Medium and Agar Plates

In-house laboratory services produce sterile autoclaved Luria Broth (LB) containing 10g/l tryptone, 5g/l yeast extract and 0.5g/l NaCl, LB-agar plates and Terrific Broth (TB) containing 12g/l tryptone, 24g/l yeast extract and 4ml/l glycerol. LB was used for small volume starter cultures and TB was used for large volume overnight cultures as there is a higher concentration of nutrients in TB which are required for low density cells that are cultured fro 18 hours. Use of TB for small volume starter cultures would result in overgrown cultures as cells are initially at relatively high density.

2.3.2. Mini-Preps

Mini-preps were made to produce small quantities of plasmids. Bacterial glycerol stocks were picked with a sterile p1000 tip and placed into 4ml of Terrific Broth (TB) containing 1% carbenicillin solution (Bioline) in a 14ml sterile Falcon tube (Scientific Laboratory Supplies) and placed in an orbital incubator at 37°C overnight. The following morning cultures were centrifuged at 2680G for 20 minutes at 4°C and the supernatant was aspirated. A commercial kit (Macherey-Nagel NucleoSpin® Plasmid) was used to produce mini-preps following the manufacturer's instructions.

2.3.3. Maxi-Preps

Plasmids that were required in larger quantities were 'maxi-prepped'. Bacterial glycerol stocks or transformed bacterial colonies were sub-cultured in 4ml of LB for approximately six hours. Depending on the density of the sub-culture, an appropriate volume was added to 300ml of TB containing 1% ampicillin (Sigma-Aldrich) in a 1 litre conical flask and incubated overnight (12-16 hours) in an orbital incubator at 37°C. Maxi-preps were produced using a commercial kit (QIAGEN® Plasmid Maxi Kit) following the manufacturer's instructions.

2.3.4. Transformation into Bacterial Competent Cells

DH5α *Escherichia coli* competent cells were used for transformation reactions. Cells were thawed on ice and 1µl of the plasmid required was added to 100µl of competent cells in a 1.5ml eppendorf tube. The cells were left on ice for 30 minutes and were then heat shocked for 45 seconds in a 42°C water bath before being quickly put back onto ice for two minutes to recover. The transformed cells were added to 500µl of LB and incubated in an orbital incubator for 1 hour at 37°C. 10µl of the transformation mixture was plated onto ampicillincontaining agar plates and incubated overnight at 37°C. The following day, single colonies were picked and individually sub-cultured for 'maxi-prepping' (described above).

2.4. Preparatory Procedures for the PtdIns5P Assay

2.4.1. Production of Neomycin-Coated Glass Beads

Beads were made as described by Schacht, 1978 [201]. In brief, two types of glass beads were prepared separately to produce two batches of neomycin-coated glass beads of 3000Å and 500Å diameter (Sigma-Aldrich). 5g of beads were oxidised to the aldehyde form by addition of 250ml of 6mM $NaIO_4$ (Sigma-Aldrich) in a glass bottle for 60 minutes. This was then aspirated and the beads were washed three times with 250ml MilliQ water (H_2O). 500ml of 40mM neomycin sulphate (Sigma-Aldrich) was adjusted to pH 9 before being added to the beads. After 20 minutes, 100mg of sodium borohydride (Sigma-Aldrich) was added and the bottle was thoroughly shaken. After a further 20 minutes this was repeated. After a further 20 minutes, the beads were washed as follows: 1x 250ml MilliQ water, 1x 250ml 1mM HCl, 1x 250ml MilliQ water and 1x 250ml 1:1 methanol (CH_3OH):water. The beads were suspended in an appropriate volume of methanol and stored at room temperature.

2.4.2. Regeneration of Neomycin Beads

Used neomycin beads were recycled when necessary. They were recycled by allowing the beads to settle in a glass bottle, aspirating the supernatant, applying a wash solution, shaking to cover the beads, allowing the beads to settle, aspirating the supernatant and applying the next wash according to the following scheme: 2x Buffer B (500ml methanol, 250ml chloroform and 200ml 2.4M HCl), 1x methanol, 2x MilliQ water, 2x 1mM HCl, 2x MilliQ water, 2x methanol:H_2O (1:1), 1x methanol, 3x Buffer A (100ml chloroform, 200ml methanol, 16.8ml MilliQ water and 3.2ml 2M ammonium formate).

2.4.3. Production of GST-Tagged PIP4K2A

A small amount of bacterial glycerol stock was picked with a p1000 sterile pipette tip, added to 10ml of LB and incubated in an orbital incubator for 2 hours at 37°C. This was then diluted to 100ml in LB containing 1% ampicillin and incubated overnight in an orbital incubator. The following morning 90ml was discarded and the remaining 10ml was made up to 100ml of LB with 1% ampicillin and incubated in an orbital incubator for six hours. This was then diluted to 1L in LB containing 1% ampicillin and incubated in an orbital incubator overnight at 30°C in the presence of 100µM IPTG (Sigma-Aldrich). The following morning the bacterial culture was centrifuged at 2680G at 4°C for 10 minutes and the pellet was washed once by resuspending in 10ml PBS and centrifuged again. Lysis buffer was prepared using 25ml of 0.1% Triton x100 in PBS and 500µl of protease inhibitor cocktail (one tablet dissolved in 1ml of MilliQ water and kept on ice) (Roche). The pellet was re-suspended in lysis buffer and transferred to 50ml falcon tubes. The cell suspension was then thoroughly sonicated and centrifuged for 10 minutes at 2680G. Cleared supernatant was transferred to a clean 50ml falcon tube. 200µl of glutathione sepharose beads were prepared by suspending in lysis buffer, centrifuging, aspirating off the supernatant and repeating several times to remove the ethanol that the beads are stored in. The beads were then added to the bacterial supernatant and left on a roller at 4°C for 2 hours. This was made up to 50ml with PBS-Triton, centrifuged at 2680G for 5 minutes and the supernatant aspirated. This was repeated three times and tubes were kept on ice throughout. A column was prepared by applying PBS-Triton, allowing this to drain and repeating several times. The beads were then washed with 1ml GST-Elution buffer (50mM Tris pH8 and 0.3M NaCl) five times. The GST-PIP4K2A was eluted into 1.5ml eppendorf tubes by applying 500µl of 10mM glutathione (Sigma-Aldrich) in GST-Elution buffer. Each elution was briefly vortexed, 5µl was transferred to 200µl of 20% BioRad Protein Assay Reagent in MilliQ water in a 96-well plate and the absorbance was measured using a FLUOstar Omega plate reader. The elutions were stored at -20°C.

2.5. PtdIns5P Assay

2.5.1. Total Lipid Extraction

Lipid extractions were performed based on the technique developed by Bligh and Dyer, 1959. For cells plated in 6-well plates, 450µl of 2.4M HCl was added to each well and cells were scrapped off with a syringe barrel. Samples were pipetted into 1.5ml eppendorf tubes.

Any left-over sample was recovered by adding 600µl of methanol and adding this to the eppendorf tubes. 500µl of chloroform was added to each tube to facilitate the formation of two phases. After vortexing, samples were centrifuged at 20238G in a bench top microfuge at room temperature so that lipids entered the bottom phase and the top phase was removed by careful pipetting. 800µl of theoretical upper phase (TUP) (235ml methanol, 245ml 1M HCl and 15ml chloroform) was added to wash the bottom phase, again tubes were vortexed and centrifuged at 20238G for one minute and the top phase was removed by pipetting. The bottom phase was transferred to a new eppendorf and samples were dried in a vacuum centrifuge dryer (GyroVap, Howe, UK) for 40 minutes.

2.5.2. Affinity Purification of Phosphoinositides Using Neomycin

Neomycin binds to phosphorylated phosphoinositides and is therefore a useful tool to selectively extract this minor subset of lipids from the entire pool of cellular lipids. 15µl of chloroform was added to each tube of dry lipids and briefly vortexed. Neomycin beads are kept in Buffer A and an appropriate volume that gives 25µl of packed beads was added to each eppendorf. This was made up to 1ml with Buffer A and put on a tube rotator for one hour. After this time, samples were centrifuged at 20238Gat room temperature to sediment the beads as much as possible. The supernatant contains all phospholipids that do not bind to neomycin. These lipids were collected by pouring the supernatant into labelled glass tubes (Culture Tubes, Fisher Scientific) in order to determine the amount of lipid phosphate (method outlined in 2.5.5.). The beads were then washed with 1ml of Buffer A, vortexed and centrifuged in a bench top microfuge at room temperature. Most of the buffer was removed by aspirating and 950µl of Buffer B (500ml methanol, 250ml chloroform and 200ml 2.4M HCl) was added to each sample. Tubes were put on a tube rotator for 45 minutes. Samples were then centrifuged in a bench top microfuge at room temperature to sediment the beads. The supernatant was then added to eppendorf tubes containing 250µl water and 250µl chloroform. The beads were left to dry and regenerated (method outlined in 2.4.2.). The samples were vortexed and centrifuged at 20238G for 1 minute. Most of the top phase was removed by gentle aspiration and the bottom phase was transferred to a clean eppendorf tube.

2.5.3. Production of the PtdIns5P standard curve

A standard curve of PtdIns5*P* was prepared by firstly making a 2pmoles/µl stock solution by adding 1µl of PtdIns5*P* (dissolved in DMSO) (Cell Signals, Columbus, OH, USA) to 500µl of MilliQ water. The PtdIns5*P* standard curve samples are; 0, 0.78, 1.56, 3.125, 6.25 and 12.5 pmoles. To each standard curve sample, 10nmoles of phosphatidylserine (PS) (SigmaAldrich) was added to optimise conditions for the following phosphorylation reaction and samples were placed in a vacuum dryer for one hour. Tubes could then be stored at -20ºC for processing at a later date.

2.5.4. Phosphorylation of PtdIns5P to Radio-Labelled PtdIns(4,5)P_2 by PIP4K2A

50µl of 10mM Tris-HCl buffer and 50µl of diethyl ether (Sigma-Aldrich) was added to each sample and each standard curve sample. Each tube was sonicated for 1.5 minutes in a Diagenode Bioruptor® Sonicator, briefly centrifuged to allow the ether to enter the top phase then dried in a vacuum dryer for 5 minutes to remove the ether. An enzyme/ATP mix was prepared as follows, per tube: 1.2µl of PIP4Kα (or the volume specified for each particular enzyme batch), 0.4µl of cold ATP (Sigma Aldrich), 47.9µl 2xPIPKIN buffer (100mM Tris pH 7.4, 20mM EGTA and 140mM KCl) and 0.5µl of γ-^{32}P ATP (Perkin Elmer) was finally added behind a perspex screen. This mixture was vortexed, spun down and 50µl was added to each tube. The reaction was incubated at 30ºC overnight. The following morning, the reaction was stopped by adding to each sample 500µl of 'stop' mixture (100ml chloroform, 100ml methanol and 2ml Bovine Brain Extract (Sigma Aldrich)) and 125µl of HCl, each tube was briefly vortexed and centrifuged. The top phase was removed and 500µl of TUP was added, again tubes were vortexed and centrifuged and the top phase was removed. The bottom phase was then transferred to a new tube and placed in a vacuum dryer for 45 minutes. Merck® Silica TLC plates were treated with a solution containing: 1.5 litres of H_2O, 1.5 litres of methanol, 2mM EDTA (Sigma-Aldrich) and 2% potassium oxalate (Sigma-Aldrich). The plates were dried at 110°C for 24 hours before use. When samples were dry, 15µl of chloroform wad added to solubilise the dried lipids and gently vortexed. Each sample was spotted on a TLC plate. Another 15µl of chloroform was added to each tube and again was spotted onto the TLC plate. The TLC plates were run inside glass tanks containing the following solution: 35ml of methanol, 45ml of chloroform, 8ml of MilliQ water and 2ml of 25% ammonia solution (Fisher Scientific). These were left to run for 2 hours. Once finished, plates were left to dry at room temperature for 30 minutes then wrapped in Saran film and placed inside a film exposure cassette. A phosphoimaging screen was placed face down onto plates and left to expose for 15 minutes. The screen was then scanned using a BioRad PharosFX molecular scanner. Radioactive spots were quantified and used for calculations (2.5.6.).

2.5.5. Phosphate assay

Phosphoric acid (H_3PO_4) was used to generate a phosphate standard curve of; 0, 5, 10, 20, 30, 40, 50, 60 and 70 nmoles. Appropriate volumes were pipetted into glass tubes. After affinity purification of phosphoinositides using neomycin (2.5.2.) the supernatants containing phospholipids that do not bind were collected as described in glass tubes. All tubes were placed in an oven at 80°C until dry. When dry, 50µl of perchloric acid (Fisher Scientific) was added to each tube to liberate the phosphates and the tubes were then placed in a heating block at 180ºC for 30 minutes. After cooling, 250µl of MilliQ water, 50µl of 2.5% ammonium molybdate tetrahydrate (Fisher Scientific) and 50µl of 10% L-ascorbic acid (Sigma-Aldrich) were added to each tube and left to develop at room temperature for up to one hour. 120µl of each standard and sample was pipetted in duplicate into a 96-well plate (Sterilin) as follows:

	1	2	3	4	5	6	7	8	9	10
A	0	5	10	20	30	40	50	60	70	
B	0	5	10	20	30	40	50	60	70	
C	1	2	3	4	5	6				
D	1	2	3	4	5	6				

Absorbance at 630nm was determined using a FLUOstar Omega plate reader (BMG) and values were used for calculations (Section 2.5.6.).

2.5.6. Calculations

An average of each duplicate standard and sample was calculated and each average was corrected by subtracting the average of the blank standard (0 nmoles). A standard curve was composed and used to calculate the nmoles of phosphate in each sample. For the TLC data, the standard values were corrected by subtracting the blank standard (0 pmoles). From this a standard curve was drawn and used to calculate the pmoles of PtdIns(4,5)P_2 in each sample. The data were normalised by dividing the pmoles of PtdIns(4,5)P_2 by the nmoles of lipid phosphate to finally obtain the pmoles of PtdIns5*P* per nmole of lipid phosphate. Each data point was presented as the mean ±SD (n=3).

2.6. In vitro PIP4K inhibition assay

AG1024 and I-OMe-AG538 were tested for their ability to inhibit GST-PIP4K2A or PIP4K2B mediated phosphorylation of PtdIns5*P in vitro*. 50µl of PS was added to a 1.5ml eppendorf tube and dried in a vacuum dryer for 20 minutes. The inhibitor of interest was prepared by making a stock solution with 1µl of inhibitor and 9µl of DMSO. From this stock the final concentrations of 10, 5, 2.5, 1, 0.5 and 0.25µM were pipetted into 1.5ml eppendorf tubes with a 1µl DMSO control. Next, 0.5µl of PIP4K in 78.5µl of 1x PIPKIN buffer was added to each tube. The tubes were kept on ice. The substrate must be added in excess to ensure it is not a limiting factor. 5µl of PtdIns5P and 95µl of 10mM Tris-HCl was added to the dried PS. This was briefly sonicated and centrifuged then 10µl was added to each tube. Next, 0.4µl of ATP and 9.1µl of 1xPIPKIN buffer were added before finally 0.5µl of ^{32}P-ATP was added to each tube behind a perspex screen. PtdIns5*P* phosphorylation was allowed to proceed for 8 minutes at 30°C in the presence of 10□Ci γ-^{32}P-ATP/20□M ATP before termination and extraction of radio-labelled PtdIns(4,5)P_2 which was separated by TLC as described in Section 2.5.4. All incubations were performed in duplicate. Dose dependent inhibition curves were calculated.

2.7. Analysis of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3

2.7.1. Analysis of PtdIns(3,4)P_2 by HPLC

U2OS cells were plated and transfected with siRNA as described in Section 2.2.3. The following day cells were washed with medium without phosphate several times before being incubated with medium containing radio-labelled orthophosphate for 90 minutes. To induce oxidative stress an appropriate volume of H_2O_2 could be added to the labelling medium. Lipids were extracted as described in Section 2.5.1. with the addition of 50µg Folsch lipids. Once the lipids had been dried they were then deacylated by the addition of 20µl butanol, 150µl methanol and 150µl methylamine per sample. Samples were sonicated for 2 minutes and incubated at 53°C for an hour. Samples were dried down and fatty acyl groups were removed by the addition of 200µl H_2O and 200µl of the mix: 2ml H_2O, 0.4ml petroleum ether, 0.1ml ethyl formate. Samples were sonicated and centrifuged and the resultant lower phase was transferred to an eppendorf tube and dried in a vacuum dryer. HPLC analysis was performed by Dr David R. Jones. The following gradient was used: 0–1M ammonium phosphate (pH 3.85) over 145 min (0–10 min, water; 10–70 min, linear gradient to 0.25M ammonium phosphate; 70–120 min, linear gradient to 1M ammonium phosphate, held at 1M ammonium phosphate for 10 min; 130–133 min, linear gradient to water; and the remaining time with

water for washout) at a flow rate of 1 ml/min ensured separation of deacylated phosphoinositides using a 5-µm pore size Partisphere 4.6- × 250-mm SAX column (Whatman).

2.7.2. Mass Spectrometry Analysis of PtdIns(3,4,5)P_3

U2OS cells were plated and transfected with siRNA as described in Section 2.2.3. and cells were treated as described in Section 2.1.8. Plates were then kept on ice, cells were scraped in 600µl 1M HCl and transferred to eppendorf tubes. Remaining cells were recovered using 600µl 1M HCl. Samples were centrifuged at 4°C for 5 minutes at 20238G, the supernatant was discarded and resultant cell pellets were kept on dry ice. Further preparation and mass spectrometry was performed by Dr. K. Anderson.

2.8. Analysis of Proteins

2.8.1. Western Blotting

Cells were lysed by directly adding LDS sample buffer (Invitrogen) with 100mM DTT (Sigma) and 1% Nonidet P-40 (Sigma) to each well, following by boiling and sonication of each sample to fully lyse. Standard western blotting techniques were utilised to analyse the expression of proteins. Invitrogen 4-12% acrylamide Bis-Tris pre-cast gels were used and wet protein transfer to nitrocellulose membranes in transfer buffer (500mM Glycine, 50mM TrisHCl, 0.01% SDS, 20% CH_2OH) at 70V for 2 hours. Membranes were then gently washed in water and blocked for 20 minutes in 10% non-fat milk powder (Marvel) in PBS-Tween. Membranes were washed 3x 5 minutes in PBS-Tween and incubated overnight at 4°C with primary antibody. The following day membranes were washed 3x 10 minutes in PBS-Tween, incubated with secondary antibody for 60 minutes at room temperature and finally washed 3x 10 minutes in PBS-Tween. Detection of protein bands was achieved using an enhanced chemiluminescence kit (Thermo Scientific) and chemiluminescence was detected using chemiluminescence-specific films (GE Healthcare). Phospho- and non-phospho- primary antibodies against PKB, p38, PRAS40, ERK, PDK1, PTEN and PIP4K2A were from Cell Signaling Technology. Actin antibody was from Millipore. HRP-linked anti-rabbit and antimouse secondary antibodies were from GE Healthcare.

Protein target	Company	Catalogue number
Phospho-PKB Thr308	Cell Signalling Technology	9275

Phospho-PKB Ser473	Cell Signalling Technology	9271
PKB	Cell Signalling Technology	9272
Phospho-p38 Thr180/Tyr182	Cell Signalling Technology	9211
p38	Cell Signalling Technology	9212
Phospho-ERK Thr202/Tyr204	Cell Signalling Technology	9101
ERK	Cell Signalling Technology	9102
Phospho-PTEN Ser380	Cell Signalling Technology	9551
PTEN	Cell Signalling Technology	9552
Phospho-PRAS40 Thr246	Cell Signalling Technology	2640
PRAS40	Cell Signalling Technology	2610
PDK1	Cell Signalling Technology	3062
PIP4K2A	Cell Signalling Technology	5527
Tubulin	Millipore	05-661
Actin	Millipore	MAB1501

2.8.2. PTEN Immunoprecipitation

Myc-tagged PTEN and control empty vector expression constructs were made up in the following mixture;; 2µg plasmid, 6µl PEI in 100µl serum free medium, and this mixture was left at room temperature for 15 minutes. $2.5x10^5$ U2OS cells were plated in a 6-well plate and a couple of hours after plating the above mixture was added to each well and incubated overnight. Following cell treatments (as described in 2.1.8), cells were lysed on ice in 100mM DTT (Sigma), 1% Nonidet P-40 (Sigma) and protease and protein phosphatase inhibitor cocktail (Roche). After being transferred to eppendorf tubes, samples were rotated at 4°C for 15 minutes and centrifuged at 16770G for 15 minutes at 4°C. A portion of the lysate was incubated with antibody and then beads for the times indicated in Figure 6.9A. After incubation, beads were obtained by centrifugation of samples at 4192G for 60 seconds, aspiration of supernatant, 3x 1ml washes with IP wash buffer after which LDS sample buffer with 100mM DTT was directly added to beads. Samples were then boiled and analysed by western blotting as described in Section 2.8.1.

2.8.3. Co-Immunoprecipitation of PIP4K2A and PTEN

Co-immunoprecipitation experiments were performed by transfecting cells with Myc-tagged PTEN and/or EE-tagged PIP4K2A and empty control vector. To each well 1µg of Myc-tagged PTEN and/or EE-tagged PIP4K2A was added and this was made up to a total of 2µg of plasmid with empty control vector where necessary. Transfections and cell treatments were performed as described in 2.8.2. Each sample was performed in duplicate to allow IP of one sample with Myc antibody and the one sample with EE antibody. After cell lysis (as described in 2.8.2.), lysates were rotated overnight at 4°C with 50µl of antibody. The following morning 10µl of protein G beads were added to each tube and tubes were rotated at 4°C for an hour. The beads were then washed 3 times with IP wash buffer. LDS sample buffer with 100mM DTT was then directly added to beads. Samples were boiled and analysed by western blotting as described in Section 2.8.1. Both the Myc and EE antibodies were produced in-house.

2.8.4. Detection of Oxidised PTEN

To detect oxidation of PTEN, U2OS cell were plated and treated as described in Section 2.1.8. and cells were lysed with the following lysis buffer:

For 2ml:

400µl 10% SDS solution

400µl 0.5M Tris

10mM *N*-ethylmaleimide (NEM)

1ml H_2O

Samples were scraped into eppendorf tubes, a volume of LDS sample buffer was added depending on the desired final protein concentration, boiled and run on 10% acrylamide Bis-Tris pre-cast gels (Invitrogen) at 150V to separate proteins as much as possible. Proteins were then transferred to nitrocellulose membranes and treated as described in Section 2.8.1.

2.8.5. His-tagged Ubiquitin Pull Down

$4x10^5$ U2OS cells were plated in 6-well plates and transfected with His-tagged ubiquitin and/or Myc-tagged PTEN as described in Section 2.8.2. Transfected cells were treated the following day as described in Section 2.1.8. Plates could be stored at -20°C for future use or immediately processed by addition of 500µl of urea buffer (8M urea, 0.2% SDS in PBS) followed by mixing at room temperature for 15 minutes. Cells were scraped into eppendorf tubes, sonicated then a portion of each sample was taken for direct western blotting and the rest was incubated with 5µl nickel resin beads in urea buffer and rotated for 2 hours at room temperature. Beads were washed 3 times with urea buffer then lysates were made by directly adding LDS sample buffer with 100mM DTT to the beads and boiling. Lysates were analysed by western blotting as described in Section 2.8.1.

2.8.6. Antibody Array Kit

A PathScan RTK Signaling Antibody Array Kit (Cell Signaling Technology) was used to analyse the phosphorylation of 39 target proteins. U2OS cells were plated and transfected as described in Section 2.2.3. and were treated as described in Section 2.1.8. Lysates were produced and the assay procedure was completed according to the manufacturers' instructions. Array images were captured using chemiluminescence-specific and spots were quantified using TINA 4.0 analysis software.

2.9. Analysis of Cell Growth Capability

2.9.1. Cell Growth Assays

8000 U2OS cells were plated in 6-well plates. Over the next 6 days the cells in the wells were trypsinised and counted each day.

2.9.2. Propidium Iodide Cell Cycle Analysis

U2OS cells were plated and transduced with shRNA constructs as described in Section 2.2.2. After puromycin selection, medium was collected and transferred to FACS tubes. The cells were washed with PBS which was added to the FACS tubes. Cells were then trypsinised and detached cells were added to the FACS tubes which were centrifuged at 240G for 3 minutes and the medium was gently aspirated. The cell pellet was washed with 500µl of 70% methanol in PBS then washed in

PBS and re-suspended in 200µl propidium iodide. Samples were then analysed by FACS analysis (performed by Dr J. T. Lynch).

2.9.3. Anchorage-Dependent Colony Assays

1000 U2OS cells were plated in 10cm dishes and incubated for 14 days. After this time the growth medium was aspirated and cell colonies were washed 3 times with PBS then stained with crystal violet solution (0.1% crystal violet, 4% formaldehyde and 50% methanol). The number of colonies was counted by eye.

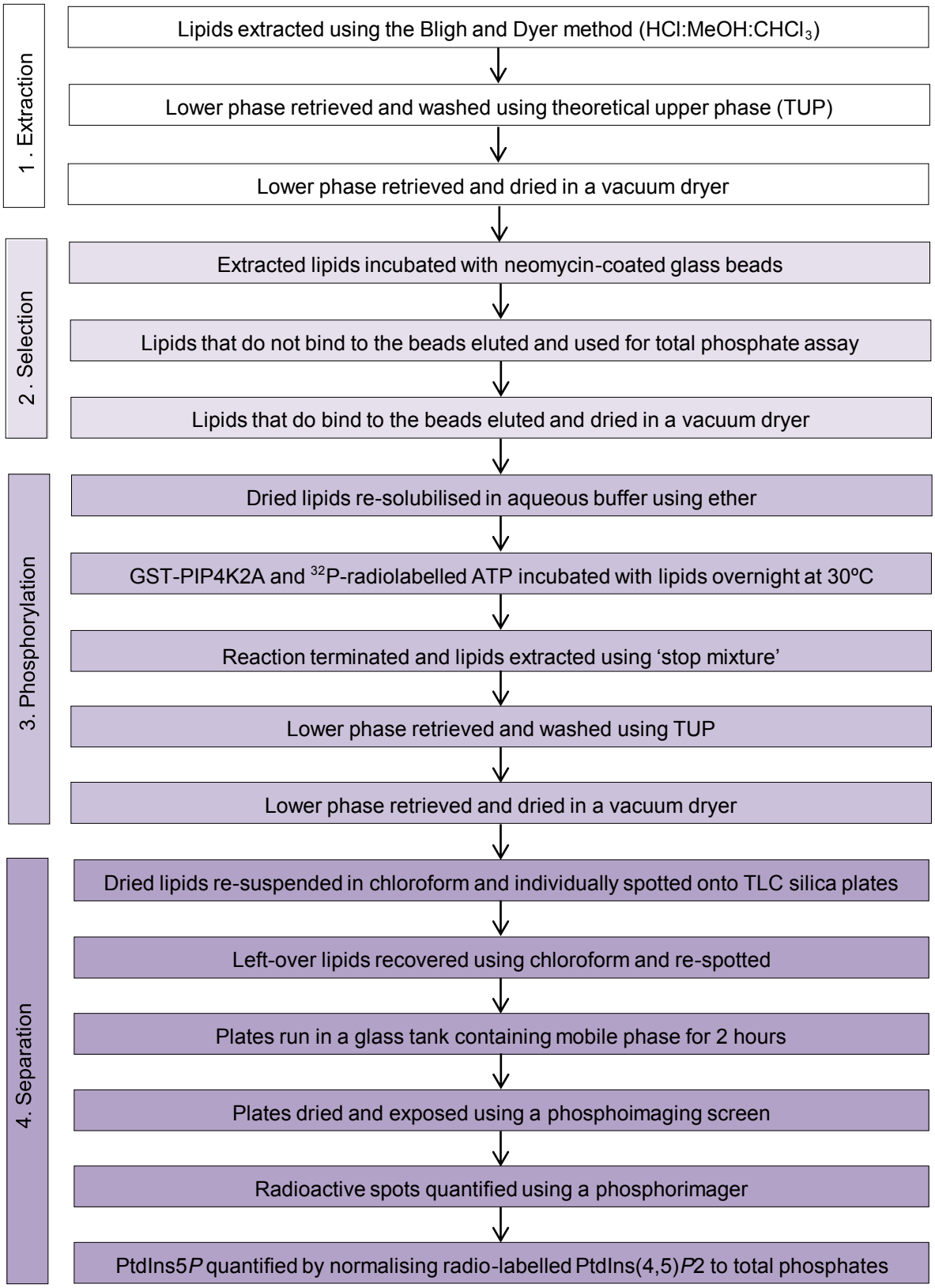


Figure 2.1. Flow diagram summarising the steps involved in PtdIns5*P* quantification.

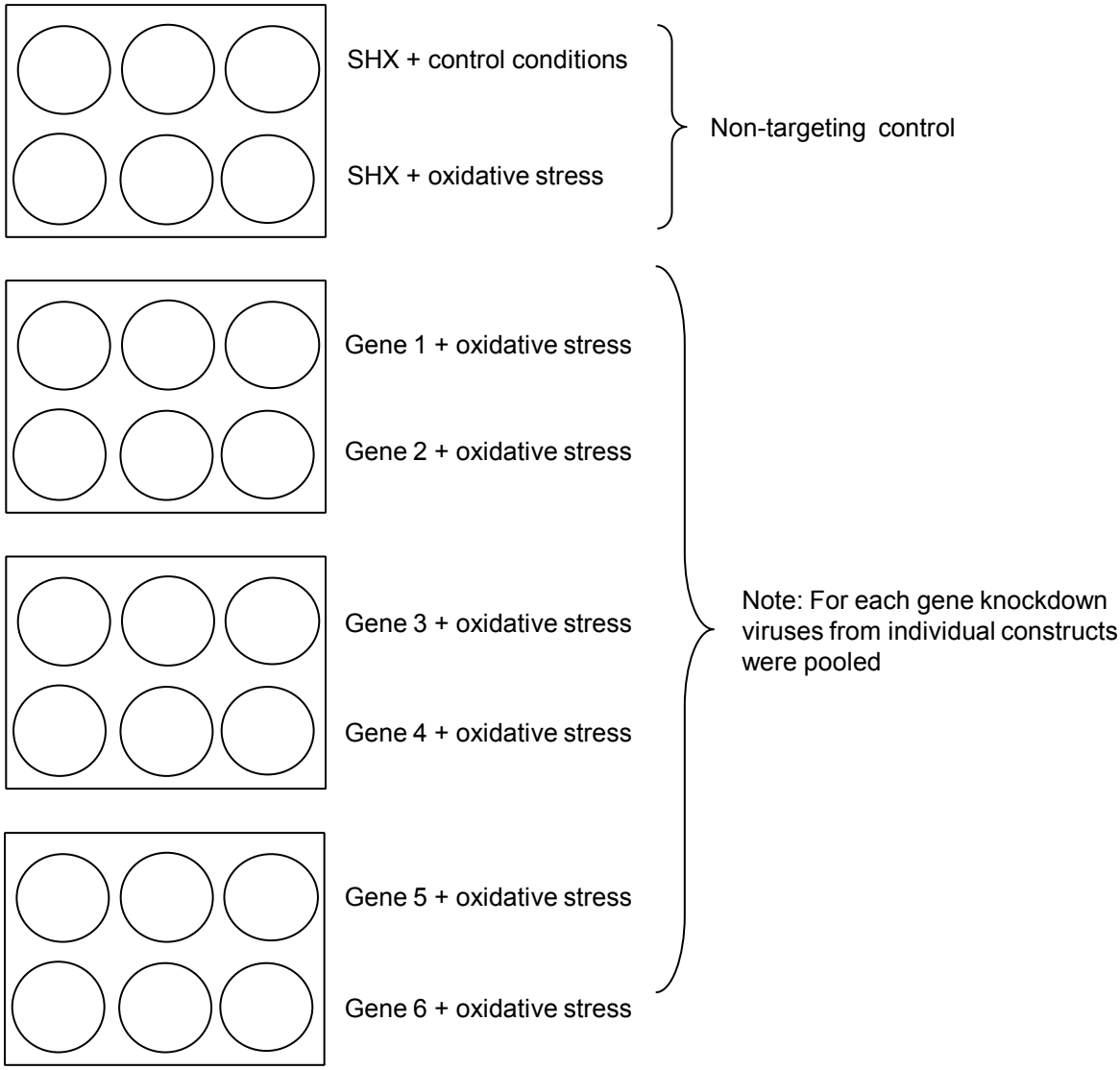


Figure 2.2. Diagram illustrating the set up of the RNAi screen.

Genes were analysed in groups of six to minimise the number of samples being handled. As a consequence a total of 17 assays was required to assess the effect of knocking down each gene of interest.

3. Identifying molecular regulators of cellular PtdIns5*P* using small molecular weight inhibitors

3.1. Introduction

PtdIns5*P* has been shown to mediate intracellular signalling in response to numerous signals. The level of PtdIns5*P* increases after thrombin stimulation of platelets [14], in response to hyperosmotic stress in plant cells [15] and during the G1 phase of the cell cycle [13]. Since its discovery, there has been no conclusive study that has definitively proved which enzymes or signalling pathways are responsible for regulating the cellular level of PtdIns5*P*. Although several enzymes have been suggested to regulate PtdIns5*P* production (Figure 1.3), some reactions have only been observed *in vitro* and may not represent the major route of synthesis *in vivo*. Studies have shown the involvement of different enzymes in both the synthesis and loss of PtdIns5*P* suggesting that the *in vivo* situation is likely to be a complex and a dynamic balance between production and depletion of PtdIns5*P* (detailed description in Chapter 1). The initial aim of the project was to investigate under which conditions the level of PtdIns5*P* is regulated and which signalling pathways are responsible for the regulation of PtdIns5*P.*

3.2. Initial observations

3.2.1. Set up of the PtdIns5P assay

There are few robust and sensitive methods to quantify cellular PtdIns5*P.* A commonly used method is high pressure liquid chromatography (HPLC) which involves extracting radiolabelled lipids, deacylating to obtain the lipid head groups and finally ion-exchange based separation. This method allows quantification of all phosphoinositide species so direct comparison of different phosphoinositides is possible. However due to the similar elution properties of PtdIns4*P* and PtdIns5*P*, the peak that represents PtdIns5*P* overlaps with the PtdIns4*P* peak making it difficult to

accurately quantify PtdIns5*P* [32]. A new HPLC methodology has been developed that allows accurate quantification of PtdIns5*P* in resting and stimulated conditions [38] which is likely to be useful in the future as the technique is attempted and optimised in different laboratories. However the HPLC methodology requires *in vivo* radio-labelling of cells with ^{32}P-orthophosphate and therefore is difficult for routine use. The most commonly used method to measure PtdIns5*P* is an enzymatic assay that involves the following steps; extraction of all cellular lipids, selection of polyphosphoinositides using neomycin coated beads and phosphorylation of PtdIns5*P* by PIP4K2A in the presence of ^{32}P-ATP. The resulting radio-labelled PtdIns(4,5)P_2 can then be quantified as a direct read-out of PtdIns5*P* [14] [13]. The drawback of this method is that only PtdIns5*P* can be quantified so it is not possible to compare the relative levels of different phosphoinositides but its ease and specificity has led to this assay being widely used. In this work an enzymatic assay based on the method described by Clarke et al. 2001 [13] was used (Chapter 2.5). It was important to firstly test the reagents required for the PtdIns5*P* assay to ensure consistent and accurate results would be obtained. Selective quantification of PtdIns5*P* is dependent on the specific enzymatic activity of PIP4K2A. GST-tagged PIP4K2A was purified from bacterial stocks and the resultant elutions (E1 and E2) were tested against a validated stock of PIP4K2A. Both new elutions E1 and E2 were able to phosphorylate PtdIns5*P* in the presence of radioactive ^{32}P-ATP to produce radio-labelled PtdIns(4,5)P_2 more efficiently than the original stock of PIP4K2A (Figure 3.1A) and importantly showed linear production of PtdIns(4,5)P_2 over a range of PtdIns5*P* concentrations.

After the initial lipid extraction, neomycin coated glass beads are used to selectively bind phosphorylated phosphoinositides which make up a minor fraction of the total cellular pool of lipids. Selective extraction of phosphorylated phosphoinositides concentrates this minor fraction of lipids and allows more sensitive quantification as much of the background lipid noise has been removed and it also allows removal of an inhibitor of the PIP4K2A enzyme. Glass beads of 3000nm and 500nm diameter were prepared with neomycin and compared. Lipids from ^{32}P-radio-labelled cell membranes were extracted and incubated with 3000nm or 500nm neomycin coated beads. The fraction of lipids that did not bind to the beads was collected and the fraction of lipids that did bind were eluted from the beads and collected. These samples were analysed using thin layer chromatography (TLC) and monophosphorylated phosphoinositides (PtdIns3*P*, PtdIns4*P* and PtdIns5*P*) were quantified. Both sets of beads were able to efficiently bind mono-phosphorylated phosphoinositides from cellular extracts with recovery of 83.4% and 75.4% for 3000nm and 500nm respectively (Figure 3.1B). Both sets of beads were also able to bind pure PtdIns5*P* (Figure 3.1C). The average recovery was 51% and 49% for 500nm and 3000nm beads respectively however use of 500nm beads provided more consistent recovery (standard deviation of 6.6 and 22.3 for 500nm and 3000nm beads respectively) and more efficiently recovered lower concentrations of PtdIns5*P* (52%

compared to 41% recovered by 3000nm beads) (Figure 3.1C) so 500nm beads were used in subsequent experiments.

3.2.2. H_2O_2-induced oxidative stress increases the level of PtdIns5P

It has been previously observed that the level of PtdIns5*P* significantly increases in response to H_2O_2-induced oxidative stress [11]. Although often considered as a toxic by-product of metabolic processes in cells, H_2O_2 is actually an important signalling molecule that can propagate activated growth factor receptor signalling through oxidation of protein targets. The most biologically relevant targets are phosphatases that become inactivated due to the formation of a reversible protein modification such as a disulphide bond. Numerous phosphatases are known to be redox regulated including; focal adhesion kinase (FAK) tyrosine phosphatase [84], Cdc25 [104] and PTEN, inhibition of which enables the accumulation of the signalling lipid PtdIns(3,4,5)P_3 and subsequent activation of downstream signalling such as the PKB protein kinase [66]. *In vivo* intracellular H_2O_2 is regulated by activated growth factor receptors and this can be mimicked experimentally by exogenously applied H_2O_2 which can freely diffuse into cells across the plasma membrane and interact with a range of intracellular signalling molecules.

H_2O_2 is known to stimulate tyrosine phosphorylation of the insulin receptor and thereby activate its receptor kinase activity [105] [106] so it is unsurprising that many signalling pathways that are activated by insulin are also activated by H_2O_2. Furthermore, insulin has been shown to induce oxidation of PTP1B, an insulin receptor phosphatase, and TC45, which de-phosphorylates many different protein tyrosine kinases, through the intracellular production of H_2O_2 [107] further demonstrating the extensive cross talk between these two signalling molecules. There is however evidence to show that H_2O_2 may also play an inhibitory role in insulin signalling [108] indicating that the relationship between insulin and H_2O_2 signalling is not straight forward. Western blotting experiments showed that numerous signalling molecules that are regulated in response to insulin are likewise regulated in response to H_2O_2 including PRAS40 and ERK whereas activation of p38 and phosphorylation of PKB on Thr308 were differentially regulated (Figure 3.2A). Interestingly, the cellular level of PtdIns5*P* was not affected when cells were stimulated with insulin or another specific signalling activator EGF for a single time point whereas the level of PtdIns5*P* dramatically increased when cells were stimulated with H_2O_2 (Figure 3.2B). Time course experiments using insulin and EGF would be required to further vaildate this observation. This effect was also seen in a number of different cell lines namely, MDA-MB-468, mouse embryonic fibroblasts (MEFs) and HT1080 (Figure 3.2C, experiment performed by Dr David Jones), suggesting that this is not a U2OS cell-specific

event. Overall this data suggests that H_2O_2 can activate a specific signal transduction event independently of insulin that results in the up-regulation of PtdIns5*P* production.

Once a stimulant that could robustly regulate PtdIns5*P* had been identified, conditions for the measurement of PtdIns5*P* were optimised to maximise the amount of PtdIns5*P* obtained and streamline the procedure as much as possible. U2OS cells were plated into 6-well plates at densities of 200,000, 300,000 or 400,000 cells per well and left overnight. The following day cells were stimulated with a high dose of H_2O_2 (4mM) for 40 minutes, a time point which has been shown to elicit maximum PtdIns5*P* production [11] and PtdIns5*P* was quantified. Cells plated at 200,000 cells per well showed an 8-fold increase in the level of PtdIns5*P* compared to a 4-fold increase in cells plated at 300,000 or 400,000 cells per well (Figure 3.3A). The duration required for cell adhesion and growth was also investigated. Cells were plated at a density of 200,000 cells per well and left for 24 or 48 hours before oxidative stress stimulation. Cells plated and left for 24 hours before oxidative stress treatment reproducibly showed an 8-fold increase in the level of PtdIns5*P* whereas cells plated and left for 48 hours gave a slightly lower and less reproducible yield of PtdIns5*P* (Figure 3.3B). Before stimulation with H_2O_2, cells are incubated in serum free medium to dampen cell signalling in order to analyse activation of signalling pathways more easily. Cells were incubated with serum free medium for either 2 or 4 hours before H_2O_2 treatment. Cells incubated in serum free conditions for 2 hours had a 5-fold increase in the level of H_2O_2-induced PtdIns5*P* whereas cells incubated for 4 hours had an 8-fold increase (Figure 3.3C). The basal level of PtdIns5*P* did not differ between 2 hours or 4 hours of serum deprivation (data not shown) further indicating that a signalling pathway downstream of H_2O_2 specifically regulates PtdIns5*P* generation. For future experiments cells were incubated for 2 hours as this time gave a measurable increase in H_2O_2-induced PtdIns5*P* and proved to be a more convenient duration for inhibitor treatments.

Oxidative stress induced by H_2O_2 was shown to increase the level of PtdIns5*P* in both a time and dose dependent manner (Figure 3.4). The maximal increase in PtdIns5*P* was seen after 40 minutes of stimulation (Figure 3.4A) and at a concentration of 4mM H_2O_2 (Figure 3.4B) similarly to previous work [11]. Another method to induce oxidative stress is with the compound tert-butylhydroquinone (TBHq) which reduces the rate of cellular ROS removal [109]. TBHq is therefore able to induce a controlled and endogenous oxidative stress reaction. Treatment with TBHq led to an increase in the level of PtdIns5*P* (Figure 3.5A). As two independent pathways of oxidative stress initiation both led to increased PtdIns5*P*, this suggests that there is a specific signalling response to oxidative stress that is responsible for up-regulating PtdIns5*P* synthesis.

The p38 mitogen-activated protein kinase (p38) is activated in response to environmental stresses [200] and protects cells from oxidative damage by inducing transcription of antioxidant genes [199]. As mentioned, p38 was shown to be strongly activated in response to H_2O_2 but not in response to insulin which suggested that p38 might be a good candidate for a H_2O_2-regulated molecule that could control PtdIns5*P* production. To test whether PtdIns5*P* is increased in response to oxidative stress due to activation of p38, U2OS cells were treated with three compounds known to strongly activate p38; anisomycin, sodium orthovanadate and arsenite. Western blot analysis confirmed that anisomycin and arsenite both strongly induce phosphorylation of p38 however unexpectidly sodium orthovanadate did not induce phosphorylation of p38 (Figure 3.5B). None of the compounds tested increased the level of PtdIns5*P* unlike H_2O_2 which caused a robust increase in the level of PtdIns5*P* (Figure 3.5C, experiment performed by Dr. David Jones) suggesting that H_2O_2 is not working through p38 activation to regulate PtdIns5*P*.

3.3. Initial round of screening using small molecular weight inhibitors

3.3.1. Introduction

In order to elucidate how PtdIns5*P* is regulated by H_2O_2, small molecular weight inhibitors known to target proteins involved in oxidative stress signalling were utilised. Information from literature searches was used to establish required concentrations for efficient inhibition of target proteins. Inhibitors were added to serum free medium and incubated with the cells for 2 hours prior to H_2O_2 treatment (with the exception of wortmannin which was added to the serum free medium 10 minutes prior to H_2O_2 treatment). A short term treatment was chosen to allow sufficient time for the compound to enter cells and selectively bind and inhibit the target therefore affecting immediate signalling capacity without affecting longer term downstream signalling outputs such as gene transcription, histone modification and cell senescence. The initial screen involved analysis of the effect of 24 small molecular weight inhibitors on the production of H_2O_2-induced PtdIns5*P* (Table 3.1.). Several interesting hits were then chosen for further analysis.

3.3.2. Inhibition of IGF1R using AG1024

As a general mechanism, H_2O_2 up-regulates tyrosine phosphorylation by inhibiting tyrosine phosphatases and one well established target is the insulin receptor. In response to oxidative stress the kinase activity of the insulin receptor is increased due to increased phosphorylation of the beta subunit [106]. Although we were unable to establish a robust increase in PtdIns5*P* in U2OS cells in

response to insulin, other studies have shown that activation of the insulin receptor can increase cellular PtdIns5*P* [18] [19]. Therefore an insulin receptor kinase inhibitor, AG1024, was used to determine whether signalling pathways downstream of receptor kinase activity were required for H_2O_2-induced PtdIns5*P* production.

In the initial round of screening, incubation of cells with AG1024 caused an increase in the level of H_2O_2-induced PtdIns5*P* of 90% (Table 3.1). In follow up validation experiments, incubation with AG1024 consistently led to approximately a 2-fold increase in the level of H_2O_2-induced PtdIns5*P* (Figure 3.6). This is particularly surprising as data from other studies would suggest that inhibition of insulin signalling would decrease PtdIns5*P* production. However it should be noted that in U2OS cells, insulin treatment did not significantly increase the level of PtdIns5*P* (Figure 3.2B) contrasting data obtained from other cell lines. This may indicate that in U2OS cells, insulin regulates an alternative set of downstream signalling molecules compared to other cell lines and therefore perturbations of insulin signalling may have a different effect on the production of PtdIns5*P*. Recently, a compound structurally similar to AG1024, I-OMe-AG538, was found to inhibit PIP4K [110]. PIP4K is an endogenous negative regulator of PtdIns5*P* and over expression of PIP4K leads to suppressed production of PtdIns5*P* in response to H_2O_2 [11]. Therefore the possibility that AG1024 can specifically inhibit PIP4K was investigated. Both I-OMe-AG538 and AG1024 inhibited the kinase activity of PIP4K2A in radio-labelling experiments but I-OMe-AG538 inhibited to a greater extent (Figure 3.7A experiment performed by Dr David Jones). Likewise, the kinase activity of PIP4K2B was inhibited by both AG1024 and I-OMe-AG538 but again I-OMe-AG538 caused greater inhibition (Figure 3.7B experiment performed by Dr David Jones). Quantification of PtdIns5*P* showed that pre-incubation of U2OS cells with I-OMe-AG538 had no effect on the level of H_2O_2-induced PtdIns5*P* in the initial screen however in validation experiments the basal level of PtdIns5*P* is reduced in I-OMe-AG538-treated cells suggesting that there may be an effect on H_2O_2-induced PtdIns5*P* (Figure 3.7C).

Although I-OMe-AG538 is clearly a superior inhibitor in kinase assays using purified enzyme in a non-cellular setting, its ability to inhibit PIP4K when added exogenously to cells has not been conclusively proven or disproven. To analyse the inhibitory action of both inhibitors in U2OS cells, cells were incubated with either AG1024 or I-OMe-AG538 and treated with 1mM H_2O_2 for various durations up to an hour. The phosphorylation status of PKB was then analysed as a read-out of PIP4K inhibition [11]. Initial results showed that in a cellular setting, AG1024 can inhibit H_2O_2-induced phosphorylation of PKB to a greater extent than I-OMe-AG538 (Figure 3.7D). To further assess whether AG1024 regulates PtdIns5*P* levels through inhibiting PIP4K in cells, endogenous PIP4K were knocked down prior to treatment with the inhibitor. Unfortunately, large differences between replicate samples makes it difficult to accurately interpret this data. In control transduced cells there was no statistically

significant increase in PtdIns5*P* in response to H_2O_2 and similarly in PIP4K knock down cells there was no statistically significant increase in PtdIns5*P* in response to H_2O_2. Notably, PtdIns5*P* levels were not further enhanced by treatment with AG1024 in PIP4K knock down cells (Figure 3.7E) however further experiments are needed to clarify whether AG1024 targets PIP4K *in vivo* to increase oxidative stress-induced PtdIns5*P*.

3.3.3. Inhibition of PIKfyve using YM201636

PIKfyve is a 5-kinase with specific activity towards PtdIns and PtdIns3*P* that has been proposed to directly generate PtdIns5*P* from PtdIns [30] or indirectly through the dephosphorylation of PtdIns(3,5)P_2 by myotubularins (MTMs), a family of 3-phosphatases [32]. Surprisingly, pharmacological inhibition of PIKfyve using the specific compound YM201636 had no effect on the level of H_2O_2-induced PtdIns5*P* (Table 3.1). Further experiments showed that the use of YM201636 at both 400nM and 800nM blocked the phosphorylation of PKB at residue Serine 473 but had no effect on the phosphorylation of ERK or p38 (Figure 3.8A, experiment performed by Dr David Jones). At a concentration of 800nM YM201636 effectively caused cytoplasmic vacuolation which is a characteristic of perturbed membrane trafficking due to loss of PtdIns(3,5)P_2 (Figure 3.8B, experiment performed by Dr David Jones). U2OS cells were treated with YM201636 prior to oxidative stress treatment and although the cellular level of PtdIns5*P* increased with increasing concentration of H_2O_2 there was no difference between DMSO or YM201636 treated cells (Figure 3.8C, experiment performed by Dr David Jones). In addition to pharmacological inhibition of PIKfyve, genetic knock down using targeted shRNA constructs was also used. Efficient knock down of PIKfyve at the mRNA level was shown by qRT-PCR (Figure 3.8D). Similarly to YM201636 treatment, cytoplasmic vacuolation was observed when PIKfyve was knocked down (Figure 3.8E). Also similarly to inhibitor treatment, in response to oxidative stress, the level of PtdIns5*P* was unaffected in PIKfyve knock down cells (Figure 3.8F).

These two approaches both discount a role for PIKfyve in the generation of H_2O_2-induced PtdIns5*P* in U2OS cells. This contrasts with the well established roles for PIKfyve as a regulator of PtdIns5*P* production in response to osmotic stress [111], insulin signalling [18] and FGF1 treatment [35]. Furthermore, complete loss of PIKfyve was reported to lead to an 85% reduction in basal cellular PtdIns5*P* level [32]. Further studies from our laboratory were not able to confirm a decrease in basal levels of PtdIns5*P* in MEFs isolated from PIKfyve knock down mice. In addition wild type and knockout MEFs showed comparable increases in PtdIns5*P* when stimulated with H_2O_2 similarly with the data shown here. The differences may reflect differences in the methodologies utilised to measure the level of PtdIns5*P*. Our studies have used the PIP4K assay which measures the total PtdIns5*P* level, while other studies have mainly utilised labelling and HPLC strategies. It is entirely

conceivable that a large pool of PtdIns5*P* turns over slowly and thus masks the measurement of any small changes in a signalling pool that might be better measured by labelling. It should also be noted that while PIKfyve can phosphorylate PtdIns it is much less efficient than the phosphorylation of PtdIns3*P* [30]. Furthermore the presence of a FYVE domain within PIKfyve may also point towards a preference for catalysing the phosphorylation of PtdIns3*P* over PtdIns as the FYVE domain can mediate specific binding to PtdIns3*P* containing intracellular vesicles [111]. Clearly PIKfyve plays an important role in the generation of PtdIns5*P* but in U2OS cells, PIKfyve does not regulate the production of oxidative stress induced PtdIns5*P*. This may be due to the variation between various cellular settings or PIKfyve may specifically produce PtdIns5*P* in response to a subset of cellular stimuli not including oxidative stress.

3.3.4. Inhibition of PI3K using PI-103, LY294002 and GDC0941

Phosphophatidylinositol-3-kinase (PI3K) is an enzyme that phosphorylates PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3 [83], a lipid that recruits the survival protein PKB to the plasma membrane to allow activation and initiation of downstream signalling [112]. PtdIns5*P* has been implicated in regulating the PI3K pathway. It is hypothesised that increased PtdIns5*P* at the plasma membrane either activates an RTK or inhibits a 3-phosphatase leading to increased levels of PtdIns(3,4,5)P_3 and enhanced recruitment and activation of PKB [164]. Three PI3K inhibitors were used to analyse the involvement of PI3K in the regulation of H_2O_2-induced PtdIns5*P* production; PI-103 which is an inhibitor of Class IA PI3K and mTOR, LY294002 which inhibits Class I PI3Ks, other PI3K-related kinases and other non-related targets and GDC0941 which inhibits all Class I PI3K isoforms. Quantification of PtdIns5*P* showed that PI-103, LY294002 and GDC0941, all caused a reduction in the level of H_2O_2-induced PtdIns5*P* by 25-30% (Table 3.1).

In further validation experiments, the efficiency of each inhibitor was assessed by using phosphorylated PKB as a read out of PI3K activity. If PI3K is efficiently inhibited it will not be able to synthesise PtdIns(3,4,5)P_3 in response to H_2O_2 and consequently PKB will not be activated at the plasma membrane. In control treated cells, as expected PKB was phosphorylated in response to H_2O_2 however each inhibitor robustly inhibited PI3K as no PKB phosphorylation was detected in inhibitor-treated cells in response to H_2O_2 (Figure 3.9A). Quantification of PtdIns5*P* in validation experiments confirmed that inhibition of PI3K reduced the level of H_2O_2-induced PtdIns5*P* but interestingly treatment with LY294002 or GDC0941 also reduced the basal level of PtdIns5*P* (Figure 3.9B and C). This indicates that PtdIns5*P* metabolism may be regulated in response to tonic PI3K signalling as well as stress-activated PI3K signalling. Inhibition of PKB signalling, a well established

downstream target for PI3K signalling, did not attenuate oxidative stress induced PtdIns5*P* production ruling out a role for the PKB pathway downstream of PI3K signalling in the regulation of PtdIns5*P* (data not shown).

Clearly, PI3K is required for complete PtdIns5*P* synthesis but in response to oxidative stress treatment the level of PtdIns5*P* does still increase in the presence of each PI3K inhibitor compared to un-stimulated control samples. One explanation is that in response to oxidative stress, PtdIns5*P* is produced in numerous cellular compartments in response to different signalling pathways. Phosphoinositides can be synthesised in discrete cellular locations and PtdIns5*P* has been shown to have various roles in the nucleus [148] [149] [197] [198]. It is possible that PI3K is able to regulate a specific pool of PtdIns5*P* hence inhibition of PI3K does not impact on all cellular pools of PtdIns5*P* and therefore an increase in PtdIns5*P* in response to H_2O_2 can be observed. The PtdIns5*P* assay is performed on total cellular lipids so it is not possible to differentiate pools of PtdIns5*P* using this method. Notably, out of 24 compounds tested in the initial round of screening, 13 changed the level of H_2O_2-induced PtdIns5*P* by 10- 40% (Table 3.1) further suggesting that multiple signalling cascades impinge upon PtdIns5*P* synthesis and inhibition of individual signalling molecules might not be able to fully prevent the production of PtdIns5*P* under oxidative stress conditions.

3.3.5. Inhibition of PTEN using VO-OHpic trihydrate

As inhibition of PI3K was shown to decrease the level of H_2O_2-induced PtdIns5*P*, the effect of pharmacologically inhibiting the enzyme responsible for antagonising PI3K activity was investigated. PTEN is a 3-phosphatase that can de-phosphorylate PtdIns(3,4,5)P_3 to produce PtdIns(4,5)P_2 thus negatively regulating PI3K signalling [184] [113]. PTEN also has protein tyrosine phosphatase activity towards numerous targets [194]. VO-OHpic trihydrate (VO-OH) is a potent and specific inhibitor of PTEN which functions in a reversible manner [196]. Incubation of U2OS cells with VO-OH for 2 hours prior to oxidative stress stimulation caused an increase in the level of PtdIns5*P* by 43% (Table 3.1). Interestingly, treatment with VO-OH also increased the basal level of PtdIns5*P* by 58% (data not shown). In further experiments, VO-OH was incubated with cells for six hours before H_2O_2 treatment and under these conditions the level of H_2O_2–induced PtdIns5*P* was increased by 80% and the basal level of PtdIns5*P* was increased by 35% (Figure 3.10A). Effective inhibition of PTEN was demonstrated as cells incubated with VO-OH had increased phosphorylation of PKB at residues Serine 473 and Threonine 308 and surprisingly there was also a dramatic increase in tyrosine phosphorylation in cells treated with both H_2O_2 and VO-OH (Figure 3.10B). It is likely that this is caused by a chemical reaction between the two compounds. The incubation of vanadate and H_2O_2 induces the formation of pervanadate which has been shown to be a potent inhibitor of tyrosine de-

phosphorylation. Therefore to directly implicate PTEN in the regulation of oxidative stress induced PtdIns5*P* production we silenced the expression of PTEN using RNAi. Importantly, in agreement with pharmacological inhibition, genetic knock down of PTEN using targeted siRNA (Figure 3.10D) and shRNA (Chapter 4 Figure 4.2) also caused an increase in the level of H_2O_2–induced PtdIns5*P.* These observations strongly implicate PTEN in the regulation of H_2O_2-induced PtdIns5*P* production.

There are several ways that PTEN could regulate the level of PtdIns5*P.* PtdIns5*P* may be a direct substrate for PTEN as PtdIns5*P* has been shown to interact with PTEN and act as an allosteric activator [114]. However, kinetic studies suggest that PtdIns5*P* is not an ideal substrate for PTEN but is a good substrate for the poorly characterised phosphatase PLIP [115]. Alternatively, PTEN could regulate PtdIns5*P* through increased PtdIns(3,4,5)P_3 signalling. In accordance with PtdIns(3,4,5)P_3 signalling playing a role, inhibition of PI3K using small molecular weight inhibitors also reduced PtdIns5*P* generation (Figure 3.9B and C). However, the PI3K inhibitors used in this study are extremely potent, demonstrated here by their ability to completely attenuate PKB activation (Figure 3.9A) but use of these inhibitors only reduced PtdIns5*P* formation by about 40%. Furthermore insulin, which induces PtdIns(3,4,5)P_3 synthesis comparably to oxidative stress stimulation, does not increase the level of PtdIns5*P*. These observations suggest that PI3K signalling impacts on PtdIns5*P* generation but it is not essential. Whether PTEN modulates PtdIns5*P* solely through PtdIns(3,4,5)P_3 signalling is not clear and could simply be tested by measuring the generation of PtdIns5*P* in PTEN knockdown cells in the presence and absence of PI3K inhibitors.

PTEN is known to be catalytically inactivated in response to oxidative stress due to the formation of a disulphide bond in the active site [66]. This would suggest that pharmacological inhibition or genetic knock down of PTEN should not affect PTEN functions in the presence of H_2O_2 as PTEN is already catalytically inactivated. In later studies I show that although PTEN rapidly becomes stoichiometrically oxidised (inactivated) by 1mM H_2O_2 it becomes re-reduced at later time points (30-120 minutes). This is in line with other studies showing a role for thioredoxin in PTEN reactivation after oxidative stress [205]. Interestingly, as also shown later, PTEN knockout does not impact on the early time points of PtdIns(3,4,5)P_3 production in response to H_2O_2 signalling but its knockdown leads to the maintenance of increased PtdIns(3,4,5)P_3 synthesis at later time points probably as a consequence of it reactivation. With respect to these data the level of PtdIns5*P* was quantified at 0 and 45 minutes after H_2O_2 stimulation, both of which show increased PtdIns(3,4,5)P_3 synthesis in PTEN knockdown cells suggesting PTEN is active at these point (Figure 6.12B). The induction of PtdIns5*P* is delayed with respect to PtdIns(3,4,5)P_3 synthesis which again might suggest that PTEN regulates PtdIns5*P* independently of PtdIns(3,4,5)P_3. If this is the case then regulation of PtdIns5*P* by PTEN might underlie its role in PtdIns(3,4,5)P_3 independent functions.

Notably, PTEN is known to regulate cell migration [116] and a mutant PTEN that shows only protein phosphatase activity can potently inhibit cell migration suggestive of a PtdIns(3,4,5)P_3-independent function although the exact mechanism remains elusive [117]. Recently, PtdIns5*P* has also been implicated in cell migration through the activation of Rac1 [35]. Therefore it can be hypothesised that PTEN may be able to regulate cellular functions such as migration through PtdIns5*P* as an alternative to PtdIns(3,4,5)P_3.

3.3.6. Inhibition of the DNA Damage Response Pathway using Nicotinamide and Olaparib

Oxidative stress is known to cause DNA damage [193] and this can lead to carcinogenesis [192]. The initiation of the DNA damage response is controlled by the DNA damage kinases; ATM, ATR and DNA-PK and by poly (ADP-ribose) polymerase 1 (PARP1). PARP1 is an enzyme which utilises NAD^+ to add an ADP-ribose group onto nuclear proteins at the site of damage [191]. In order to determine if the DNA damage pathway might impact on PtdIns5*P* production inhibitors of the DNA damage kinases and of PARP1 were utilised. Inhibition of DNA-PK or of ATM/ATR did not attenuate oxidative stress induced PtdIns5*P* generation (Table 3.1.). In contrast nicotinamide, which inhibits PARP1 activity, significantly attenuated PtdIns5*P* generation.

Nicotinamide is also known to inhibit the activity of the nuclear deacetylase SIRT1 [190] which has also been implicated in DNA damage pathways. To define which enzyme might be important in PtdIns5*P* generation cell were treated with olaparib. Olaparib is a PARP-1 specific inhibitor which is currently being used in a number of clinical trials to treat BRCA mutated cancers by inducing sensitivity to chemotherapy [189]. In the initial round of screening, nicotinamide reduced the level of H_2O_2–induced PtdIns5*P* by 50% and olaparib by 35% (Table 3.1). In validation experiments nicotinamide reduced the level of H_2O_2–induced PtdIns5*P* by 45% and olaparib by 33% (Figure 3.11A). In both the initial round of screening and in follow up experiments, the level of H_2O_2–induced PtdIns5*P* was lower in cells treated with nicotinamide than those treated with olaparib, however when both nicotinamide and olaparib were used in combination there was no clear additive effect on the suppression of PtdIns5*P* production (Figure 3.11B) suggesting that both inhibitors are blocking the same signalling pathway. It is therefore likely that activation of PARP1 by oxidative damage in part underlies the increase in PtdIns5*P*. Although nicotinamide can inhibit PARP1, olaparib is a much better inhibitor but does not attenuate PtdIns5*P* production as well as nicotinamide. This could suggest that rather than activation of PARP1 being the essential link to oxidative stress induced PtdIns5*P* generation, a common metabolite regulated by olaparib and nicotinamide might be important. During oxidative stress the levels of NAD^+ and NADH decrease as a consequence of extensive PARP1 activation. The addition of high extracellular concentrations of nicotinamide can be

used by the salvage pathway to regenerate NAD^+/NADH [187] [188]. Thus nicotinamide not only inhibits PAPR1 but also can regenerate the pool of NAD^+/NADH. Thus PtdIns5*P* generation might occur in response to a change in the cellular concentration of these intracellular metabolites. In order to fully elucidate the role of intracellular NAD^+/NADH levels on the production of PtdIns5*P*, it will be necessary to measure the level of NAD^+/NADH in response to H_2O_2 in the presence and absence of nicotinamide and olaparib. Inhibition of components of the salvage pathway by the use of either inhibitors or RNAi could also be used to define if PtdIns5*P* responds to maintenance of NAD^+/NADH levels (Figure 3.12).

3.4. Conclusions

Evidently, the production of PtdIns5*P* in response to oxidative stress is regulated by a complex and overlapping system of signalling networks. This conclusion is not surprising considering the pleotropic nature of H_2O_2 and the plethora of signalling systems that are activated in response to oxidative stress. Determination of exactly which pathways impact on PtdIns5*P* is also confounded by the inability to easily measure PtdIns5*P* in different cellular compartments. There is evidence to suggest that oxidative stress increases PtdIns5*P* in both cellular membranes as well as in the nucleus (Jones et al. unpublished). To elucidate the contribution of each system on the production of PtdIns5*P* is likely to be a difficult task due to the many feedback loops employed by the cell to control signalling cascades and the unequal input of various signalling pathways that each seem to converge upon PtdIns5*P*. However the data presented here suggest that at least two pathways are important in regulating PtdIns5*P* generation in response to oxidative stress. The first is the PI3K/PTEN pathway while the other is the PARP/NAD pathway. Whether these two pathways regulate different intracellular pools of PtdIns5*P* remains to be determined.

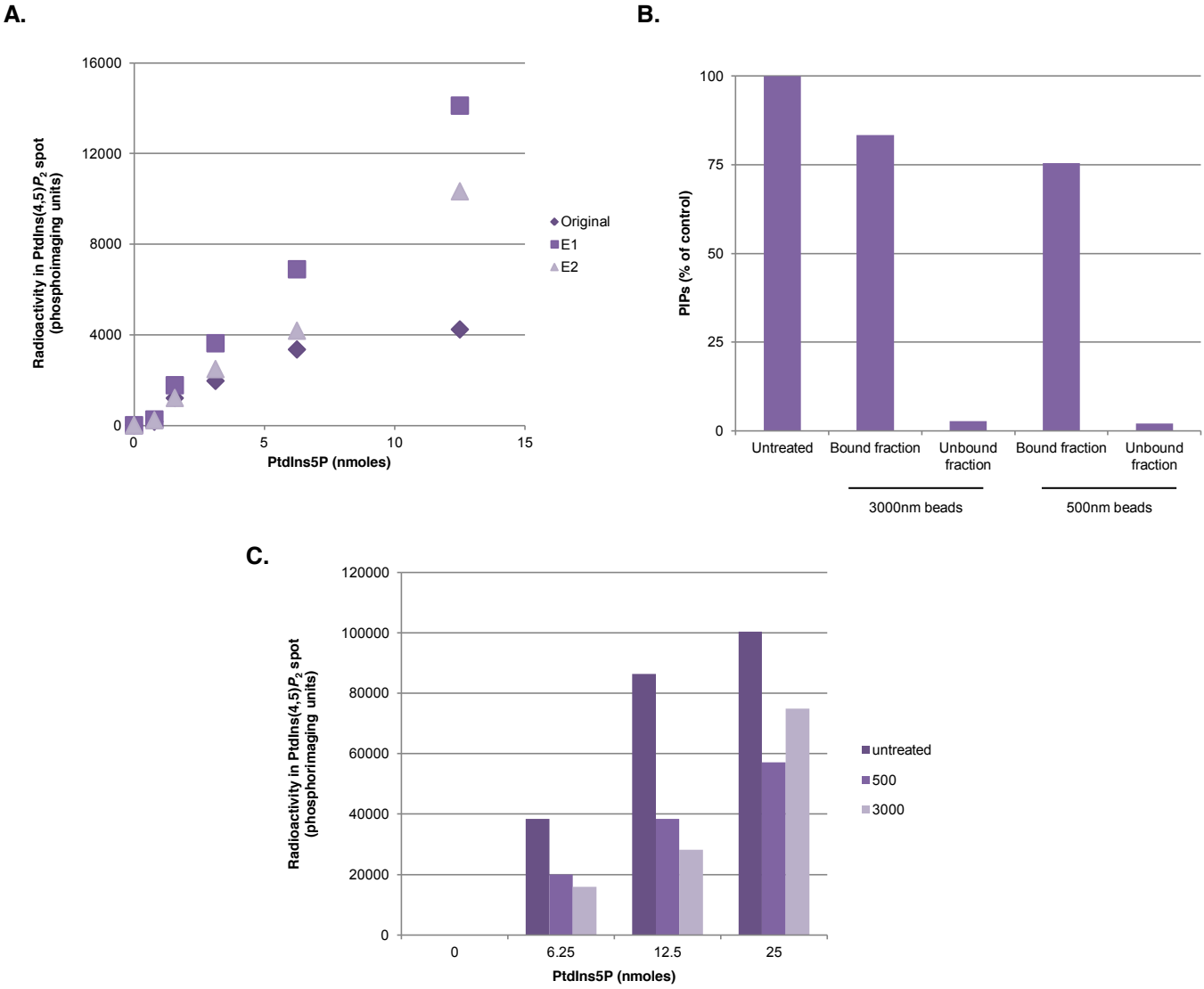


Figure 3.1. Set up of the PtdIns5*P* assay.

A. Purified GST-tagged PIP4K2A elutions 1 and 2 (E1 and E2) were used to phosphorylate a standard curve of PtdIns5*P* and the resultant radioactive PtdIns(4,5)P_2 was measured. **B.** 3000nm and 500nm glass beads were prepared by coating with neomycin and were then used to selectively extract polyphosphoinositides (PPIns). The fraction of lipids that bound to the beads and the fraction that did not bind to the beads were quantified. **C.** Both 500nm and 3000nm neomycin-coated glass beads were used in a PtdIns5*P* assay with a standard curve of PtdIns5*P* to assess the ability of each set of beads to bind pure PtdIns5*P*.

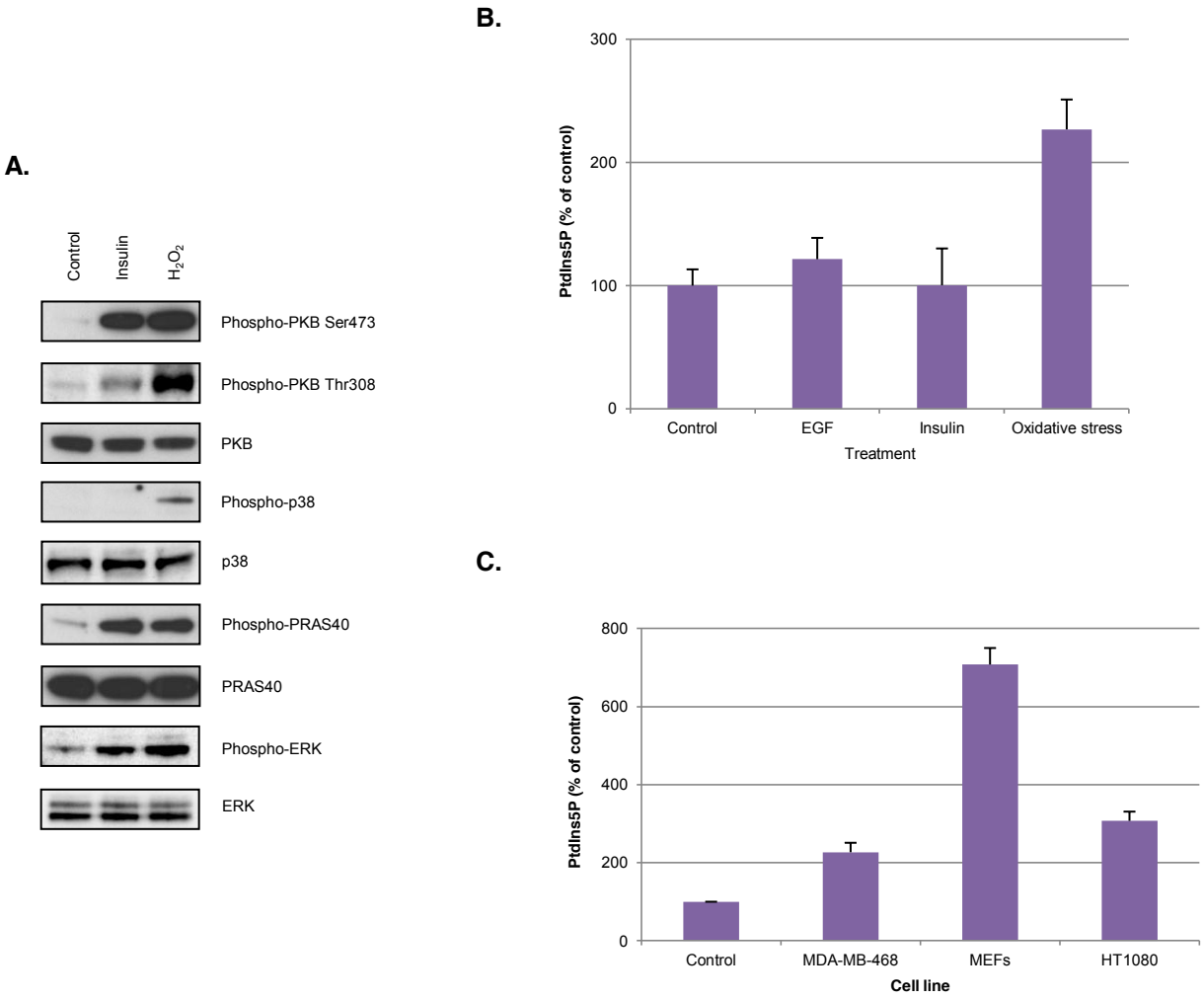


Figure 3.2. Oxidative stress increases the cellular level of PtdIns5*P*.

A. U2OS cells were serum starved for 2 hours followed by control treatment or stimulated with 1mg/ml insulin or 1mM H_2O_2 for 15 minutes. Lysates were produced and the activation of several signalling pathways was analysed by western blotting. **B.** U2OS cells were maintained as controls or stimulated with 1µg/ml EGF, 1mg/ml insulin or 1mM oxidative stress for 40 minutes, lipids were extracted and the level of PtdIns5*P* was measured. Only treatment with H_2O_2 caused a robust increase in the level of PtdIns5*P*. **C.** A number of different cell lines were stimulated with 1mM H_2O_2 for 45 minutes and the level of cellular PtdIns5*P* was quantified. In each cell line H_2O_2 robustly increased the level of PtdIns5*P* compared to an un-stimulated control. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3).

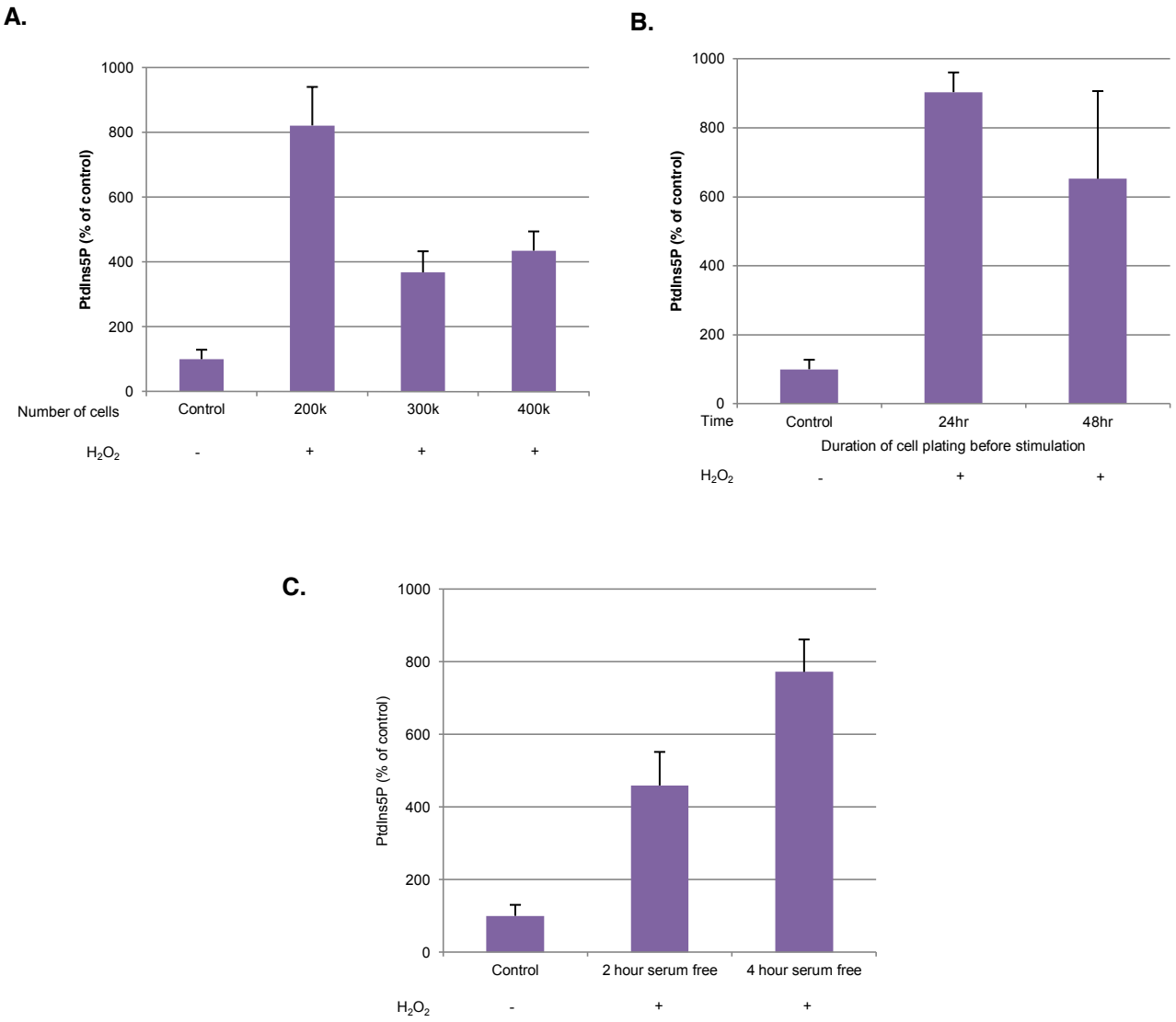


Figure 3.3. Optimising conditions for the PtdIns5*P* assay.

A. PtdIns5*P* was quantified from U2OS cells that were plated at different densities and left overnight before 45 minutes 1mM oxidative stress treatment. **B.** U2OS cells were plated and left for 24 hours or 48 hours before control or 45 minutes 1mM oxidative stress treatment, lipids were extracted and PtdIns5*P* was measured. **C.** U2OS cells were serum starved for 2 or 4 hours before control or 45 minutes 1mM oxidative stress treatment, lipids were extracted and PtdIns5*P* was measured. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3).

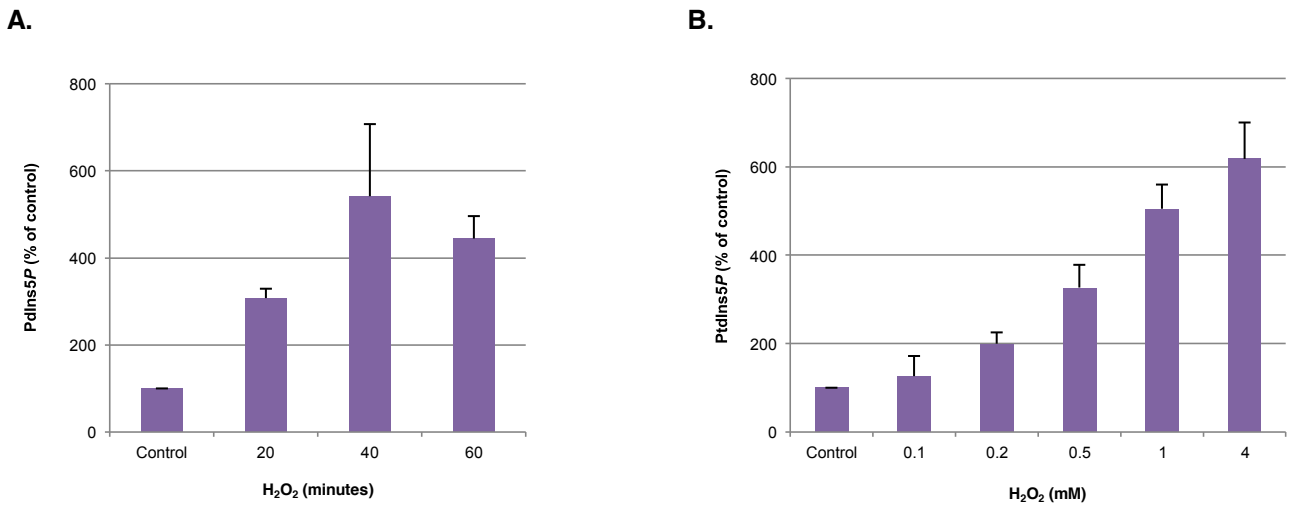


Figure 3.4. H_2O_2 increases the cellular level of PtdIns5*P* in a time and dose dependent manner.

A. PtdIns5*P* was quantified from U2OS cells that were kept in control conditions or stimulated with 1mM H_2O_2 for 20, 40 or 60 minutes. The production of PtdIns5*P* was maximal after 40 minutes of stimulation. **B.** PtdIns5*P* was quantified from U2OS cells that were kept in control conditions or stimulated with increasing doses of H_2O_2 for 60 minutes. The level of PtdIns5*P* increased in a dose-dependent manner. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3).

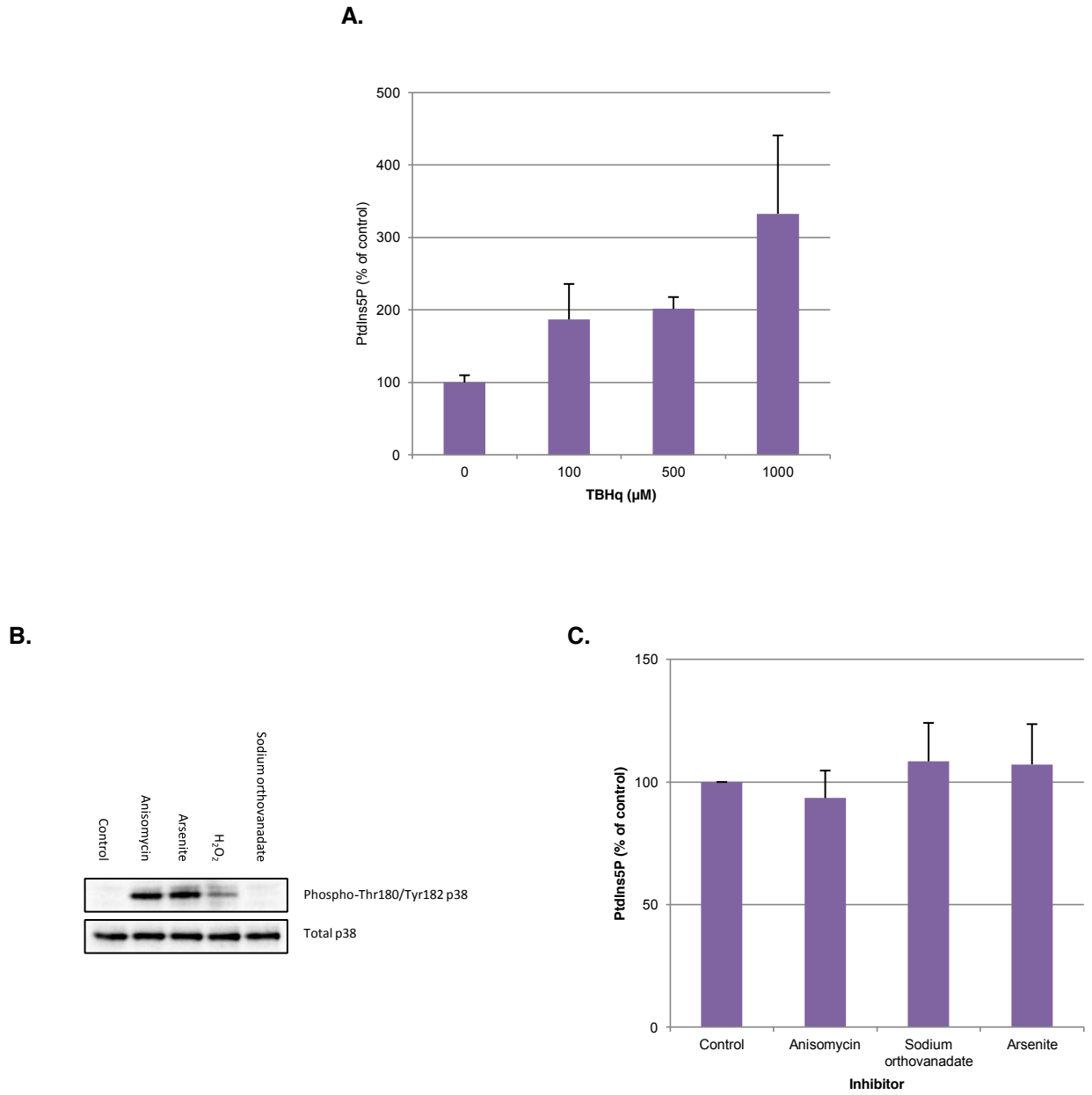


Figure 3.5. Oxidative stress specifically increases the level of PtdIns5*P*.

A. U2OS cells were maintained as controls or treated with increasing doses of the mitochondrial oxidative stress inducer tert-butylhydroquinone (TBHq) for 60 minutes, lipids were extracted and PtdIns5*P* was quantified. The level of PtdIns5*P* increased in response to TBHq. **B.** U2OS cells were treated with DMSO control or compounds that strongly activate p38 activity for 40 minutes at concentrations of 10μg/ml anisomysin, 1mM sodium orthovanadate or 100μM sodium arsenite for 40 minutes. Lysates were separated by SDS-PAGE and blotted with the antibodies indicated. **C.** U2OS cells were treated with DMSO control or compounds that strongly activate p38 activity for 40 minutes. (same concentrations as B.) Lipids were extracted and PtdIns5*P* was quantified. None of these activators were able to increase the level of cellular PtdIns5*P*. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3).

Inhibitor	Target	Final concentration	Effect on H_2O_2-induced PtdIns5*P* level (nearest 5%)
AG1024	IR/ IGF-1R	2μM	+90%
AG1478	EGFR	2μM	+60%
R59949	DGK	1μM	+50%
GSK 2334470	PDK1	10nM	+50%
CGK-733	ATM/ ATR	10μM	+40%
VO-OH	PTEN	500nM	+40%
PP2	Src kinase	1μM	+30%
Wortmannin	PI3Ks	100nM	+25%
PP1	Src kinase	1μM	+10%
DMSO	Control treatment	control	100%
PD98059	MEK1	1μM	None (100%)
CRT359	PIP5K	12μM	None (100%)
Merk-7	PDK1	1μM	None (100%)
NU-7441	DNA-PK	8μM	None (100%)
Genistein	Tyrosine Kinases	10μM	None (100%)
YM 201636	PIKfyve	800nM	None (100%)
KU-55933	ATM	5μM	-15%
LY 294002	PI3Ks	20μM	-25%
Curcumin	PIKfyve	1μM	-25%
SB203580	p38 MAPK	5μM	-25%
PI-103	PI3K	1μM	-25%
GDC 0941	PI3K	1μM	-30%
Rapamycin	mTOR	100nM	-30%
Olaparib	PARP	2μM	-35%
Nicotinamide	PARP	1μM	-50%

Table 3.1. Initial round of screening using small molecular weight inhibitors.

A library of small molecular weight inhibitors were used to treat U2OS cells prior to oxidative stress treatment. The effect on the oxidative stress induced cellular level of PtdIns5*P* in inhibitor treated samples compared to DMSO control treated samples is shown as a percentage change when the DMSO control sample for each individual experiment is set at 100%. Numbers are rounded to the nearest 5%.

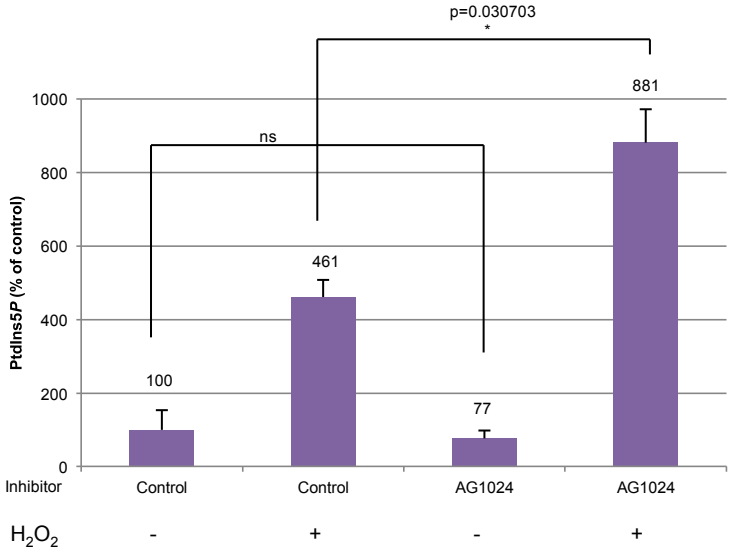
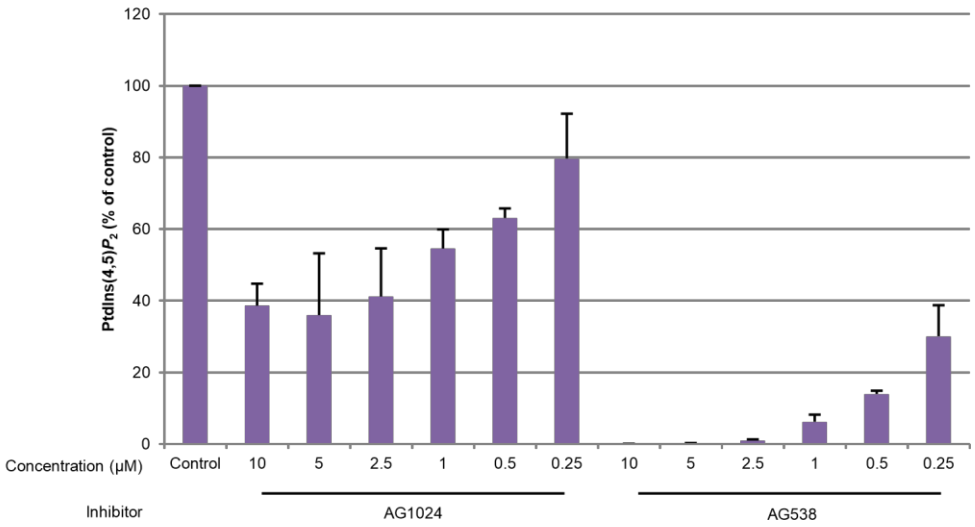


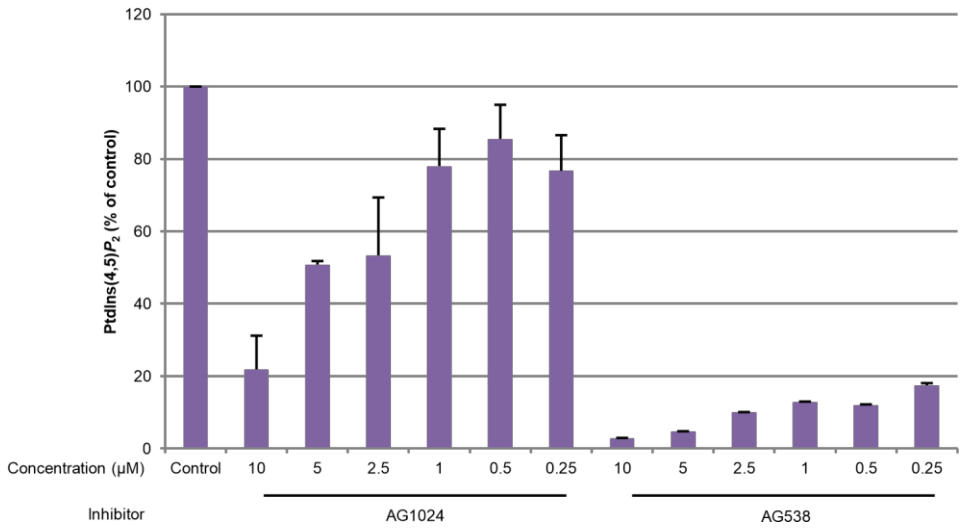
Figure 3.6. AG1024 treatment increases the production of H_2O_2-induced PtdIns5*P*.

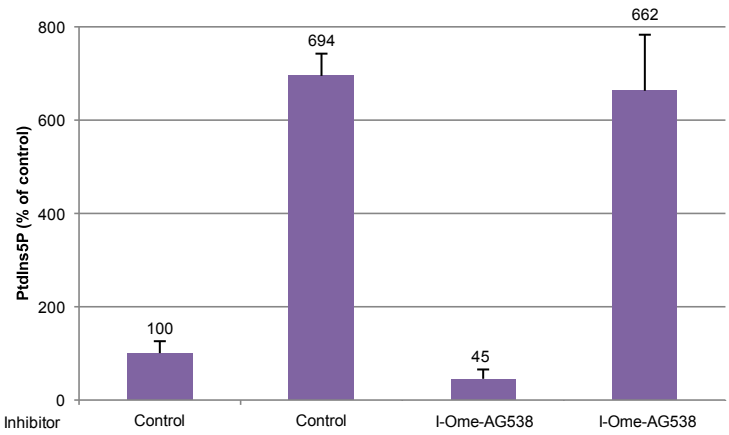
U2OS cells were treated with DMSO or 2μM AG1024 for 2 hours during serum free incubation and maintained in control conditions or stimulated with 1mM H_2O_2 for 45 minutes before lipid extraction and quantification of PtdIns5*P*. The level of H_2O_2-induced PtdIns5*P* was higher in cells treated with AG1024 than DMSO control and the basal level was slightly lower in AG1024 treated cells. Data represents the average value plus the standard deviation of a triplicate (n=3). Student's t-test indicates data is statistically significant. (ns= not significant).

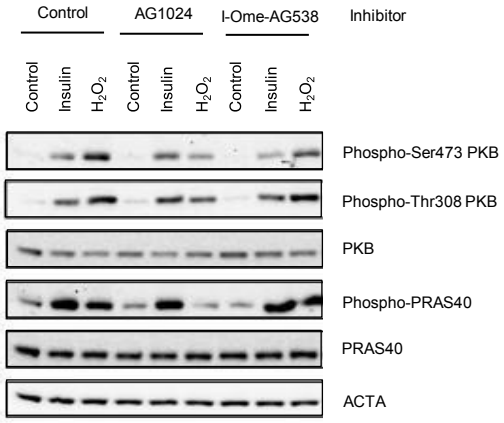
A.


120
100
80
60
40
20
0
PtdIns(4,5)P2 (% of control)
Concentration (μM)
Control
10
5
2.5
1
0.5
0.25
10
5
2.5
1
0.5
0.25
Inhibitor
AG1024
AG538

B.


120
100
80
60
40
20
0
PtdIns(4,5)P2 (% of control)
Concentration (μM)
Control
10
5
2.5
1
0.5
0.25
10
5
2.5
1
0.5
0.25
Inhibitor
AG1024
AG538





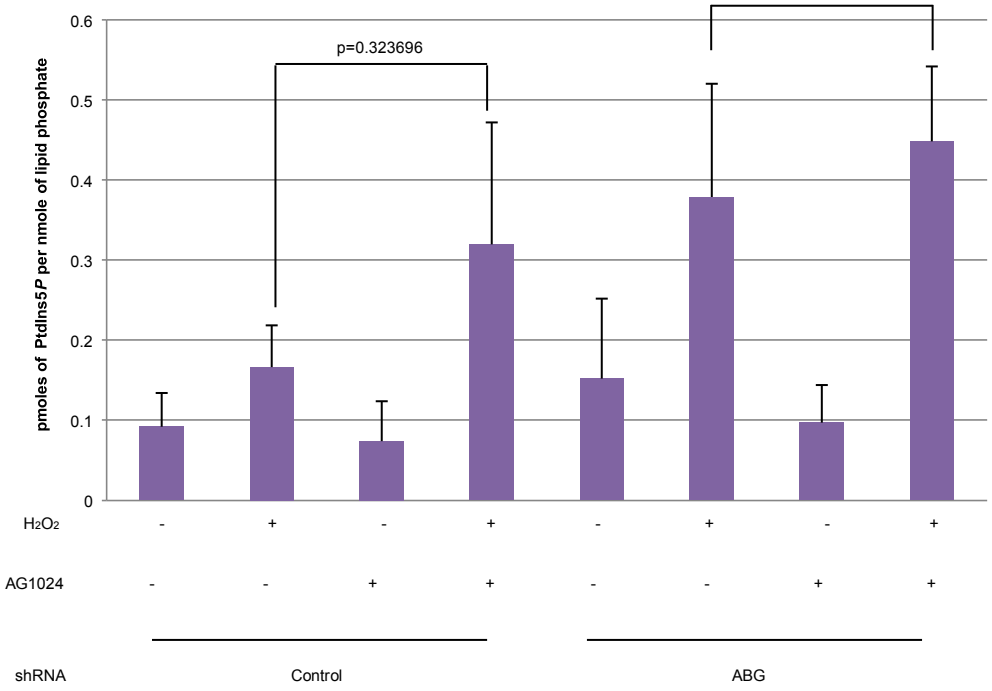


Figure legend on following page.

Figure 3.7. AG1024 treatment inhibits the enzymatic activity of PIP4K2A and PIP4K2B.

A. Purified PIP4K2A was incubated with the indicated concentrations of AG1024 or AG538. Enzymatic activity was measured by the ability of PIP4K2A to phosphorylate PtdIns5*P* to PtdIns(4,5)P_2 in the presence of radiolabelled ATP. Both inhibitors reduced the enzymatic activity of PIP4K2A but AG538 caused greater inhibition. **B.** The same experiment was performed using purified PIP4K2B. Similarly, both inhibitors reduced the enzymatic activity of PIP4K2B but again AG538 caused greater inhibition. **C.** U2OS cells were treated with DMSO or 2μM I-Ome-AG538 for 2 hours during serum free incubation and kept as controls or stimulated with 1mM H_2O_2 for 45 minutes before quantification of PtdIns5*P*. The level of H_2O_2-induced PtdIns5*P* was not affected by I-Ome-AG538. Data represents the average value plus the standard deviation of a triplicate. **D.** U2OS cells were pre-incubated with 2μM of the indicated inhibitors and kept in control conditions or treated with 1mM H_2O_2 or 1μg/ml insulin for 15 minutes. Lysates were separated by SDS-PAGE and blotted with the antibodies indicated. **E.** U2OS cells were transduced with control shRNA constructs or shRNA targeting each isoform of PIP4K (ABG). These cells were then pre-incubated with DMSO or 2μM AG1024 and maintained in control conditions or treated with 1mM H_2O_2 for 45 minutes and the level of PtdIns5*P* was measured. In each graph the data represents the average value plus the standard deviation of a triplicate (n =3). Student's t-test indicates data are not statistically significant. (ns= not significant).

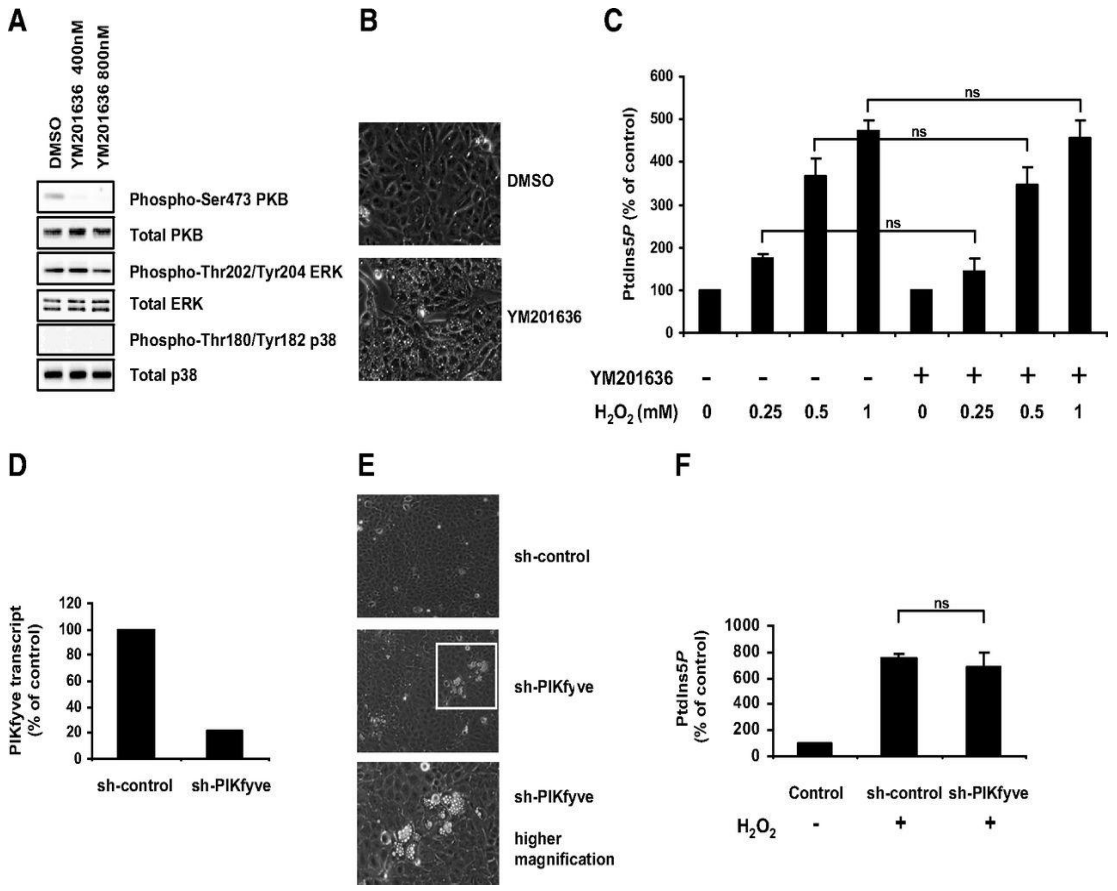


Figure 3.8. Both pharmacological inhibition of and lentiviral-mediated knockdown of PIKfyve protein have no effect on the level of oxidative stress-induced PtdIns5P in U2OS cells.

A. U2OS cells were washed free of serum and treated with YM201636 for 30 min at the indicated concentrations. Phosphorylation status of PKB, ERK, and p38 was determined by Western blotting. **B.** Vacuole formation in DMSO (control) and 800 nM YM201636-treated cells. Micrographs show vaculolation of cells after 2 h of treatment with YM201636. **C.** U2OS cells were incubated with 800 nM YM201636 for 2 h in serum-free conditions, followed by treatment with the indicated concentrations of H_2O_2 for 40 min. Cellular PtdIns5*P* was quantitated. **D.** RT-PCR was performed to quantitate endogenous PIKfyve mRNA in U2OS cells transduced with control vector (sh-control) or with a vector targeting PIKfyve mRNA (sh-PIKfyve). **E.** Vacuole formation in sh-control and sh-PIKfyve cells. Bottom panel shows a higher magnification of the sh-PIKfyve cells within the boxed area in the middle panel. **F.** sh-Control and sh-PIKfyve U2OS cells were serum starved for 2 h, followed by treatment with 1 mM H_2O_2 for 40 min. Cellular PtdIns5*P* was quantitated. Bars represent the average + sd of a triplicate. n.s., not significant (*P*>0.05); ANOVA with *post hoc* Tukey's test (*C*) or Student's *t* test (*F*).

Jones D R et al. FASEB J 2013;27:1644-1656 The FASEB Journal

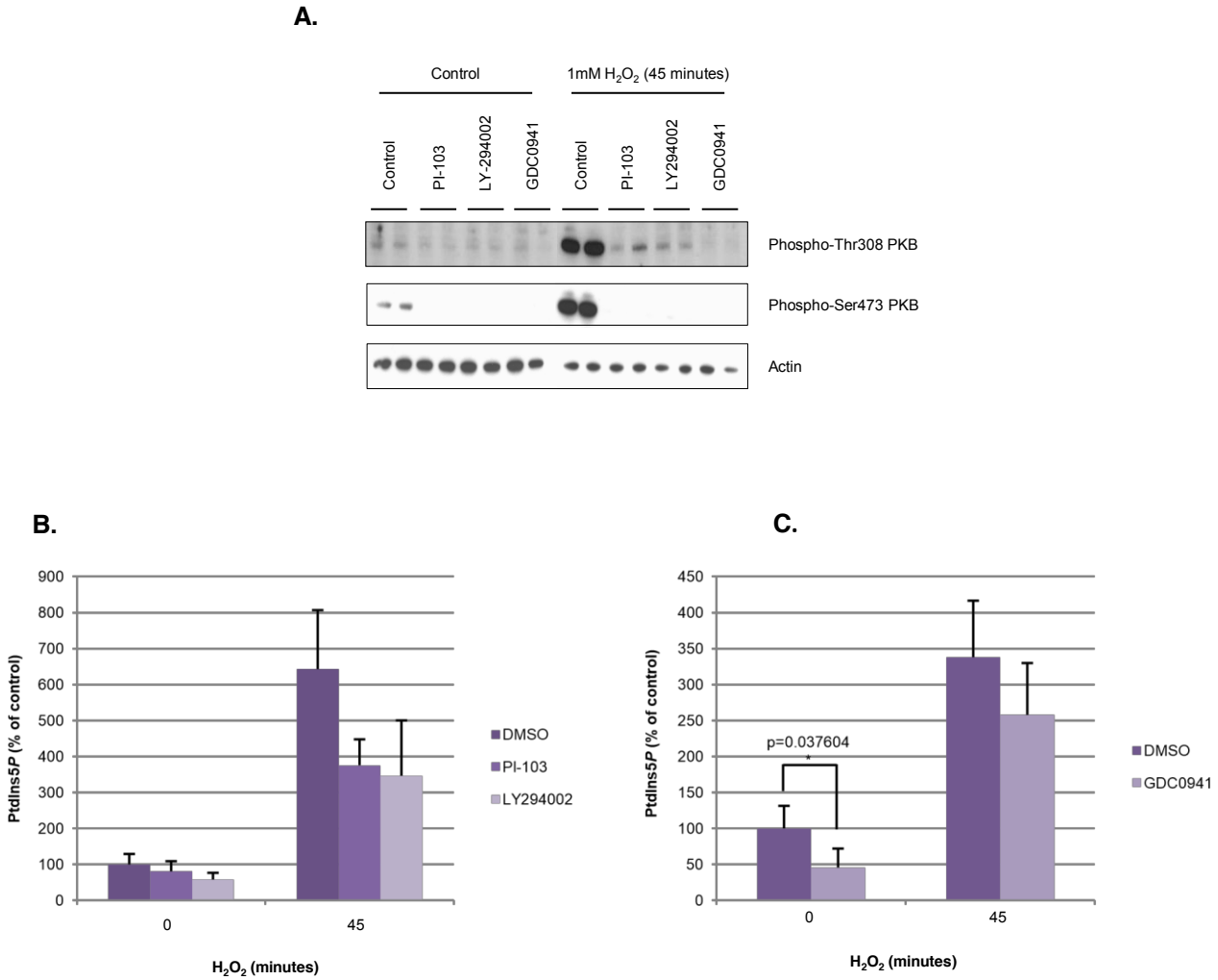


Figure 3.9. Inhibition of PI3K causes a reduction in the level of PtdIns5*P*.

A. U2OS cells were treated with DMSO or a PI3K inhibitor for 2 hours during serum free incubation followed by control treatment or 40 minutes 1mM H2O2 treatment. Lysates were made an western blot analysis was used to determine effective inhibition of PI3K based on phosphorylation of PKB. **B.** Two inhibitors of PI3K, PI-103, and LY294002, were used to treat U2OS cells for two hours prior to control or H_2O_2 treatment. Lipids were extracted and PtdIns5*P* was quantified. Both inhibitors effectively reduced the level of PtdIns5*P* in un-stimulated conditions and also in oxidative stress conditions. Each bar represents the average value plus the standard deviation for a triplicate (n=3). **C.** The same experiment was conducted as described in A. using another PI3K inhibitor, GDC0941. This inhibitor effectively reduced the level of PtdIns5*P* in un-stimulated and also in response to H_2O_2. All concentrations can be found in Table 3.1.

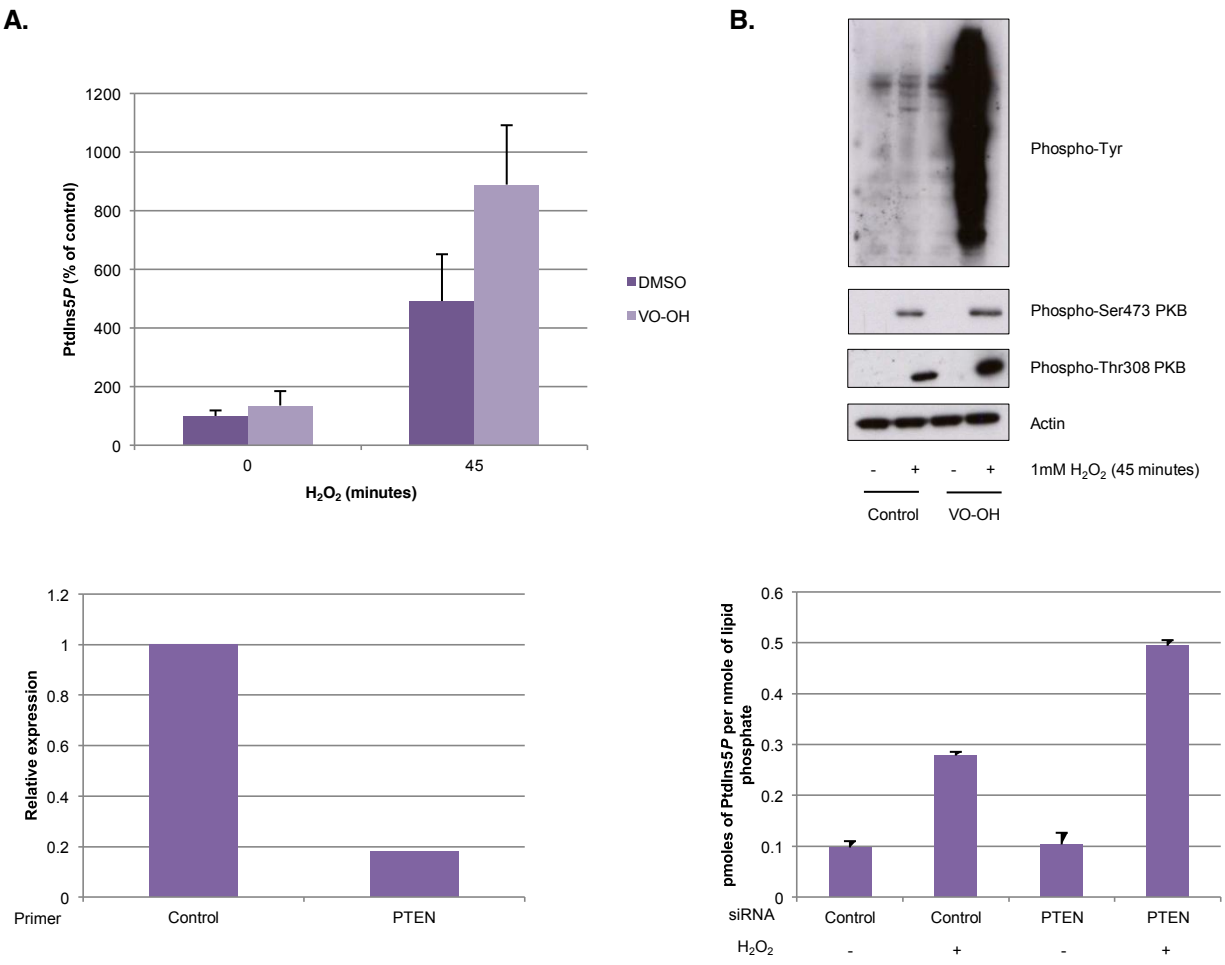


Figure 3.10. Inhibition of PTEN leads to increased PtdIns5*P*.

A. The PTEN inhibitor VO-OHpic trihydrate (VO-OH) was incubated with U2OS cells for 6 hours at 500nM before control treatment or 45 minutes 1mM H_2O_2. PtdIns5*P* quantification showed enhanced H_2O_2-induced PtdIns5*P* in the presence of this compound. **B.** U2OS cells were treated with DMSO or 500nM VO-OH for 2 hours during serum free incubation followed by control or 45 minutes 1mM H_2O_2 treatment. Lysates were made and western blot analysis was used to determine effective inhibition of PTEN based on phosphorylation of PKB. **C.** qRT-PCR was used to measure the mRNA level of PTEN in control transduced and siPTEN transduced cells. **D.** U2OS cells were transduced with non-targeting siRNA (siControl) or siRNA against PTEN (siPTEN) and maintained in control conditions or treated with 1mM H_2O_2 for 45 minutes. Quantification of PtdIns5*P* showed that knock down of PTEN caused enhanced PtdIns5*P* production in response to H_2O_2. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3).

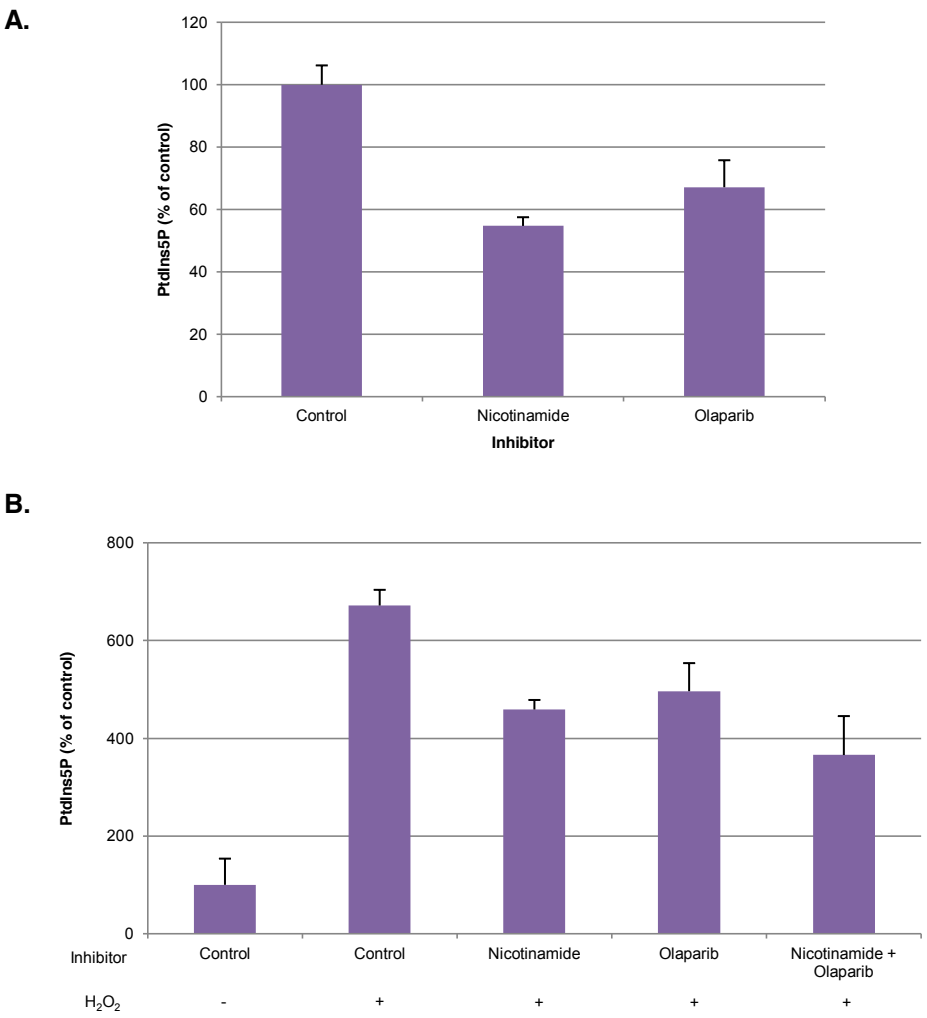


Figure 3.9. Inhibition of the PARP-1 pathway leads to reduced PtdIns5*P* in response to oxidative stress. **A.** Two inhibitors of PARP-1, nicotinamide and olaparib, were used to treat U2OS cells prior to oxidative stress treatment at 1μM and 2μM respectively. Lipids were extracted and PtdIns5*P* quantification showed that both inhibitors effectively reduced the level of H_2O_2-induced PtdIns5*P.* **B.** An experiment was performed similar to that described in A. with the inclusion of U2OS cells treated with both nicotimamide and olaparib in combination at 1μM and 2μM respectively. Cells treated with a combination of nicotinamide and olaparib showed a similar reduction in the level of PtdIns5*P* as cells treated with each inhibitor alone confirming that they work on the same signalling pathway. In each graph the data represents the average value plus the standard deviation of a triplicate (n=3). Student's t-test shows statistically significant results. ns= not significant.

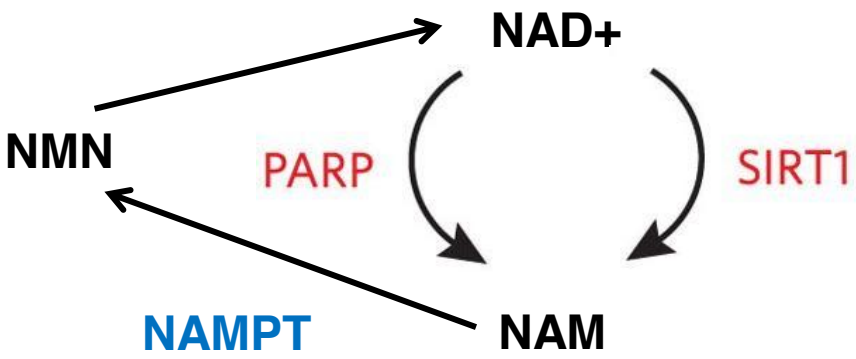


Figure 3.12. Diagram of NAD+ Usage.

Nicotinamide (NAM) is a product of NAD^+ usage that can be used to re-synthesise NAD^+ by nicotinamide phosphoribosyltransferase (NAMPT). NAMPT produced nicotinamide which is then used to produce nicotinamide mononucleotide (NMN), which is then converted to NAD^+ by nicotinamide mononucleotide adenylyltransferases. NAD^+ is an electron carrier used by many different enzymes including PARP and SIRT1.

4. Targeting phosphoinositide metabolic enzymes to study cellular production of PtdIns5*P*

4.1. Introduction

PtdIns5*P* remains the most elusive of the phosphoinositide family of lipids. For each phosphoinositide there are clearly defined pathways of synthesis and removal which are tightly controlled by specific kinases and phosphatases with the help of a range of accessory proteins. Many of these pathways have been thoroughly characterised which has enabled the discovery of unique signalling roles for each phosphoinositide in distinct cellular compartments. There is, however, still no firm evidence that conclusively proves which enzymes synthesise cellular PtdIns5*P*. Despite PtdIns5*P* being relatively poorly understood, as more research is being undertaken in an attempt to define its functions it is becoming clear that PtdIns5*P* is a key regulator in many signalling pathways, particularly those that arise from perturbations in the external cell environment. Such external signals affect the PtdIns5*P* level which then engenders the cellular response. By understanding the route of PtdIns5*P* synthesis it will be possible to fully appreciate which pathways impact on its synthesis, how PtdIns5*P* fulfils its diverse range of roles in the cell and how these roles may contribute to human disease development in particular with respect to cancer.

To determine which enzymes are responsible for H_2O_2-induced PtdIns5*P* production, a library of shRNA constructs that target all known phosphoinositide kinases, phosphatases, phospholipases and accessory proteins was used to genetically knock down individual proteins. The ability of each protein to regulate the production of PtdIns5*P* in response to H_2O_2 stimulation was analysed. In total 101 proteins were targeted.

4.2. Optimisation of the screening protocol

4.2.1. Introduction

Due to the labour intensive nature of the PtdIns5*P* assay (Figure 2.1), it was not possible for the RNAi screen to be conducted in a high-throughput manner. For this reason it was particularly important to initially optimise the screening protocol to establish a reproducible and accurate methodology that would guarantee consistency throughout the screen.

4.2.2. Optimisation of lentiviral production and transduction protocols using SHX-GFP

The use of shRNA constructs over other RNAi technologies to knock down target genes was chosen due to the heritable and selectable nature of the system. Upon transduction with shRNA constructs, the shRNA can stably integrate into the genome and is therefore passed onto progenitor cells during cell division. This type of RNAi construct commonly contains a selectable marker such as GFP or puromycin resistance, allowing selection of successfully transduced cells [118]. The library of shRNA constructs was selected based on literature searches to include constructs that targeted all known regulators of phosphoinositide metabolism. For each gene target, either two validated constructs or five non-validated constructs were selected. In total 345 shRNA constructs were chosen. The library was purchased in the form of bacterial glycerol stocks which required DNA plasmid isolation to obtain the shRNA plasmid. Each plasmid was prepared and purified individually to ensure maximum yield with minimum contamination as this would allow production of high quality lentiviral particles. Optimisation of the plasmid preparation protocol allowed isolation of plasmid DNA at a concentration of ≥100ng/µl.

The ideal method of delivery for shRNA constructs is by lentiviral transduction [119]. The packaging cell line chosen to generate lentiviral particles was 293FT cells as they are fast growing and easy to transfect. The lentiviral particles that have been produced in the cells are released into the medium which is collected. In order to produce active lentiviral particles, in addition to the shRNA-containing plasmid, plasmids encoding viral components are also required; VSVG (which encodes viral envelope protein) and GAG-POL (which encodes packaging proteins). The ratio of each plasmid was optimised (data not shown) and the optimum transfection ratio was 4:2:1 (shRNA:GAG-POL:VSVG) in a total of 2µg plasmid per well when cells are plated in a 6 well plate.

Transfection reagents are needed to allow efficient uptake of plasmid DNA into target cells. Polyethylenimine (PEI) and Fugene are two commonly used transfection reagents that were

compared to determine which reagent would allow most efficient production of lentiviral particles. 293FT cells were transfected with a non-targeting shRNA that also contains a GFP gene (SHX-GFP) using either PEI or Fugene at a ratio of 3:1 (transfection reagent: total DNA (µg)). Expression of GFP can be detected and quantified using flow cytometry. Various volumes of the medium containing the lentiviral particles were then used to transduce U2OS cells. The number of cells expressing GFP was measured and the efficiency of PEI and Fugene mediated transfection was compared. The level of transduction achieved using PEI as the transfection reagent was significantly higher than the level achieved using Fugene. For example, the number of cells within the M1 gated region (expressing GFP) using 1000µl of 'Fugene supernatant' is lower than the number of cells within this region using only 250µl of 'PEI supernatant' (Figure 4.1A).

Further optimisation experiments were aimed to determine the volume of lentiviral supernatant and the duration of incubation with lentiviral supernatant that would give maximum transduction of target cells. Increasing volumes of SHX-GFP lentiviral supernatant were incubated with U2OS cells overnight and the percentage of GFP expressing cells was quantified using Flow Cytometry. As little as 100µl of lentiviral supernatant resulted in 75% of cells expressing GFP (Figure 4.1B). Further to this, 200µl of SHX-GFP lentiviral supernatant was incubated with U2OS cells for various times from one hour to 18 hours. Incubation of cells for 18 hours gave the maximum transduction efficiency as over 98% of cells expressed GFP after this duration of incubation (Figure 4.1C). Therefore in further experiments, 200µl of lentiviral supernatant was incubated with target cells for 18 hours.

4.2.3. Determining the reproducibility of PtdIns5P quantification in SHX-transduced cells

As mentioned, the RNAi screen was aimed at elucidating regulators of PtdIns5*P* production in response to H_2O_2-induced oxidative stress. Therefore it was necessary to test whether the extent of the cellular PtdIns5*P* increase was comparable in SHX-transduced U2OS cells compared to non-transduced U2OS cells. This experiment was performed in replicates of 6 samples, therefore 12 samples were transduced with SHX, six of which received 4mM H_2O_2 for 45 minutes and six received no H_2O_2. The raw data shows that between replicates there is some variability (Figure 4.2A). Analysis of average values indicated that there was approximately a 5-fold increase in the level of PtdIns5*P* in response to H_2O_2 in SHXtransduced cells which is significant and comparable to non-transduced U2OS cells (Figure 4.2B). This finding highlighted the importance of thoroughly checking the PtdIns5*P* measurements obtained from the SHX-transduced samples in the screen as excessive variation between control samples could result in misleading data.

4..3. RNAi screen

4.3.1. Set up of the RNAi Screen

The screen was performed in sets of six target genes to ensure efficient, safe and proper handling of all the samples, therefore in total 17 assays were required to evaluate the effect of all 101 targets. In each assay, 6 wells of U2OS cells were transduced with non-targeting SHX, 3 of these wells were kept in control conditions (no H_2O_2) and 3 wells were treated with 4mM H_2O_2 for 45 minutes. All cells transduced with targeting constructs received pooled constructs and were treated with 4mM H_2O_2 for 45 minutes. No cells transduced with targeting constructs were kept in control conditions as this would have resulted in too many samples that could not have been easily handled. Therefore, each assay involved analysis of 24 samples. An additional well was transduced for each target gene to allow qRT-PCR analysis of RNA expression and ensure knock down efficiency (Figure 4.3.).

4.3.2. Analysis of Knock Down Samples

In each assay the level of PtdIns5*P* in each knock down sample was calculated as a percentage of the H_2O_2-stimulated SHX-transduced sample (% change in the level of PtdIns5*P* = pmoles of PtdIns5*P* in sample of interest/ pmoles of PtdIns5*P* in the H_2O_2stimulated SHX-transduced sample x 100). This allowed collective analysis of all 17 assays. Within the panel of selected phosphoinositide kinases, phosphatases, phospholipases and accessory proteins, some candidates have previously been described as regulators of PtdIns5*P* and therefore acted as internal controls. A well established negative regulator of PtdIns5*P* is PIP4K and targeted knock down of each isoform, PIP4K2A, PIP4K2B and PIP4K2C, led to increased H_2O_2-induced PtdIns5*P* by 39.5%, 162.1% and 355.7% respectively (Figure 4.4 and Table 4.1). This is an interesting observation as PIP4K2B has been shown to have 2000-fold lower enzymatic activity than PIP4K2A [44] yet knock down of PIP4K2B had a greater effect on the level of PtdIns5*P* than knock down of PIP4K2A. Knock down of PIP4K2C was recently shown to increase the level of PtdIns5*P* to a greater extent that knock down of PIP4K2A or PIP4K2B [38]. In future experiments the role of PIP4K2A in the regulation of H_2O_2-induced PtdIns5*P* was shown to be particularly significant and was analysed in greater detail (Chapter 5). Never the less, this result indicated that under the conditions of the RNAi screen it should be possible to isolate regulators of oxidative stressinduced PtdIns5*P* generation.

Similarly to the inhibitor screen, over half of the samples transduced with targeted knock down constructs (56/101) showed a change in the level of H_2O_2-induced PtdIns5*P* but the change was by

less than 30% (Figure 4.4 and Table 4.1). This provides further evidence that there are likely to be a number of distinct signalling pathways that all contribute to the regulation of PtdIns5*P* production. Interestingly, far more knock down samples led to enhanced rather than decreased PtdIns5*P* production. Out of the 101 samples, 32 caused an increase in the level of PtdIns5*P* by 30% or more whereas only 13 caused a decrease by 30% or more. This may suggest that the cell has more negative regulatory mechanisms in place to control the cellular level of PtdIns5*P* as opposed to mechanisms that can synthesise PtdIns5*P*.

One family of enzymes that seem to have a role in the regulation of PtdIns5*P* generation is the PI3K family. Knock down of each catalytic subunit of the Class I PI3Ks caused an increase in the level of PtdIns5*P*. The catalytic subunits; PIK3CA and PIK3CB (analysed in the same assay), PIK3CD and PIK3CG (analysed in the same assay), caused an increase of 76%, 20%, 254% and 185% respectively. These enzymes are members of the Class I family of PI3Ks that are responsible for the phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 at the plasma membrane in response to extracellular signals [83]. These observations contradict data obtained using pharmacological inhibition of PI3K as use of three different PI3K inhibitors caused a decrease in the level of H_2O_2-induced PtdIns5*P* (Chapter 3, Figure 3.9). Importantly, RNAi-mediated knock down causes reduction of target protein over 3 days and would allow up-regulation of compensatory signalling molecules whereas use of pharmacological compounds causes acute inhibition of enzymatic activity and therefore causes short term cellular effects. Unlike specific targeted shRNA constructs, each of the PI3K inhibitors are effective against numerous molecular targets; PI103 is an inhibitor of Class IA PI3K, mTOR and DNA damage activated kinases, LY294002 inhibits Class I PI3Ks, other PI3K-related kinases and other non-related targets and GDC0941 inhibits all Class I PI3K isoforms and mTOR. This suggests that although genetic and pharmacological methods can both target PI3K there can be differing outcomes which might be related to the off target effects of small molecular weight inhibitors. For example inhibition of PI3K activity can lead to increased autophagy while many of the inhibitors of PI3K also attenuate the induction of autophagy as they can block the synthesis of PtdIns3*P* which is required for autophagy induction. This might suggest that modulation of PtdIns5*P* generation is a consequence of a process that is regulated by PtdIns(3,4,5)P_3 rather than as a requirement for the generation of this lipid as a precursor.

Knock down of the Class II PI3Ks also increased the level of H_2O_2-induced PtdIns5*P*. There are three Class II kinases that phosphorylate PtdIns to make PtdIns3*P* and phosphorylate PtdIns4*P* to produce PtdIns(3,4)P_2 [120]; PIK3C2A, PIK3C2B and PIK3C2G. Knock down of these enzymes increased PtdIns5*P* production by 44% (analysed in one assay), 207% and 150% (analysed in a separate assay) respectively. The activation, localisation and function of each Class II PI3K remains poorly

understood and various roles have been attributed to this family of enzymes. In resting conditions PtdIns3*P* decorates endosomal membranes and facilitates recruitment of effector proteins that bind via a PtdIns3*P* interaction domain such as the PX or FYVE domain. One particularly relevant endosomal protein is PIKfyve, a 5kinase that phosphorylates PtdIns3*P* to produce PtdIns(3,5)P_2. PIKfyve has been implicated in the synthesis of PtdIns5*P* by direct phosphorylation of PtdIns or by producing PtdIns(3,5)P_2 which can be de-phosphorylated by myotubularins to produce PtdIns5*P* [32]. Significantly, comparative genomics suggested that since PtdIns3*P* is located on early endosomes, PtdIns(3,5)P_2 is located on late endosomes and PtdIns(4,5)P_2 is located on the plasma membrane, PtdIns5*P* could be predicted to be involved in the transport of late endosomal vesicles to the plasma membrane [37]. In this scenario, the production of PtdIns5*P* would require Class II PI3Ks to synthesise PtdIns3*P* which would ultimately act as a substrate for PtdIns5*P* synthesis via PtdIns(3,5)P_2 however as knock down of Class II PI3Ks caused increased PtdIns5*P* production this is unlikely to be a relevant function.

PIP5K is a 5-kinase whose main role in the cell is to phosphorylate PtdIns4*P* to produce PtdIns(4,5)P_2 although to a lesser extent PIP5K is also able to phosphorylate PtdIns, PtdIns3*P* and PtdIns(3,4)P_2 [23] [24] [25]. There are three isoforms of PIP5K; PIP5K1A, PIP5K1B and PIP5K1C. Distinct cellular localisations strongly imply that each isoform performs different functions and are likely to regulate separate pools of PtdIns(4,5)P_2. In the RNAi screen, knock down of both PIP5K1A and PIP5K1B resulted in enhanced PtdIns5*P* production by 283% and 273% respectively. Knock down of PIP5K1C led to a slight decrease in PtdIns5*P* production, 81% compared to SHX-transduced cells. The most likely mechanism for PIP5K-induced negative regulation of PtdIns5*P* is through the disruption of phosphoinositides in the plasma membrane. For example, in response to H_2O_2-induced oxidative stress, PIP5K1B can phosphorylate PtdIns(3,4)P_2 to produce PtdIns(3,4,5)P_3 [25] which could have a knock-on effect on the level of PtdIns5*P*. Alternatively, PIP5K could be regulating a nuclear pool of PtdIns5*P*. PIP5K1A has been found in nuclear speckles [121] and PtdIns5*P* has long been known to reside in the nucleus [13]. There is no obvious or direct role in the negative regulation of PtdIns5*P* production that can be attributed to PIP5K. In order to further dissect the role of PIP5K it would be necessary to ascertain in which cellular compartment PIP5K is having an effect and whether the level of PtdIns5*P* is specifically being regulated in that compartment.

Several members of the myotubularins (MTMs) have previously been implicated in the regulation of PtdIns5*P*. This family of 3-phosphatases can de-phosphorylate PtdIns(3,5)P_2 to make PtdIns5*P* [122] but they also de-phosphorylate PtdIns3*P* to produce PtdIns [123]. In total there are 15 MTMs, 8 of which are active enzymes and 6 are catalytically dead. Each MTM is capable of forming a heterodimer, often consisting of one active and one inactive enzyme. [124]. It is thought that the role

of the catalytically dead MTMs is to act as a substrate binding competitor and therefore negatively regulate the activity of the active phosphatase [125] but the exact functional role of many MTMs remains elusive. In the RNAi screen, genetic knock down of each MTM had varying effects on the level of H_2O_2-induced PtdIns5*P* as knock down of eight of the MTMs had no significant effect, knock down of two of the MTMs led to a decrease and knock down of four of the MTMs caused an increase. This is an interesting observation itself as all 15 enzymes regulate the de-phosphorylation of the same lipid yet clearly these enzymes can fulfil very different roles in the regulation of PtdIns5*P* production. This may be explained by the various cellular localisations and tissue expression patterns of different MTMs which allows regulation of specific pools of phosphoinositides [126].

Of the 4 MTMs that caused an increase in H_2O_2-induced PtdIns5*P*, 3 were catalytically active namely; MTMR4, MTMR7 and MTMR8 and one was catalytically inactive; MTMR11. The level of PtdIns5*P* was increased by 246%, 265%, 263% and 176% respectively. Knock down of catalytically active MTMs would be expected to decrease the production of PtdIns5*P* and clearly this is not the case for MTMR4, MTMR7 or MTMR8. MTMR11 is not known to heterodimerise with any of the catalytically active MTMs, in fact very little is known generally about the cellular function of MTMR11 making it very difficult to speculate how knock down of this protein could be affecting the regulation of PtdIns5*P* production. Knock down of the catalytically active MTMR6 led to 34% less PtdIns5*P* being produced and knock down of the pseudophosphatase MTMR10 caused a reduction of 60% compared to SHXtransduced cells. As mentioned, knock down of a catalytically active myotubularin could decrease the level of PtdIns5*P* as a consequence of reduced de-phosphorylation of PtdIns(3,5)P_2, a function that could be fulfilled by MTMR6. Interestingly, the initial reaction rate for MTMR6 towards PtdIns3*P* is increased in the presence of PtdIns5*P* suggesting that PtdIns5*P* may act in a positive feedback loop as an allosteric activator [127]. MTMR10 was chosen for further validation due to the fact that knock down of this protein caused the greatest reduction in the cellular level of H_2O_2-induced PtdIns5*P* and that the main aim of the RNAi screen was to identify regulators of PtdIns5*P* synthesis. In total, five candidate genes were chosen for further validation as knock down of each led to decreased synthesis of PtdIns5*P*, suggestive of a role in the synthesis of H_2O_2-induced PtdIns5*P*.

4.3.3. Candidate Genes

Five genes from the RNAi screen were chosen for further evaluation; MTMR10, FAN1, CDIPT, ITPKC and PI4K2A. These were selected based on the observation that knock down of these genes reduced the level of H_2O_2-induced PtdIns5*P* by ≥40% compared to SHXtransduced samples. None have been

directly associated with PtdIns5*P* synthesis previously. In the initial screen, MTMR10 knock down led to a 60% reduction in the level of H_2O_2-induced PtdIns5*P* (Figure 4.5A). Efficiency of knock down at the mRNA level was analysed in samples transduced with pooled lentiviral supernatant and individual shRNA constructs. Analysis by qRT-PCR showed that MTMR10 expression was reduced by approximately 50% by both pooled lentiviral supernatant and each individual shRNA construct (Figure 4.5B). There is very little known about the function of MTMR10. It is a catalytically dead MTM that has not been shown to interact with any other MTM. Consequently, its potential role in the regulation of PtdIns5*P* is unclear.

FAN1 knock down led to a 56% reduction in the level of H_2O_2-induced PtdIns5*P* which was comparable to the basal level of PtdIns5*P* in SHX-transduced samples (Figure 4.6A). Quantification of FAN1 RNA by qRT-PCR showed that pooled lentiviral supernatant reduced FAN1 expression by at least 90% and each individual shRNA construct reduced FAN1 expression by at least 50% (Figure 4.6B). Although there was some variation in the knock down efficiency of each individual construct, pooling all five constructs gave the best knock down so pooled lentiviral supernatant was used for following validation experiments. FAN1 was included in the library of phosphoinositide-related proteins as it was initially named MTMR15. It was recently confirmed as a nuclease involved in the repair of DNA interstrand crosslinks and renamed FAN1 [128] [129]. This is a particularly noteworthy observation as PtdIns5*P* has already been implicated in the initiation of DNA damage (Chapter 3.3.6).

Knock down of CDIPT in the initial screen led to a 41% reduction in the level of H_2O_2induced PtdIns5*P* (Figure 4.7A). Analysis by qRT-PCR showed that pooled lentiviral supernatant very efficiently reduced CDIPT expression as less than 1% remained. Each individual shRNA construct also efficiently reduced CDIPT expression by at least 92% (Figure 4.7B). Again, pooling all five constructs gave the best knock down so pooled lentiviral supernatant was used for following validation experiments. CDIPT is a crucial enzyme in the synthesis of PtdIns. It catalyses the addition of myo-inositol to CDPdiacylglycerol producing PtdIns and CMP [130] and its catalytic activity is concentrated in the endoplasmic reticulum and Golgi body [204]. Interestingly, PtdIns5*P* is also thought to reside in the Golgi body and vesicular network, adding weight to the idea that CDIPT could regulate PtdIns5*P* in this cellular compartment. Restricted PtdIns availability could limit the production of PtdIns5*P* and if this is the case, there must also be a 5-kinase capable of phosphorylating PtdIns to form PtdIns5*P*, a role that both PIKfyve and PIP5K have been shown to perform *in vitro* however this specific activity has never been formally identified *in vivo*. More likely however is that as knock down of CDIPT will affect the production of all phosphoinositides, disruption of general phosphoinositide availability will lead to altered PtdIns5*P* levels.

In the initial screen, ITPKC knock down led to a 55% reduction in the level of H_2O_2-induced PtdIns5*P* (Figure 4.8A). Pooled lentiviral supernatant efficiently knocked down ITPKC by at least 82%. Each individual shRNA construct also efficiently reduced ITPKC expression by at least 65% (Figure 4.8B). ITPK enzymes are a family of 3-kinases responsible for the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 [131]. Both of these second messengers are very important in the regulation of calcium signalling. There are three isoforms of ITPK; ITPKA, ITPKB and ITPKC. In the screen ITPKA knock down led to a 43% increase in the production of PtdIns5*P* which was not significant enough to warrant further investigation. Both ITPKB and ITPKC caused a decrease in the production of PtdIns5*P* by 45% and 55% respectively. When further validation of knock down efficiency of shRNA constructs was performed, it was shown that all three constructs targeting ITPKB were inefficient and therefore ITPKB was excluded from further analysis. ITPKC is most highly expressed in the skeletal muscle [132] and was shown to reside exclusively in the cytoplasm [133] but has more recently been shown to shuttle between the nucleus and the cytoplasm [134]. The direct involvement of PtdIns5*P* in calcium signalling has never been reported but notably PtdIns5*P* is a precursor of PtdIns(4,5)P_2 which can be cleaved to form Ins(1,4,5)P_3. Furthermore, PLC, the enzyme responsible for cleaving PtdIns(4,5)P_2 to form DAG and Ins(1,4,5)P_3, has been shown to also directly cleave PtdIns5*P* in high calcium conditions [135], further implicating calcium signalling in the regulation of PtdIns5*P*. It is therefore entirely possible that disruption of enzymes and inositol lipids involved in calcium signalling may affect the synthesis of PtdIns5*P*.

PI4K2A knock down led to a 49% reduction in the level of H_2O_2-induced PtdIns5*P* (Figure 4.9A). RNA quantification confirmed that pooled lentiviral supernatant reduced PI4K2A expression by at least 94% and each individual shRNA construct reduced PI4K2A expression by at least 84% (Figure 4.9B). The presence of 4-kinase activity towards PtdIns has long been known [136] but the enzyme was not cloned until many years later when PI4K2A was sequenced and the existence of further isoforms was identified [137]. The enzyme was shown to be strongly associated with membranes due to palmitoylation [138]. PI4K2B was later cloned and found to have different expression patterns and catalytic activity to PI4K2A although both isoforms were found to be associated with endosomal vesicle membranes [139]. In the RNAi screen, only PI4K2A knock down had a significant effect on the level of PtdIns5*P*, suggesting that each isoform has a distinct role in the cell. PI4K2A is involved in trafficking in the Trans-Golgi Network (TGN) where PtdIns4*P* is abundant [140], [141]. This is a particularly relevant observation due to the predicted role of PtdIns5*P* in membrane trafficking [37]. Therefore PI4K2A represented a good candidate for a PtdIns5*P* regulator.

4.3.4. Validation of Candidate Genes using Transient Transduction

In order to validate and further characterise the effect of knocking down each candidate gene on the level of PtdIns5*P*, U2OS cells were transiently transduced for 48 hours with the same batch of pooled lentiviral supernatant as the initial screen. Initially, samples were treated with 4mM H_2O_2 similarly to the initial screen. For each experiment, samples were kept for qRT-PCR analysis of RNA expression and in each experiment qRT-PCR analysis revealed that target gene RNA expression was reduced by ≥70%. Quantification of PtdIns5*P* surprisingly showed that in two experiments, there was no significant effect of knocking down any of the five candidate genes on the level of PtdIns5*P* in response to H_2O_2 (data not shown). In response to increasing doses of H_2O_2 the level of PtdIns5*P* was shown to increase (Figure 3.4) which is why a dose of 4mM was used in the initial screen, as this gave a maximal level of PtdIns5*P* without causing obvious morphologic changes to the cells. Treatment of cells with 1mM H_2O_2 was shown to cause a robust increase in PtdIns5*P* and in the literature concentrations of 1mM and below are more commonly used therefore in further experiments samples were treated with 1mM H_2O_2. Again, in two experiments using 1mM H_2O_2, there was no significant effect of knocking down any of the five candidate genes on the level of H_2O_2-induced PtdIns5*P* (data not shown). In one further experiment, samples transduced with targeting shRNA constructs were also kept in un-stimulated conditions to allow analysis of basal PtdIns5*P* in all genetic knock down backgrounds. There was no consistent decrease in the level of either basal or H_2O_2-induced PtdIns5*P* in knock down samples, in fact knock down of both CDIPT and PI4K2A led to an increase in the level of basal and H_2O_2-induced PtdIns5*P* in the representative experiment shown (Figure 4.10A).

4.3.5. Validation of Candidate Genes using Stable Cell Lines

Transient transduction allows enough time for stable integration of shRNA constructs into the genome of target cells which are then used immediately. Stable cell lines are produced by transducing target cells with shRNA constructs followed by selection of transduced cells with puromycin as the shRNA constructs encode a gene conferring puromycin resistance. Transduced U2OS cells were cultured for 14 days in medium containing puromycin before H_2O_2 treatment and quantification of cellular PtdIns5*P*. The knock down efficiency of stably integrated shRNA constructs was analysed using qRT-PCR which showed that all five target genes were expressed at ≤30% compared to SHX-transduced cells (Figure 4.11B). In two separate experiments there was no significant effect on the level of H_2O_2-induced PtdIns5*P* in any of the five stable cell lines (Figure 4.11A).

4.4. Conclusions

The characterisation of H_2O_2-induced PtdIns5*P* in SHX-transduced samples and rigorous set up of the RNAi screen was intended to ensure that data obtained from the screen would be reliable and accurate across all assays. Transduction of shRNA into U2OS cells was optimised to ensure maximum transduction efficiency (Figure 4.1). Initial experiments indicated that differences in the level of PtdIns5*P* between replicate un-stimulated samples were minimal and although there was some variation between replicate stimulated samples the differences were considered to be acceptable (Figure 4.2).

Throughout the screen, differences between replicate samples were monitored. Of the 5 candidate genes chosen for further analysis, all 5 showed high similarity between replicates of SHX-transduced samples and replicates of targeted knock down samples (Figures 4.5- 4.9). Additionally all 5 candidate genes were shown to be statistically significant using a Student's T-test (Figures 4.5- 4.9). The following validation experiments were performed in an attempt to replicate the effect these 5 candidate genes showed in the initial screen:

- Transient transduction of cells and 4mM H_2O_2 treatment
- Transient transduction of cells and 1mM H_2O_2 treatment
- Transient transduction of cells and 1mM H_2O_2 treatment, wild type U2OS cells included
- Transient transduction of cells and 1mM H_2O_2 treatment, analysis of basal PtdIns5*P* for each candidate gene also included
- Analysis of basal and 1mM H_2O_2-induced PtdIns5*P* in stable cell lines for each candidate gene

None of these experiments reproduced the effects seen in the initial screen. There are several possible explanations for this. It has been shown that shRNA constructs are heterogeneously processed in the cell by Dicer to form biologically active siRNAs and this can lead to off target effects [142]. Precautions were taken to ensure efficient knock down of target genes as at least two different validated constructs or five different non-validated constructs were selected. Due to the large number of constructs being used, shRNA constructs were pooled and used to transduce target U2OS cells as transducing individual constructs would have resulted in an enormous number of samples. Off-target effects are unlikely to account for the differences observed between the initial screen and the first validations experiments as the same batch of pooled lentiviral supernatant was used to transduce the cells.

Analysis of PtdIns5*P* levels from control and H_2O_2-treated SHX samples from each of the 17 assays revealed that there was noticeable variation when the data is directly compared between assays (Figure 4.12A) although importantly there was clearly a robust H_2O_2induced increase in PtdIns5*P* in each assay. There are a number of possible explanations for this variation. Due to the high number of samples, 17 assays were required and this process took approximately 3 months to complete. Cell age or passage number may affect the status of the cells which could potentially influence the response to oxidative stress. Alternatively it could be due to a problem with the standard curve as the quantification of PtdIns5*P* is defined by a set of standard PtdIns5*P* samples within each assay. During processing, standard samples are dried and re-solubilised into ether, any differences in this step between assays could affect the final concentration of PtdIns5*P* determined in each standard sample and consequently affect the calculated quantification of PtdIns5*P* in the samples.

It is important to consider however, that as each assay was done on an individual basis, every sample within each assay was incubated with serum free medium for the same duration, received the same batch of prepared H_2O_2 for the same duration and the PtdIns5*P* quantification was performed using the same reagents. Therefore we assumed it was safe to compare the samples transduced with targeting constructs to the SHX-transduced samples in each assay regardless of the variation between assays. However retrospectively the variability in the ratio of H_2O_2-stimulated SHX-transduced samples: control samples across the entire screen was too great. Quantification of the fold change in the level of PtdIns5*P* between control and H_2O_2-stimulated samples showed that the average fold change was approximately 4-fold, the highest was 7.5-fold and the lowest was 2-fold (Figure 4.12C). This high variability is likely to have led to the identification of a high number of false positives.

Due to the difficult nature of the PtdIns5*P* assay (Figure 2.1), triplicate samples were used for quantification which led to a large number of samples being processed, over 500 in total. Due to the high number of samples, the entire screen was only performed once. Clearly, a more high-throughput screening method would allow replicate screens to be performed and this would provide a clearer view of which candidate hits are reproducible. The most effective way to improve the screening procedure would involve developing a highthroughput assay to quantify PtdIns5*P*. Recently a new high pressure liquid chromatography (HPLC) method has been developed that is able to separate PtdIns5*P* from PtdIns4*P* even in basal conditions where the level of PtdIns5*P* is as low as 1% of the level of PtdIns4*P* [38]. Further development of this method of PtdIns5*P* quantification would be very useful for a screening protocol and may allow multiple rounds of screening in order to confirm the reproducibility of candidate hits.

There were however some candidate PtdIns5*P* regulators from the initial screen that could be validated. Knock down of PTEN in the initial screen led to a 65% increase in H_2O_2induced PtdIns5*P* (Figure 4.4 and Table 4.1). Further to this, both pharmacological inhibition and genetic knock down of PTEN was shown to cause an increase in the production of PtdIns5*P* in response to H_2O_2 stimulation (Figure 3.10). This result is very encouraging as it indicates not only that an important tumour suppressor may be involved in the regulation of PtdIns5*P* production but also that despite the seemingly high false discovery rate, further optimisation of the screening procedure could successfully uncover novel PtdIns5*P* regulators. Another interesting candidate gene is PIP4K2A. As mentioned, knock down of PIP4K2A increased H_2O_2-induced PtdIns5*P* by 39.5% in the initial screen (Figure 4.4 and Table 4.1). More detailed analysis of the effect of PIP4K2A knock down over a time course of H_2O_2 stimulation later confirmed an important role for PIP4K2A in the regulation of H_2O_2induced PtdIns5*P* again restoring confidence that fundamentally the RNAi screening approach works but requires further optimisation to reduce experimental variation.

An interesting observation that arose from the initial screen is that suppression of a number of components that impact on autophagy appears to increase PtdIns5*P* production. Knock down of each catalytic and regulatory subunit of the Class I PI3Ks caused an increase in the level of PtdIns5*P*. Knock down of the catalytic subunits; PIK3CA, PIK3CB, PIK3CD and PIK3CG all caused an increase in PtdIns5*P* production as did knock down of the Class II PI3Ks; PIK3C2A, PIK3C2B and PIK3C2G. Finally knock down of PIP5K which have also been shown to be involved in autophagic processes [143] [144] also increased oxidative stress induced PtdIns5*P* generation. This might suggest that autophagic processes increase the substrate which is utilised to produce PtdIns5*P* during oxidative stress.

Overall, despite failing to uncover a novel regulator of PtdIns5*P* synthesis, the RNAi screen has highlighted the apparent involvement of numerous enzymes in PtdIns5*P* production. Similarly to the inhibitor screen, many enzymes seem to have an effect on the PtdIns5*P* level without affecting the entire cellular pool of PtdIns5*P*. This adds weight to the idea that various cellular pools of PtdIns5*P* could each be regulated by a specific subset of enzymes therefore suggesting that PtdIns5*P* plays a unique role in several sub-cellular localisations. It also highlights the vital role PtdIns5*P* plays as a signalling molecule in its own right as the cell utilises many signalling cascades to regulate the availability of PtdIns5*P*.

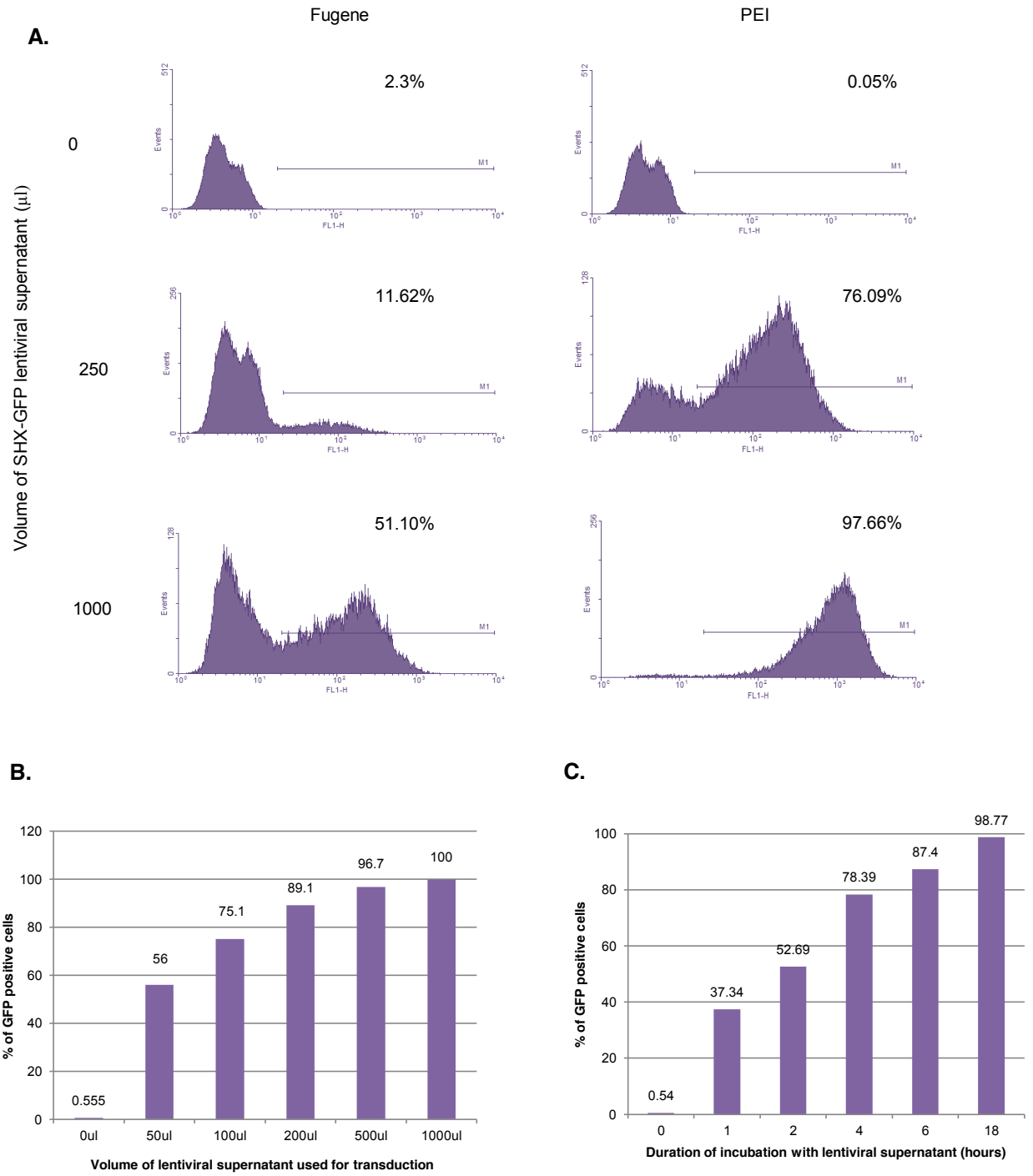


Figure 4.1. Optimisation of lentiviral production and transduction protocols using SHX-GFP.

A. U2OS cells were transduced with non-targeting SHX-GFP using either Fugene or PEI. Following overnight incubation and 24 hours recovery in normal medium, cells were analysed for GFP expression by FACS. **B.** U2OS cells were transduced with different volumes of medium containing SHX-GFP lentiviral particles overnight and after 24 hours recovery GFP expression was measured by FACS. **C.** 200µl of medium containing SHX-GFP lentiviral particles was incubated with U2OS cells for different durations followed by 24 hours recovery and GFP expression was analysed by FACS.

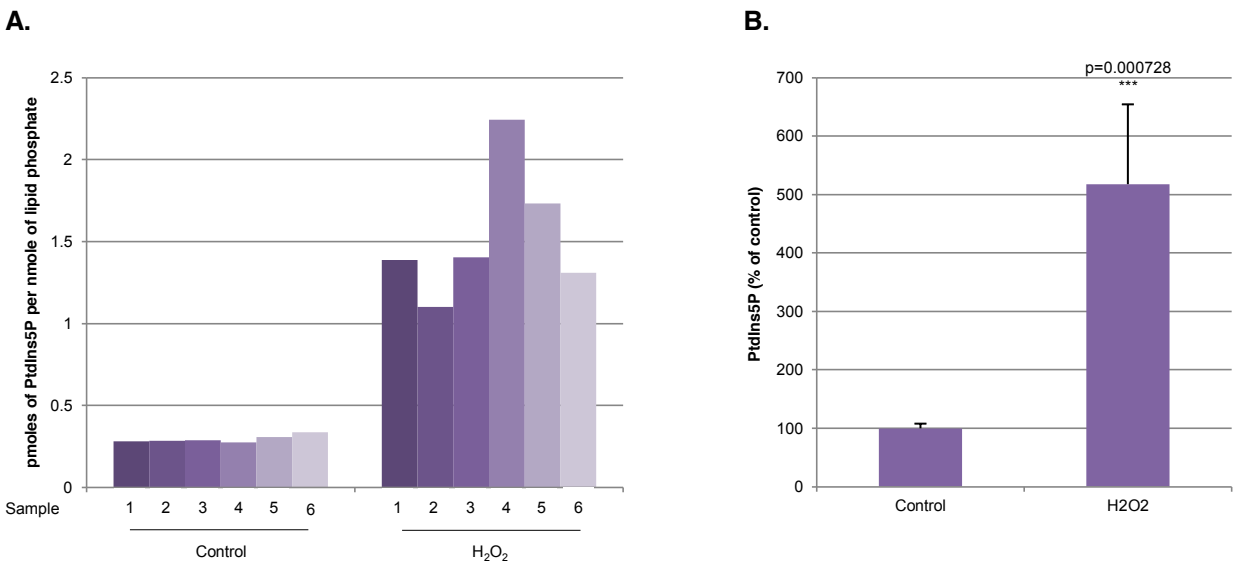


Figure 4.2. Testing the reproducibility of PtdIns5*P* quantification using non-targeting construct SHX. **A.** U2OS cells were transduced with a non-targeting shRNA construct (SHX). Six samples were kept in control conditions and six samples were stimulated with 4mM H_2O_2 for 45 minutes and PtdIns5*P* was quantified. Data is shown as the raw measurements for each sample. **B.** Average values from A. plus the standard deviation. Data presented as fold increase over un-stimulated samples set at 100%. Student's t-test indicates data is highly statistically significant.

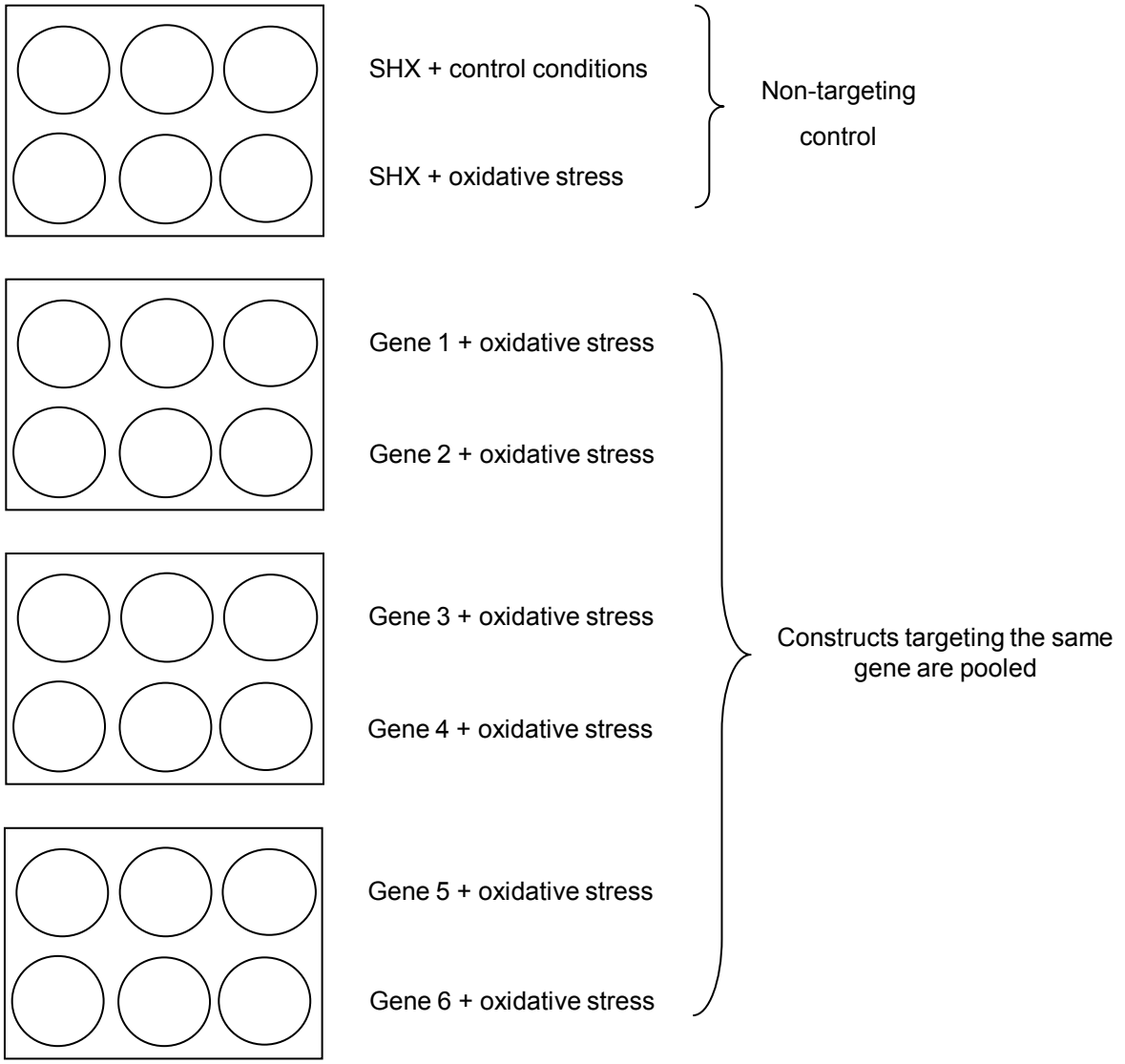


Figure 4.3. Schematic Set up of the RNAi Screen.

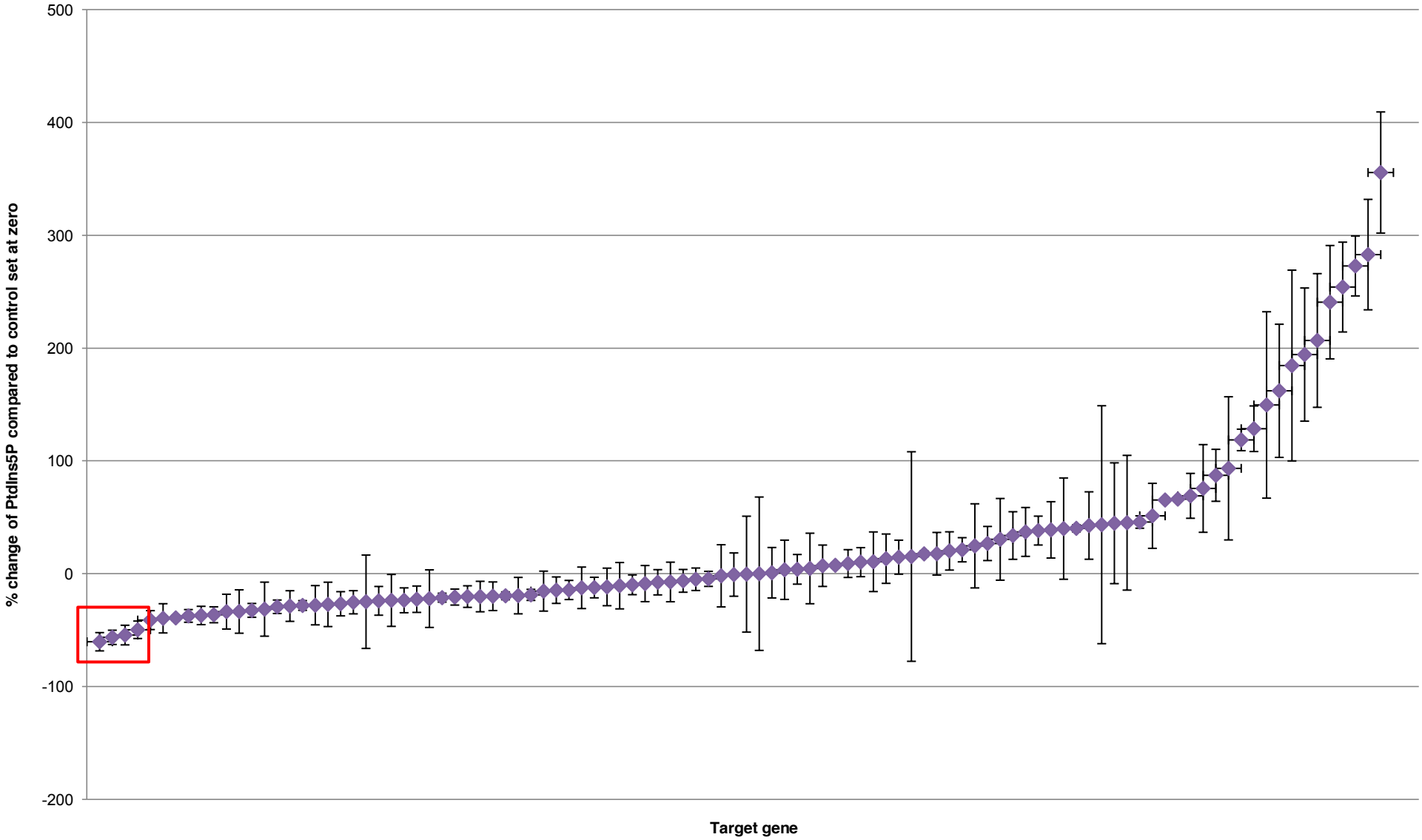


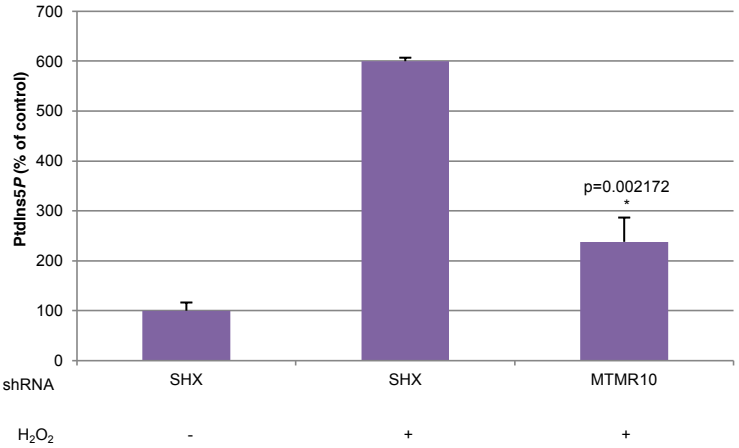
Figure 6. Results from the RNAi screen

102 phosphoinositide related enzymes and co-factors were knocked down in U2OS cells by RNAi and oxidative stress induced PtdIns5*P* was measured. Each sample was done in triplicate and the data is shown as a percentage of U2OS cells transduced with a non-targeting control construct set at zero.

Library name	Gene name	No. of shRNA constructs	Gene description	% of PtdIns5P compared to control set at 100%
PIP5K2C	PIP4K2C	2	phosphatidylinositol-5-phosphate 4-kinase, type II, gamma	455.7
PIP5K1A	PIP5K1A	4	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	382.9
PIP5K1B	PIP5K1B	5	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	372.8
PIK3CD	PIK3CD	2	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	354.1
PIK3C2B	PIK3C2B	3	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta	306.8
PIK3CG	PIK3CG	2	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	284.6
MTMR7	MTMR7	5	myotubularin related protein 7	265.2
MTMR8	MTMR8	5	myotubularin related protein 8	263.5
PIP5K2B	PIP4K2B	4	phosphatidylinositol-5-phosphate 4-kinase, type II, beta	262.2
IHPK2	IP6K2	2	inositol hexakisphosphate kinase 2	257
PIK3C2G	PIK3C2G	3	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma	249.6
MTMR4	MTMR4	2	myotubularin related protein 4	246.3
IPPK	IPPK	2	inositol 1,3,4,5,6-pentakisphosphate 2-kinase	222
MGEA5	MGEA5	2	meningioma expressed antigen 5 (hyaluronidase)	187.2
MTMR11	MTMR11	2	myotubularin related protein 11	176.3
PIK3CA	PIK3CA	2	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	175.6
ITPK1	ITPK1	2	inositol-tetrakisphosphate 1-kinase	172.4
PIK3R3	PIK3R3	4	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	172.1
PLCD4	PLCD4	5	phospholipase C, delta 4	169
OGT	OGT	2	O-linked N-acetylglucosamine (GlcNAc) transferase	166.1
PTEN	PTEN	2	phosphatase and tensin homolog	164.9
PIK3R2	PIK3R2	2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)	152.9
PIK3R5	PIK3R5	2	phosphoinositide-3-kinase, regulatory subunit 5	151.3
PIK3C3	PIK3C3	2	phosphatidylinositol 3-kinase, catalytic subunit type 3	145.8
PIK3C2A	PIK3C2A	5	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha	144.7
SYNJ1	SYNJ1	2	synaptojanin 1	144.6
ITPKA	ITPKA	5	inositol-trisphosphate 3-kinase A	142.7
RICTOR	RICTOR	2	RPTOR independent companion of MTOR, complex 2	140.2
PIP5K2A	PIP4K2A	5	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	139.5
FRAP1	MTOR	2	mechanistic target of rapamycin (serine/threonine kinase)	138.9
PLCB1	PLCB1	5	phospholipase C, beta 1 (phosphoinositide-specific)	138.2
PITPNM1	PITPNM1	5	phosphatidylinositol transfer protein, membrane-associated 1	137
PIK3R4	PIK3R4	2	phosphoinositide-3-kinase, regulatory subunit 4	133.8
PIK3R1	PIK3R1	5	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	126.8
SBF2	MTMR13	5	myotubularin related protein 13	121.2
PIK3CB	PIK3CB	2	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	120.2
INPP4B	INPP4B	4	inositol polyphosphate-4-phosphatase, type II, 105kDa	117.7
MARCKS	MARCKS	5	myristoylated alanine-rich protein kinase C substrate	117.6
PIB5PA	INPP5J	2	inositol polyphosphate-5-phosphatase J	114.7
PIK4CB	PI4KB	2	phosphatidylinositol 4-kinase, catalytic, beta	113.3
TPTE2	TPTE2	5	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2	110.6
PLCZ1	PLCZ1	5	phospholipase C, zeta 1	110.3
TMEM55B	TMEM55B	5	transmembrane protein 55B	110.1
HISPPD2A	PPIP5K1	5	diphosphoinositol pentakisphosphate kinase 1	109
C17orf38	PIK3R6	5	phosphoinositide-3-kinase, regulatory subunit 6	107.4
INPP4A	INPP4A	5	inositol polyphosphate-4-phosphatase, type I, 107kDa	107.1
MTMR2	MTMR2	5	myotubularin related protein 2	106.1
INPP1	INPP1	2	inositol polyphosphate-1-phosphatase	105.3
INPP5D	INPP5D	5	inositol polyphosphate-5-phosphatase, 145kDa	104.6

MTMR9	MTMR9	5	myotubularin related protein 9	103.9
INPPL1	INPPL1	2	inositol polyphosphate phosphatase-like 1	103.5
LOC400924	MTMR3	5	myotubularin related protein 3	100.9
AKT3	AKT3	2	v-akt murine thymoma viral oncogene homolog 3	99.2
PI4K2B	PI4K2B	4	phosphatidylinositol 4-kinase type 2 beta	98.1
PLCG2	PLCG2	2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	96.2
PLCH2	PLCH2	5	phospholipase C, eta 2	95.4
PLCB3	PLCB3	2	phospholipase C, beta 3 (phosphatidylinositol-specific)	95.1
PLCD3	PLCD3	5	phospholipase C, delta 3	93.7
INPP5E	PMPCA	2	inositol polyphosphate-5-phosphatase, 72 kDa	92.7
MTM1	MTM1	2	myotubularin 1	92.4
PIP5K3	PIKFYVE	2	phosphoinositide kinase, FYVE finger containing	91.2
MTMR1	MTMR1	2	myotubularin related protein 1	91.1
INPP5A	INPP5A	2	inositol polyphosphate-5-phosphatase, 40kDa	90.2
INPP5F	INPP5F	2	inositol polyphosphate-5-phosphatase F	89.5
SKIP	INPP5K	2	inositol polyphosphate-5-phosphatase K	89.4
PITPNA	PITPNA	2	phosphatidylinositol transfer protein, alpha	88.3
PITPNM2	PITPNM2	5	phosphatidylinositol transfer protein, membrane-associated 2	87.7
IHPK3	IP6K3	5	inositol hexakisphosphate kinase 3	85.6
KIAA1303	RPTOR	2	regulatory associated protein of MTOR, complex 1	85.4
OCRL	OCRL	2	oculocerebrorenal syndrome of Lowe	84.6
IHPK1	IP6K1	2	inositol hexakisphosphate kinase 1	81.9
PIP5K1C	PIP5K1C	4	phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	81.2
PIK4CA	PI4KA	2	phosphatidylinositol 4-kinase, catalytic, alpha	80.4
PRKAA2	PRKAA2	5	protein kinase, AMP-activated, alpha 2 catalytic subunit	80
MTMR12	MTMR12	5	myotubularin related protein 12	79.7
PLCG1	PLCG1	5	phospholipase C, gamma 1	79.7
IMPK	IMPK	5	inositol polyphosphate multikinase	79.3
PITPNM3	PITPNM3	5	PITPNM family member 3	78.8
SBF1	MTMR5	2	myotubularin related protein 5	77.9
PLCH1	PLCH1	5	phospholipase C, eta 1	76.4
AKT1	AKT1	2	v-Akt Murine Thymoma Viral Oncogene Homolog 1	76.3
SMG1	SMG1	5	SMG1 phosphatidylinositol 3-kinase-related kinase	76
PLCD1	PLCD1	2	phospholipase C, delta 1	74.7
C3orf29	MTMR14	2	myotubularin related protein 14	73.3
PTPMT1	PTPMT1	2	protein tyrosine phosphatase, mitochondrial 1	72.8
SYNJ2	SYNJ2	5	synaptojanin 2	71.3
PLCB4	PLCB4	2	phospholipase C, beta 4	70.8
INPP5B	INPP5B	5	inositol polyphosphate-5-phosphatase, 75kDa	70.7
TPTE	TPTE	5	transmembrane phosphatase with tensin homology	67.5
MTMR6	MTMR6	2	myotubularin related protein 6	66.4
IMPAD1	IMPAD1	5	inositol monophosphatase domain containing 1	63.6
PLCB2	PLCB2	5	phospholipase C, beta 2	63
AKT2	AKT2	2	v-akt murine thymoma viral oncogene homolog 2	62.6
GFPT1	GFPT1	2	glutamine--fructose-6-phosphate transaminase 1	60.8
PRKAA1	PRKAA1	5	protein kinase, AMP-activated, alpha 1 catalytic subunit	60.4
CDIPT	CDIPT	5	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	58.9
ITPKB	ITPKB	3	inositol-trisphosphate 3-kinase B	54.6
PI4KII	PI4K2A	2	phosphatidylinositol 4-kinase type 2 alpha	51.3
ITPKC	ITPKC	2	inositol-trisphosphate 3-kinase C	45.5
KIAA1018	FAN1	5	FANCD2/FANCI-associated nuclease 1	43.6
MTMR10	MTMR10	2	myotubularin related protein 10	39.7

A.



B.

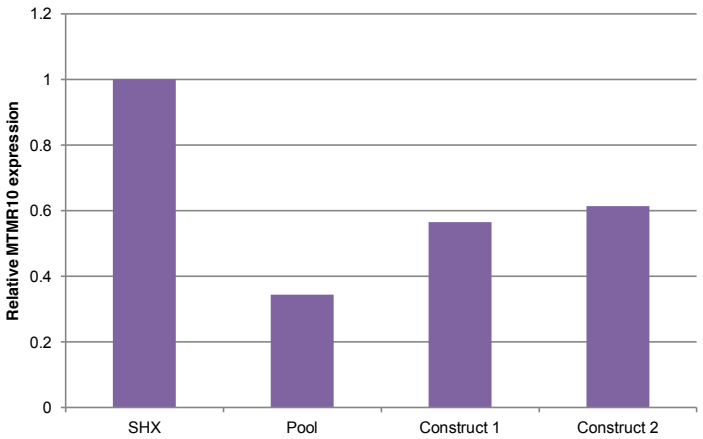
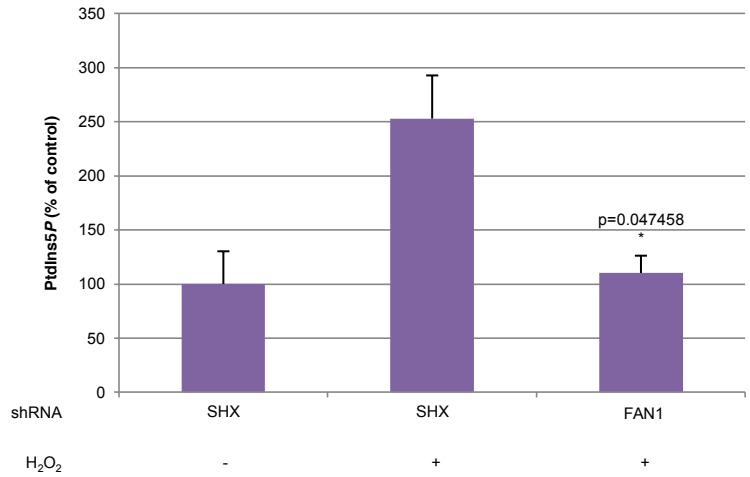


Figure 4.5. Data from the original screen: MTMR10.

A. shRNA mediated knock down of MTMR10 in the original screen caused a significant reduction in the production of PtdIns5*P* in response to oxidative stress. Data represents the average value plus the standard deviation of a triplicate. Student's t-test indicates data is statistically significant. **B.** qRT-PCR was used to analyse the expression of MTMR10 mRNA in cells transduced with pooled constructs (Pool) or each individual shRNA construct, each of which are present in the pooled lentiviral supernatant.

A.



B.

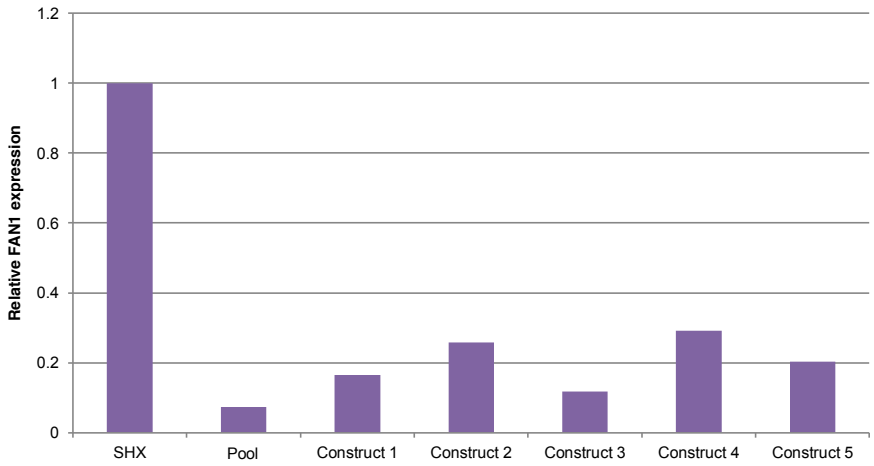
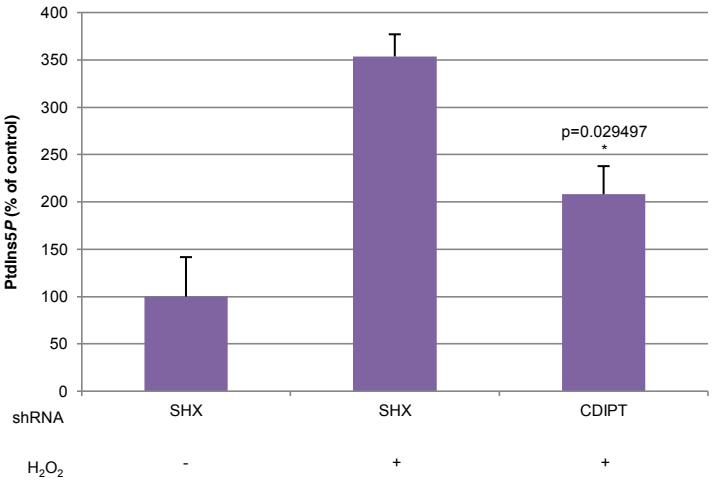


Figure 4.6. Data from the original screen: FAN1.

A. shRNA mediated knock down of FAN1 in the original screen caused a significant reduction in the production of PtdIns5*P* in response to oxidative stress. Data represents the average value plus the standard deviation of a triplicate. Student's t-test indicates data is statistically significant. **B.** qRT-PCR was used to analyse the expression of FAN1 mRNA in cells transduced with pooled constructs (Pool) or each individual shRNA construct, each of which are present in the pooled lentiviral supernatant.

A.



B.

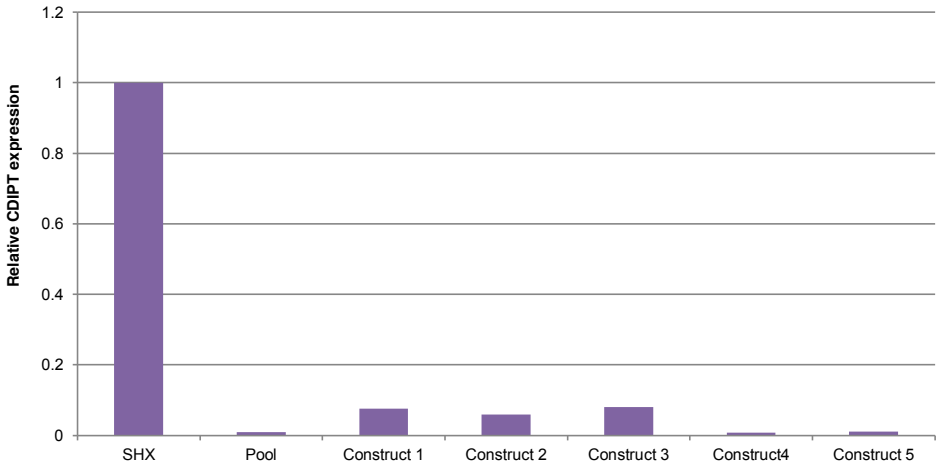
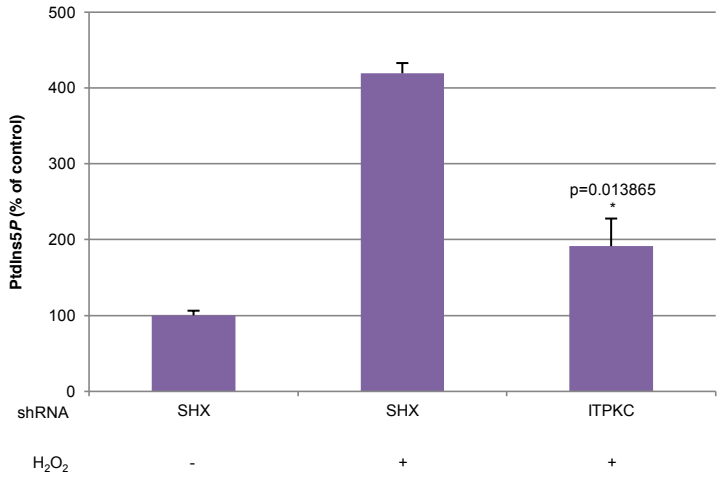


Figure 4.7. Data from the original screen: CDIPT.

A. shRNA mediated knock down of CDIPT in the original screen caused a significant reduction in the production of PtdIns5P in response to oxidative stress. Data represents the average value plus the standard deviation of a triplicate. Student's t-test indicates data is statistically significant. **B.** qRT-PCR was used to analyse the expression of CDIPT mRNA in cells transduced with pooled constructs (Pool) or each individual shRNA construct, each of which are present in the pooled lentiviral supernatant.

A.



B.

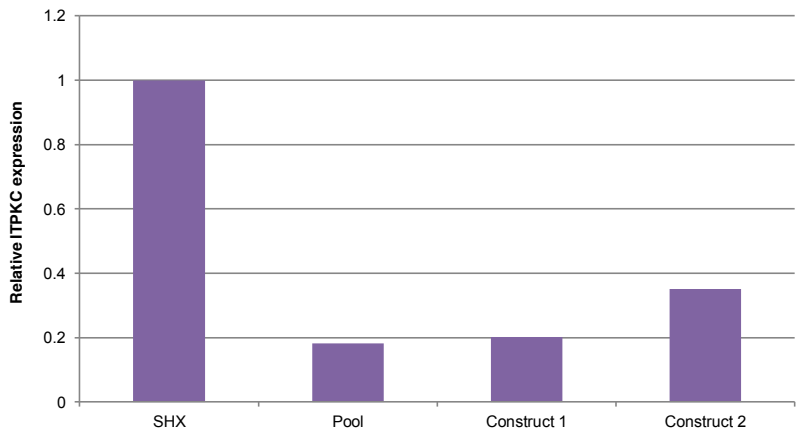
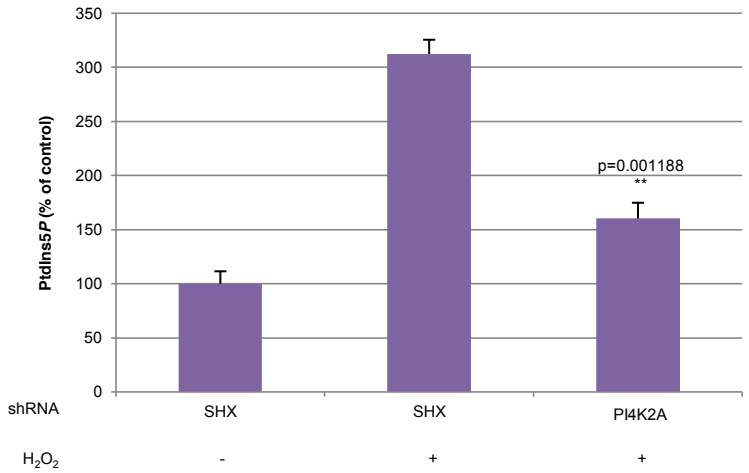


Figure 4.8. Data from the original screen: ITPKC.

A. shRNA mediated knock down of ITPKC in the original screen caused a significant reduction in the production of PtdIns5P in response to oxidative stress. Data represents the average value plus the standard deviation of a triplicate. Student's t-test indicates data is statistically significant. **B.** qRT-PCR was used to analyse the expression of ITPKC mRNA in cells transduced with pooled constructs (Pool) or each individual shRNA construct, each of which are present in the pooled lentiviral supernatant.

A.



B.

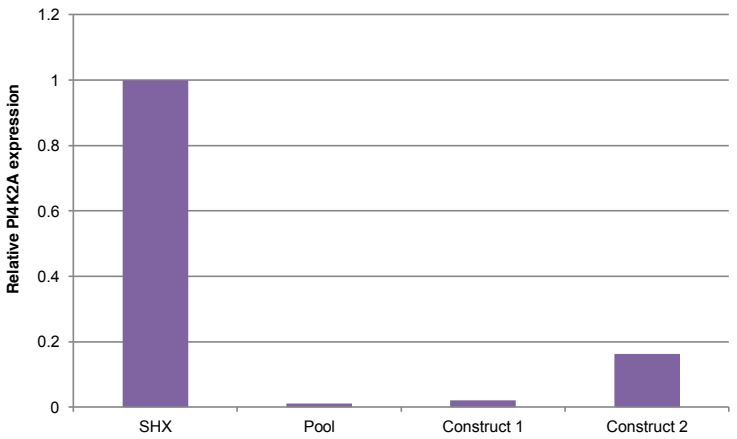


Figure 4.9. Data from the original screen: PI4K2A.

A. shRNA mediated knock down of PI4K2A in the original screen caused a significant reduction in the production of PtdIns5P in response to oxidative stress. Data represents the average value plus the standard deviation of a triplicate. Student's t-test indicates data is statistically significant. **B.** qRT-PCR was used to analyse the expression of PI4K2A mRNA in cells transduced with pooled constructs (Pool) or each individual shRNA construct, each of which are present in the pooled lentiviral supernatant.

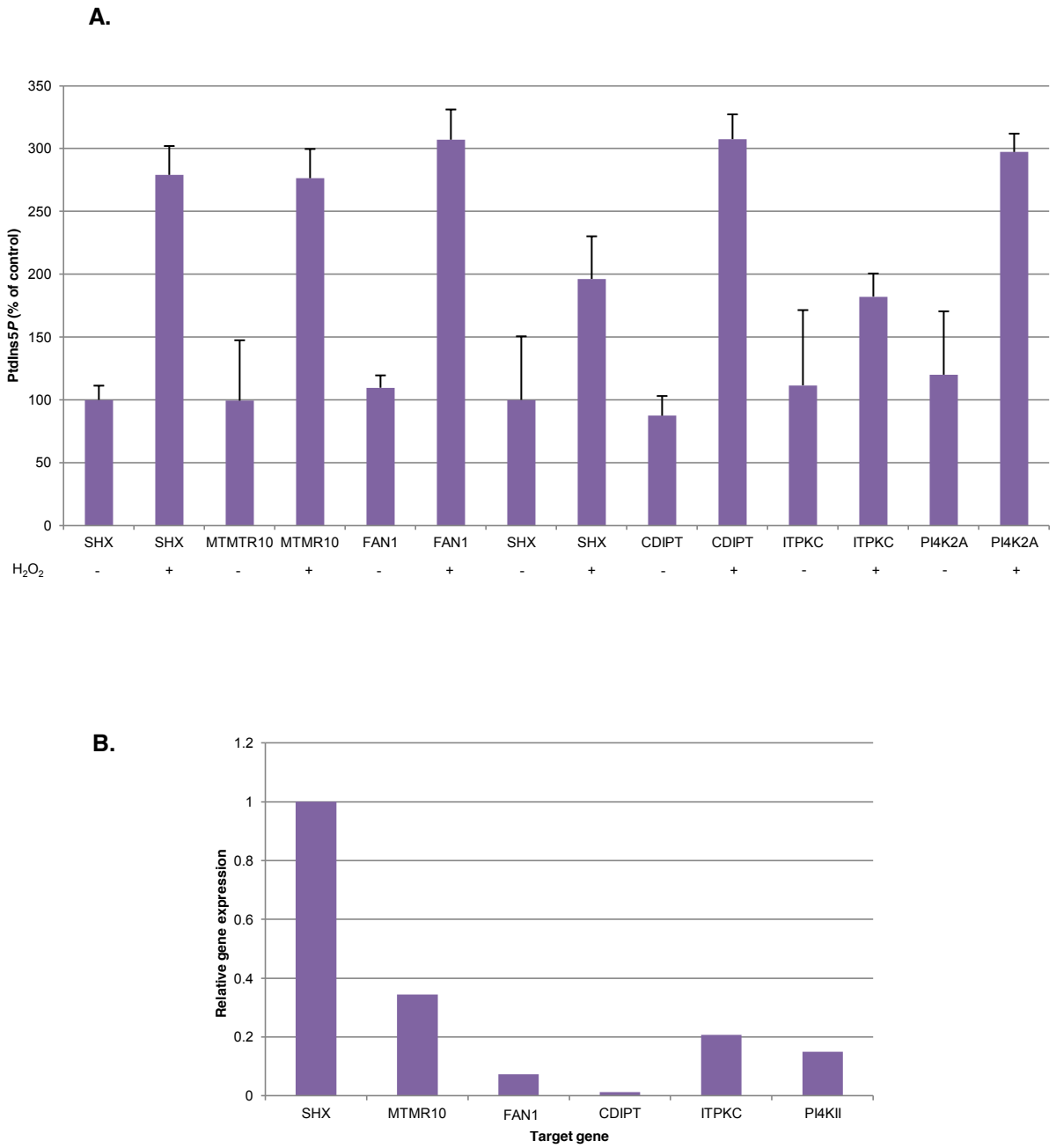


Figure 4.10. Validation of candidate hits using transient transduction.

A. U2OS cells were transduced with shRNA constructs targeting each of the candidate hits from the initial screen. Following overnight incubation and 24 hours recovery the cells were stimulated with 4mM H_2O_2 for 45 minutes and the level of PtdIns5*P* was measured. Data represents the average value plus the standard deviation of a triplicate. **B.** qRT-PCR was used to analyse the mRNA level of the indicated genes in the corresponding transduced cell (e.g. MTMR10 mRNA quantified in cells transduced with shRNA against MTMR10). The data shows that each candidate gene was efficiently knocked down at the RNA level.

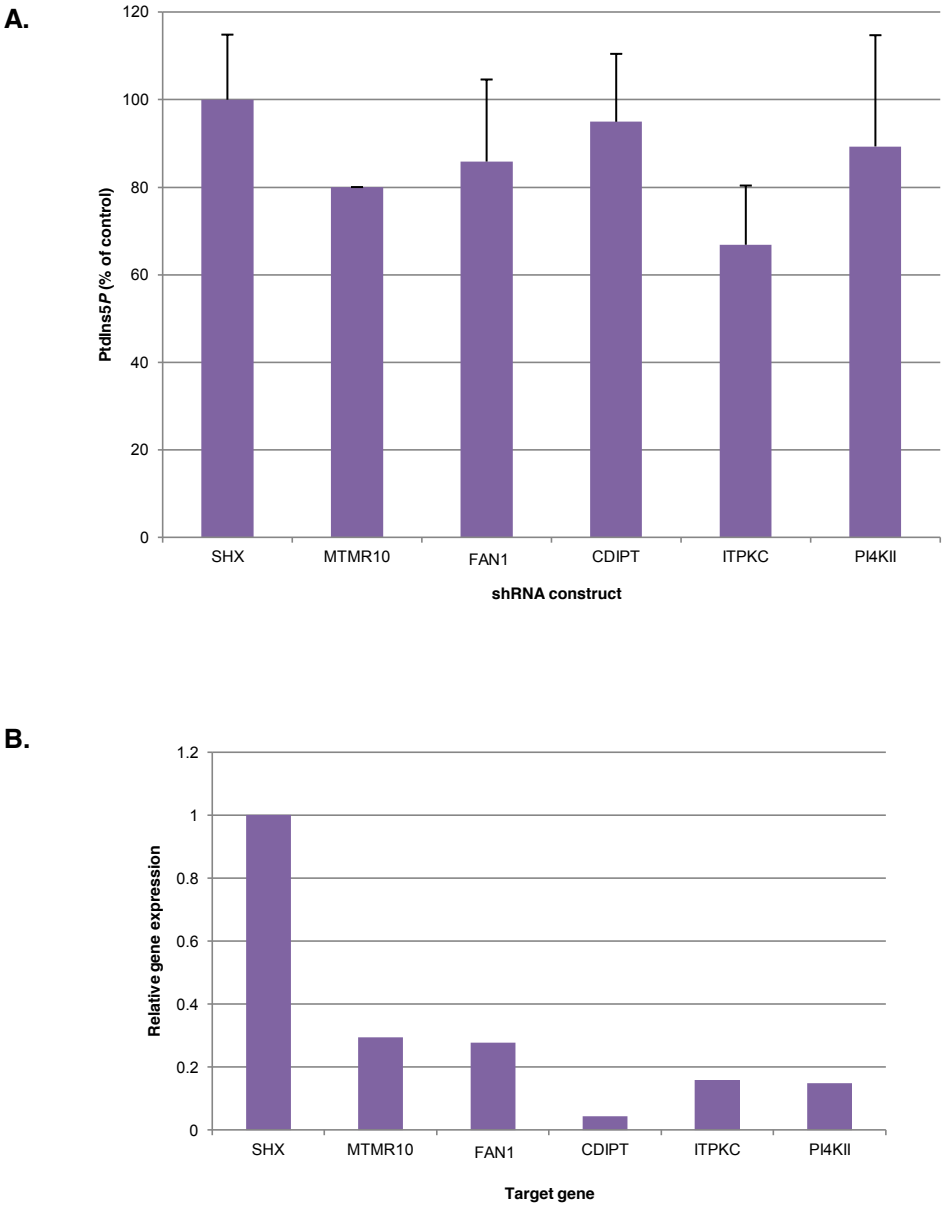


Figure 4.11. Validation of candidate hits using stable cell lines.

A. Stable cells lines were made by transducing U2OS cells with shRNA constructs targeting each of the candidate hits and culturing the cells in selective puromycin-containing medium for a minimum of 14 days. Stably transduced cells were then used in a PtdIns5*P* assay after stimulation with 4mM H_2O_2 for 45 minutes. Data represents the average value plus the standard deviation of a triplicate. **B.** Similarly to Figure 4.10B., qRT-PCR was used to analyse the expression of the indicated genes. The data shows efficient knock down of each candidate gene to a similar extent as transient transduction (Figure 4.10B).

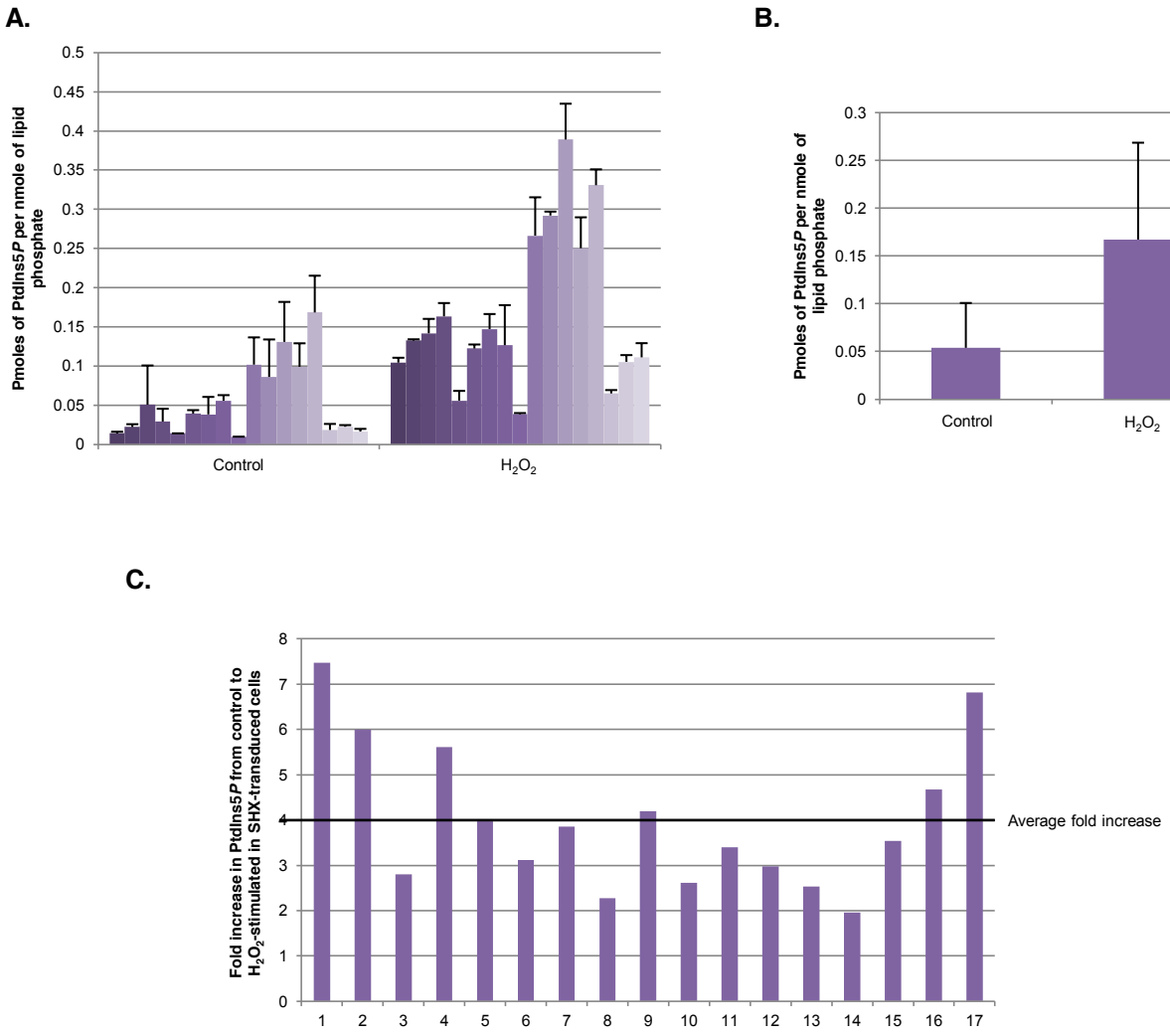


Figure 4.12. Analysis of SHX data from the RNAi screen.

A. The level of PtdIns5*P* in un-stimulated and H_2O_2-treated SHX-transduced cells from all 17 assays of the RNAi screen were directly compared and revealed that there was high variability in the amount of PtdIns5*P* quantified in SHX-transduced cells between each of the 17 assays. Values indicate average values plus the standard deviation from triplicate samples. **B**. The average PtdIns5*P* level in SHX-transduced cells from all 17 assays of the RNAi screen was calculated and reveals that there is a robust increase in PtdIns5*P* in response to oxidative stress, however the large standard deviation shows there is high variability between assays. Values indicate average values plus the standard deviation from 17 sets of triplicate samples **C.** Data shows the fold change of PtdIns5*P* induced by H_2O_2 treatment in SHX-transduced cells from all 17 assays of the RNAi screen. Across all 17 assays the average fold increase in PtdIns5*P* was 4-fold.

5. The Role of PIP4K in the Regulation of PtdIns5*P* and PKB

5.1. Introduction

There is strong evidence to show that PIP4Ks play a role in the negative regulation of PtdIns5*P*. There are three isoforms of PIP4K; PIP4K2A, PIP4K2B and PIP4K2C (also known as PIP4Kα, β and γ) each of which, when knocked down, gave an increase in the level of PtdIns5*P* in the initial RNAi screen (Table 4.1 Figure 4.4). PIP4K2A is known to become localised at the plasma membrane upon tyrosine kinase receptor (TKR) stimulation and its subsequent activation leads to the production of PtdIns(4,5)P_2 and consequently a loss of PtdIns5*P* [42]. PIP4K2B has been implicated in the regulation of a nuclear pool of PtdIns5*P* [148]. When cells are stimulated with UV light, p38 becomes activated and phosphorylates PIP4K2B on Serine 326. This modification inhibits the enzymatic activity of PIP4K2B and therefore allows the level of nuclear PtdIns5*P* to accumulate as PIP4K2B is no longer able to phosphorylate PtdIns5*P* to PtdIns(4,5)P_2. It is interesting to note that PIP4K2B has 2000-fold lower enzymatic activity than PIP4K2A and may act to support the more enzymatically active PIP4K2A [44]. PIP4K2C was the last PIP4K isoform to be discovered and was shown to be the least similar of the PIP4Ks; it has 61.1% sequence homology to PIP4K2A and 63.3% to PIP4K2B whereas between PIP4K2A and PIP4K2B there is 76.6% homology [41]. The exact role of PIP4K2C in the regulation of PtdIns5*P* is still unclear but it has been implicated in the regulation of a smooth endoplasmic reticulum-Golgi pool of PtdIns5*P* [38]. To further investigate the role of PIP4K in the regulation of PtdIns5*P*, stable U2OS cell lines were produced by transducing cells with shRNA constructs targeting all combinations of the three PIP4K isoforms and culturing cells in selective puromycin medium therefore allowing stable integration of the constructs into the genome of the cells.

5.2. Knock Down of PIP4K Isoforms Leads to Enhanced PtdIns5P Production

To evaluate the role of each PIP4K isoform in the regulation of PtdIns5*P*, shRNA was utilised to knock down each isoform individually and in combination so in total seven cell lines were produced. In order to obtain the most efficient knock down in validation experiments, knock down efficiency of each construct was analysed using qRT-PCR and only the two most efficient constructs were pooled and used in the following experiments. Analysis of mRNA expression in each cell line showed that each targeted isoform was expressed at ≤50% compared to SHX-transduced U2OSs (Figure 5.1A).

Observations from this experiment are informative of the significance of each PIP4K isoform in the regulation of PtdIns5*P*. Each cell line with PIP4K2A knocked down, whether alone or in combination with another isoform, showed slightly enhanced basal PtdIns5*P* but more significantly showed enhanced H_2O_2-induced PtdIns5*P* (Figure 5.1B). PIP4K2A is largely cytosolic, one study showed that the distribution is 60% cytosolic, 40% nuclear and that the entire cytosolic pool is associated with membranes [145]. Only knock down of PIP4K2A and not the other isoforms causes enhanced PtdIns5*P* production in response to tyrosine phosphorylation suggesting that PIP4K2A is responsible for regulating the extranuclear pool of PtdIns5*P* [42]. Despite many studies describing PIP4K2A as a predominantly cytosolic enzyme, some evidence does implicate it in the nucleus as PIP4K2B can heterodimerise with PIP4K2A and transport the enzymatically active PIP4K2A to the nucleus [44].

Knock down of PIP4K2B alone also caused an increase in PtdIns5*P* production and the additive effect of knocking down both PIP4K2A and PIP4K2B together was shown to be statistically significant when compared to SHX-transduced cells using a Student's t-test (Figure 5.1B). This suggests that PIP4K2B has a direct role in the regulation of PtdIns5*P*. If PIP4K2B acted simply as a molecular chaperone to direct the localisation of PIP4K2A, there would not be an additive effect of knocking down both isoforms compared to knocking down either PIP4K2A alone or PIP4K2B alone. It is possible that PIP4K2B provides most of the nuclear PtdIns5*P*-4 kinase activity as it is mainly localised in the nucleus [146] due to the presence of an alpha helix structure which is specific to PIP4K2B [147]. Furthermore, studies have demonstrated a number of roles for PIP4K2B in the regulation of nuclear PtdIns5*P* [148] [149]. However, like PIP4K2A, the localisation of PIP4K2B is not exclusive to a single cellular compartment. In fact, PIP4K2B was initially discovered due to its interaction with p55 TNF receptor at the plasma membrane [150]. Clearly the roles of PIP4K2A and PIP4K2B are not entirely independent but both isoforms exert an independent effect on the level of PtdIns5*P* due to the additive effect of knocking down both isoforms. PIP4K2C and PIP4K2B/PIP4K2C

cell lines showed no significant change in the level of PtdIns5*P* despite PIP4K2C knock down giving the largest increase in the initial screen (Figure 5.1B). Unlike PIP4K2A and PIP4K2B, there were only two shRNA constructs in the library that targeted PIP4K2C as both of these constructs had been validated therefore it is difficult to understand how in the initial experiment knock down of PIP4K2C led to such a dramatic up-regulation of PtdIns5*P* which could not be repeated in two validation experiments. Like PIP4K2B, PIP4K2C is able to interact with PIP4K2A [45] and like PIP4K2B, it has been suggested that PIP4K2C directs the localisation of PIP4K2A to specific cellular locations. This is unlikely to be relevant during H_2O_2-induced PtdIns5*P* production as double knock down of both PIP4K2B and PIP4K2C had no effect on the PtdIns5*P* level (Figure 5.1B). If either isoform was required to guide PIP4K2A it would be expected that knock down of both would lead to an up-regulation of PtdIns5*P*. Knock down of PIP4K2A and PIP4K2C led to approximately the same increase in PtdIns5*P* as knock down of PIP4K2A alone suggesting that PIP4K2C does not strongly contribute to the regulation of PtdIns5*P* in response to oxidative stress.

The triple knock down cell line (termed 'ABG') had the greatest effect causing a 2-fold increase in H_2O_2-induced PtdIns5*P* (Figure 5.1B). The simplest interpretation of all the data is that each isoform regulates an independent pool of cellular PtdIns5*P* therefore knock down of all isoforms will cause an increase in PtdIns5*P* in numerous cellular locations and the global level will significantly increase. It should be noted that in ABG cells the knock down of PIP4K2A was much less efficient than in the PIP4K2A knock down cells. In following experiments knock down of all three isoforms was used to further elucidate the role of PIP4K in the regulation of H_2O_2-induced PtdIns5*P*.

5.3. Investigating the Regulation of PKB Activation by PIP4K

5.3.1. Introduction

PKB (also known as Akt) was originally discovered as an acquired cellular oncogene in the genome of the AKT8 transforming retrovirus [69]. Since its discovery, thousands of papers describing the regulation, downstream targets and functions of PKB have been published. PKB is activated downstream of PI3K signalling. In response to various extracellular signals, PKB translocates to the plasma membrane [151] due to recruitment by PtdIns(3,4)P_2 [72] and PtdIns(3,4,5)P_3 [71] via the PH domain of PKB. At the plasma membrane, phosphorylation at two residues is required for full activation; Threonine 308 (Thr308) in the activation loop which is phosphorylated by PDK1 [73] and Serine 473 (Ser473) in the Cterminal hydrophobic region which is phosphorylated by mTORC2 [74]. Detection of phosphorylation at these two residues is commonly used as a measure of PKB activation. Fully activated PKB can initiate pro-survival signalling and cell growth and proliferation by

phosphorylating a plethora of downstream targets. Most known targets of PKB are negatively regulated such as the pro-apoptotic protein BAD [80] and glycogen synthase kinase-3 (GSK-3) [81] and inactivation of these targets leads to enhancement of cell growth and survival. The regulation of PKB is frequently altered in many types of cancer leading to enhanced cell growth, proliferation and survival. This mis-regulation can occur upstream of PKB through expression of a constitutively activated PI3K or loss of PTEN activity both of which cause increased levels of PtdIns(3,4,5)P_3. Much research effort has been focused on understanding the mechanisms involved in PKB signalling in an attempt to develop inhibitors that could potentially be used clinically to treat cancer.

PtdIns5*P* has been implicated in the regulation of PKB signalling by several different groups. PIP4K2B$^{-/-}$ mice are hypersensitive to insulin and display enhanced PKB phosphorylation [102] suggesting that PIP4K2B negatively regulates insulin signalling and activation of PKB possibly through removal of PtdIns5*P*. IpgD is a 4-phosphatase that produces PtdIns5*P* from PtdIns(4,5)P_2 at the plasma membrane and is utilised by *Salmonella flexneri* for invasion into host cells. Expression of IpgD increases the level of PtdIns5*P* in the plasma membrane which co-localises with phosphorylated PKB [164]. In this paper, increased PtdIns5*P* was suggested to attenuate PKB de-phosphorylation and to induce ligand independent activation of the EGF receptor both of which would lead to enhanced PKB activation. PtdIns5*P* has also been shown to induce accumulation of active EGFR in early endosomes which leads to maintained PKB activation [36]. Another study showed that activation of the PtdIns5*P* pathway using PIP4K over-expression in cells reduces the production of PtdIns(3,4,5)P_3 in response to insulin and therefore causes reduced PKB activation. The authors hypothesised that PtdIns5*P* might regulate the de-phosphorylation of PtdIns(3,4,5)P_3 [155]. PtdIns5*P* has also been implicated directly in the regulation of PKB phosphorylation as PtdIns5*P*dependent negative phosphorylation of PP2A prevents de-phosphorylation of PKB [103]. Finally, knock down of PIP4K2A and PIP4K2B in the BT474 cell line leads to enhanced PKB activation and an associated increase in ROS and cellular senescence however this was shown to be PI3K independent [68]. Clearly the exact mechanism is still unclear but there is substantial evidence implicating PtdIns5*P* and PIP4K in the regulation of PKB signalling.

5.3.2. Optimisation of Conditions for Protein Detection of Phosphorylated PKB

H_2O_2 is able to robustly increase the cellular level of PtdIns5*P* and is also known to cause PKB activation and was therefore an appropriate agonist to study the role of PtdIns5*P* in the regulation of PKB. In order to ascertain optimum conditions for the detection of PKB phosphorylation U2OS cells were plated at various densities; 50,000, 75,000 or 100,000 cells per well in a 12-well plate, and were treated with either 500μM or 1mM H_2O_2. Analysis of Thr308 by western blotting showed

that maximum phosphorylation was obtained by plating 100,000 cells and treating with 1mM H_2O_2, a concentration of H_2O_2 commonly used in studies on the cell signalling functions of H_2O_2 (Figure 5.2). These conditions were therefore chosen for future experiments.

5.3.3. Knock Down of PIP4K Leads to Enhanced PKB Activation in Response to Oxidative Stress

As described, knock down of all three isoforms of PIP4K leads to increased PtdIns5*P* production therefore triple knock down of all PIP4K isoforms was used to manipulate the level of PtdIns5*P* and allow analysis of the effect of PtdIns5*P* on PKB activation. Initial experiments utilised siRNA constructs targeting each isoform and quantification of RNA by qRT-PCR confirmed that each isoform was knocked down by ≥60% (Figure 5.3A). In nontargeting siRNA control transfected U2OS cells (control), treatment with H_2O_2 led to phosphorylation of PKB at both Thr308 and Ser473 after 5 minutes which peaked at 15 minutes and steadily decreased thereafter (Figure 5.3B). In ABG cells phosphorylation of both Thr308 and Ser473 was enhanced after 5 minutes of treatment compared to control cells and increased phosphorylation was evident at 45 minutes in ABG cells compared to control cells (Figure 5.3B). This experiment was repeated using shRNA constructs targeting each PIP4K isoform and similar results were obtained (data not shown). The use of two different RNAi technologies gives confidence that the results obtained are not due to offtarget effects.

In experiments utilising both shRNA and siRNA it is interesting to note that the effect of PIP4K knock down on PKB phosphorylation is most apparent early and late in the time course (5 minutes and onwards from 45 minutes) and maximal phosphorylation at 15 minutes is less affected by knock down of PIP4K. This suggests that PIP4K may suppress phosphorylation of PKB initially and may enhance de-phosphorylation of PKB in response to longer durations of H_2O_2 stimulation. Additionally, in both sets of experiments the effect on the phosphorylation of Thr308 was greater than Ser473 in PIP4K knock down cells. Often, only one of these two residues is analysed as a read-out of PKB activation as it is assumed that phosphorylation of each residue is interdependent. However, as mentioned, each residue is phosphorylated by a specific kinase so each phosphorylation is an independent event. Furthermore, evidence points to differential phosphorylation of Thr308 and Ser473 being responsible for the specificity of downstream target substrates. For example genetic knockdown of ER-stress induced PKB-binding protein GRP78 increases phosphorylation of Ser473 without affecting Thr308 thereby increasing the ratio of Ser473/Thr308 which results in altered PKB substrate specificity [152]. In proceeding experiments the phosphorylation of both residues were considered and carefully analysed.

Insulin is known to elicit a comparable signalling response to H_2O_2, although insulin does not induce changes in the level of PtdIns5*P* in U2OS cells. Experiments to analyse insulininduced activation of PKB in cells with PIP4K isoforms knocked down by siRNA or shRNA were conducted. As expected, treatment of cells with insulin led to phosphorylation of PKB at Thr308 and Ser473 but there was no reproducible difference between control cells and ABG cells knocked down by siRNA (Figure 5.4B) or shRNA (data not shown). Despite H_2O_2 being described as an insulinmimetic [153] there is much conflicting data which suggests that H_2O_2 can in fact inhibit insulin signalling [154] [108] which may explain why H_2O_2 and insulin treatment lead to different effects in this cellular setting. Furthermore, H_2O_2 causes a robust increase in the level of PtdIns5*P* whereas the effect of insulin on PtdIns5*P* production is minimal (Figure 3.2B). Clearly in U2OS cells the signalling cascades activated in response to H_2O_2 and insulin are significantly different and this may be due to the differential production of PtdIns5*P* which consequently leads to activation of specific downstream signalling molecules such as PKB.

5.3.4. Knock Down of PIP4K Isoforms Leads to Differential Effects on the Phosphorylation of PKB in Response to Oxidative Stress

To determine the involvement of each PIP4K isoform in the regulation of PKB, each isoform was genetically knocked down individually and in combination by shRNA, similarly to the experiment described in Figure 5.1. Analysis by qRT-PCR showed that knock down of each targeted isoform was efficient as RNA expression was reduced by ≥80% compared to SHXtransduced cells (Figure 5.5A). As expected, H_2O_2 treatment induced phosphorylation of Thr308 and Ser473 in control SHX-transduced cells after just 5 minutes, the peak of activation was at 20 minutes and phosphorylation of both residues returned to the basal state by 60 minutes (Figure 5.5B).

Knock down of each PIP4K isoform individually and in combination showed marked differences in the ability of each isoform to regulate the phosphorylation of PKB. At 40 minutes of H_2O_2 treatment all combinations of PIP4K knock down caused enhanced phosphorylation of Thr308 and to a lesser extent Ser473 with the exception of PIP4K2B knock down alone. This is surprising as knock down of PIP4K2B led to increased production of PtdIns5*P* in response to 45 minutes of H_2O_2 treatment (Figure 5.1B) and if an increase in PtdIns5*P* is predicted to enhance PKB phosphorylation knock down of PIP4K2B would be expected to cause enhanced phosphorylation of PKB. However, if PIP4K2B regulates only a nuclear rather than plasma membrane associated pool of PtdIns5*P* then knock down of PIP4K2B may not influence PKB activation.

All other combinations of PIP4K knock down enhanced phosphorylation of PKB to a similar extent at the peak of PKB phosphorylation (20 minutes) suggesting that PIP4K may be responsible for suppressing further phosphorylation of PKB when it is already highly activated. It is difficult to establish the exact contribution of each isoform as for example knock down of PIP4K2A alone and PIP4K2C alone caused enhanced PKB phosphorylation yet knock down of both these isoforms together did not have an additive effect. This could be due to PKB being maximally activated in PIP4K2A or PIP4K2C knock down cells. In order to address this hypothesis, further experiments analysing PKB phosphorylation in PIP4K knock down cells in response to lower doses of H_2O_2 would be required. Initial experiments analysing the phosphorylation of PKB in PIP4K2A knock down and ABG cells in response to 500μM and 1mM H_2O_2 indicate that knock down of all three PIP4K isoforms does not cause an additive effect compared to knock down of PIP4K2A alone when cells are treated with 500μM H_2O_2 (data not shown).

At 40 and 60 minutes of H_2O_2 treatment the level of PKB phosphorylation in SHX-transduced cells was largely returned to basal levels whereas all knock down samples had maintained maximal PKB phosphorylation except PIP4K2B knock down alone. This suggests that not only is PIP4K likely to have a role in suppressing further phosphorylation of PKB when it is already highly activated but may also positively regulate the de-phosphorylation of PKB. Noticeably each genetic background that included knock down of PIP4K2A showed enhanced PKB phosphorylation (A, AB, AG, ABG) (Figure 5.5B). The plasma membrane localisation of PIP4K2A is likely to be of great significance as several groups have shown that PtdIns5*P* produced at the plasma membrane can regulate the activation of PKB [155] [36]. Furthermore, in response to 60 minutes treatment in PIP4K2A knock down, AG and ABG cells, PKB is activated to a comparable extent suggesting that loss of PIP4K2A is exerting the effect. All of these data strongly points to PIP4K2A being a major regulator of H_2O_2-induced PKB activation. Similarly to the experiment described in Figure 5.3, the effect of PIP4K knock down on the phosphorylation of Thr308 was greater than Ser473 at each time point analysed. This further suggests that each residue is independently phosphorylated however PIP4K clearly controls both phosphorylation sites which suggests that regulation is likely to occur through a common upstream regulator.

In order to demonstrate that increased phosphorylation of PKB, induced by PIP4K knock down, results in increased activation of PKB, the phosphorylation of a known PKB downstream target was assessed. The Proline-Rich Akt-Substrate of 40kDa (PRAS40) was initially isolated as a 14-3-3 binding protein and later shown to be a component of the mTORC1 complex. Although PRAS40 could be phosphorylated by mTORC1 at Thr246 [156], mouse embryonic fibroblasts that lack Akt1 and Akt2 have 70% less PDGF-stimulated Thr246 phosphorylation suggesting that PKB is the major

in vivo kinase [157]. Phosphorylation of PRAS40 was induced concomitantly with the induction of PKB phosphorylation (Figure 5.5B), although its phosphorylation more closely resembled the phosphorylation of Ser473 rather than Thr308. This may suggest that phosphorylation of Thr308 and Ser473 can each direct the substrate specificity of PKB or that both resides need to be phosphorylated for PKB to being fully active [73].

The main conclusion of this part of the project is that out of the three PIP4K isoforms, PIP4K2A is likely to be the major isoform involved in the regulation of H_2O_2-induced PKB activation. Considering the predicted role for PtdIns5*P* in PKB activation, the predominant localisation of PIP4K2A at the plasma membrane and its high enzymatic activity towards PtdIns5*P*, PIP4K2A is a good candidate based on previous data. Therefore, future experiments aimed to elucidate how PIP4K2A regulates PKB activation through the regulation of PtdIns5*P*.

5.4. Investigating the Role of PIP4K2A in the Regulation of PKB Activation

5.4.1. Knock Down of PIP4K2A by siRNA Leads to Enhanced PtdIns5P Production and Activation of PKB in Response to Oxidative Stress

In order to obtain the most consistent knock down possible from experiment to experiment siRNA was utilised. Production of lentiviral particles to transduce cells with shRNA often leads to varying viral titres which can affect the extent of knock down in target cells. shRNAmediated knock down of PIP4K2A also led to variable increases in the expression of PIP4K2B and PIP4K2C. However these increases did not correlate with the extent of PIP4K2A knockdown as assessed by qRT-PCR. On the other hand siRNA transfection involves addition of the same volume and concentration of constructs in each experiment therefore promoting more consistent knock down. PIP4K2A was knocked down using siRNA and the mRNA levels of the three different isoforms were assessed by qRT-PCR. The expression of PIP4K2A was reduced by 60%, while the levels of PIP4K2B and PIP4K2C were slightly elevated (Figure 5.6A). While the increase in PIP42B and PIP4K2C might lead to a compensatory increase in PIP4K activity, as illustrated in Figure 5.5B, PKB activation was comparable in PIP4K2A single knockdown cells and in ABG knockdown cells.

Similarly to shRNA-mediated knock down of PIP4K2A (Figure 5.5), siRNA-mediated knock down also caused enhanced phosphorylation of Thr308 and Ser473, particularly before and after maximal PKB phosphorylation (5 minutes and 30 minutes onwards) again suggesting that PIP4K2A may suppress the initial phosphorylation and/or positively regulate the dephosphorylation of PKB. Also similarly to shRNA experiments, siRNA-mediated loss of PIP4K2A had a greater impact on the phosphorylation

of Thr308 than Ser473. The reproducibility between two different knock down technologies strongly implies that the observed phenotypes are the result of on-target knock down.

Previous experiments to quantify the level of PtdIns5*P* evaluated the effect of knocking down all combinations of PIP4K isoforms in response to a 45 minute H_2O_2 treatment (Figure 5.1) so to assess the effect of PIP4K2A knock down in more detail PIP4K2A knock down cells were treated with H_2O_2 for various amounts of time and PtdIns5*P* was quantified. In response to 15 minutes of H_2O_2 treatment the level of PtdIns5*P* was not different between PIP4K2A knock down and control cells (Figure 5.6C). However, there was a 3.4-fold increase in PtdIns5*P* after 60 minutes which was sustained for 2 hours of H_2O_2 treatment. This again highlights the problem of selecting a single time point for analysis as only quantifying PtdIns5*P* produced in response to 45 minutes of H_2O_2 treatment showed that knock down of PIP4K2A had minimal effect yet a more detailed time course has revealed that PIP4K2A knock down has a dramatic effect on the production of PtdIns5*P* at slightly later time points. The increase in PtdIns5*P* especially at later time points correlates with increased PKB activation observed with knockdown of PIP4K2A suggesting that PtdIns5*P* might impact directly on PKB activation or its maintenance.

We were unable to measure differences in PtdIns5*P* at the early time point of H_2O_2 stimulation (5 minutes) in PIP4K2A knockdown cells although we observed enhanced PKB activation at this time point. This may reflect the inability to measure low levels of PtdIns5*P* accurately however we were able to measure differences in the basal levels of PtdIns5*P* before stimulation. More likely H_2O_2 induces early changes in PtdIns5*P* that are not regulated by PIP4K2A which might be present in different sub-cellular compartments.

5.4.2. Over-expression of PIP4K2A Leads to Suppressed PtdIns5P Production and PKB Phosphorylation in Response to Oxidative Stress

In order to further investigate if the level of PtdIns5*P* impacts upon the regulation of PKB activation, U2OS cells stably expressing a doxycycline-inducible Myc-tagged PIP4K2A were generated to suppress the increase in PtdIns5*P* in response to oxidative stress. Doxycycline induction of Myc-PIP4K2A resulted in a time-dependent increase in the expression and activity of Myc-PIP4K2A (Figure 5.7A, experiment performed by Dr. David Jones). Based on optimisation experiments, doxycycline was incubated with cells for 18 hours to induce significant expression of Myc-PIP4K2A. PtdIns5*P* levels were assessed in the presence and absence of doxycycline to determine if over-expression of PIP4K2A could suppress of H_2O_2induced PtdIns5*P* generation. At all time points

assessed PIP4K2A expression suppressed PtdIns5*P* although it did not completely prevent an increase in PtdIns5*P* in response to H_2O_2.

As Myc-PIP4K2A suppressed H_2O_2-induced PtdIns5*P* (Figure 5.7B) the phosphorylation status of PKB in doxycycline treated Myc-PIP4K2A expressing cells (Myc-PIP4K2A cells) was examined. As expected, in control cells phosphorylation of both Thr308 and Ser473 was induced upon H_2O_2 treatment and at each time point examined, phosphorylation of both Thr308 and Ser473 was attenuated in cells expressing Myc-PIP4K2A (Figure 5.7B). Interestingly, phosphorylation of p38, ERK and PTEN was not affected by the expression of Myc-PIP4K2A suggesting that the effect of PIP4K2A over-expression does not generally affect pathways that are activated by H_2O_2 but may specifically regulate the activation of PKB. Over-expression of PIP4K2A appeared to suppress both Ser473 and Thr308 phosphorylation to approximately the same extent, which is slightly different to the results obtained with knockdown of PIP4K2A. This might reflect the possibility that over-expression of PIP4K2A can impact on all pools of PtdIns5*P*, whereas knock down will be limited to the pool of PtdIns5*P* that is endogenously regulated by PIP4K2A. These data strongly suggest that changes in PtdIns5*P* induced by oxidative stress impact on PKB activation.

5.4.3. Knock Down of PIP4K2A Leads to Enhanced Production of Both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in Response to Oxidative Stress

As differential expression of PIP4K2A affects the phosphorylation of both Thr308 and Ser473 it is likely that a common upstream activator is regulated. Both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 can recruit PKB to the plasma membrane leading to its activation [72] [80, 158]. Previous work using U2OS cells stably expressing doxycycline-inducible Myc-tagged PIP4K2A demonstrated that in response to H_2O_2 treatment Myc-PIP4K2A cells have significantly reduced PtdIns4*P* and PtdIns(3,4)P_2 as well as reduced PtdIns5*P* [11]. Suppression of PtdIns(3,4)P_2 production may explain the attenuation of PKB phosphorylation in Myc-PIP4K2A expressing cells as less PKB can be recruited to the plasma membrane and consequently the phosphorylation of Thr308 and Ser473 is reduced. Based on this it was hypothesised that knock down of PIP4K2A would cause an increase in the level of H_2O_2-induced PtdIns(3,4)P_2.

To test this hypothesis siRNA was used to knock down PIP4K2A in U2OS cells which were then radio-labelled with ^{32}P-orthophosphate and treated with 1mM H_2O_2 for various times. Due to the much larger abundance of PtdIns(4,5)P_2 and the isomeric nature of PtdIns(3,4)P_2 it cannot easily be quantified using mass spectrometry. Radio-labelled phosphoinositides were extracted, deacylated to obtain the head groups which were then analysed by High Pressure Liquid Chromatography (HPLC).

Quantification of PtdIns(3,4)P_2 revealed a 2-fold increase in PIP4K2A knock down cells in response to 20 and 60 minutes H_2O_2 treatment (Figure 5.8A). In basal conditions and in response to 5 minutes H_2O_2 treatment there was no difference between control and PIP4K2A knock down cells (Figure 5.8A). This is an important observation as enhanced PKB phosphorylation in PIP4K2A knock down cells was particularly apparent after maximal PKB (Figure 5.6B). Enhancement of PtdIns(3,4)P_2 production could therefore be responsible for prolonging the phosphorylation of PKB. However, the phosphorylation of PKB was also enhanced in response to 5 minutes H_2O_2 treatment in PIP4K2A knock down cells although the level of PtdIns(3,4)P_2 was not affected by PIP4K2A knock down at this time point.

Recruitment and activation of PKB can also be mediated by PtdIns(3,4,5)P_3 [71] and PtdIns(3,4,5)P_3 is thought to be acutely produced in response to PI3K activation. It was therefore hypothesised that knock down of PIP4K2A could cause enhanced production of PtdIns(3,4,5)P_3 in response to acute H_2O_2 treatment. Attempts to quantify PtdIns(3,4,5)P_3 using HPLC were unsuccessful therefore to evaluate the level of PtdIns(3,4,5)P_3 in PIP4K2A knock down cells extracted lipid samples were analysed using Mass Spectrometry (MS protocol performed by Karen Anderson). In basal conditions there was a small increase in the level of PtdIns(3,4,5)P_3 in PIP4K2A knock down cells but after 5 minutes of H_2O_2 treatment there was a significant increase in the level of PtdIns(3,4,5)P_3 in PIP4K2A knock down cells of almost 2-fold. In response to 20, 40 and 60 minutes of H_2O_2 treatment there was no difference between control-transfected cells and PIP4K2A knock down cells (Figure 5.8B). This data strongly implicates PIP4K2A in the regulation of two PKB-recruiting lipids, PtdIns(3,4,5)P_3 in response to acute H_2O_2 stimulation and PtdIns(3,4)P_2 in response to longer term stimulation which consequently allows regulation of PKB phosphorylation in response to both acute and longer term H_2O_2 treatment.

5.5. Knock Down of PIP4K Isoforms Causes Reduced Growth Capability

PKB has long been recognised as a potent activator of cell growth, division and survival [69] however recent work has shown that hyperactivated PKB can also induce premature cell senescence [159]. To investigate the effect of knocking down PIP4K on cell growth capability, stable cell lines with each isoform knocked down individually and in combination were produced. Analysis by qRT-PCR demonstrated that targeted genes were knocked down by at ≥95% and similarly to the experiment described in Figure 5.6, non-targeted isoforms were up-regulated by up to 70% (Figure 5.9A). Growth capability was analysed by; propidium iodide based cell cycle analysis (Figure 5.9B), anchorage dependent clonogenic growth assays (Figure 5.9C) and cell growth assays (Figure 5.9D). Each analytical method showed that, with the exception of PIP4K2B knock down alone, knock down of

each PIP4K isoform individually or in combination induced G1 growth arrest, reduced clonogenic potential and suppressed cell growth (Figure 5.9B, C and D and respectively).

The reduced growth capability of U2OS cells lacking PIP4K could be explained by the presence of hyperactivated PKB that has been shown to induce premature senescence in response to oncogenic and ROS-mediated apoptosis partly by increasing intracellular ROS [159]. A recent study showed that knocking down both PIP4K2A and PIP4K2B in the breast cancer cell line BT474 caused reduced cell growth and induced cellular senescence, cells also had increased ROS and displayed elevated Ser473 but treatment with the PI3K inhibitor GDC0941 did not rescue these effects, suggesting that PIP4K-mediated growth deficiency is not dependent on PKB [68].

5.6. Conclusions

Knock down experiments have demonstrated that PIP4Ks are endogenous negative regulators of H_2O_2-induced PtdIns5*P* production. Of the three isoforms, PIP4K2A was shown to have a particularly important role as knock down of this isoform alone led to enhanced PtdIns5*P* production and over-expression of PIP4K2A suppressed the production of PtdIns5*P* in response to H_2O_2. PtdIns5*P* has been implicated in the regulation of PKB activation and experiments utilising genetic manipulation of PIP4K2A in order to adjust the cellular level of PtdIns5*P* has added weight to this proposal. Knock down of PIP4K2A led to enhanced PKB phosphorylation at both Thr308 and Ser473. Conversely, over-expression of PIP4K2A led to suppression of PKB phosphorylation at both Thr308 and Ser473. Knock down of PIP4K2A caused greater enhancement of Thr308 phosphorylation compared to Ser473 suggesting that the phosphorylation of each residue may be independently regulated although importantly both were regulated by PIP4K2A knock down suggesting that PIP4K2A regulates a common regulator of both sites of phosphorylation.

A regulator of PKB that has previously been linked to PtdIns5*P* is the phosphatase PP2A that can de-phosphorylate PKB at both Thr308 and Ser473 [160, 161]. PP2A was shown to be inhibited due to a PtdIns5*P*-dependent phosphorylation which allows accumulation of phosphorylated PKB [103]. However this is unlikely to explain how PKB becomes phosphorylated more rapidly in response to acute H_2O_2 stimulation in PIP4K2A knock down cells. Other possible upstream regulators of PKB are the lipids PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 which are both able to recruit PKB to the plasma membrane. PtdIns5*P* has been implicated in maintaining activation of PKB signalling through the negative regulation of a PtdIns(3,4,5)P_3 phosphatase [155]. Quantification of PtdIns(3,4,5)P_3 in PIP4K2A knock down cells showed that in basal conditions there was an increase in the level of PtdIns(3,4,5)P_3 but after 5 minutes of H_2O_2 treatment there was a significant increase of almost 2-

fold. Additionally, quantification of PtdIns(3,4)P_2 revealed a 2-fold increase in PIP4K2A knock down cells in response to 20 and 60 minutes H_2O_2 treatment. PIP4K2A-mediated regulation of these two lipids neatly corresponds with the observed enhancement of PKB phosphorylation in response to acute and long term H_2O_2 treatment.

An interesting hypothesis to explain how PIP4K2A through PtdIns5*P* can regulate the availability of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 is by regulation of growth factor receptors. PtdIns5*P* induced by the bacterial phosphatase IpgD has been shown to cause accumulation of ligand independent activated EGFR in early endosomes and prevention of its degradation which consequently leads to prolonged EGF signalling and PKB activation [36]. The idea that PtdIns5*P* acts as a mediator of receptor signalling fits with data obtained in the inhibitor screen as inhibition of a number of signalling pathways changed the level of H_2O_2-induced PtdIns5*P* by 10-40% (Table 3.1) suggesting that multiple signalling cascades impinge upon PtdIns5*P* synthesis and inhibition of individual signalling molecules will not fully abrogate production of PtdIns5*P* in response to H_2O_2 stimulation. Many activated growth receptors have docking sites for downstream signal transducers such as PI3K including the insulin receptor [162] which places PI3K at the plasma membrane and in proximity of its substrate, PtdIns(4,5)P_2. It is possible that PtdIns5*P* is employed by the cell at the plasma membrane to co-ordinate the activation of growth factor receptors and resultant recruitment and activation of PI3K.

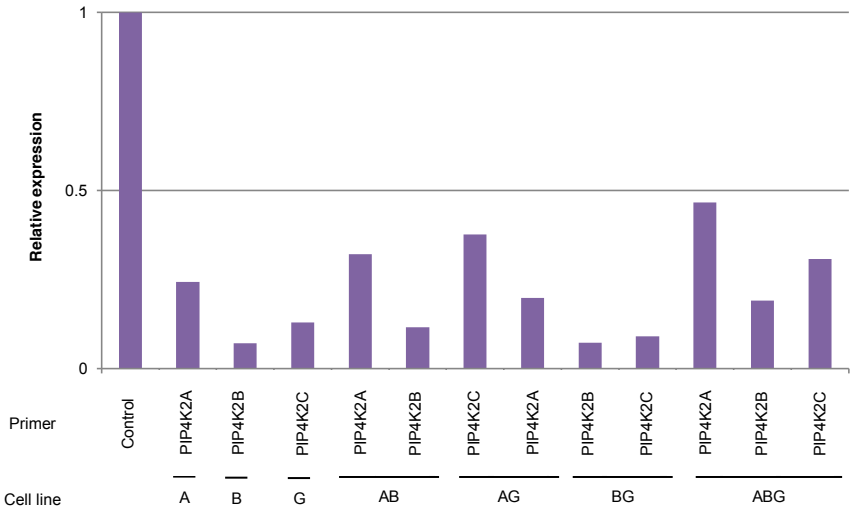
Enhanced activation of PKB is often associated with cell growth and survival yet PIP4K knock down cells displayed growth deficient phenotypes, similarly to another study that demonstrated that although knock down of PIP4K2A and PIP4K2B leads to enhanced activation of PKB cells lacking PIP4K2A and PIP4K2B display reduced cell growth [68]. In that instance the growth defect appeared to be independent of increased PKB activation however whether the growth defect is dependent on PtdIns5*P* accumulation is not yet clear.

Understanding the relationship between growth control and PtdIns(3,4,5)P_3/PKB activation when PIP4K2A is knocked down is of utmost importance. Inhibition of PIP4K activity has been shown to reduce tumour incidence in mice that lack p53 and inhibitors of PIP4K might therefore be useful to specifically inhibit the growth of p53 null tumour cells. However if growth suppression and PKB activation induced by PIP4K knock down are dependent on separate pathways then once tumour cells bypass the growth control defect they would be presented with unfettered PtdIns(3,4,5)P_3/PKB activation to stimulate cell proliferation. Understanding how PIP4K regulates both processes might enable specific inhibition of growth without inducing PtdIns(3,4,5)P_3 activation. With respect to PIP4K inhibition and tumour growth Emerling et al showed that inhibition of both PIP4K2A and PIP4K2B was

required in order to attenuate growth of breast cancer cells. In this study only inhibition of PIP4K2A was required. These observations are important as previous studies from our laboratory and from others [163] have shown that inhibition of PIP4K2B leads to the suppression of the expression of the tumour suppressor protein E-cadherin. Suppression of E-cadherin levels is strongly associated with enhanced metastatic characteristics of tumour cells. Studies in our laboratory strongly showed, in leukaemia cells and in 16 other tumour cells lines (including the one used in the Emerling study), that knockdown of only PIP4K2A strongly suppresses tumour cell growth with little or no additive effect by knockdown of PIP4K2B [43]. These data suggest that exploiting isoform specific inhibitors of PIP4Ks will likely be more useful than pan specific inhibitors.

Although the precise mechanism of action remains unclear, this work has demonstrated a role for PIP4K2A-regulated PtdIns5*P* in H_2O_2-induced PKB activation. Studies to determine how this might occur suggest that at early time points of stimulation there is an increase in PtdIns(3,4,5)P_3, while at later time points there is an increase in PtdIns(3,4)P_2. Both of these lipids are well established upstream regulators of PKB activation. Interestingly, although we were unable to demonstrate an increase in PtdIns5*P* production when cells were stimulated with insulin, we did observe a two-fold increase in PtdIns(3,4,5)P_3 formation in cells with knocked down PIP4K2A (Figure 5.10). Surprisingly, we were unable to demonstrate an increase in insulin-induced PKB activation in PIP4K2A knock down cells compared to control cells. It may be that in PIP4K2A knockdown cells insulin can now induce a PtdIns5*P* response and the inability to measure changes in PKB activation might reflect the maximal-like doses of insulin used in this study. Nevertheless these data show that PIP4K2A is a strong regulator of PtdIns(3,4,5)P_3 formation in cells. In fact the increase in insulin induced PtdIns(3,4,5)P_3 in PIP4K2A knock down cells was comparable to that observed when PTEN was knockdown (see Chapter 6). How exactly knockdown of PIP4K2A impacts on PtdIns(3,4,5)P_3 levels is not clear. It might impact on synthesis or attenuate degradation perhaps by inhibiting PTEN activity. This was further studied in the next chapter.

A.



Cell line	PIP4K isoforms knocked down
A	PIP4K2A
B	PIP4K2B
G	PIP4K2C
AB	PIP4K2B PIP4K2B
AG	PIP4K2A PIP4K2C
BG	PIP4K2B PIP4K2C
ABG	PIP4K2A PIP4K2B PIP4K2C

B.

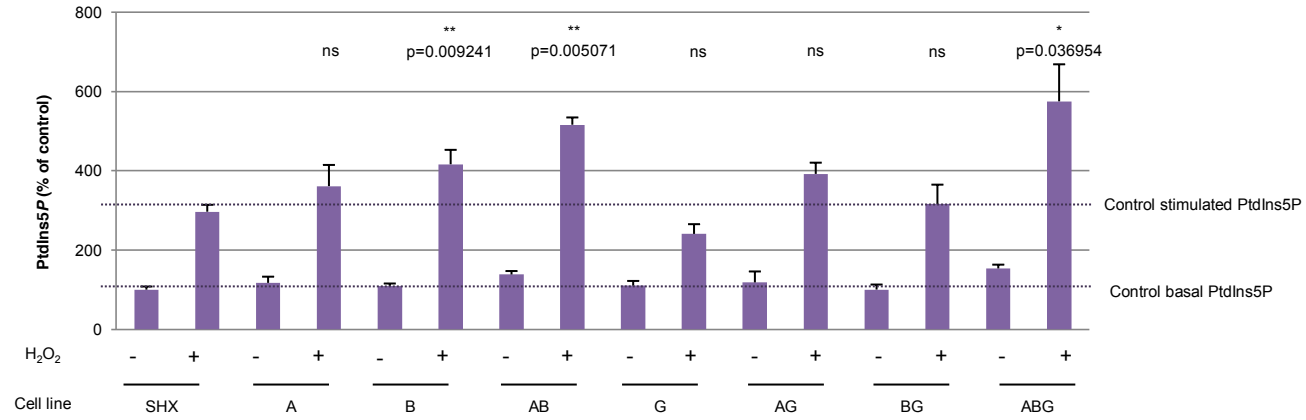


Figure 5.1. Knock down of PIP4K isoforms leads to enhanced PtdIns5*P* production in response to H_2O_2. **A.** q RT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K isoforms as described in the table. **B.** Control cells or cells with knocked down isoforms of PIP4K (as described in the table) were maintained as controls or treated with 1mM H_2O_2. Lipids were extracted and PtdIns5*P* was quantified and showed that loss of PIP4K isoforms causes enhanced production of PtdIns5*P* in response to 45 minutes of oxidative stress treatment. Each bar represents the average plus the standard deviation of a triplicate. A paired student's t-test was used to determine significance compared to SHX-transduced H_2O_2-stimulated control.

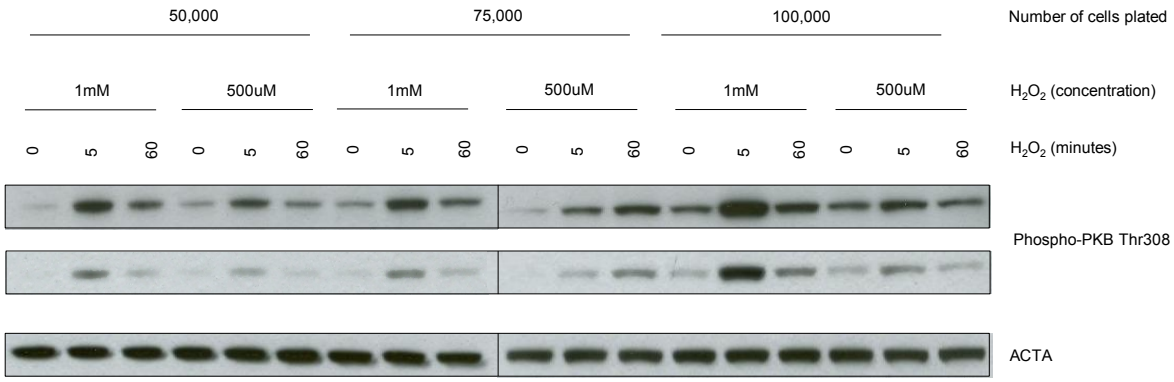


Figure 5.2. Optimisation of conditions for detection of phosphorylated PKB.

U2OS cells were plated at different densities and stimulated for various timepoints with either 1mM or 500μM H_2O_2. Lysates were separated by SDS-PAGE and blotted with the antibodies indicated to determine the optimal set of conditions for studying the activation of PKB. 100,000 cells per 4cm^2 (12 well plate) stimulated with 1mM H_2O_2 was chosen for future experiments.

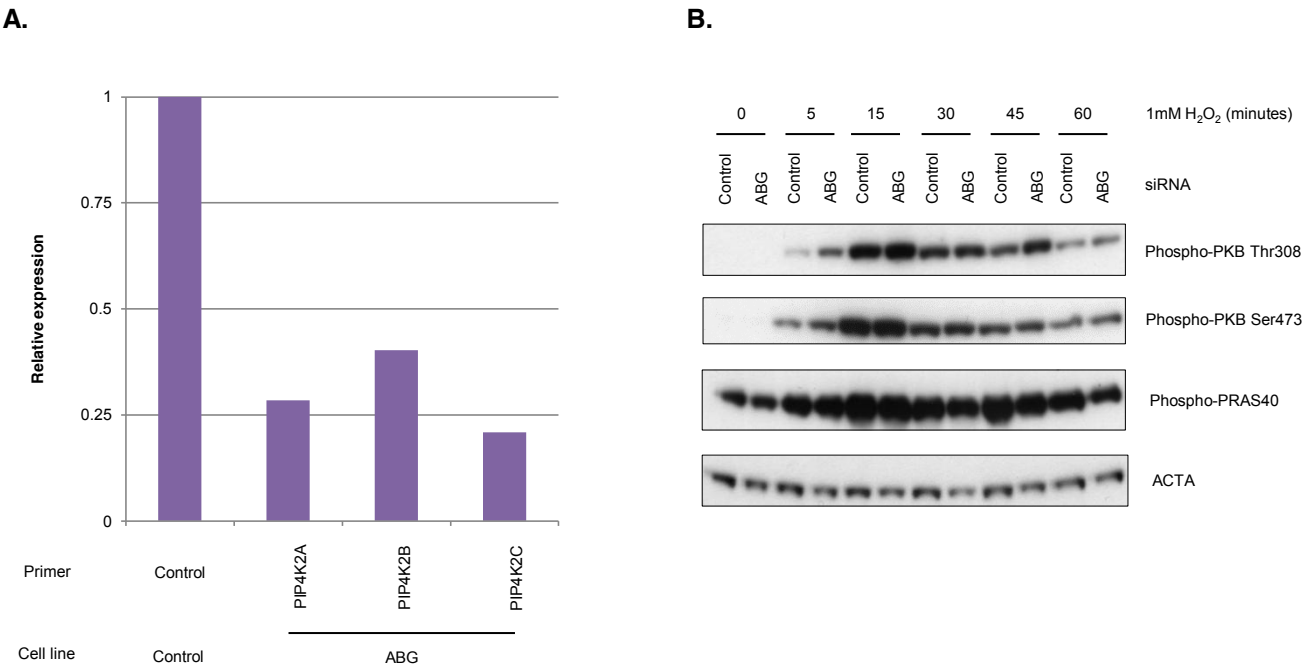


Figure 5.3. PIP4K knock down leads to enhanced PKB phosphorylation in response to oxidative stress. **A.** q RT-PCR was used to measure the mRNA level of the genes indicated in control and cells knocked down for PIP4K2A, PIP4K2B and PIP4K2C (ABG). **B.** Lysates were prepared from control cells or ABG before and after treatment with 1mM H_2O_2. Lysates were separated by SDS-PAGE and phosphorylated PKB was detected by western blotting and showed that PKB activation is enhanced in ABG cells.

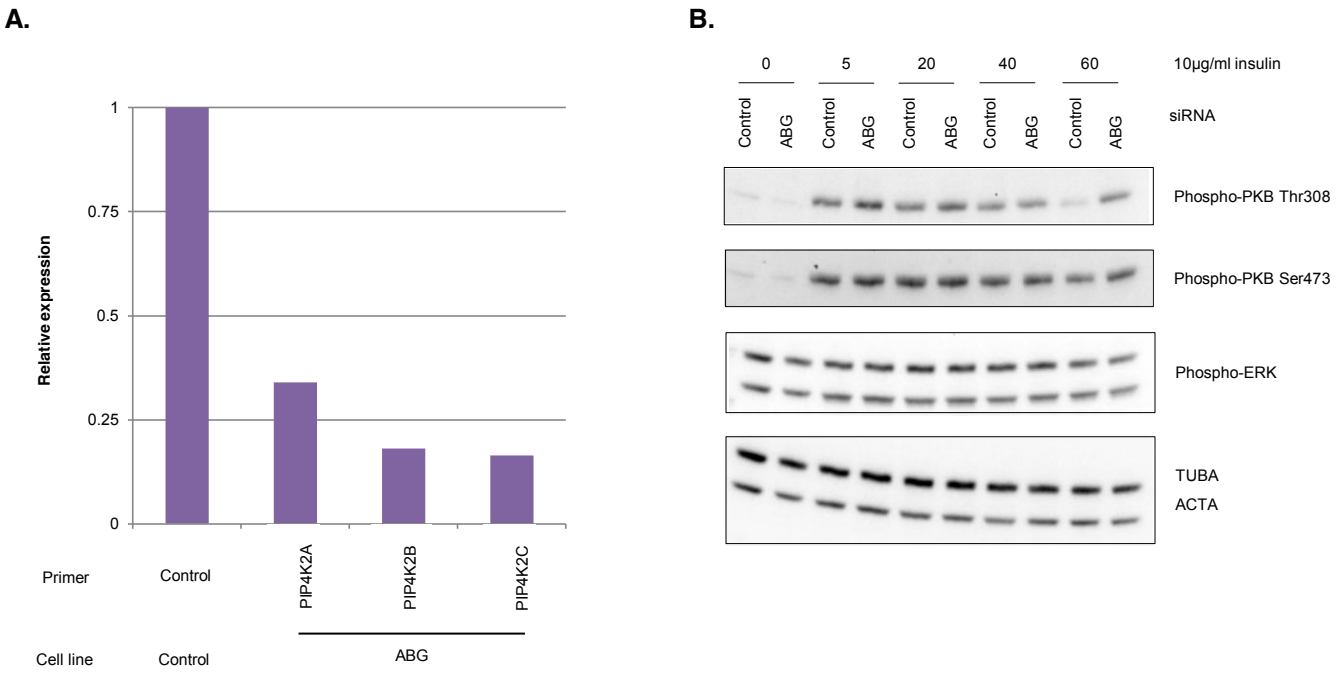
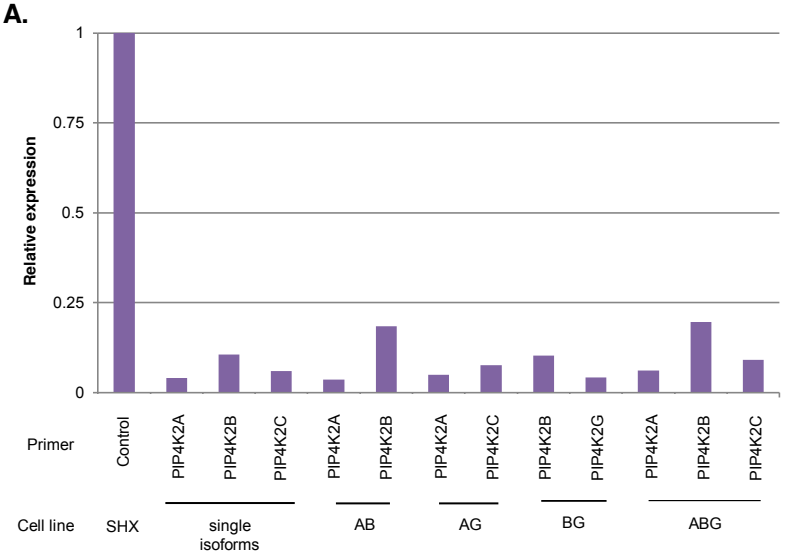


Figure 5.4. PIP4K knock down does not leads to enhanced PKB phosphorylation in response to insulin stimulation.

A. q RT-PCR was used to measure the mRNA level of the indicated genes in control and ABG knock down cells. **B.** Lysates were prepared from cells transfected with siRNA targeted against PIP4K2A, PIP4K2B and PIP4K2C (ABG) and treated with 10µg/ml insulin. Lysates were separated by SDS-PAGE and phosphorylated PKB was detected by western blotting and showed that insulin-induced PKB activation is not affected in ABG cells.

A.



Cell line	PIP4K isoforms knocked down
A	PIP4K2A
B	PIP4K2B
G	PIP4K2C
AB	PIP4K2B PIP4K2B
AG	PIP4K2A PIP4K2C
BG	PIP4K2B PIP4K2C
ABG	PIP4K2A PIP4K2B PIP4K2C

B.

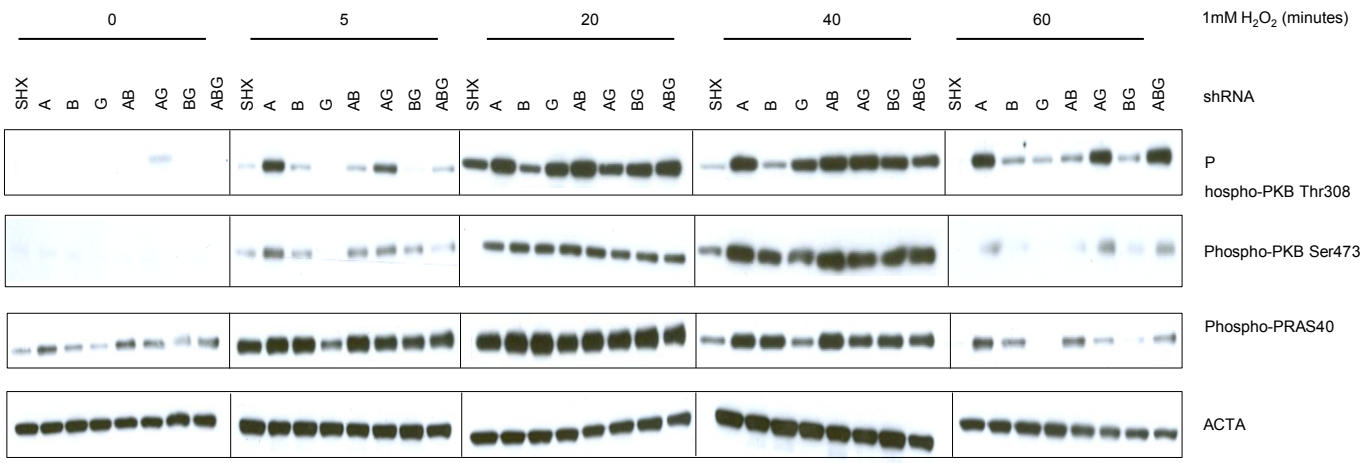


Figure 5.5. Knock down of PIP4K2A alone is sufficient to enhance PKB phosphorylation.

A. q RT-PCR was used to measure the mRNA level of targeted genes in control cells or those knocked down for PIP4K isoforms as described in the table. **B.** Control cells or cells transduced with shRNA targeting each isoform of PIP4K individually and in combination were maintained as controls or were treated with1mM H_2O_2. Lysates were separated by SDS-PAGE and blotted with the antibodies indicated on the right.

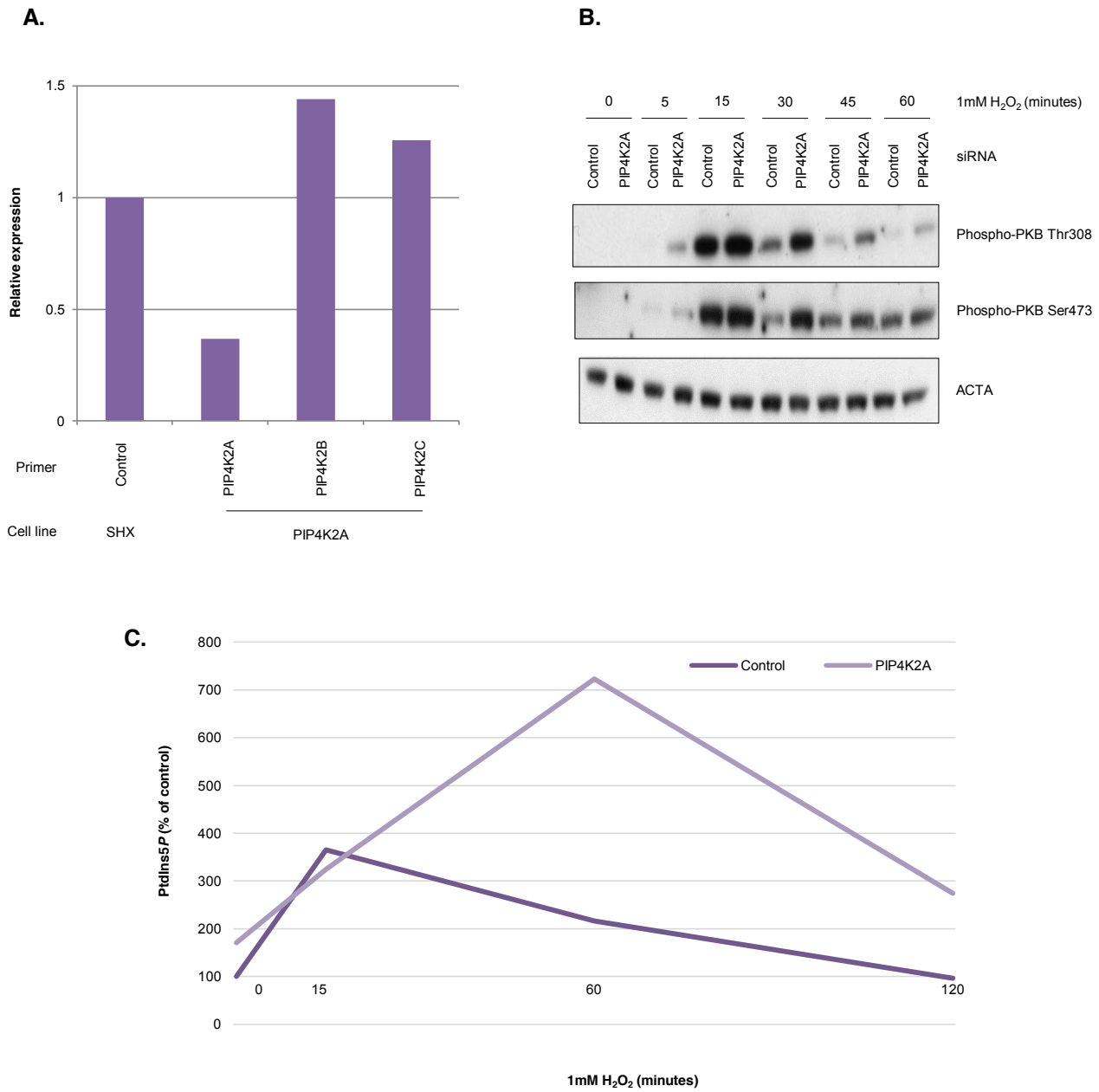


Figure 5.6. PIP4K2A knock down alone by siRNA is sufficient to enhance PKB phosphorylation and enhance the production of PtdIns5*P*.

A. q RT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K2A. **B.** Control cells or cells transfected with siRNA targeting PIP4K2A were maintained as controls or were treated with 1mM H_2O_2 for the times indicated. Cell lysates were separated by SDS-PAGE and phosphorylated PKB was detected by western blotting. Phosphorylation of both Thr308 and Ser473 were enhanced at numerous time points in PIP4K2A knock down cells. **C.** Control cells or cells with knocked down PIP4K2A were maintained as controls or treated with 1mM H_2O_2. Lipids were extracted and PtdIns5*P* was quantified and showed that loss of PIP4K2A causes enhanced production of PtdIns5*P* in response to 60 minutes of oxidative stress treatment.

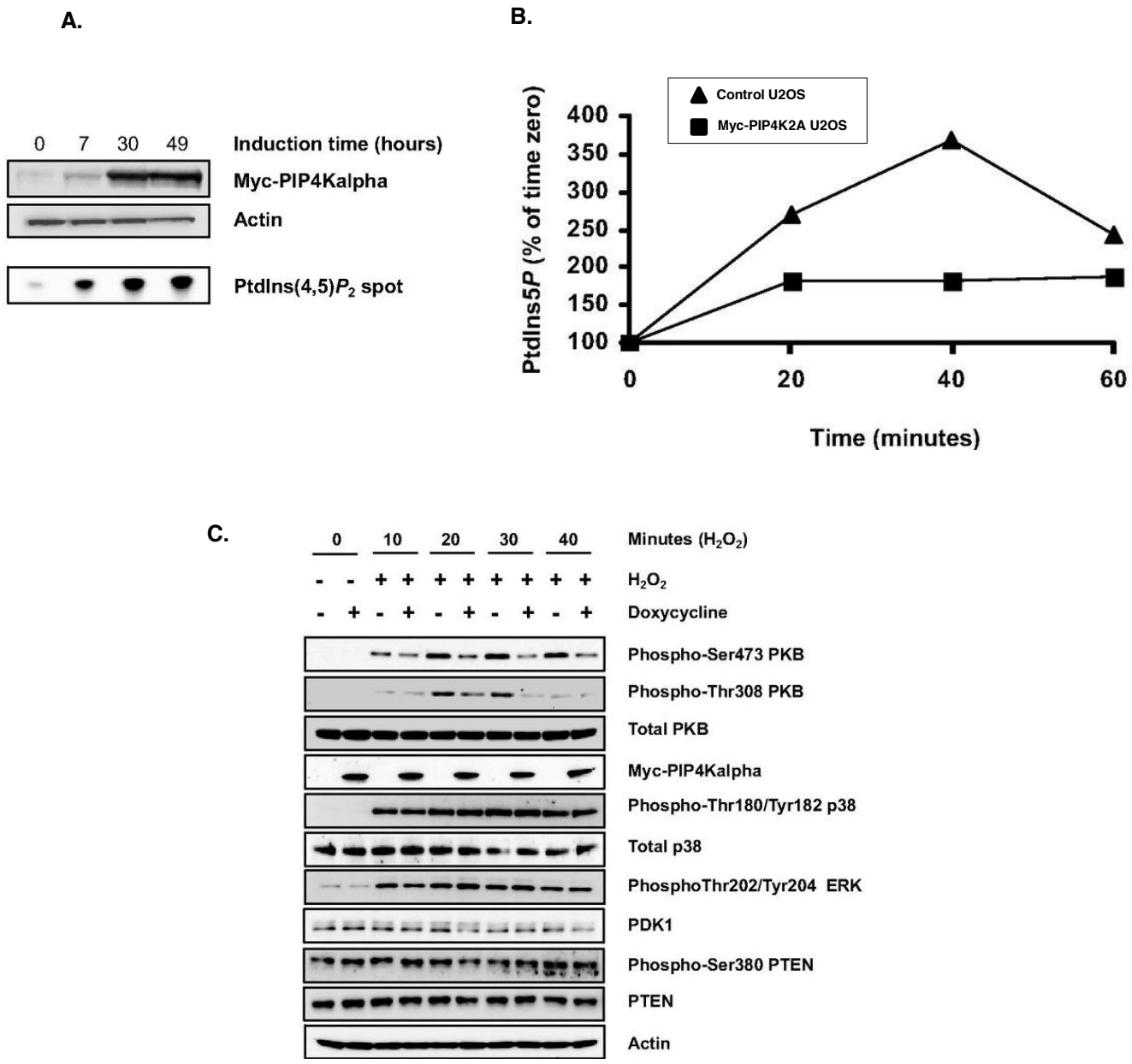
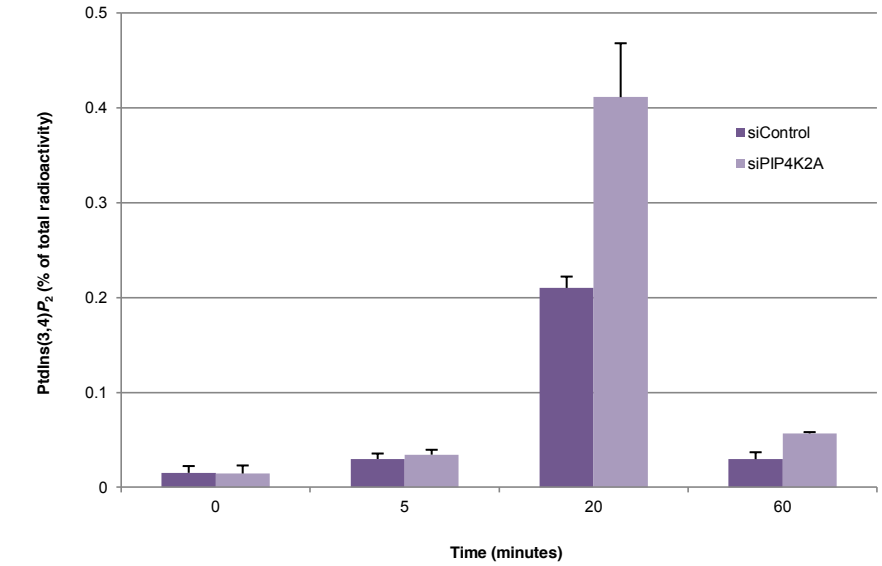


Figure 5.7. PIP4K2A regulates the level of H_2O_2-induced PtdIns5*P* and leads to attenuated PKB activation.

A. Induction of PIP4K2A leads to robustly increased expression of enzymatically active PIP4K2A. **B.** Control cells or Myc-PIP4K2A cells were treated with doxycycline overnight and stimulated with 1mM H_2O_2 for the times indicated. Lipids were extracted and the level of PtdIns5*P* was quantitated. The production of H_2O_2-induced PtdIns5*P* is repressed but not completely prevented in PIP4K2A over-expressing cells compared to control treated cells in response to oxidative stress. **C.** Lysates were made from cells over-expressing PIP4K2A that had been treated with 1mM H_2O_2. Western blot analysis revealed that phosphorylation of PKB at activation residues Ser473 and Thr308 is attenuated in PIP4K2A over-expressing cells in response to oxidative stress but the phosphorylation status of other signalling molecules are not affected.



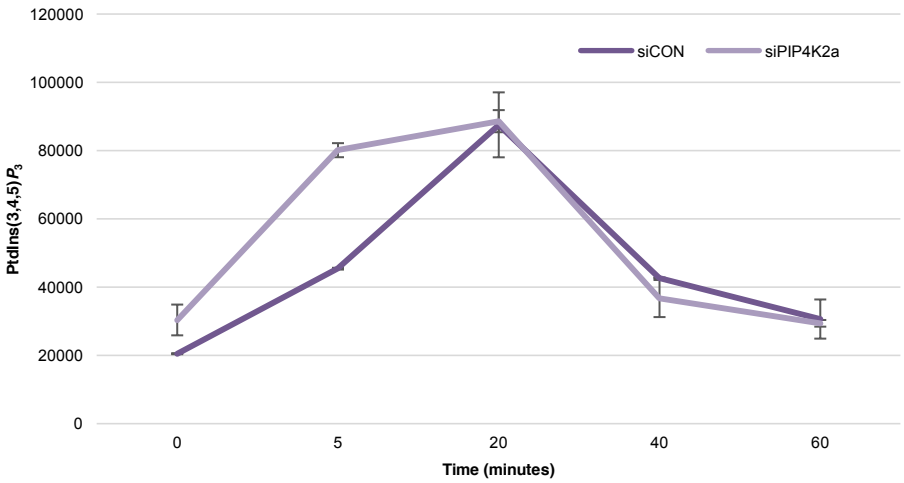
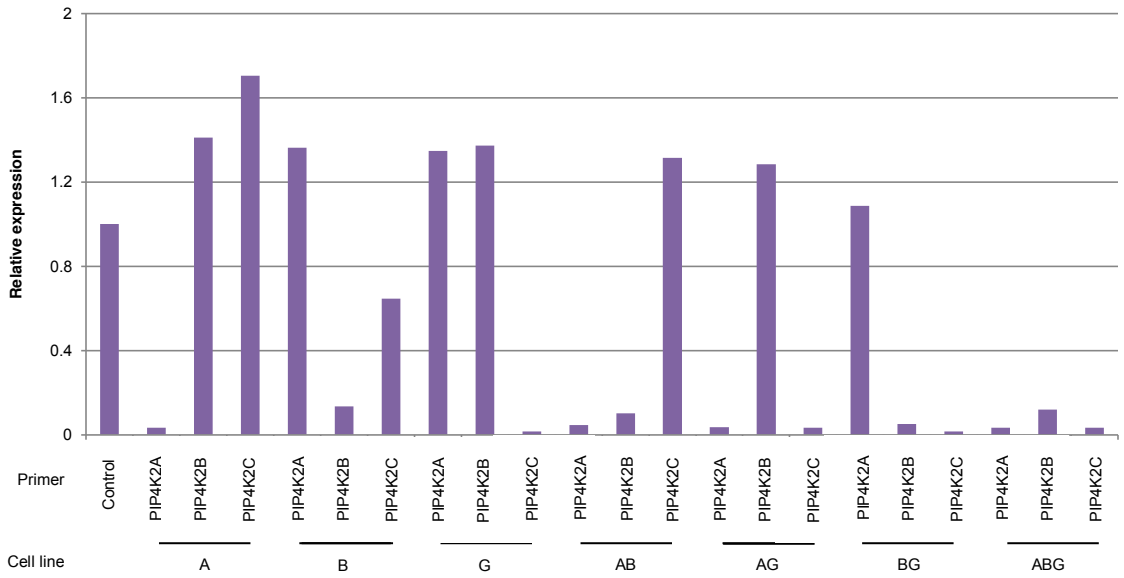
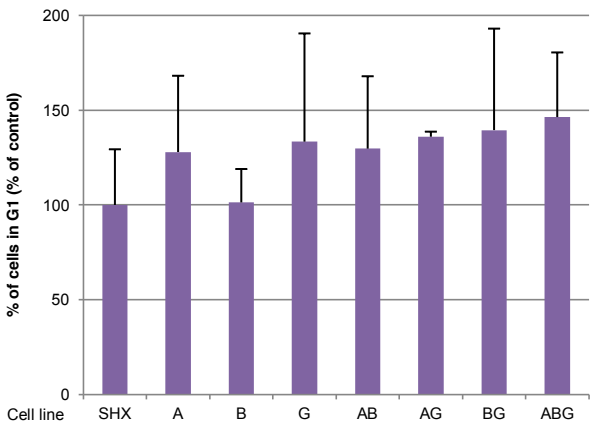


Figure 5.8. PIP4K2A knock down causes enhanced production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. **A.** Control or PIP4K2A knock down cells were radio-labelled ^{32}P-orthophosphate for 90 minutes and were maintained as controls or treated with 1mM H_2O_2 for the times indicated. Lipids were extracted, deacylated and the head groups were analysed by HPLC. **B.** Control cells or PIP4K2A knock down cells were treated with 1mM H_2O_2 for the various time points indicated after which lipids were extracted and PtdIns(3,4,5)P_3 levels were measured by mass spectrometry.

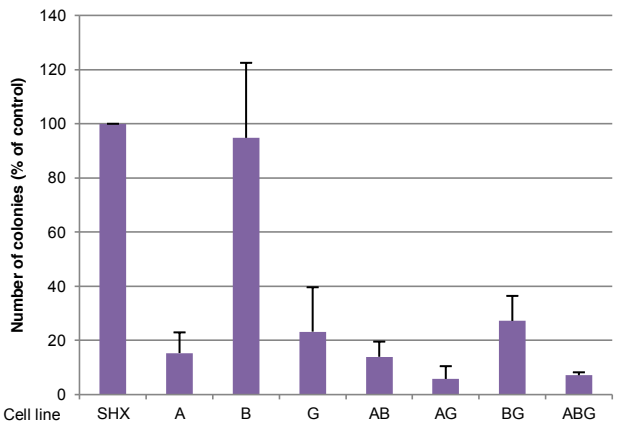
A.


Relative expression
2
1.6
1.2
0.8
0.4
0
Primer
Control
PIP4K2A
PIP4K2B
PIP4K2C
Cell line
A
B
G
AB
AG
BG
ABG

B.


% of cells in G1 (% of control)
200
150
100
50
0
Cell line
SHX
A
B
G
AB
AG
BG
ABG

C.


Number of colonies (% of control)
140
120
100
80
60
40
20
0
Cell line
SHX
A
B
G
AB
AG
BG
ABG

D.

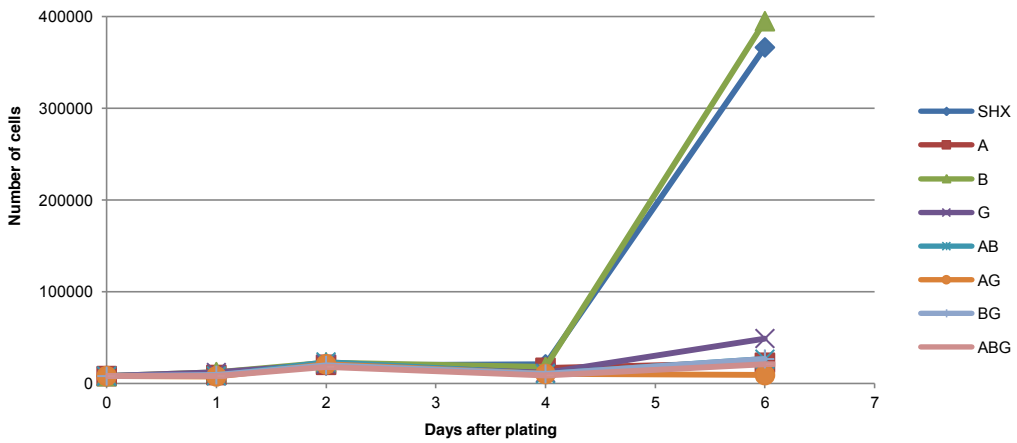

Number of cells
400000
300000
200000
100000
0
0
1
2
3
4
5
6
7
Days after plating
SHX
A
B
G
AB
AG
BG
ABG

Figure 5.9. Knock down of PIP4K isoforms leads to reduced growth capability.

A. q RT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K isoforms. **B, C & D.** Stable cell lines with integrated shRNA constructs targeting each PIP4K isoform singly and in combination were evaluated for growth phenotypes; cell counting experiments to determine cell growth (B), FACS analysis to determine the percentage of cells in G1 of the cell cycle (C) and anchorage dependent clonogenic assays to determine colony forming ability (D).

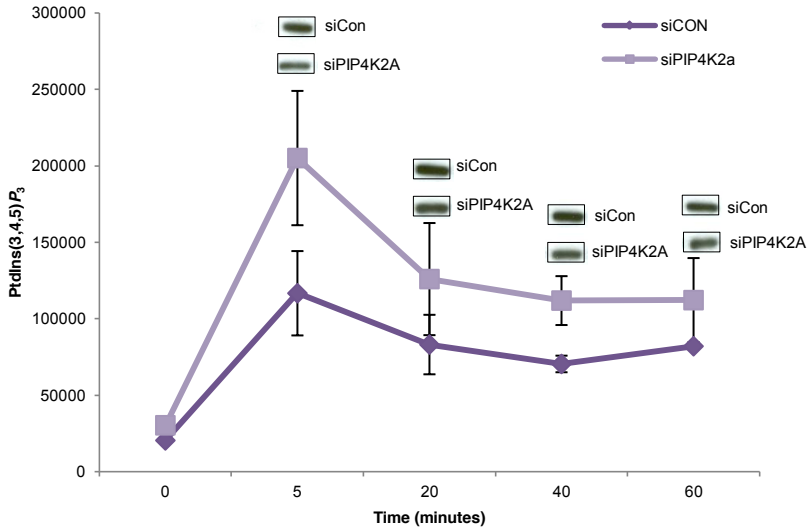


Figure 5.10. PIP4K2A knock down causes enhanced production of PtdIns(3,4,5)P_3 in response to insulin. Control and PIP4K2A knock down cells were maintained in control conditions or treated with 10µg/ml insulin for the time points indicated and analysis by mass spectrometry shows that the level of PtdIns(3,4,5)P_3 is higher in PIP4K2A knock down cells compared to control-transfected cells. Lysates of similar PIP4K2A knock down cells were analysed for expression of phosphorylated PKB at residue Ser473 as an indicator of PKB activation, bands represents samples stimulated with 10µg/ml insulin for the corresponding time points. Despite the increased PtdIns(3,4,5)P_3 in PIP4K2A knock down cells there is no change in phosphorylation of PKB.

6. The Role of PTEN in PtdIns5*P*-Mediated PKB Activation

6.1. Introduction

The mechanism of PtdIns5*P*-mediated activation of PKB is not well understood. Studies in Chapter 5 suggest that PIP4K2A regulates the level of both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 which are two phosphoinositides intimately linked with the activation of PKB. Knock down of PIP4K2A caused increased production of PtdIns5*P* (Figure 5.6C), an early increase in PtdIns(3,4,5)P_3 (Figure 5.8B) and a later increase in PtdIns(3,4)P_2 (Figure 5.8A) with the temporal changes in the levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 corresponding with enhanced PKB phosphorylation. In contrast over-expression of Myc-PIP4K2A suppressed the increase in the levels of both PtdIns5*P* (Figure 5.7C) and PtdIns(3,4)P_2 [11] while we did not measure the level of PtdIns(3,4,5)P_3. Knock down of PIP4K2A might increase the synthesis of PtdIns(3,4,5)P_3 or could attenuate phosphatase activity. As we did not observe a decrease in PtdIns(3,4)P_2 at early time points and therefore the increased PtdIns(3,4,5)P_3 is unlikely to be produced through 5-phosphosphorylation of PtdIns(3,4)P_2 we hypothesised that PIP4K might regulate PTEN activity and therefore regulate PtdIns(3,4,5)P_3 through inhibition of 3-phosphatase activity towards PtdIns(3,4,5)P_3.

PTEN is a lipid 3-phosphatase with activity towards PtdIns(3,4,5)P_3 [184] [113] but later shown to also de-phosphorylate the 3- position of PtdIns(3,4)P_2 [114]. PTEN negatively regulates PKB dependent cell survival [113] and tumours in PTEN$^{+/-}$ mice show loss of heterozygosity of the wild type allele and increased phosphorylated PKB [183]. Thus regulation of PTEN by PIP4K2A could explain the observed changes in phosphoinositides. Additionally, both pharmacological and genetic inhibition of PTEN was shown to increase the level of H_2O_2-induced PtdIns5*P* (Figure 3.10) which made PTEN a good candidate for a cooperative enzyme in the enhancement of PtdIns5*P*-mediated PKB phosphorylation.

6.2. Does PtdIns5P Regulate PKB Activation through PTEN?

6.2.1. PTEN Knock Down Enhances PKB Phosphorylation in Response to Oxidative Stress

To assess whether PtdIns5*P* could be working through PTEN to regulate H_2O_2-induced PKB activation, all three PIP4K isoforms and PTEN were genetically knocked down individually (ABG, PTEN) or in combination (ABG + PTEN). Quantification of RNA using qRT-PCR showed that all genes targeted by shRNA were knocked down by ≥65% except in the ABG background where knock down of PTEN was less efficient and was expressed at 60% of its control value (Figure 6.1A). This phenomenon was observed across all shRNA and siRNA-mediated knock down experiments. In response to H_2O_2, as expected, shRNA-mediated knock down of PIP4K (ABG) caused enhanced activation of PKB compared to SHX transduced cells at each time point analysed (Figure 6.1B). Knock down of PTEN also led to enhancement of H_2O_2-induced PKB activation at each time point and interestingly when PIP4K and PTEN were knocked down in combination (ABG + PTEN) there was no clear additive effect on the level of PKB phosphorylation (Figure 6.1B) suggesting that PIP4K and PTEN may be working through the same pathway. Similar results were obtained using siRNA-mediated knock down (data not shown). It should be noted that the intensities of the protein bands are very strong, therefore it may be difficult to observe any additive effects (Figure 6.1B).

Experiments were performed to analyse the effect of PTEN and PIP4K knock down on insulin-mediated PKB activation. Treatment of SHX-transduced cells with insulin led to phosphorylation of PKB at Thr308 and Ser473 but knock down of PIP4K or PTEN by shRNA (Figure 6.2B) or siRNA (data not shown) did not lead to enhanced PKB phosphorylation unlike treatment with H_2O_2. This data emphasises the potential role for PtdIns5*P* in PTENmediated PKB regulation as H_2O_2 causes a robust increase in the level of PtdIns5*P* whereas the effect of insulin on PtdIns5*P* production is minimal (Figure 3.2B). It should be noted that the dose of insulin used in these experiments in near maximal (10µg/ml) which may make it difficult to observe any differences in the activation of PKB. To further confirm the observations made here it would be necessary to repeat these experiments using various doses of insulin and to also measure the level of PtdIns5*P* in PIP4K knock down cells in response to insulin, an initial experiment has shown that the level of PtdIns5*P* is not increased over a time course of insulin and that there is no effect of knocking down PIP4K (data not shown). Overall the data presented here suggests that PTEN and PIP4K are relevant to PKB activation in response to H_2O_2 but not insulin in U2OS cells.

6.2.2. There is No Additive Effect of Knocking Down Both PTEN and PIP4K2A on the Enhancement of PKB Phosphorylation in Response to Oxidative Stress

As demonstrated, knock down of PIP4K2A alone is sufficient to enhance the production of PtdIns5*P* (Figure 5.6C) and the phosphorylation of PKB in response to H_2O_2 (Figure 5.6B). This may be due to the predominantly plasma membrane localisation of PIP4K2A and its high enzymatic activity. PTEN is thought to exert its lipid phosphatase effect mainly at the plasma membrane therefore it was hypothesised that PIP4K2A alone could co-operate with PTEN to regulate H_2O_2-induced activation of PKB. Knock down mediated by siRNA resulted in ≥90% reduction in RNA expression for each targeted gene, again with the exception of PTEN knock down when in combination with PIP4K2A knock down where 28% RNA expression remained (Figure 6.3A). Over numerous experiments the knock down efficiency of siRNA constructs proved to be more consistent than shRNA constructs so in all future transient knock down experiments, siRNA was used. In concurrence with previous experiments, knock down of PIP4K2A led to enhanced PKB phosphorylation at Thr308 and Ser473 in response to H_2O_2 and the enhancement of Thr308 phosphorylation was more pronounced than Ser473 (Figure 6.3B). Knock down of PTEN also caused enhancement of H_2O_2-induced PKB phosphorylation at each time point and knock down of both PIP4K and PTEN in combination (PTEN + PIP4K2A) had no clear additive effect on the level of PKB phosphorylation at Thr308 or Ser473 (Figure 6.3B). As seen in previous experiments, knock down of PIP4K2A caused enhanced PKB phosphorylation in response to acute H_2O_2 treatment (5 minutes) and longer term treatment (40 and 60 minutes) (Figure 6.3B). In previous experiments maximal PKB phosphorylation was not enhanced as strongly by PIP4K2A knock down but in Figure 6.3B it is not easy to establish at which point maximal activation occurs, this highlights the danger of picking specific time points to assess an ongoing event. Additionally, the exact time of maximal PKB phosphorylation varied slightly between experiments, although it was clear from a further experiment that maximal activation occurs between 15 and 20 minutes and knock down of PIP4K2A, PTEN or PTEN + PIP4K2A did not significantly enhance the maximal phosphorylation of PKB compared to control-transduced cells (data not shown).

To assess the possibility that the level of PKB phosphorylation in PTEN + PIP4K2A knock down cells is saturated and this is why there was no clear additive effect, stimulation with a lower concentration of H_2O_2, 500μM, was compared to 1mM treatment. In addition to a comparison between 500μM and 1mM H_2O_2 treatment, the effect of PIP4K2A knock down was compared to knock down of all three PIP4K isoforms (ABG) with and without PTEN knock down. Overall, when cells were stimulated with 500μM H_2O_2 PKB was phosphorylated to a lesser degree than with 1mM but the pattern of phosphorylation was the same across the time course of H_2O_2 treatment and across the different genetic knock down backgrounds (Figure 6.4B) suggesting that 500μM H_2O_2 elicits the same cell

signalling response as 1mM. Importantly, in response to both 500μM and 1mM H_2O_2, knock down of PTEN or PIP4K2A caused enhanced phosphorylation of PKB at Thr308 and Ser473 and knock down of PTEN and PIP4K2A in combination (PTEN + PIP4K2A) did not have an additive effect on PKB phosphorylation (Figure 6.4B). Using a lower dose of stimulant to elicit a weaker signalling response has therefore confirmed that there is no additive effect on the phosphorylation of PKB of knocking down both PTEN and PIP4K2A in combination further suggesting that these enzymes may be working on the same signalling pathway to regulate PKB or that they work at a point upstream of a rate limiting step in PKB phosphorylation.

Previous experiments have demonstrated that knock down of all three PIP4K isoforms in combination causes enhanced H_2O_2-induced PKB phosphorylation and that the extent of enhancement is comparable between PIP4K2A knock down alone and ABG knock down (Figure 5.5B). In response to 1mM H_2O_2 the phosphorylation of PKB is up-regulated to a very similar extent in PIP4K2A knock down alone and ABG knock down cells whereas in response to 500μM H_2O_2 the effect of knocking down PIP4K2A alone is suppressed when PIP4K2B and PIP4K2C are also knocked down (Figure 6.4B). This data provides further evidence that knock down of PIP4K2A alone is sufficient to enhance the phosphorylation of PKB and that the effect of PIP4K2A knock down might be suppressed in ABG knock down cells. Secondly this data strengthens the theory that PIP4K2A and PTEN may be working on the same signalling pathway to regulate PKB and suggests that PIP4K2A may be an upstream regulator of PTEN as there is no clear additive effect of knocking down both PIP4K2A and PTEN together. It is important to note however that the extent of PTEN knock down is greater when PTEN is knocked down alone compared to in combination with PIP4K2A knock down (23% remaining expression compared to 37% respectively) (Figure 6.4A). This was observed in all similar experiments and when PTEN was knocked down in combination with PIP4K2A its remaining expression could be up to 50%. Furthermore, differences in the protein level of PTEN could also be observed (Figure 6.4B). The genetic dosage of PTEN is known to be an important factor in the progression of prostate cancer and in knock-out mice, decreasing expression of PTEN strongly correlated with increasing expression of phosphorylated PKB [100]. For this reason, qRT-PCR data and protein levels were carefully scrutinised in each experiment to ensure consistent knock down of PTEN.

6.2.3. Both PIP4K2A and PTEN Regulate the Level of H_2O_2-Induced PtdIns5P

Knock down of PIP4K2A has been shown to increase the level of H_2O_2-induced PtdIns5*P* (Figure 5.6C) and both genetic and pharmacological inhibition of PTEN has also been shown to increase the level of H_2O_2-induced PtdIns5*P* (Figure 3.10). If PIP4K2A and PTEN are working on the same

signalling pathway to regulate PKB activation through PtdIns5*P*, knock down of PIP4K2A and PTEN in combination would be expected to increase the level of H_2O_2-induced PtdIns5*P* to the same extent as knock down of PIP4K2A alone or PTEN alone. Analysis of knock down samples by qRT-PCR revealed that the RNA expression of target genes was reduced by ≥80% with the exception of PTEN knock down when in combination with knock down of PIP4K2A which was reduced by 58% (Figure 6.5A). Knock down of PTEN alone caused a 1.8-fold increase, PIP4K2A knock down caused a 1.7-fold increase and knock down of PTEN and PIP4K2A in combination caused a 1.6-fold increase in H_2O_2-induced PtdIns5*P* (Figure 6.5B). This data implies that PTEN and PIP4K2A regulate a common signalling pathway that is responsible for controlling PtdIns5*P* production in response to H_2O_2.

The hypothesis developed based on this part of the project was that PIP4K2A and PTEN may both regulate a common signalling pathway that is activated by H_2O_2 and regulates activation of PKB through the up-regulation of PtdIns5*P*. Following experiments aimed to elucidate the relationship between PIP4K2A and PTEN.

6.3. Can PIP4K2A Regulate the Activity of PTEN in Response to Oxidative Stress?

6.3.1. PIP4K2A and PTEN Do Not Directly Interact

To establish whether PIP4K2A and PTEN directly interact co-immunoprecipitation experiments were performed in U2OS cells expressing Myc-tagged PTEN and EE-tagged PIP4K2A. Cells were treated with H_2O_2 for 0, 20 or 60 minutes to determine whether an interaction between PIP4K2A and PTEN could be induced by H_2O_2 signalling. Samples immunoprecipitated with antibody targeting the Myc tag (PTEN) were blotted with a PIP4K2A antibody and samples immunoprecipitated with an EE tag antibody (PIP4K2A) were probed with a PTEN antibody. Under the conditions tested no clear interaction between PTEN and PIP4K2A could be observed (Figure 6.6).

Interestingly, PTEN has been shown to physically interact with PtdIns5*P* which activates PTEN activity [114]. To investigate the possibility that PtdIns5*P* regulates PKB activation through a direct interaction with PTEN, a co-localisation study utilising a PtdIns5*P* specific binding domain molecular probe would be necessary. One such probe is the GFP-PHDx3 probe which is comprised of a GFP protein fused to 3 repeats of the PHD domain from ING2 which was used to demonstrate the co-localisation of PtdIns5*P* with phosphorylated PKB [164]. This method of visualising PtdIns5*P* in the cell and determining co-localisation of PtdIns5*P* with protein interactors is very useful and informative but there are issues with the specificity of probes as many phosphoinositide binding domains are known to be promiscuous and can bind to more than one phosphoinositide. This means that the

specificity of binding is never guaranteed. Development of highly specific binding probes will allow precise and exhaustive analysis of PtdIns5*P* interactors.

6.3.2. PIP4K2A Does Not Affect the Oxidation of PTEN in Response to Oxidative Stress

PTEN is known to be catalytically inactivated by H_2O_2 due to the formation of a disulphide bond between two critical cysteine residues in the active site [66] and it has been suggested that this mechanism allows the accumulation of PtdIns(3,4,5)P_3 and subsequent activation of PKB in response to H_2O_2 [165]. To verify that PTEN becomes oxidised by H_2O_2 in U2OS cells, wild type cells were treated with H_2O_2 for various times up to two hours and the redox state of PTEN was analysed by western blotting. After 5 minutes of 1mM H_2O_2 the PTEN band had shifted down indicative of PTEN oxidation and by 120 minutes the majority of PTEN appeared to become re-activated as determined by the presence of the reduced state (Figure 6.7A). Between 5 minutes and 120 minutes there was a gradual increase in the amount of reduced PTEN present in the sample although the lower oxidised PTEN band was still apparent even after 60 minutes of H_2O_2. This suggests that part of the cellular stock of PTEN is maintained in an oxidised state while PTEN is gradually reduced back to its native form. To demonstrate the effect of oxidation on the catalytic activity of PTEN, U2OS cells were transduced with a Myc-tagged PTEN expression construct, treated with 1mM H_2O_2 for various durations and cell lysates were analysed for PKB phosphorylation by western blotting. Catalytically active PTEN would prevent the accumulation of PtdIns(3,4,5)P_3 and suppress activation of PKB. In response to H_2O_2, PKB was phosphorylated at both Thr308 and Ser473 but there was no suppression of PKB phosphorylation in the presence of over-expressed PTEN despite PTEN being very highly expressed in cells transfected with the Myc-tagged PTEN expression construct (Figure 6.7B). In response to 10µg/ml insulin treatment, phosphorylation of PKB was suppressed in PTEN over-expressing cells compared to control cells demonstrating that H_2O_2-induced oxidation of PTEN specifically causes inhibition of its catalytic activity (Figure 6.7C).

In order to regulate the activation of PKB, PIP4K2A could regulate the oxidation of PTEN. Over-expression of PIP4K2A could suppress PKB activation by protecting PTEN from oxidation and therefore preventing catalytic inactivation of PTEN, preventing accumulation of PtdIns(3,4,5)P_3 and consequently suppressing the activation of PKB. Conversely knock down of PIP4K2A could lead to increased oxidation of PTEN. To test this hypothesis, cells stably expressing a doxycycline-inducible Myc-tagged PIP4K2A and PIP4K2A knock down cells were treated with H_2O_2 and the redox state of PTEN was analysed by western blotting. Over-expression of PIP4K2A did not significantly change the rate or extent of PTEN oxidation or reduction in response to H_2O_2 (Figure 6.7D) and similarly knock down of PIP4K2A had no significant effect (Figure 6.7E). As only a small number of time points were

selected for analysis it is possible that PIP4K2A may be able to regulate the redox state of PTEN at a precise time point that was not analysed therefore a more detailed time course using various doses of H_2O_2 may be required to observe an effect. However from this work the differential expression of PIP4K2A does not prevent oxidation of PTEN and does not cause PTEN to be reduced more rapidly, therefore regulation of PTEN oxidation may not be a mechanism for PIP4K2A-mediated regulation of H_2O_2-induced PKB activation.

The ability of H_2O_2 to catalytically inactivate PTEN suggests that knock down of PTEN would have no effect on H_2O_2-induced PKB phosphorylation however it has been shown that phosphorylation of both Thr308 and Ser473 is enhanced when PTEN is knocked down. As the PI3K antagonistic activity of PTEN is unlikely to account for this effect, there must be an alternative role for PTEN in the regulation of PKB. The protein phosphatase activity of PTEN was actually described before its more well known lipid phosphatase activity [96] but the role of its protein phosphatase activity in the regulation of PKB remains controversial. It has been reported that a tumour derived PTEN protein phosphatase dead mutant is able to suppress PtdIns(3,4,5)P_3 production and PKB phosphorylation similarly to wild type PTEN but lack of protein phosphatase activity prevents inhibition of cell invasion, suggesting that some of the tumour suppressive functions of PTEN are PtdIns(3,4,5)P_3-independent [166]. Overall, this work proposed that the protein phosphatase activity of PTEN is not required for regulation of PKB activation. A more recent study has implicated the protein phosphatase activity of PTEN in the regulation of insulin receptor-mediated PKB activation [95]. Specifically, in cells lacking NEDD4, a negative regulator of PTEN, phosphorylation of PKB is lost and this effect is rescued by knocking down PTEN. It was shown that PTEN directly de-phosphorylates IRS1 which negatively regulates PKB phosphorylation in response to IGF1 and insulin signalling. This newly established role for PTEN is particularly interesting considering that the IGF1-R/ InsR inhibitor AG1024 inhibits H_2O_2-induced activation of PKB (Chapter 3). Together this data suggests that PTEN and PtdIns5*P* may work through the insulin receptor to regulate the activation of PKB, however the mechanism that links them directly is still unclear.

6.3.3. PIP4K2A Does Not Affect the Stability of PTEN

Due to its critical activity PTEN is tightly regulated in a number of different ways. Post translational modifications play an important role in controlling the activity and localisation of PTEN. Phosphorylation of Serine 380, Threonine 382 and Threonine 383, which cluster in the C-terminal tail, inhibits the activity of PTEN while maintaining the protein in a stable state [181]. This cluster of phosphorylations has been shown to inhibit the localisation of PTEN at the plasma membrane thereby preventing the degradation of PtdIns(3,4,5)P_3 [182]. It has been suggested that

phosphorylation of the C-terminal tail causes PTEN to form a closed conformation due to the formation of intra-molecular bonds [180]. To analyse the stability of PTEN, U2OS cells were treated with 1mM H_2O_2 for various durations up to an hour and cell lysates were probed with antibodies specific to PTEN and phosphorylated Serine 380 on PTEN. If PTEN is degraded in response to H_2O_2 the amount of PTEN protein should decrease. Over an hour of H_2O_2 treatment the amount of PTEN and Ser380 was maintained showing that PTEN is not degraded and that the phosphorylation status of PTEN in respect to Serine 380 is not changed in response to H_2O_2 (Figure 6.8A). Interestingly, in the presence of H_2O_2 a band was visible at a higher molecular weight than PTEN when lysates were probed with an antibody targeted to phosphorylated Serine 380. This band was not present in cells treated with siRNA targeted against PTEN (Figure 6.8A, final lane, siPTEN) suggesting that it is PTEN or a close derivative. The molecular weight of PTEN is 54kDa and the H_2O_2-induced higher molecular weight band represents a protein between 64-98kDa. PTEN has recently been shown to exist as a catalytically active homodimer [101] however preservation of a dimer structure requires cell lysates to be processed in non-reducing and non-denaturing conditions. Alternatively, this high molecular weight band may represent the recently identified 75kDa Long-PTEN which is secreted from cells and is able to enter other cells and inhibit PI3K signalling [167]. It has not been shown whether oxidative stress induces alternative translation to produce Long-PTEN but it is interesting to note that H_2O_2 could act as an intercellular signalling molecule through the induction of Long-PTEN.

Oxidative stress did not directly affect PTEN stability but the possibility that PIP4K2A could regulate the stability of PTEN as a mechanism of PKB regulation was investigated. Knock down of PIP4K2A could enhance degradation of PTEN therefore allowing accumulation of PtdIns(3,4,5)P_3 and consequently leading to increased activation of PKB, conversely over expression of PIP4K2A could suppress PKB activation by enhancing the stability of PTEN. To test this hypothesis, Myc-PIP4K2A cells and PIP4K2A knock down cells were treated with H_2O_2 and cell lysates were probed with antibodies targeted to PTEN and Ser380. In neither PIP4K2A knock down cells (Figure 6.8B) nor Myc-PIP4K2A cells (Figure 6.8C) was the stability of PTEN or phosphorylation of Ser380 different compared to control cells. Interestingly, the higher molecular weight PTEN band was also induced by H_2O_2 treatment in both of these sets of experiments, confirming its existence in different cellular settings. Overall it is clear that PIP4K2A does not have a role in regulating the phosphorylation status or stability of PTEN in resting conditions or in response to H_2O_2.

6.3.4. PIP4K2A May Regulate the Ubiquitination of PTEN

Recently the ubiquitination of PTEN has been highlighted as an important regulatory mechanism. *In vitro* membrane targeting of PTEN enhances its ubiquitination and subsequently inhibits its

phosphatase activity towards PtdIns(3,4,5)P_3 [168]. Polyubiquitination of PTEN by NEDD4-1 leads to its proteasomal degradation [178] whereas monoubiquitination controls the nuclear localisation of PTEN [177]. Numerous E3 ubiquitin ligases are now known to ubiquitinate PTEN including WWP2 [93] and RFP [94]. Of particular interest is the finding that the ability of PTEN to regulate IGF1/ insulin-mediated activation of PKB is NEDD4 dependent [95] demonstrating the importance of this posttranslational modification in the regulation of PTEN activity.

Initial experiments were undertaken to optimise the detection of ubiquitinated PTEN from cells. The first experiment attempted involved immunoprecipitation (IP) of PTEN from wild type U2OS cells and western blot analysis using an antibody that recognises ubiquitinated protein. Firstly optimisation of PTEN IP was required to establish the minimum volume of PTEN antibody, duration of lysate incubation with PTEN antibody and duration of lysate incubation with protein G agarose beads. The maximum recovery of PTEN was obtained by incubating protein G agarose beads with 5µl of PTEN antibody for 2 hours then incubating the protein G agarose beads with the cell lysate for 2 hours (Figure 6.9A). Wild type U2OS cells were treated with 1mM H_2O_2 for various time points up to an hour and PTEN was immunoprecipitated according to the optimal conditions established. An antibody targeted to ubiquitinated proteins was used to detect ubiquitinated PTEN and although smeared bands indicative of ubiquitinated PTEN were present, overnight exposure was required to obtain clear bands (Figure 6.9B). This suggested that of the cellular pool of PTEN that was successfully selected by IP, only a minor fraction was ubiquitinated. This is not surprising considering that membrane-targeted PTEN was shown to be preferentially ubiquitinated [168], suggesting that different pools of PTEN are likely to be differentially ubiquitinated. Another observation from this experiment is that the level of total PTEN in immunoprecipitated samples appears to be lower in cells stimulated with H_2O_2 for 5, 20 and 40 minutes, time points that correlate with H_2O_2-induced oxidation of PTEN which raises the possibility that oxidised PTEN is not efficiently immunoprecipitated by a PTEN antibody.

Due to the difficulties in obtaining clear bands representing endogenous ubiquitinated PTEN and possible issues with immunoprecipitating oxidised PTEN, another method was attempted. PIP4K2A knock down cells were transfected with His-tagged ubiquitin and/or Myc-tagged PTEN expression constructs. Cells were then treated with 1mM H_2O_2 for 60 minutes and lysates were incubated with nickel beads to pull down His-tagged ubiquitin. The amount of ubiquitinated PTEN in the lysates was analysed using a PTEN antibody. Western blot analysis of whole cell lysates (that were not incubated with nickel beads) showed that PIP4K2A was knocked down at the protein level and that PTEN was highly expressed only in the samples transfected with Myc-PTEN however the expression of PTEN was variable between these samples (Figure 6.10A). Analysis of His-tagged proteins affinity purified with the nickel beads (His pull down) using a PTEN antibody showed that multiple bands indicative of

ubiquitinated PTEN were only present in samples transfected with both Histagged ubiquitin and Myc-tagged PTEN expression constructs. Quantification of ubiquitinated PTEN was achieved using TINA analysis software and involved quantifying all of the multiple bands of ubiquitinated PTEN in a sample and normalising this to the total PTEN in the corresponding whole cell lysate. This evaluation showed that in cells transfected with non-targeting control siRNA, H_2O_2 induced a decrease in the amount of ubiquitinated PTEN (Figure 6.10B). In PIP4K2A knock down cells the level of ubiquitinated PTEN was slightly higher both in resting conditions and in response to H_2O_2 treatment (Figure 6.10B).

PIP4K2A was then over-expressed to assess if suppressing the increase in H_2O_2-induced PtdIns5*P* affects ubiquitination of PTEN. Western blot analysis of whole cell lysates showed that PIP4K2A was over-expressed at the protein level and showed that PTEN was highly expressed only in the samples transfected with Myc-PTEN. Again the expression of PTEN was variable between these samples (Figure 6.11A). Analysis of proteins affinity purified by the nickel columns with a PTEN antibody showed multiple bands of ubiquitinated PTEN only in samples transfected with both His-tagged ubiquitin and Myc-tagged PTEN expression constructs. Quantification of ubiquitinated PTEN using TINA analysis software revealed that in control cells (not treated with doxycycline), H_2O_2 induced a 2-fold increase in the amount of ubiquitinated PTEN (Figure 6.11B). Strikingly, in Myc-PIP4K2A cells (treated with doxycycline) the level of ubiquitinated PTEN was severely suppressed in resting conditions and in response to H_2O_2 treatment (Figure 6.10B). Unfortunately, experimental problems have hampered the dissection of the role of PtdIns5*P* in PTEN ubiquitination so far. A continuing problem in these experiments is ensuring equal expression of each overexpressed component. As demonstrated by western blotting, the level of over-expressed PTEN varied between samples and preliminary results strongly indicate that His-ubiquitin was also expressed to variable degrees across samples (data not shown). In order to validate these initial results and further determine the significance of PTEN ubiquitination in PtdIns5*P*-mediated PKB activation a method that can precisely and reproducibly quantify the level of ubiquitinated PTEN will need to be utilised.

Despite differences in the effect of H_2O_2 on PTEN ubiquitination in both sets of control samples, these experiments implicate PIP4K and possibly PtdIns5*P* in the regulation of PTEN ubiquitination. This is a particularly attractive concept considering the recent publication describing the importance of NEDD4-dependent PTEN regulation in IGFmediated PKB activation [95]. H_2O_2 is known to induce insulin receptor-mediated phosphorylation of IRS1 leading to recruitment of the PI3K regulatory subunit p85 [169]. Studies of PTEN oxidation by H_2O_2 have concentrated on the lipid phosphatase activity of PTEN, leaving the possibility that PTEN maintains protein phosphatase activity in the oxidised state. Therefore it could be suggested that PtdIns5*P* is employed by the cell in response to H_2O_2 to induce ubiquitination of PTEN thus preventing de-phosphorylation of IRS1 and allowing PKB

activation. To test this hypothesis the enzymatic activity of NEDD4 should be assessed in response to H_2O_2 treatment in a PIP4K2A knock down background. This could be achieved by measuring the ubiquitination of PTEN, by analysing the interaction of NEDD4 and PTEN using co-immunoprecipitation techniques or by performing cell-free enzymatic assays using NEDD4 purified from H_2O_2-treated PIP4K2A knock down cells. If PtdIns5*P* regulates NEDD4-mediated ubiquitination of PTEN, one would expect to see enhanced PTEN ubiquitination in PIP4K2A knock down cells.

6.3.5. Knock Down of PIP4K2A and PTEN Leads to Enhanced Production of PtdIns(3,4,5)P_3 in Response to Oxidative Stress

Recruitment of PKB to the plasma membrane and subsequent activation are mediated by the availability of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. As described, knock down of PIP4K2A increased the level of H_2O_2-induced PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Figure 5.8) which was suggested to be a mechanism for PIP4K2A-mediated PKB activation as PIP4K2A could be regulating two PKB-recruiting lipids, PtdIns(3,4,5)P_3 in response to acute H_2O_2 stimulation and PtdIns(3,4)P_2 in response to longer term stimulation which consequently allows regulation of PKB phosphorylation. If PIP4K2A and PTEN are working on the same pathway to regulate PKB activation through upstream phosphoinositides, knock down of PIP4K2A or PTEN individually would be expected to increase the level of PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3 and knock down of PIP4K2A and PTEN in combination would be predicted to have no additive effect on the level of either PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. To test this hypothesis PTEN, PIP4K2A and PTEN + PIP4K2A knock down cells were radiolabelled with ^{32}P-orthophosphate and treated with 1mM H_2O_2 for 20 minutes. This time point was chosen as in a previous experiment PIP4K2A knock down caused a 2-fold increase in the level of PtdIns(3,4)P_2 in response to 20 minutes of H_2O_2 (Figure 5.8A). Radio-labelled extracted lipids were deacylated and analysed using High Pressure Liquid Chromatography (HPLC, equipment-based protocol performed by Dr David Jones) which revealed that knock down of PTEN had no significant effect on the level of H_2O_2-induced PtdIns(3,4)P_2, while knock down of PIP4K2A led to a 1.6-fold increase in the level of PtdIns(3,4)P_2. Knock down of PTEN and PIP4K2A (PTEN + PIP4K2A) also led to a 1.6-fold increase in the level of PtdIns(3,4)P_2 (Figure 6.12A). This clearly demonstrates that knock down of PIP4K2A is responsible for the increase in the level of PtdIns(3,4)P_2 in PTEN + PIP4K2A knock down and that PTEN does not regulate the level of H_2O_2-induced PtdIns(3,4)P_2 making it unlikely that PIP4K2A regulates the level of PtdIns(3,4)P_2 through PTEN.

To evaluate the level of PtdIns(3,4,5)P_3 samples were analysed using Mass Spectrometry (Figure 6.12B, MS protocol performed by Karen Anderson). In control transfected cells the level of PtdIns(3,4,5)P_3 was increased in response to 5 minutes of H_2O_2, the level peaked after 20 minutes,

was significantly decreased after 40 minutes and had returned to almost basal by 60 minutes. In PTEN knock down cells the basal level of PtdIns(3,4,5)P_3 was 2-fold higher than control cells. The increase of PtdIns(3,4,5)P_3 initiated by H_2O_2 was suppressed as after 5 minutes of H_2O_2 treatment the level of PtdIns(3,4,5)P_3 was the same in PTEN knock down compared to control. The increased PKB observed in PTEN knock down cells in response to 5 minutes of H_2O_2 treatment does not correlate with increased PtdIns(3,4,5)P_3 however it is possible that due to the increased basal level of PtdIns(3,4,5)P_3, PKB is already localised at the plasma membrane and immediately ready to be phosphorylated in response to H_2O_2 treatment. After 20 minutes there was a slight increase but after 40 and 60 minutes although the level of PtdIns(3,4,5)P_3 had started to decrease it was maintained at a significantly higher level than in control cells (Figure 6.12B).

These observations tie in with the known function of PTEN as a negative regulator of PtdIns(3,4,5)P_3. In un-stimulated conditions PTEN is likely to regulate the basal pool of PtdIns(3,4,5)P_3 therefore knock down of PTEN would be expected to cause an increase in the basal level of PtdIns(3,4,5)P_3. Upon H_2O_2 stimulation inactivation of PTEN leads to increased PtdIns(3,4,5)P_3 accumulation. As PTEN appears to be stoichiometrically oxidised (see Figure 6.7A) and therefore inactivated it is not surprising that knock down of PTEN at this point does not affect PtdIns(3,4,5)P_3 accumulation. At later time points oxidised PTEN becomes reactivated (Figure 6.7A) and is able to degrade PtdIns(3,4,5)P_3 to reduce its accumulation. In agreement with this hypothesis, knock down of PTEN induces the accumulation of PtdIns(3,4,5)P_3 in response to H_2O_2 stimulation at later time points. A more detailed appraisal of the does curves of H_2O_2 stimulation and PtdIns(3,4,5)P_3 accumulation is likely to confirm these observations.

In PIP4K2A knock down cells the basal level of PtdIns(3,4,5)P_3 was slightly higher than in control cells and by 5 minutes the level of PtdIns(3,4,5)P_3 was almost 2-fold higher. Interestingly, this increase was not maintained as in response to 20, 40 and 60 minutes of H_2O_2 the level of PtdIns(3,4,5)P_3 in PIP4K2A knock down cells was comparable to control cells (Figure 6.12B). This is a very interesting result as knock down of PIP4K2A has the opposite effect on the level of PtdIns(3,4,5)P_3 to knock down of PTEN. PTEN seems to regulate the basal level of PtdIns(3,4,5)P_3 and is responsible for removing PtdIns(3,4,5)P_3 once it has been able to accumulate. PIP4K2A appears to regulate a subset of basal PtdIns(3,4,5)P_3 and the pool of PtdIns(3,4,5)P_3 produced in response to acute H_2O_2 stimulation but is not involved in the removal of accumulated PtdIns(3,4,5)P_3.

Similarly to PTEN knock down, knock down of PTEN and PIP4K2A in combination (PTEN + PIP4K2A) caused a 2-fold increase in the basal level of PtdIns(3,4,5)P_3. After 5 minutes the average level of PtdIns(3,4,5)P_3 was higher than control cells but due to the large variation between triplicate

samples this difference was not significant. After 20 minutes of H_2O_2 the level of PtdIns(3,4,5)P_3 was higher in PTEN + PIP4K2A cells and similarly to PTEN knock down although the level of PtdIns(3,4,5)P_3 decreased it was maintained at a significantly higher level than in control cells after 40 and 60 minutes of H_2O_2. Clearly PTEN and PIP4K2A contribute to the regulation of H_2O_2-induced PtdIns(3,4,5)P_3 production independently and at different stages of H_2O_2 signalling. Furthermore, the effect of PTEN + PIP4K2A knock down at each time point analysed is likely to be mediated by the knock down of either PTEN or PIP4K2A. The increased level of PtdIns(3,4,5)P_3 in basal conditions and the maintenance of PtdIns(3,4,5)P_3 after maximal production in PTEN + PIP4K2A cells reflects the effects of PTEN knock down alone and are therefore likely to be due to knock down of PTEN. Although based on this one experiment the increased PtdIns(3,4,5)P_3 observed in PTEN + PIP4K2A cells after 5 minutes of H_2O_2 was shown to be insignificant, assuming that further experiments would show that this increase is in fact significant, this would likely be due to loss of PIP4K2A as only knock down of PIP4K2A alone and not knock down of PTEN caused an increase in the level of PtdIns(3,4,5)P_3 at this time point.

Unlike previous experiments, this data strongly suggests that PIP4K2A and PTEN function independently to regulate the level of PtdIns(3,4,5)P_3. Knock down of PIP4K2A alone has been shown to increase the level of PtdIns(3,4,5)P_3 in response to acute H_2O_2 treatment and increase the level of PtdIns(3,4)P_2 in response to longer term H_2O_2 treatment. Considering the role of both of these lipids in the recruitment and activation of PKB it is likely that PIP4K2A is able to regulate PKB activation through the availability of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at different stages of H_2O_2 signalling. There is data that suggests that PIP4K over-expression decreases PKB activation by increasing the degradation of PtdIns(3,4,5)P_3. In that study the authors also observed an increase in PtdIns(3,4)P_2 levels suggesting that PIP4K regulates a PtdIns(3,4,5)P_3-5-phosphatase [155]. While our data in part agrees with this there are aspects that are not clear. For example in both oxidative stress stimulation and in insulin stimulation (Figures 6.12B and 6.14) there is no additive effect of PIP4K2A knock down and PTEN knock down on PtdIns(3,4,5)P_3 accumulation. This may not be surprising during oxidative stress treatment as PTEN is inactivated. However, PTEN knock down leads to a two-fold accumulation of PtdIns(3,4,5)P_3 upon insulin stimulation as does knock down of PIP4K2A. If PIP4K2A knock down suppresses the activity of a PtdIns(3,4,5)P_3-5phosphatase one would expect a clear additive accumulation of PtdIns(3,4,5)P_3. Furthermore the suppression of a PtdIns(3,4,5)P_3-5-phosphatase would not account for an increase in PtdIns(3,4)P_2 at later time points. Clearly the regulation of kinases and phosphatase by PIP4K2A that impact on PtdIns(3,4,5)P_3 accumulation is complex and warrants further detailed study.

This work utilises PIP4K2A as a cellular tool to manipulate the level of PtdIns5*P* but in order to substantiate the idea that PtdIns5*P* is responsible for regulation of PtdIns(3,4)P_2, experiments directly evaluating the effect of PtdIns5*P* would be required. Currently, fluorescently labelled PtdIns5*P* can be delivered into cells with a cationic polyamine carrier [52]. The development of protocols that allow the addition of soluble PtdIns5*P* into cells may prove to be very informative. However it would be important to ensure that the cellular location of exogenous PtdIns5*P* could be controlled as the existence and significance of discrete cellular pools of PtdIns5*P* with various cellular functions is known to be important and ideally, targeting exogenous PtdIns5*P* to the plasma membrane would allow specific evaluation of PtdIns5*P*mediated PKB activation.

Overall, considering that at each time point analysed knock down of either PTEN or PIP4K2A is likely to have independently mediated the effect of PTEN + PIP4K2A knock down on the level of PtdIns(3,4,5)P_3, it is probable that the enhancement of PKB phosphorylation in PTEN + PIP4K2A knock down cells is also due to knock down of either PTEN or PIP4K2A. Data obtained in western blotting experiments showed that there was no additive effect of knocking down both PTEN and PIP4K2A on the activation of PKB and there are several possible explanations for this. Firstly, due to the dynamic regulation of phosphoinositide metabolism, two independent signalling pathways that converge on a single signalling molecule are likely to be affected by one another due to feedback loops. Potentially, knock down of both PIP4K2A and PTEN could cause PKB to be so highly activated that a negative feedback loop is initiated to suppress further activation. Secondly, as described, knock down of PTEN when in combination with PIP4K2A knock down was always less efficient that PTEN knock down alone. The dose of PTEN has been shown to be very important in cancer progression [100] and if the cellular level of PTEN is slightly higher in PTEN + PIP4K2A knock down cells compared to PTEN knock down alone this may be adequate to suppress the level of PtdIns(3,4,5)P_3 and cause reduced activation of PKB.

In conclusion, PTEN and PIP4K2A contribute to the regulation of H_2O_2-induced PtdIns(3,4,5)P_3 production independently and at different stages of H_2O_2 signalling and only PIP4K2A is able to regulate the level of H_2O_2-induced PtdIns(3,4)P_2 accumulation. It is therefore likely that PTEN and PIP4K2A perform independent but complementary functions to regulate the activation of PKB.

6.4. Knock Down of PIP4K2A Leads to Maintained Phosphorylation of Numerous Signalling Molecules

Due to the pleotropic nature of H_2O_2, it can activate or inhibit a range of signalling pathways. In order to identify other pathways that might be regulated by PtdIns5*P* and to determine if increased

PtdIns5*P* acts as a hub for H_2O_2 signalling, an antibody array kit evaluating phosphorylation of Receptor Tyrosine Kinases was used (Figure 6.13). This comprised of 39 antibodies that specifically recognise phosphorylated proteins such as the insulin receptor (InsR), fibroblast growth factor receptor 1 (FGFR1), ephrin receptors (Eph) and PKB, many of which are phosphorylated in response to H_2O_2. PIP4K2A knock down cells were treated with 1mM H_2O_2 and lysates were produced. Similarly to previous experiments, knock down of PIP4K2A caused enhanced phosphorylation of PKB at Thr308 and Ser473 in response to H_2O_2, and the enhancement of Thr308 phosphorylation was greater than that of Ser473. These targets acted as internal controls and proved that in respect of PKB, knock down of PIP4K2A acted as expected. In basal conditions most of the target proteins were phosphorylated to a similar degree in control and PIP4K2A knock down cells. In response to 5 and 20 minutes of H_2O_2 treatment a subset of proteins were more highly phosphorylated in PIP4K2A knock down cells and after 60 minutes many of the proteins showed little or no phosphorylation in control cells but phosphorylation was maintained in PIP4K2A knock down cells. This data actually suggests that over a period of H_2O_2 stimulation, the phosphorylation of many signalling molecules decreases in control cells whereas in PIP4K2A knock down cells the phosphorylation is not necessarily increased but is maintained. An interesting example of this is the set of molecules involved in IGF1 and insulin signalling. In control cells the phosphorylation of InsR, IRS-1 and IGF-1R all steadily decrease upon H_2O_2 stimulation and after 60 minutes the phosphorylation of each is not detectable. In PIP4K2A knock down cells the phosphorylation of InsR is maintained throughout H_2O_2 treatment and the phosphorylation of IRS1 and IGF-1R is increased in response to 5 minutes and after a slight drop is then maintained. Overall 25 out of 39 target proteins showed enhanced phosphorylation in response to 60 minutes H_2O_2 in PIP4K2A knock down cells compared to control cells.

This experiment provides preliminary evidence to suggest that PtdIns5*P* plays a role in the regulation of H_2O_2-activated PKB at the level of plasma membrane receptors. By regulating numerous plasma membrane receptors that are all able to converge on PKB, PtdIns5*P* would be able to induce a rapid and precise response to oxidative stress. It has been shown that the ability of H_2O_2 to initiate PKB phosphorylation is dependent on IGF-1R/ InsR signalling using the AG1024 inhibitor and this antibody array has shown that when the level of PtdIns5*P* is increased by PIP4K2A knock down, phosphorylation of InsR, IGF-1R and IRS1 is maintained which could explain the maintenance of PKB phosphorylation observed in PIP4K2A knock down cells. Other signalling molecules that appear to be regulated in PIP4K2A knock down cells include; platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor 2 (VEGFR2), the T-cell antigen receptor kinase Zap70 and the non-receptor tyrosine kinase Src, all of which are associated with the activation of PKB. Overall, this data implicates PtdIns5*P* in the regulation of PKB through activation and maintenance of receptor signalling similarly to the described roles for PtdIns5*P* in the maintenance of EGF signalling [36].

6.5. Conclusions

Preliminary data indicated that PIP4K2A could regulate the activation of PKB through PTEN. Knock down of PIP4K2A or PTEN led to enhanced PKB phosphorylation and increased PtdIns5*P* in response to H_2O_2 and knock down of both PIP4K2A and PTEN in combination had no additive effect on either the phosphorylation of PKB or the level of PtdIns5*P*. Experiments aimed to elucidate the relationship between PIP4K2A and PTEN showed that they do not directly interact, PIP4K2A does not affect the stability, phosphorylation or H_2O_2-induced oxidation of PTEN but may regulate the ubiquitination of PTEN. However, quantification of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, two lipids responsible for the recruitment of PKB to the plasma membrane that are regulated by PTEN activity, strongly suggested that PIP4K2A and PTEN function independently of each other to regulate the levels of these two signalling lipids. PIP4K2A but not PTEN was able to regulate the level of H_2O_2-induced PtdIns(3,4)P_2 and the regulation of H_2O_2-induced PtdIns(3,4,5)P_3 production was regulated by either PIP4K2A or PTEN at different stages of H_2O_2 signalling.

Further to this, analysis of PtdIns(3,4,5)P_3 levels revealed that knock down of PIP4K2A and PTEN alone or in combination led to enhanced production of PtdIns(3,4,5)P_3 in response to 10µg/ml insulin (Figure 6.12B, MS analysis performed by Karen Anderson) however this did not equate to an increase in the phosphorylation of PKB (Figure 6.2B). This data shows that there is some disconnect between the level of PtdIns(3,4,5)P_3 and the activation of PKB. It is possible that the disconnect is a consequence of the high level insulin utilised in this study or that in fact at high levels of PtdIns(3,4,5)P_3 PtdIns5*P* can collaborate to help to activate PKB. Thus in insulin signalling, increased levels of PtdIns(3,4,5)P_3 observed with either PIP4K2A knock down or PTEN knock down are not translated into enhanced PKB activation because of a lack of a PtdIns5*P* signal. This is a particularly interesting observation considering that PtdIns5*P* has been previously linked to insulin signalling [18] and considering that insulin signalling machinery has been shown to be required for H_2O_2-induced PKB activation. This suggests that H_2O_2 is able to elicit a signalling output through the insulin signalling machinery that is distinct from that induced by insulin signalling itself and perhaps it is PtdIns5*P* that determines the specific downstream signalling specificity. A recent publication has described PTEN as a negative regulator of IRS1 as PTEN is able to directly dephosphorylate IRS1 and terminate downstream signalling, an effect which is negatively regulated by NEDD4, a PTEN ubiquitin ligase [95]. Preliminary data suggests that differential expression of PIP4K2A may regulate the ubiquitination of PTEN (Figure 6.11) therefore it can be hypothesised that PtdIns5*P* regulates signalling through IRS1 by mediating the ubiquitination of PTEN however substantial further work is required to investigate this hypothesis. Importantly this hypothesis indicates that PtdIns5*P* could

regulate the protein phosphatase activity of PTEN rather than the more commonly described lipid phosphatase activity.

Overall, this work has provided evidence that PIP4K2A specifically regulates PKB signalling though PtdIns5*P* in response to H_2O_2 and that this regulation is likely to occur at the plasma membrane through the regulation of H_2O_2-sensitive receptors leading to downstream regulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Furthermore, PtdIns5*P* seems to regulate PtdIns(3,4,5)P_3 in response to acute H_2O_2 treatment and PtdIns(3,4)P_2 in response to longer term H_2O_2 treatment in order to efficiently enhance initial PKB activation and also prolong maintenance of PKB signalling. Despite PtdIns(3,4,5)P_3 being regulated in PIP4K2A knock down cells in response to both H_2O_2 and insulin, only under conditions that increase the cellular level of PtdIns5*P* does increased PtdIns(3,4,5)P_3 lead to enhanced PKB activation, implicating PtdIns5*P* as a vital regulator of PKB.

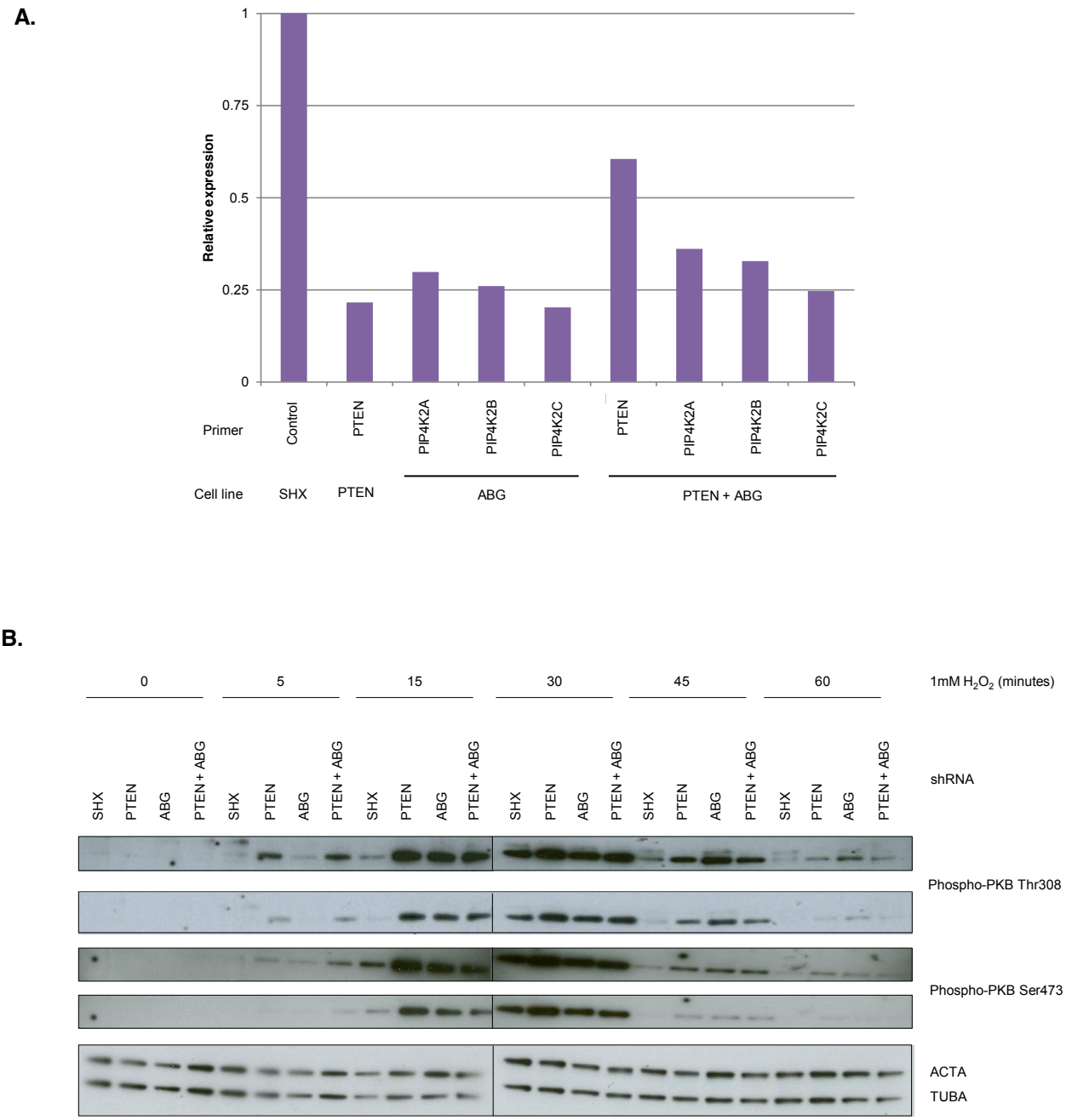
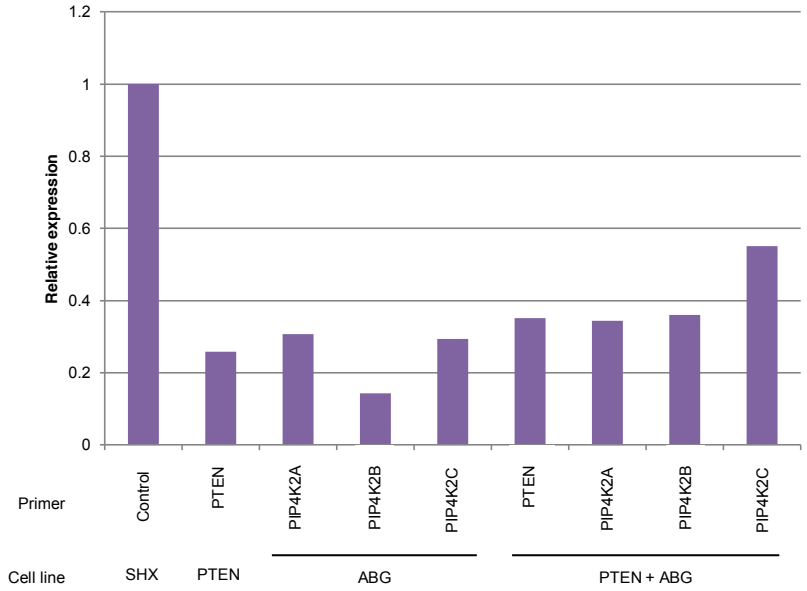


Figure 6.1. Knock down of PTEN regulates the activation of PKB in combination with PIP4K knock down. **A.** q RT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K2A, PIP4K2B and PIP4K2C (ABG) and/or PTEN. **B.** Lysates were prepared from cells transfected with shRNA targeting PIP4K2A, PIP4K2B and PIP4K2C (ABG) and/ or PTEN and kept in control conditions or treated with 1mM H_2O_2. Phosphorylated PKB was detected by western blotting and showed that phosphorylation of PKB at residues Ser473 and Thr308 is enhanced in PTEN knock down cells in response to oxidative stress similarly to PIP4K knock down cells. Knock down of PTEN and PIP4K in combination has no additive effect on the activation of PKB.

A.



B.

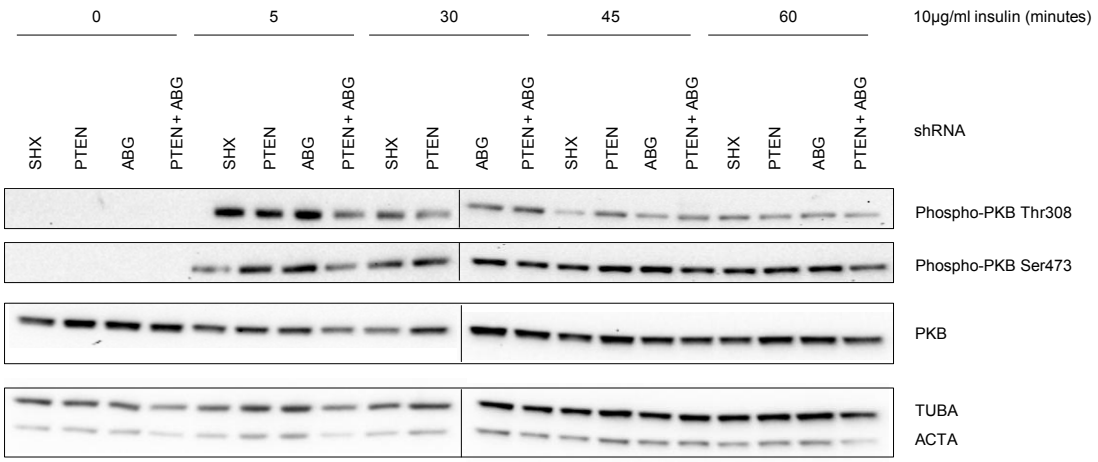
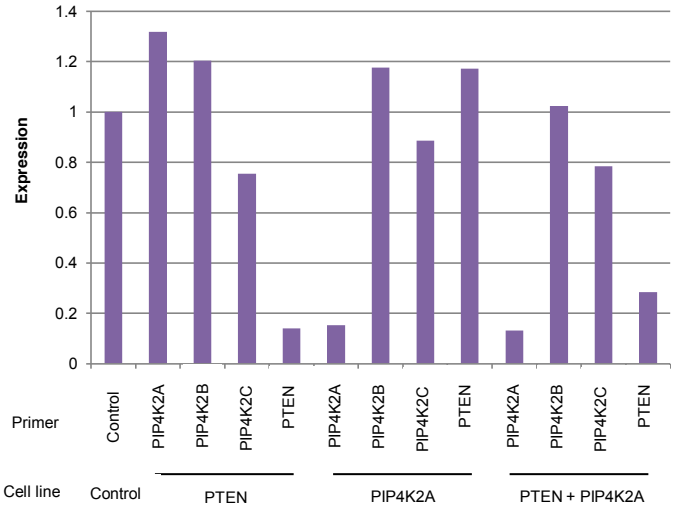


Figure 6.2. PTEN knock down has no effect on the activation of PKB in response to insulin.

A. q RT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K2A, PIP4K2B and PIP4K2C (ABG) and/or PTEN. **B.** Lysates were prepared from cells transfected with siRNA targeting PIP4K2A, PIP4K2B and PIP4K2C (ABG) and/ or PTEN and left in control conditions or treated with 10µg/ml insulin. Phosphorylated PKB was detected by western blotting and showed that phosphorylation of PKB at residues Ser473 and Thr308 is not affected in PTEN or PIP4K2A knock down cells. Knock down of PTEN and PIP4K in combination also had no effect on the activation of PKB.

A.



B.

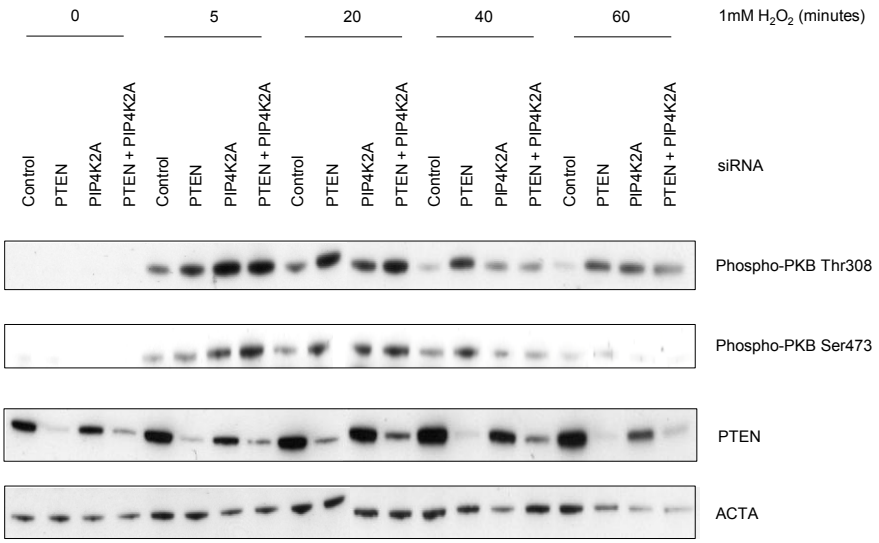


Figure 6.3. PTEN knock down regulates the activation of PKB in combination with PIP4K2A knock down. **A.** qRT-PCR was used to measure the mRNA level of the genes indicated in control cells or cells knocked down for PIP4K2A and/or PTEN. **B.** Control cells or cells transfected with siRNA targeting PIP4K2A and/or PTEN were maintained as controls or were treated with 1mM H_2O_2 for the times indicated. Cell lysates were separated by SDS-PAGE and phosphorylated PKB was detected by western blotting and showed that phosphorylation of PKB at residues Ser473 and Thr308 is enhanced in PTEN and PIP4K2A knock down cells in response to oxidative stress. Knock down of PTEN and PIP4K2A in combination showed no additive effect on the activation of PKB.

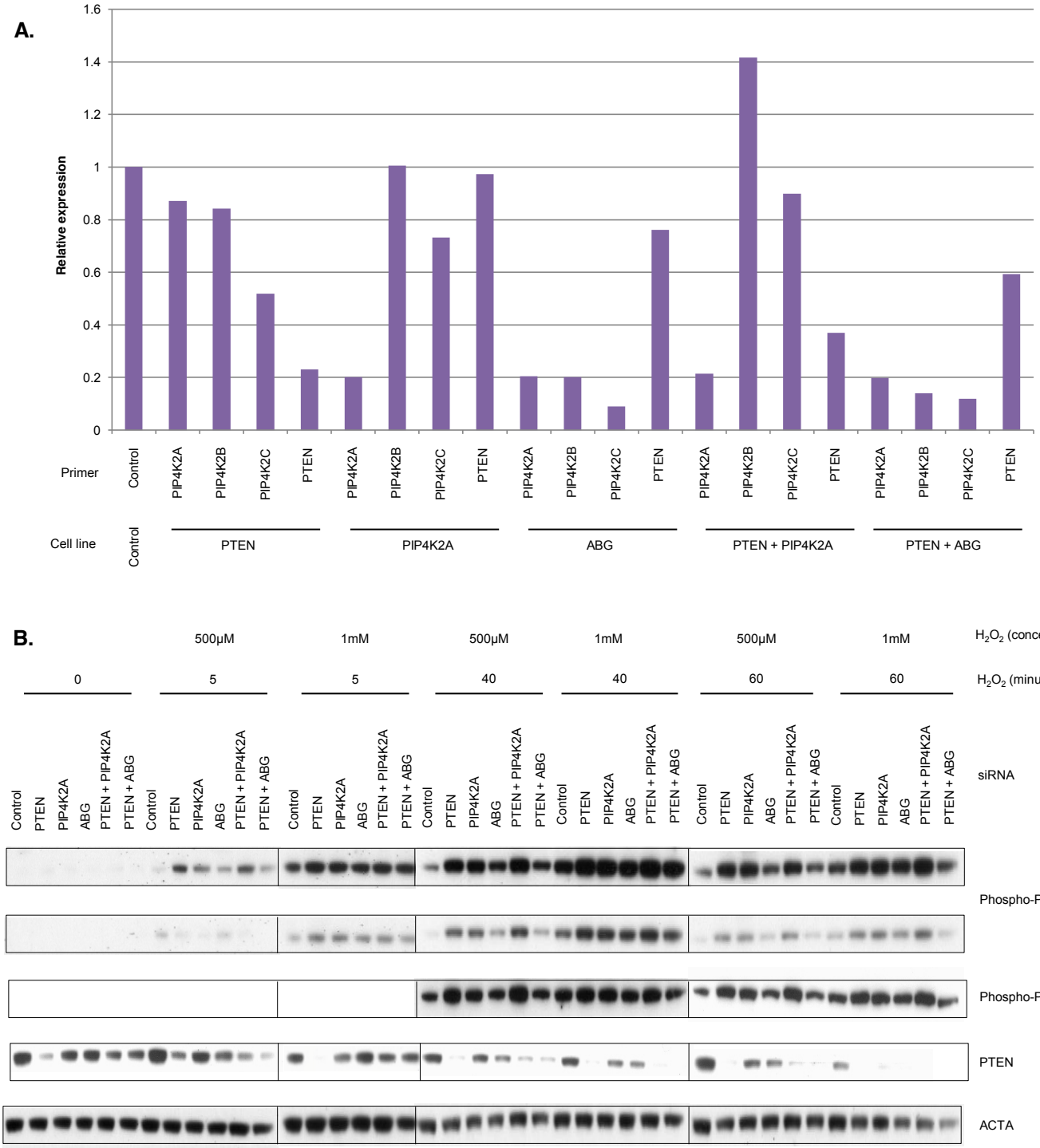


Figure 6.4. PTEN knock down regulates the activation of PKB in combination with PIP4K2A knock down more efficiently than in combination with the knock down of other isoforms of PIP4K.

A. qRT-PCR was used to measure the mRNA level of the indicated genes in control cells or cells knocked down for PTEN, PIP4K2A or ABG as stated. **B.** Control cells or cell transfected with siRNA targeting all PIP4K isoforms (ABG), PIP4K2A and/ or PTEN were kept in control conditions or treated with 500µM or 1mM H_2O_2 for the indicated times. Cell lysates were seperated by SDS-PAGE and phosphorylated PKB was detected by western blotting and showed that phosphorylation of PKB at residues Ser473 and Thr308 is enhanced in PTEN knock down cells in response to oxidative stress similarly to ABG and PIP4K2A knock down cells. Knock down of PTEN in combination with ABG or PIP4K2A knock down had no additive effect on the activation of PKB.

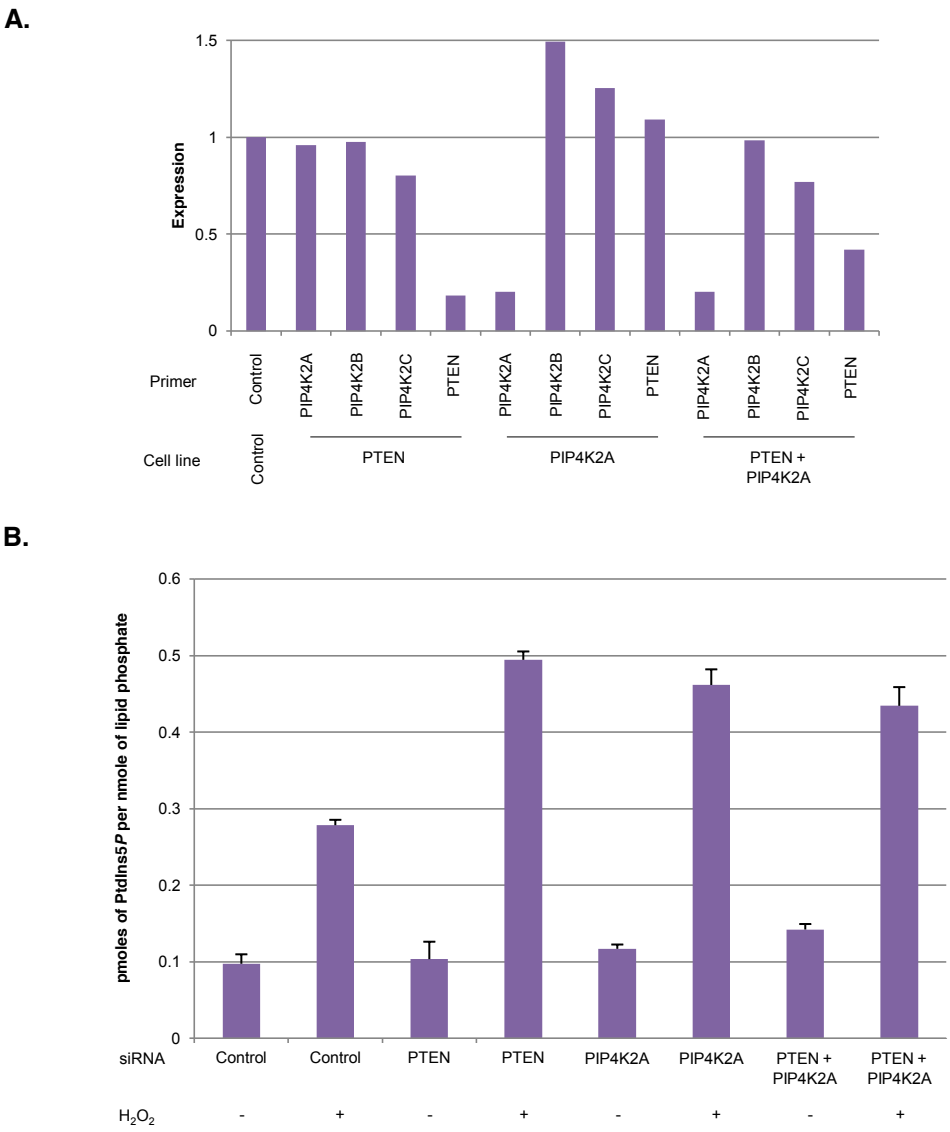


Figure 6.5. Knock down of PTEN and PIP4K2A increases the production of PtdIns5*P* in response to oxidative stress.

A. qRT-PCR was used to measure the mRNA level of the indicated genes in control cells or cells knocked down for PTEN and/or PIP4K2A. **B.** siRNA was used to knock down PIP4K2A, PTEN or both in combination. Cells were kept in control conditions or stimulated with 1mM H_2O_2 for 45 minutes. Lipids were extracted and the cellular level of PtdIns5*P* was quantified which revealed that knock down of PTEN, PIP4K2A or PTEN and PIP4K2A in combination increase the level of H_2O_2-induced PtdIns5*P* to a similar extent.

A.

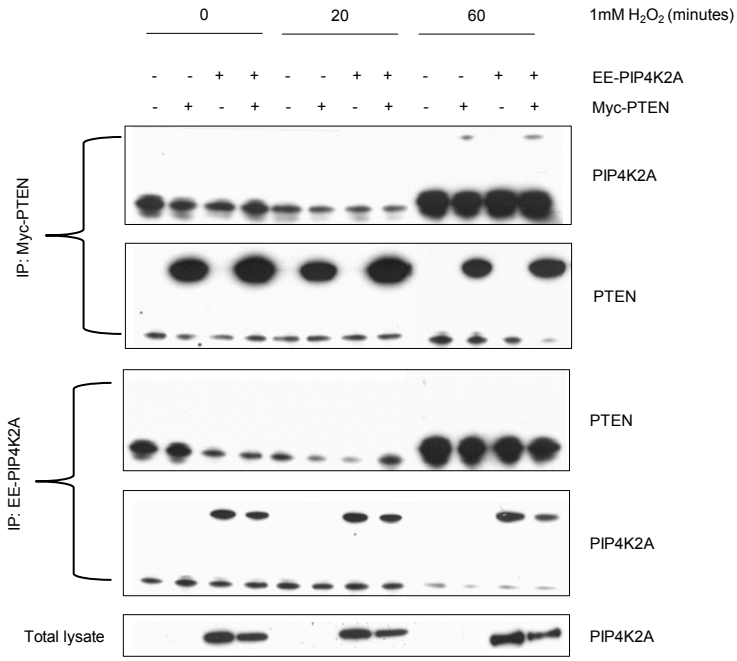


Figure 6.6. PIP4K2A and PTEN do not directly interact.

Co-immunoprecipitation experiments were performed with cells expressing EE-tagged PIP4K2A and Myc-tagged PTEN which were maintained as controls or were treated with 1mM H_2O_2 for the times indicated. In samples pulled down with a PTEN antibody (IP: Myc-PTEN), PTEN can be detected only in samples where Myc-PTEN was expressed however there was no consistent expression of PIP4K2A in samples expressing Myc-PTEN. Lower molecular weight bands may represent antibody components. In samples pulled down with a PIP4K2A antibody (IP: EE-PIP4K2A), PIP4K2A can be detected only in samples where PIP4K2A was expressed but PTEN was not detected in samples expressing EE-PIP4K2A. Overall analysis of immunoprecipitations showed that PIP4K2A and PTEN do not directly interact.

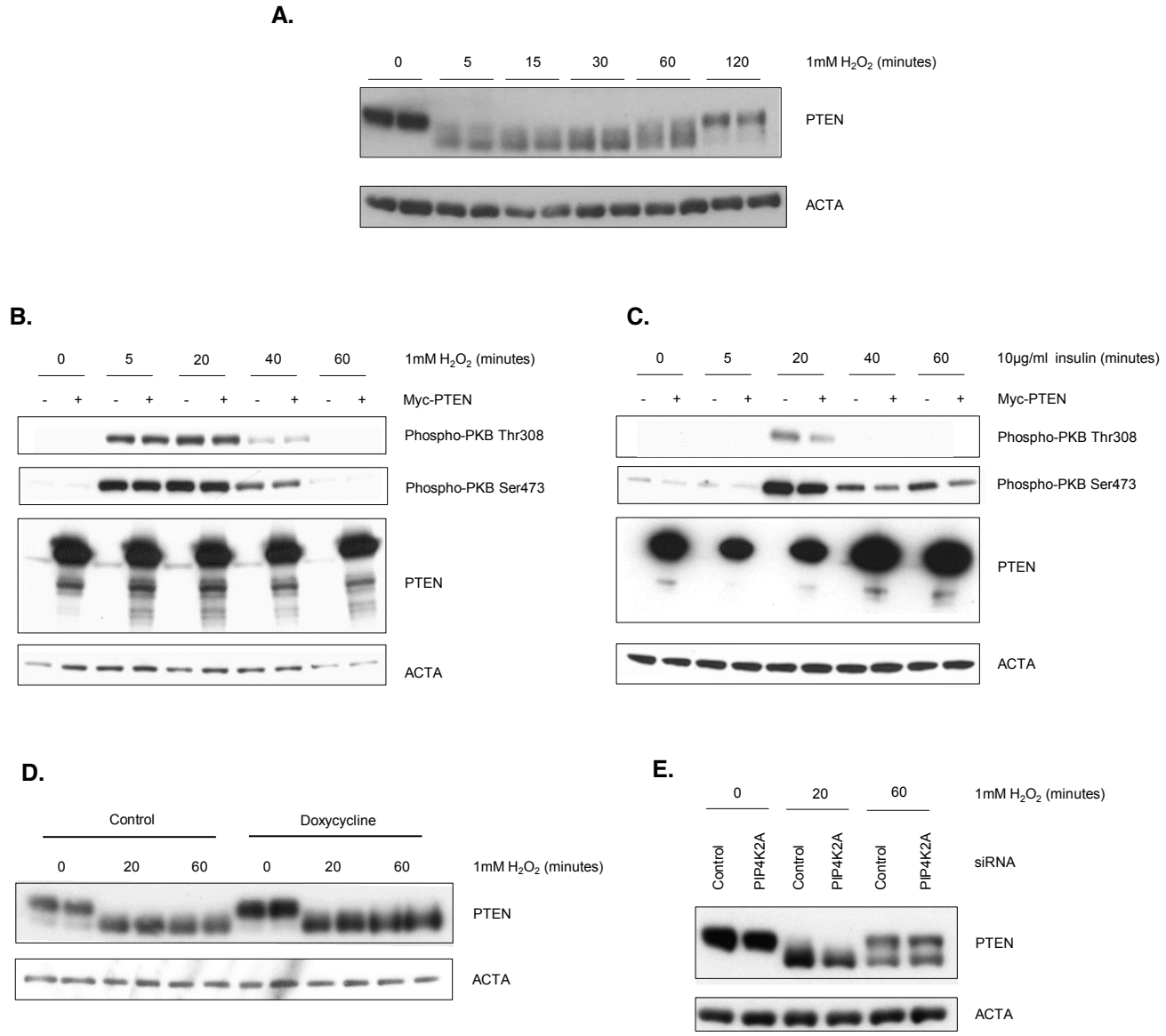


Figure 6.7. Knock down or over-expression of PIP4K2A does not affect H_2O_2-induced oxidation of PTEN. **A.** U2OS cells were kept as controls or treated with 1mM H_2O_2 for the indicated times. Cell lysates were treated with NEM, a small compound that forms stable, covalent thioether bonds with reduced cysteines which permanently blocks the formation of disulfide bonds in response to H_2O_2, and were separated by SDS-PAGE. Western blot analysis showed that the PTEN band shifts down in response to oxidative stress indicating that PTEN becomes oxidised. Each condition is represented by duplicate samples. **B.** Myc-PTEN was expressed in U2OS cells which were then kept in control conditions or treated with 1mM H_2O_2 for the indicated times. Cell lysates were separated by SDS-PAGE and western blotting revealed that over-expression of PTEN is unable to suppress the activation of PKB in these conditions. **C.** The same experiment as that described in B. was conducted with cells kept in control untreated conditions or treated with10µg/ml insulin. **D.** Control or PIP4K2A over-expressing cells were kept as control or treated with 1mM H_2O_2 for the indicated times and cell lysates were prepared and treated with NEM. Western blotting showed that in response to oxidative stress the oxidation of PTEN is not affected by PIP4K2A over-expression **E.** The same experiment as that described in D. was conducted with control or PIP4K2A knock down cells. Oxidation of PTEN is not affected by PIP4K2A knock down.

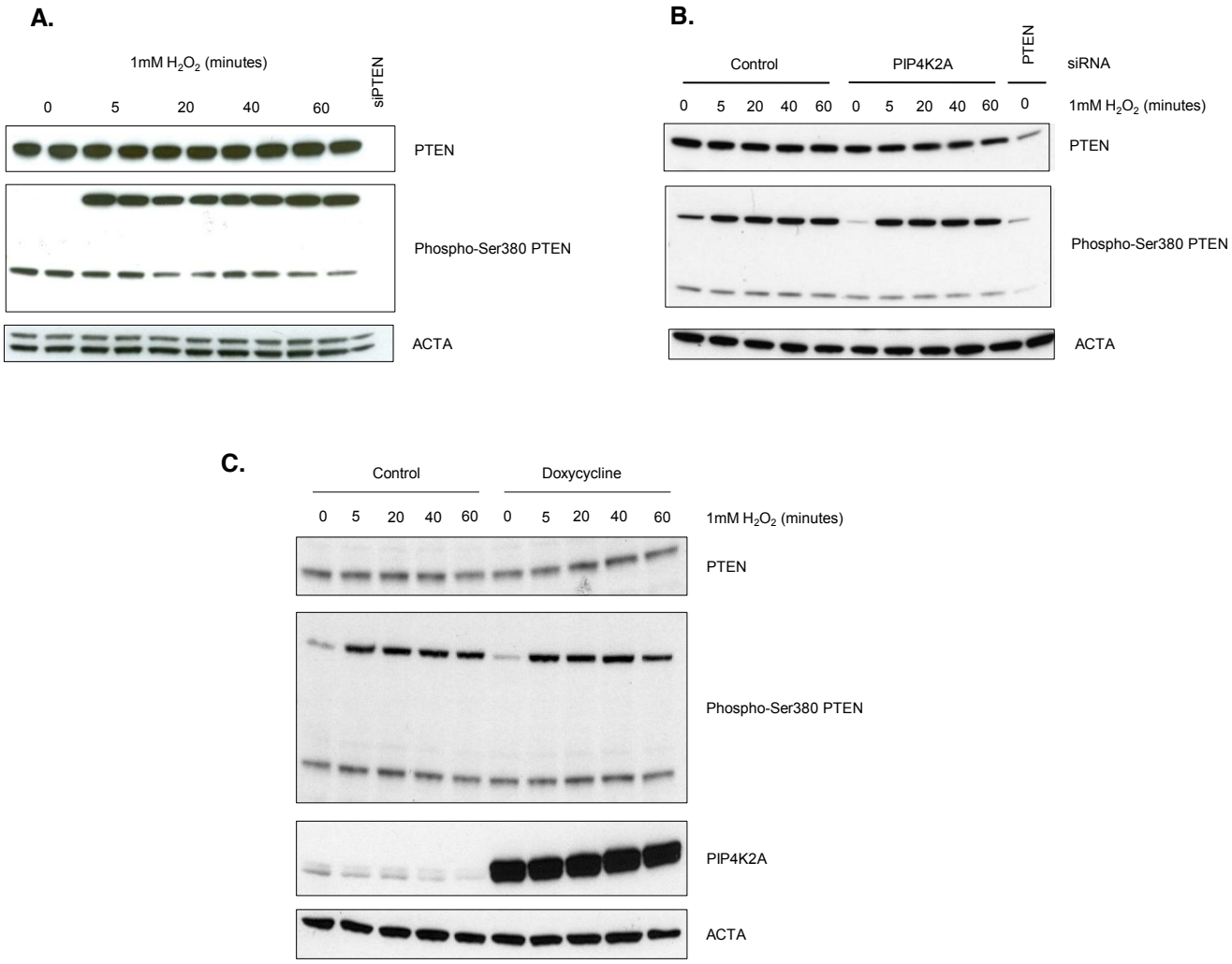
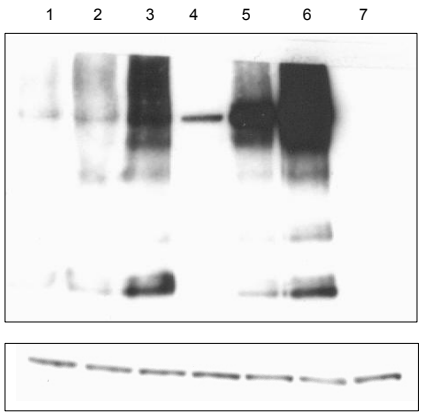


Figure 6.8. Knock down or over-expression of PIP4K2A does not affect the stability of PTEN in response to H_2O_2.

A. U2OS cells were maintained as controls or treated with 1mM H_2O_2 for the times indicated and the protein level of PTEN in cell lysates was analysed by western blotting which showed that the total level of PTEN does not change in response to oxidative stress treatment. **B.** Control and PIP4K2A knock down cells were kept in control conditions or treated with 1mM H_2O_2 for the times indicated . Cell lysates were separated by SDS-PAGE and PTEN and phosphorylated PTEN were detected by western blotting which revealed that knock down of PIP4K2A does not affect the stability of PTEN in response to oxidative stress. **C.** The same experiments as that described in B. was conducted using control and PIP4K2A over expressing cells. The stability of PTEN in response to oxidative stress treatment was not affected by PIP4K2A over-expression.

A.



1. Lysate + 1ul PTEN antibody O/N then 2 hours with beads following day
2. Lysate + 2ul PTEN antibody O/N then 2 hours with beads following day
3. Lysate + 5ul PTEN antibody O/N then 2 hours with beads following day
4. Beads + 1ul PTEN antibody for 2 hours then beads + lysate for 2 hours
5. Beads + 2ul PTEN antibody for 2 hours then beads + lysate for 2 hours
6. Beads + 5ul PTEN antibody for 2 hours then beads + lysate for 2 hours
7. Beads + no antibody for 2 hours then beads + lysate for 2 hours

B.

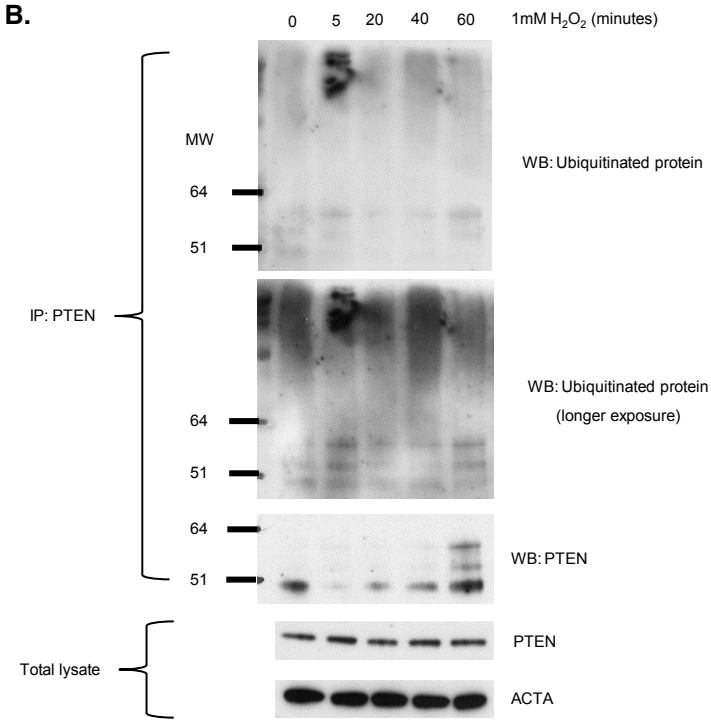


Figure 6.9. Optimisation of endogenous ubiquitin pull down assay.

A. Different conditions were tested for optimum PTEN pull down. Protocol number 6 was chosen for future experiments. **B.** Wild type U2OS cells were stimulated with 1mM H_2O_2 for the times indicated and PTEN was immunoprecipitated using protocol 6 from A. Cell lysates were then separated by SDS-PAGE and probed for endogenous ubiquitin but no smear was readily detected.

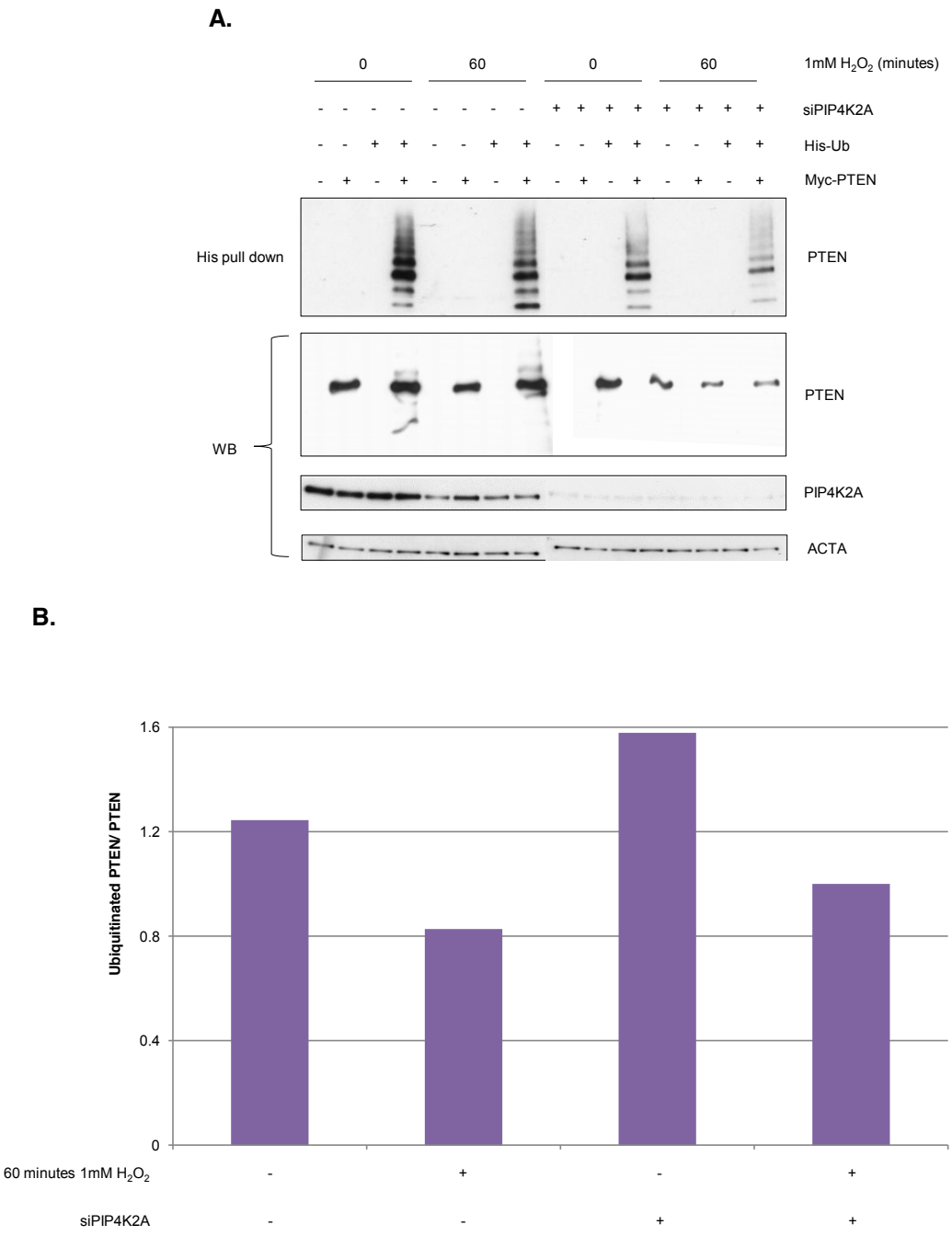


Figure 6.10. PTEN ubiquitination is not affected in PIP4K2A knock down cells.

A. His-tagged ubiquitin and Myc-tagged PTEN were expressed in control and PIP4K2A knock down U2OS cells. Following 60 minutes1mM H_2O_2 treatment, His-tagged ubiquitin was affinity purified with nickel beads and lysates were probed for PTEN. The amount of ubiquitinated PTEN detected in PIP4K2A knock down cells did not change compared to control treated cells . **B.** Quantification of A. using TINA software.

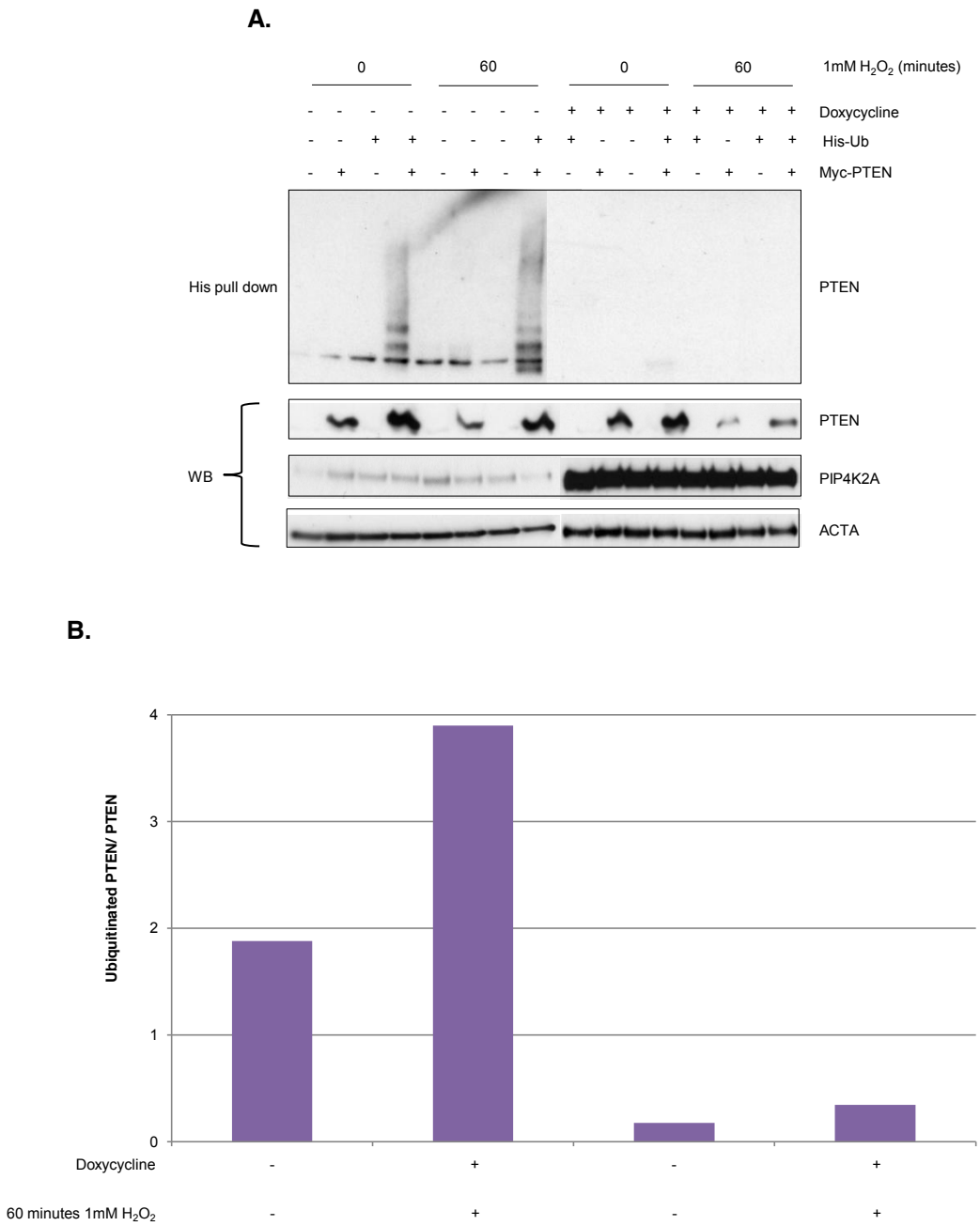


Figure 6.11. PTEN ubiquitination is reduced in cells over-expressing PIP4K2A.

A. His-tagged ubiquitin and Myc-tagged PTEN were expressed U2OS cells which inducibly express PIP4K2A when treated with doxycycline. Cells were treated or not with doxycycline as indicated for 24 hours and were then treated with 1mM H_2O_2 for 60 minutes. His-tagged proteins were affinity purified on nickel beads and bound proteins were probed for PTEN by western blotting. The amount of ubiquitinated PTEN detected in PIP4K2A over-expressing cells was reduced compared to control treated cells under oxidative stress conditions.

B. Quantification of A. using TINA software.

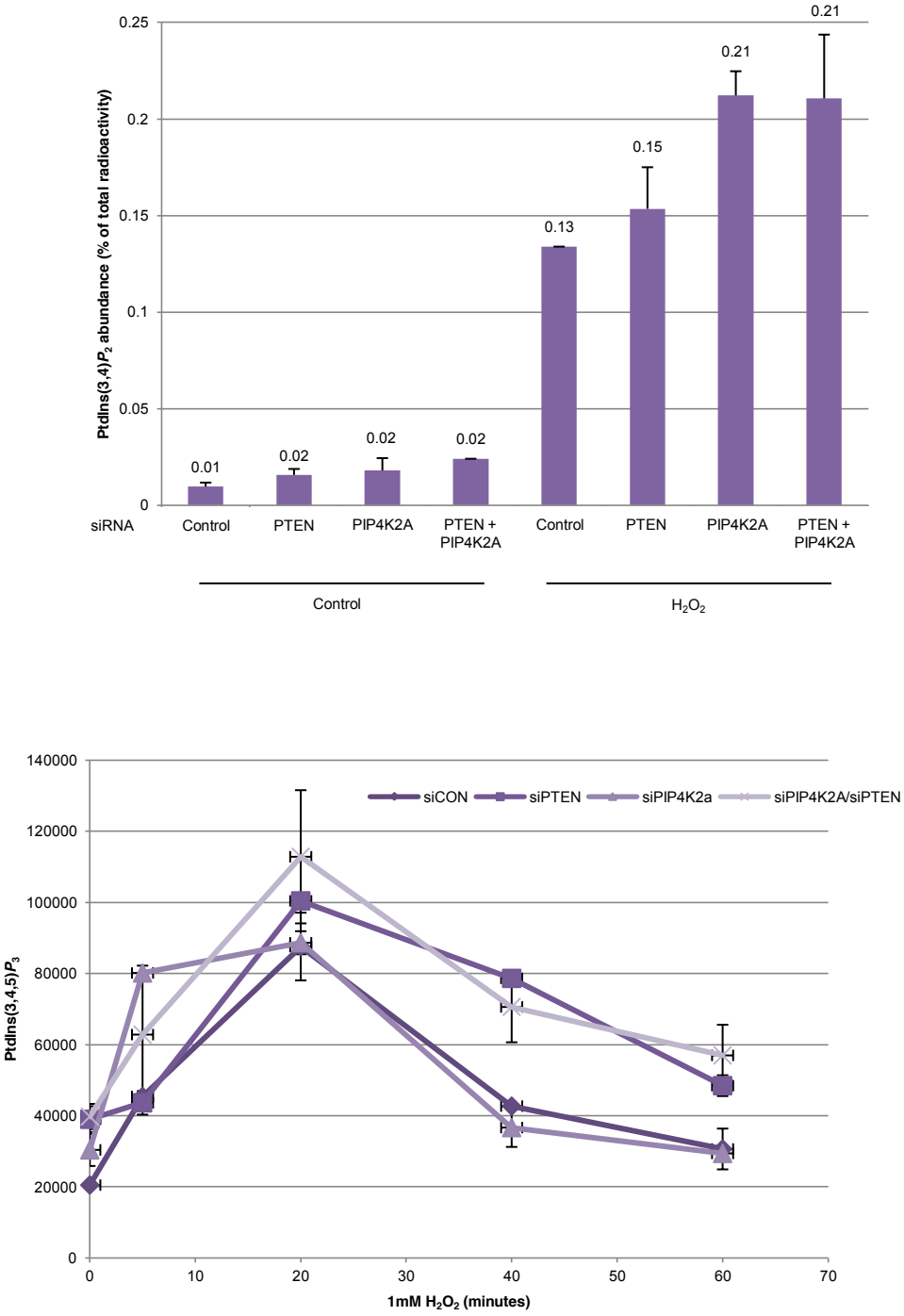


Figure 6.12. Knock down of PIP4K2A enhances production of H_2O_2-induced PtdIns(3,4)P_2 and knock down of PIP4K2A or PTEN independently regulate H_2O_2-induced PtdIns(3,4,5)P_3.

A. Knock down U2OS cells were radio-labelled with ^{32}P-orthosphosphate, treated with 1mM H_2O_2 for 20 minutes and extracted lipids were deacylated and the lipid head groups were analysed by HPLC. In PIP4K2A knock down cells the cellular level of PtdIns(3,4)P_2 is higher after 20 minutes of oxidative stress but there was no effect of PTEN knock down. **B.** Knock down U2OS cells were treated with 1mM H_2O_2 for the indicated times and the level of PtdIns(3,4,5)P_3 was analysed by mass spectrometry. Knock down of PIP4K2A or PTEN affect the level of H_2O_2-induced PtdIns(3,4,5)P_3 at different time points.

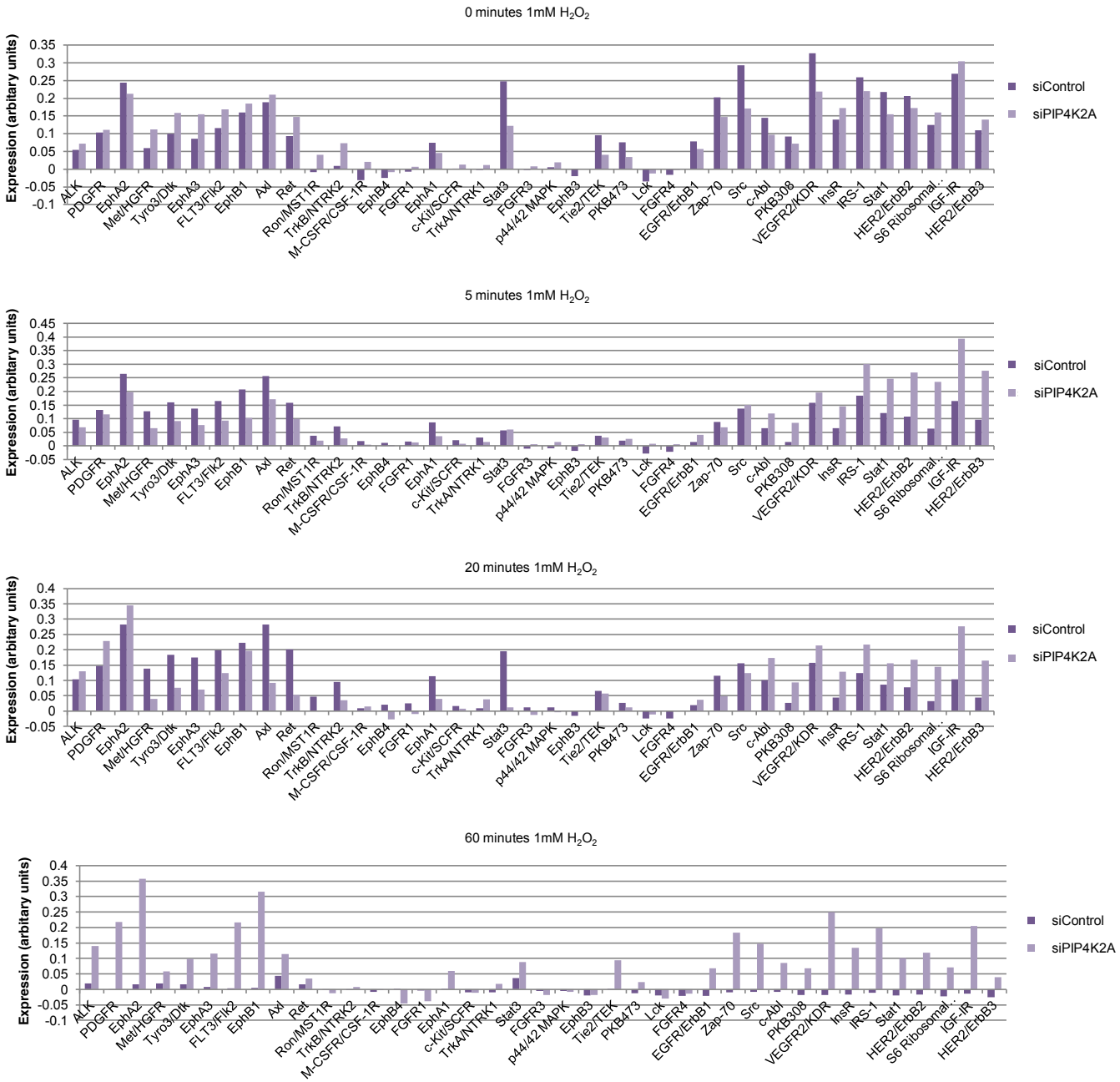


Figure 6.13. PIP4K2A knock down affects the phosphorylation of numerous signalling molecules in response to H_2O_2.

Using an antibody array kit, control or knock down PIP4K2A cells were kept in control conditions or stimulated with 1mM H_2O_2 for 5, 20 or 60 minutes and lysates were used to probe antibody arrays containing 39 different phospho-signalling molecules. In response to H_2O_2 the phosphorylation of many signalling molecules is maintained at higher levels in PIP4K2A knock down cells compared to control cells.

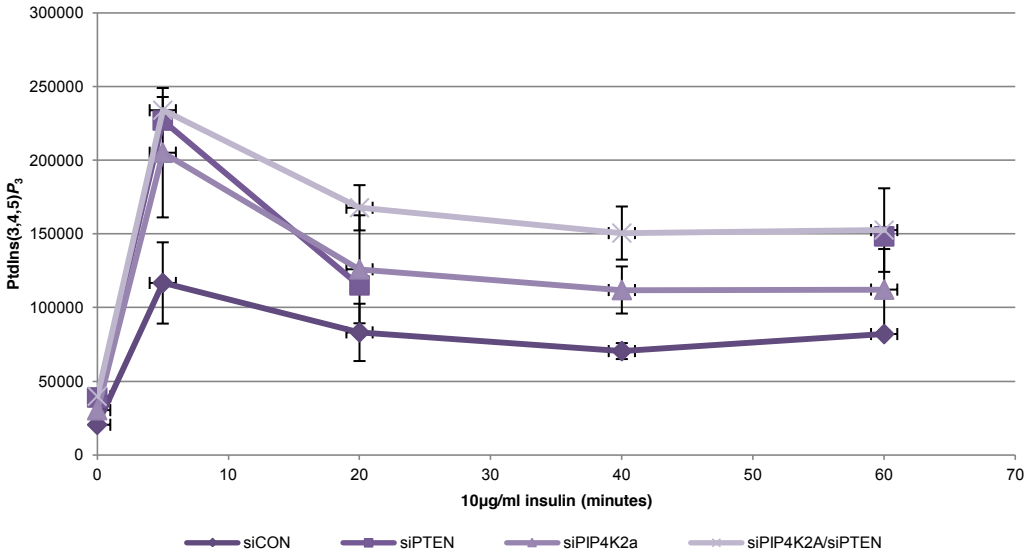


Figure 6.14. Knock down of PTEN or PIP4K2A enhances the level of PtdIns(3,4,5)P_3.
Control cells and cells knocked down for PIP4K2A, PTEN or PIP4K2A and PTEN were kept in control conditions or treated with 10µg/ml insulin for the indicated times and the level of PtdIns(3,4,5)P_3 was analysed by mass spectrometry. Knock down of PIP4K2A and PTEN enhanced the level of insulin-induced PtdIns(3,4,5)P_3.

7. Discussion

In this study I have attempted to understand how the PIP4K/PtdIns5*P* pathway is regulated in response to oxidative stress and what pathways downstream of changes in oxidative stress might be regulated. Oxidative stress is thought to be one of the major causative agents that underlies the pathologies that occur as a consequence of ageing and is also important in some instances in the development of insulin resistance and the development of diabetes. There is also an increased incidence of cardiac failure in mice when cardiomyocytes are subject to specific increases in oxidative stress probably as a consequence of the induction of accelerated senescence [170]. Furthermore the strong link between oxidative stress, senescence, cancer and pathologies of aging suggest that it is of global importance to identify cellular pathways that are specifically regulated by increased oxidative stress and how these might impact on downstream signalling.

In collaboration with Dr. D. Jones I found that levels of PtdIns5*P* were strongly increased in cells that have increased accumulation of reactive oxygen species (ROS). The increase was induced by inhibiting endogenous pathways that are involved in ROS detoxification. This increase in PtdIns5*P* could be mimicked by treating cell with exogenous H_2O_2. Importantly H_2O_2 treatment inhibits cell proliferation, which could be alleviated to some extent by reducing the levels of PtdIns5*P* generated in response to H_2O_2 treatment [11]. These data suggest that PtdIns5*P* induced by oxidative stress impacts on cellular outcomes associated with that stress. H_2O_2 is a pleotropic agent as it is able to cross the plasma membrane and de-regulate a number of intracellular pathways. It impacts on tyrosine kinase receptor signalling through its ability to inactive tyrosine phosphatases and can impact on metabolism through its ability to oxidise critical substrates such as NADH. However, nothing is known about how it impacts on PtdIns5*P* generation. In my studies I used a panel of well classified inhibitors to attempt to define which pathways downstream of H_2O_2 treatment might regulate the level of PtdIns5*P*. Inhibition of the p38 MAP kinase pathway or chemical activation of this pathway had little impact on stress-induced PtdIns5*P* generation which was surprising as previous studies have implicated this pathway in PtdIns5*P* generation [148]. Analysis of the inhibitors that were used suggests that PIP5K and PIKfyve, two enzymes that have been suggested to generate PtdIns5*P*, are not involved in stress-induced PtdIns5*P* generation. We also carried out genetic knock down and over-expression experiments to confirm the lack of a role for PIKfyve in H_2O_2-induced PtdIns5*P* generation. A number of inhibitors appeared to subtly affect the production of PtdIns5*P* suggesting

that there might be different pools of PtdIns5*P* that are regulated by different pathways in response to H_2O_2. Data obtained using a library of shRNA constructs targeting phosphoinositide-regulating enzymes also indicated that PtdIns5*P* is likely to be regulated by numerous phosphoinositide dependent pathways. Across the two screens, inhibition or knock down of numerous targets led to a change in the level of H_2O_2-induced PtdIns5*P* of approximately 30% compared to control treated cells. The existence of cellular pools of PtdIns5*P* that are regulated by distinct subsets of enzymes that are localised in the same or different sub-cellular compartment suggests that in response to oxidative stress the level of PtdIns5*P* is increased in various sub-cellular locations in order to engender a cellular response. It is possible that measuring PtdIns5*P* in different sub-cellular compartments might have led to the identification of a strong regulator of stress-induced PtdIns5*P* however this would have been extremely difficult to determine using the assays available.

Three pathways that apparently regulate stress-induced PtdIns5*P* generation warrant further discussion. AG1024 is a relatively ineffective inhibitor of the IGF1R (7μM) and the insulin receptor (57μM) tyrosine kinase. Surprisingly AG1024 increased H_2O_2-induced PtdIns5*P* suggesting that inhibition of insulin signalling increases PtdIns5*P*. However, further analysis showed that in fact AG1024 is also an inhibitor of PIP4K activity *in vitro*. In accordance, a structurally similar molecule I-OMe-AG538 was recently also found to inhibit PIP4K activity [7, 110], and indeed the data shows that I-OMe-AG538 is a much more potent PIP4K inhibitor compared to AG1024 *in vitro*. Surprisingly however I-OMe-AG538 does not increase cellular PtdIns5*P* levels *in vivo* in response to H_2O_2 while AG1024 does. This might suggest that I-OMe-AG538 does not enter cells or that it is unable to inhibit PIP4K *in vivo* or that treatment with H_2O_2 disrupts its activity. Nonetheless my studies unveil for the first time that acute inhibition of PIP4K leads to an increase in cellular PtdIns5*P* levels. That AG1024 functions through PIP4K inhibition was suggested by a lack of effect of the inhibitor on oxidative stress-induced PtdIns5*P* levels when all three PIP4K isoforms were knocked down.

Both chemical and genetic inhibition of PTEN led to increased oxidative stress-induced PtdIns5*P*. The acute chemical inhibition probably rules out a long term effect of PTEN downregulation on gene transcription and its subsequent effect on the level of PtdIns5*P*. PTEN might regulate PtdIns5*P* directly or might regulate another phosphoinositide that impacts on PtdIns5*P* generation. Alternatively it might be a lipid phosphatase-independent but protein phosphatase-dependent mechanism. Interestingly knock down of PTEN together with PIP4K2A did not lead to enhanced PtdIns5*P*

generation compared to knock down of PIP4K2A alone. While care should be taken interpreting these data, they might suggest that PTEN regulates PtdIns5*P* through inhibition of PIP4K2A activity. It is possible that PtdIns(3,4,5)P_3 induced by PTEN knock down might impact on PtdIns5*P* generation and inhibition of the PI3K pathway with three structurally different chemical inhibitors suppressed PtdIns5*P* generation induced by H_2O_2. Taken together this might suggest that PTEN induced PtdIns(3,4,5)P_3 production can lead to the inhibition of PIP4K2A activity which in part enables H_2O_2 induced PtdIns5*P* generation. This link between PIP4K2A and PTEN is further illustrated by the ability of PIP4K2A to regulate PtdIns(3,4,5)P_3 accumulation in response to both insulin and H_2O_2. At early time points of insulin-induced PtdIns(3,4,5)P_3 generation, knock down of PIP4K2A and PTEN are clearly not additive. At later time points PtdIns(3,4,5)P_3 generation does appear to be additive. The scenario in H_2O_2 stimulation is more complex and difficult to interpret as H_2O_2 stimulation reversibly inactivates PTEN by oxidation. However, the data might suggest that PIP4K2A knock down impacts on PtdIns(3,4,5)P_3 accumulation in both a PTEN dependent and independent manner. Further studies to investigate how PIP4K2A might impact on PTEN revealed that PIP4K2A might regulate PTEN ubiquitination. The role of ubiquitination on PTEN function is complex and has been suggested to regulate its localisation, its lipid phosphatase activity and its protein phosphatase activity. For example, insulin/IGF1-mediated NEDD4 ubiquitination of PTEN prevents PTEN from de-phosphorylating activated IRS1 [95]. Activated IRS1 leads to enhanced PtdIns(3,4,5)P_3 signalling and activation of other pathways downstream of IRS1. Interestingly, data obtained using an antibody array kit showed that H_2O_2-mediated phosphorylation of IRS1 is strongly maintained in PIP4K2A knock down cells compared to control cells, suggesting that PtdIns5*P* could regulate the protein phosphatase activity of PTEN on IRS1. Further studies will be required to understand how the interplay between PTEN and PIP4K2A is regulated and how it might impacts on downstream signalling.

The third pathway that deserves mention is illustrated by inhibition by nicotinamide. Nicotinamide can inhibit the SIRT deacetylases, inhibit PARP1 and is a substrate that can be used to re-fill pools of NAD^+/NADH. In order to distinguish between these possibilities we assessed the inhibition of PARP1 using the specific inhibitor olaparib. Inhibition of PARP1 also decreased H_2O_2-induced PtdIns5*P* generation and was not additive with nicotinamide treatment suggesting that they both act through the same pathway. Treatment with H_2O_2 induces DNA breaks which leads to the activation of PARP1 and sustained poly-ADP ribosylation of DNA, which leads to a decrease in cellular levels of NAD^+/NADH. The decrease in NAD^+/NADH can be blocked by treatment with a PARP1 inhibitor. The fact that olaparib and nicotinamide might work through the same pathway suggests that either PARP1 activity or the loss of NAD^+/NADH plays a role in regulating stress-induced PtdIns5*P* generation. Other experiments aimed at manipulating the NAD^+/NADH levels might reveal which

aspect is the most important. Understanding how PARPs and PIP4K/PtdIns5*P* might interact could be important in defining populations of patients that might have enhanced sensitivity to PARP inhibitors.

How PtdIns5*P* is regulated is not clear. Previous studies have indicated that PIKfyve is a critical regulator of PtdIns5*P* levels however our studies were unable to demonstrate a role for this enzyme in regulating stress-induced PtdIns5*P*. I therefore tried to discover which phosphoinositide modulators might play a role in PtdIns5*P* generation by using an shRNA approach targeting all known phosphoinositide modulators. This screen revealed that the PIP4K family and PTEN could regulate stress-induced PtdIns5*P* accumulation suggesting that the approach adopted was suitable to find regulators of PtdIns5*P* generation. However, although a number of targets were discovered that appeared to be required for PtdIns5*P* generation in response to H_2O_2 treatment, they could not be subsequently validated. This was surprising as the initial screen was carried out on three separate biological samples which in most cases gave very good statistical differences from the SHX control. Given that we utilised the same virus batch to attempt to validate the screen it is unlikely that the inability to confirm the results represents an off-target effect. More likely it is the inconsistency between the assays that led to the problems. The assay was carried out in sets of 24 samples, three control samples (SHX), three control transduced samples treated with H_2O_2 (SHX) and six different targeted shRNA samples in triplicates stimulated with H_2O_2. In order to screen the library, 17 sets of assays were carried out over approximately 3 months. While the assay performed well on replicates within individual assays (Figure 4.2), it appeared much worse when the control and H_2O_2-treated SHX samples were compared across the 17 sets of assays (Figure 4.11). The only way to correct this would be to perform the entire screen in duplicates or alternatively in hind sight it may have been better to carry out only single points and repeat the whole screen. The development of better assays that are not so labour intensive with so many steps will likely allow a much better screen for regulators of PtdIns5*P*. Currently there are no simple tools that would enable the visualisation of PtdIns5*P* in cells that could be used as a screening methodology.

By genetic knock down or over-expression of PIP4K2A to manipulate the cellular level of PtdIns5*P* this study has implicated PtdIns5*P* in the regulation of PKB activation. In PIP4K2A knock down cells activation of PKB was observed at early time points and again at later points. The later time points coincided with the peak of PtdIns5*P* generated in response to oxidative stress. Activation of PKB was assessed by monitoring the phosphorylation of Ser473 and Thr308, which are phosphorylated by mTORC2 and PDK1 respectively. As the phosphorylation of both sites was increased in response to PIP4K2A knock down we hypothesised that an upstream regulator was modulated by PIP4K2A knock down. The levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, two lipids that are critical for the activation of PKB and can regulate the kinase ability of mTORC2 and PDK1, were increased in PIP4K2A knock

down cells. At early time points PtdIns(3,4,5)P_3 was increased while at later time points PtdIns(3,4)P_2 was increased, which coincided with the peak of PtdIns5*P*. A similar analysis during insulin stimulation showed that PIP4K2A knock down strongly increased early PtdIns(3,4,5)P_3 accumulation and also decreased the removal of PtdIns(3,4,5)P_3 at later time points. Surprisingly, we did not observe increased PKB phosphorylation in response to insulin. This might reflect that we were unable to observe any increases in PtdIns5*P* accumulation in wild type or PIP4K2A knock down cells in response to insulin or that the increase in PtdIns(3,4,5)P_3 driven by the insulin dose used (10ug/ml) was maximal for PKB stimulation but not for PtdIns(3,4,5)P_3 synthesis. Nonetheless taken together these data leave us in a paradoxical situation. Many studies including the ones from this laboratory [11] have implicated PtdIns5*P* in the regulation of PtdIns(3,4,5)P_3 and PKB activity however my time course data suggests a disconnect between PtdIns(3,4,5)P_3/PKB activation and increased PtdIns5*P* synthesis with the extreme of no observable PtdIns5*P* increase with insulin stimulation yet a strong increase in PtdIns(3,4,5)P_3 synthesis. Given the many caveats, which might include small changes in PtdIns5*P* that occur within sub-cellular contexts that cannot be measured with this assay, we might suggest that PIP4K2A impacts on PtdIns(3,4,5)P_3/PKB activation in a lipid kinase dependent and independent manner. Further studies aimed at elucidating the contribution of the kinase activity might include genetic knock-in of a kinase dead allele, or over-expression of wild type and kinase inactive PIP4K2A to determine how each suppresses the observed activation of PKB upon knock down of PIP4K2A.

Knock down of PIP4K2A also induced strong cell growth defects leading to a cessation of proliferation with an increased number of cells in G1. This attenuation of growth appears paradoxical considering the well-established role for PKB in enhancing cell growth. However, it should be noted that hyper-activated PKB can induce premature senescence and sensitise cells to ROS-mediated apoptosis [159]. We did not check if the cessation in proliferation was dependent on PKB as U2OS cell growth is strongly dependent on continued PtdIns(3,4,5)P_3 and PKB activation [11]. A different study also found that knock down of PIP4K2A and PIP4K2B together in breast cancer cell lines caused enhanced PtdIns(3,4,5)P_3 production, phosphorylation of PKB and cell senescence [68]. In that instance the induction of cell senescence was shown to be independent of the PI3K/PKB pathway but dependent on cellular accumulation of ROS. An analysis of which isoforms of PIP4K induce either PKB activation or cessation of proliferation showed that loss of PIP4K2A induced both phenotypes, while loss of PIP4K2C did not affect PtdIns(3,4,5)P_3 signalling but did lead to a cessation of proliferation. PIP4K2C has been shown to have very low PIP4K activity but can recruit PIP4K2A by interaction. Whether this interaction impacts on any given phenotype is completely unclear. Given that PIP4K2C is predominantly localised to endomembranes [45] and that PIP4K2A is localised to the cytoplasm/plasma membrane it is possible that the activation of PtdIns(3,4,5)P_3/PKB and the cessation of proliferation occur as a consequence of the regulation of PIP4K2A at two different

localisations. Thus PtdIns(3,4,5)P_3/PKB activation might occur as a consequence of PIP4K2A regulation at the plasma membrane and regulation of PIP4K2A at the endomembranes as a consequence of its interaction with PIP4K2C may determine cell growth. Studies by Clarke et al [171] have determined how to convert the very low activity of PIP4K2C to an enzyme with PIP4K activity that is not highly dissimilar to PIP4K2A using a minimal number of mutations. Use of this mutant might by-pass the requirement for PIP4K2C mediated recruitment of PIP4K2A enabling the demonstration that the PtdIns(3,4,5)P_3 accumulation and the cessation of proliferation are regulated in two distinct ways. If this is the case then it would be the first demonstration of the requirement for heterodimerisation of the PIP4K isoforms in a physiological process [44]. Furthermore, two studies have implicated PIP4K as essential for tumour growth and importantly demonstrate that they are not essential for normal cell growth [68] [43]. This suggests that loss of PIP4K acts synthetically with something that is present in tumour cells that prevents cell growth. Given that PtdIns5*P* is strongly regulated by oxidative stress and that oncogenic mutations generally increase the cellular accumulation of ROS it might be that the critical factor that synthetically combines with PIP4K inhibition is the accumulation of reactive oxygen species [68].

The possibility that interaction between different phenotypes of PIP4K might play different roles in cell growth and proliferation is an important and relevant observation from the point of view of drug development. Keune et al. showed that low expression of PIP4K2B in breast tumours (which would be expected to cause increased PtdIns5*P*) is associated with increased tumour size, development of metastases and worse patient prognosis [172]. In contrast knock down of PIP4K2A in leukaemia cells and in human breast cancer cells [43] inhibits cell growth, attenuates tumour development and importantly does not appear to effect normal cell growth. These data suggest that specific inhibitors of PIP4K would be useful as therapeutic targets and my studies would implicate that the development of PIP4K isoform specific inhibitors might be better for more precise targeted therapy.

Overall this work has provided evidence to suggest that PIP4K are important regulators of cellular PtdIns5*P* and that their de-regulation impacts on PtdIns(3,4,5)P_3/PKB activation and cell proliferation. How exactly these phenotypes are engaged is not clear but the data presented here suggest that there may be a role for both a scaffolding function of PIP4K and for their PIP4K activity. Further studies should be aimed at resolving the contribution of the kinase activity to the different phenotypes as this will determine how this family of enzymes can be targeted therapeutically to benefit cancer patients.

Reference List

1. Balla, T., Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol Rev, 2013. 93(3): p. 1019-137.
2. Di Paolo, G. and P. De Camilli, Phosphoinositides in cell regulation and membrane dynamics. Nature, 2006. 443(7112): p. 651-7.
3. Balla, T., Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J Cell Sci, 2005. 118(Pt 10): p. 2093-104.
4. Ford, M.G., et al., Curvature of clathrin-coated pits driven by epsin. Nature, 2002. 419(6905): p. 361-6.
5. Maffucci, T., et al., Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. J Cell Biol, 2005. 169(5): p. 789-99.
6. Burd, C.G. and S.D. Emr, Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol Cell, 1998. 2(1): p. 157-62.
7. Ellson, C.D., et al., PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40(phox). Nat Cell Biol, 2001. 3(7): p. 679-82.
8. Patki, V., et al., Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A, 1997. 94(14): p. 7326-30.
9. Jones, A.T. and M.J. Clague, Phosphatidylinositol 3-kinase activity is required for early endosome fusion. Biochem J, 1995. 311 (Pt 1): p. 31-4.
10. Mills, I.G., A.T. Jones, and M.J. Clague, Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. Curr Biol, 1998. 8(15): p. 881-4.
11. Jones, D.R., et al., PtdIns5P is an oxidative stress-induced second messenger that regulates PKB activation. The FASEB Journal, 2013. 27(4): p. 1644-1656.
12. Rameh, L.E., et al., A new pathway for synthesis of phosphatidylinositol-4,5bisphosphate. Nature, 1997. 390(6656): p. 192-6.
13. Clarke, J.H., et al., Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. Biochem J, 2001. 357(Pt 3): p. 905-10.
14. Morris, J.B., et al., Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. FEBS Lett, 2000. 475(1): p. 57-60.
15. Meijer, H.J., et al., Identification of a new polyphosphoinositide in plants, phosphatidylinositol 5-monophosphate (PtdIns5P), and its accumulation upon osmotic stress. Biochem J, 2001. 360(Pt 2): p. 491-8.
16. Coronas, S., et al., Elevated levels of PtdIns5P in NPM-ALK transformed cells: implication of PIKfyve. Biochem Biophys Res Commun, 2008. 372(2): p. 351-5.

17. Dupuis-Coronas, S., et al., The nucleophosmin-anaplastic lymphoma kinase oncogene interacts, activates, and uses the kinase PIKfyve to increase invasiveness. J Biol Chem, 2011. 286(37): p. 32105-14.
18. F, D., et al., Role for a novel signaling intermediate, phosphatidylinositol 5phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology, 2004. 145(11): p. 4853-65.
19. Grainger, D.L., et al., Involvement of phosphatidylinositol 5-phosphate in insulinstimulated glucose uptake in the L6 myotube model of skeletal muscle. Pflugers Arch, 2011. 462(5): p. 723-32.
20. Oppelt, A., et al., Production of phosphatidylinositol 5-phosphate via PIKfyve and MTMR3 regulates cell migration. EMBO Rep, 2013. 14(1): p. 57-64.
21. Viaud, J., et al., Phosphatidylinositol 5-phosphate regulates invasion through binding and activation of Tiam1. Nat Commun, 2014. 5: p. 4080.
22. Puhar, A., et al., A Shigella effector dampens inflammation by regulating epithelial release of danger signal ATP through production of the lipid mediator PtdIns5P. Immunity, 2013. 39(6): p. 1121-31.
23. Zhang, X., et al., Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. J Biol Chem, 1997. 272(28): p. 17756-61.
24. Tolias, K.F., et al., Type I phosphatidylinositol-4-phosphate 5-kinases synthesize the novel lipids phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 5phosphate. J Biol Chem, 1998. 273(29): p. 18040-6.
25. Halstead, J.R., et al., A novel pathway of cellular phosphatidylinositol(3,4,5)trisphosphate synthesis is regulated by oxidative stress. Current Biology, 2001. 11(6): p. 386-395.
26. Wang, Y., et al., Loss of PIP5KIgamma, unlike other PIP5KI isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes. J Clin Invest, 2008. 118(2): p. 812-9.
27. Sasaki, J., et al., Regulation of anaphylactic responses by phosphatidylinositol phosphate kinase type I {alpha}. J Exp Med, 2005. 201(6): p. 859-70.
28. Di Paolo, G., et al., Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature, 2004. 431(7007): p. 415-22.
29. Shisheva, A., D. Sbrissa, and O. Ikonomov, Cloning, characterization, and expression of a novel Zn2+-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. Mol Cell Biol, 1999. 19(1): p. 623-34.
30. Sbrissa, D., O.C. Ikonomov, and A. Shisheva, PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J Biol Chem, 1999. 274(31): p. 21589-97.

31. Jefferies, H.B., et al., A selective PIKfyve inhibitor blocks PtdIns(3,5)P(2) production and disrupts endomembrane transport and retroviral budding. EMBO Rep, 2008. 9(2): p. 164-70.
32. Zolov, S.N., et al., In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. Proc Natl Acad Sci U S A, 2012. 109(43): p. 17472-7.
33. Laporte, J., et al., A gene mutated in X-linked myotubular myopathy defines a new putative tyrosine phosphatase family conserved in yeast. Nat Genet, 1996. 13(2): p. 175-82.
34. Haugsten, E.M., A. Oppelt, and J. Wesche, Phosphatidylinositol 5-phosphate is a second messenger important for cell migration. Commun Integr Biol, 2013. 6(5): p. e25446.
35. Oppelt, A., et al., PIKfyve, MTMR3 and their product PtdIns5P regulate cancer cell migration and invasion through activation of Rac1. Biochem J, 2014. 461(3): p. 38390.
36. Ramel, D., et al., Shigella flexneri infection generates the lipid PI5P to alter endocytosis and prevent termination of EGFR signaling. Sci Signal, 2011. 4(191): p. ra61.
37. Lecompte, O., O. Poch, and J. Laporte, PtdIns5P regulation through evolution: roles in membrane trafficking? Trends in Biochemical Sciences. 33(10): p. 453-460.
38. Sarkes, D. and L.E. Rameh, A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. Biochem J, 2010. 428(3): p. 375-84.
39. Divecha, N., et al., The cloning and sequence of the C isoform of PtdIns4P 5-kinase. Biochem J, 1995. 309 (Pt 3): p. 715-9.
40. Castellino, A.M., et al., A novel interaction between the juxtamembrane region of the p55 tumor necrosis factor receptor and phosphatidylinositol-4-phosphate 5-kinase. J Biol Chem, 1997. 272(9): p. 5861-70.
41. Itoh, T., T. Ijuin, and T. Takenawa, A Novel Phosphatidylinositol-5-phosphate 4Kinase (Phosphatidylinositol-phosphate Kinase IIγ) Is Phosphorylated in the Endoplasmic Reticulum in Response to Mitogenic Signals. Journal of Biological Chemistry, 1998. 273(32): p. 20292-20299.
42. Wilcox, A. and K.A. Hinchliffe, Regulation of extranuclear PtdIns5P production by phosphatidylinositol phosphate 4-kinase 2α. FEBS Letters, 2008. 582(9): p. 13911394.
43. Jude, J.G., et al., A targeted knockdown screen of genes coding for phosphoinositide modulators identifies PIP4K2A as required for acute myeloid leukemia cell proliferation and survival. Oncogene, 2014.
44. Bultsma, Y., W.J. Keune, and N. Divecha, PIP4Kbeta interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kalpha. Biochem J, 2010. 430(2): p. 223-35.
45. Clarke, J.H., P.C. Emson, and R.F. Irvine, Localization of phosphatidylinositol phosphate kinase IIgamma in kidney to a membrane trafficking compartment within specialized cells of the nephron. Am J Physiol Renal Physiol, 2008. 295(5): p. F1422-30.

46. Merlot, S., et al., A PTEN-related 5-phosphatidylinositol phosphatase localized in the Golgi. J Biol Chem, 2003. 278(41): p. 39866-73.
47. Zhang, J., et al., Mitochondrial phosphatase PTPMT1 is essential for cardiolipin biosynthesis. Cell Metab, 2011. 13(6): p. 690-700.
48. Gozani, O., et al., The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell, 2003. 114(1): p. 99-111.
49. Bua, D.J., et al., Nuclear phosphatidylinositol-5-phosphate regulates ING2 stability at discrete chromatin targets in response to DNA damage. Sci Rep, 2013. 3: p. 2137.
50. Qian, C. and M.M. Zhou, SET domain protein lysine methyltransferases: Structure, specificity and catalysis. Cell Mol Life Sci, 2006. 63(23): p. 2755-63.
51. Milne, T.A., et al., MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell, 2002. 10(5): p. 1107-17.
52. Alvarez-Venegas, R. and Z. Avramova, SET-domain proteins of the Su(var)3-9, E(z) and trithorax families. Gene, 2002. 285(1-2): p. 25-37.
53. Gelato, K.A., et al., Accessibility of different histone H3-binding domains of UHRF1 is allosterically regulated by phosphatidylinositol 5-phosphate. Mol Cell, 2014. 54(6): p. 905-19.
54. Lewis, A.E., et al., Identification of nuclear phosphatidylinositol 4,5-bisphosphateinteracting proteins by neomycin extraction. Mol Cell Proteomics, 2011. 10(2): p. M110.003376.
55. Jungmichel, S., et al., Specificity and commonality of the phosphoinositide-binding proteome analyzed by quantitative mass spectrometry. Cell Rep, 2014. 6(3): p. 57891.
56. Guittard, G., et al., Cutting edge: Dok-1 and Dok-2 adaptor molecules are regulated by phosphatidylinositol 5-phosphate production in T cells. J Immunol, 2009. 182(7): p. 3974-8.
57. Guittard, G., et al., Evidence for a positive role of PtdIns5P in T-cell signal transduction pathways. FEBS Lett, 2010. 584(11): p. 2455-60.
58. Konradt, C., et al., The Shigella flexneri type three secretion system effector IpgD inhibits T cell migration by manipulating host phosphoinositide metabolism. Cell Host Microbe, 2011. 9(4): p. 263-72.
59. Babior, B.M., R.S. Kipnes, and J.T. Curnutte, Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest, 1973. 52(3): p. 741-4.
60. Cook, S.A., P.H. Sugden, and A. Clerk, Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. Circ Res, 1999. 85(10): p. 940-9.
61. Du, G.J., et al., Bcl-2 switches the type of demise from apoptosis to necrosis via cyclooxygenase-2 upregulation in HeLa cell induced by hydrogen peroxide. Cancer Lett, 2006. 232(2): p. 179-88.
62. Sundaresan, M., et al., Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science, 1995. 270(5234): p. 296-9.

63. Bae, Y.S., et al., Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J Biol Chem, 1997. 272(1): p. 217-21.
64. Schreck, R., P. Rieber, and P.A. Baeuerle, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J, 1991. 10(8): p. 2247-58.
65. Konishi, H., et al., Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. Proc Natl Acad Sci U S A, 1997. 94(21): p. 11233-7.
66. Lee, S.R., et al., Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem, 2002. 277(23): p. 20336-42.
67. Keune, W.J., et al., Regulation of phosphatidylinositol-5-phosphate signaling by Pin1 determines sensitivity to oxidative stress. Sci Signal, 2012. 5(252): p. ra86.
68. Emerling, B.M., et al., Depletion of a putatively druggable class of phosphatidylinositol kinases inhibits growth of p53-null tumors. Cell, 2013. 155(4): p. 844-57.
69. Staal, S.P., Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A, 1987. 84(14): p. 5034-7.
70. Walker, K.S., et al., Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha. Biochem J, 1998. 331 (Pt 1): p. 299-308.
71. James, S.R., et al., Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. Biochem J, 1996. 315 (Pt 3): p. 709-13.
72. Klippel, A., et al., A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. Molecular and Cellular Biology, 1997. 17(1): p. 338-344.
73. Alessi, D.R., et al., Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol, 1997. 7(4): p. 261-9.
74. Sarbassov, D.D., et al., Phosphorylation and regulation of Akt/PKB by the rictormTOR complex. Science, 2005. 307(5712): p. 1098-101.
75. Burnett, P.E., et al., RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A, 1998. 95(4): p. 1432-7.
76. Dan, H.C., et al., Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J Biol Chem, 2002. 277(38): p. 35364-70.
77. Inoki, K., et al., TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol, 2002. 4(9): p. 648-57.
78. Potter, C.J., L.G. Pedraza, and T. Xu, Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol, 2002. 4(9): p. 658-65.

79. Manning, B.D., et al., Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell, 2002. 10(1): p. 151-62.
80. del Peso, L., et al., Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science, 1997. 278(5338): p. 687-9.
81. Cross, D.A., et al., Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature, 1995. 378(6559): p. 785-9.
82. Zhou, B.P., et al., HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol, 2001. 3(11): p. 973-82.
83. Hawkins, P.T., T.R. Jackson, and L.R. Stephens, Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. Nature, 1992. 358(6382): p. 157-159.
84. Chiarugi, P., et al., Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. J Cell Biol, 2003. 161(5): p. 933-44.
85. Skolnik, E.Y., et al., Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell, 1991. 65(1): p. 83-90.
86. Stephens, L., et al., A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell, 1994. 77(1): p. 83-93.
87. Chaussade, C., et al., Evidence for functional redundancy of class IA PI3K isoforms in insulin signalling. Biochem J, 2007. 404(3): p. 449-58.
88. Samuels, Y., et al., High frequency of mutations of the PIK3CA gene in human cancers. Science, 2004. 304(5670): p. 554.
89. Campbell, I.G., et al., Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res, 2004. 64(21): p. 7678-81.
90. Karakas, B., K.E. Bachman, and B.H. Park, Mutation of the PIK3CA oncogene in human cancers. Br J Cancer, 2006. 94(4): p. 455-9.
91. Burris, H.A., Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. Cancer Chemother Pharmacol, 2013. 71(4): p. 829-42.
92. Akinleye, A., et al., Phosphatidylinositol 3-kinase (PI3K) inhibitors as cancer therapeutics. J Hematol Oncol, 2013. 6(1): p. 88.
93. Maddika, S., et al., WWP2 is an E3 ubiquitin ligase for PTEN. Nat Cell Biol, 2011. 13(6): p. 728-33.
94. Lee, J.T., et al., RFP-mediated ubiquitination of PTEN modulates its effect on AKT activation. Cell Res, 2013. 23(4): p. 552-64.
95. Shi, Y., et al., PTEN is a protein tyrosine phosphatase for IRS1. Nat Struct Mol Biol, 2014. 21(6): p. 522-7.

96. Li, J., et al., PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 1997. 275(5308): p. 1943-7.
97. Teng, D.H., et al., MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res, 1997. 57(23): p. 5221-5.
98. Wiencke, J.K., et al., Methylation of the PTEN promoter defines low-grade gliomas and secondary glioblastoma. Neuro Oncol, 2007. 9(3): p. 271-9.
99. Rasheed, B.K.A., et al., PTEN Gene Mutations Are Seen in High-Grade but not in Low-Grade Gliomas. Cancer Research, 1997. 57(19): p. 4187-4190.
100. Trotman, L.C., et al., Pten dose dictates cancer progression in the prostate. PLoS Biol, 2003. 1(3): p. E59.
101. Papa, A., et al., Cancer-associated PTEN mutants act in a dominant-negative manner to suppress PTEN protein function. Cell, 2014. 157(3): p. 595-610.
102. Lamia, K.A., et al., Increased insulin sensitivity and reduced adiposity in phosphatidylinositol 5-phosphate 4-kinase beta-/- mice. Mol Cell Biol, 2004. 24(11): p. 5080-7.
103. Ramel, D., et al., PtdIns5P protects Akt from dephosphorylation through PP2A inhibition. Biochem Biophys Res Commun, 2009. 387(1): p. 127-31.
104. Sohn, J. and J. Rudolph, Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. Biochemistry, 2003. 42(34): p. 10060-70.
105. Hayes, G.R. and D.H. Lockwood, Role of insulin receptor phosphorylation in the insulinomimetic effects of hydrogen peroxide. Proc Natl Acad Sci U S A, 1987. 84(22): p. 8115-9.
106. Koshio, O., Y. Akanuma, and M. Kasuga, Hydrogen peroxide stimulates tyrosine phosphorylation of the insulin receptor and its tyrosine kinase activity in intact cells. Biochem J, 1988. 250(1): p. 95-101.
107. Meng, T.C., et al., Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. J Biol Chem, 2004. 279(36): p. 37716-25.
108. Hansen, L.L., et al., Insulin signaling is inhibited by micromolar concentrations of H(2)O(2). Evidence for a role of H(2)O(2) in tumor necrosis factor alpha-mediated insulin resistance. J Biol Chem, 1999. 274(35): p. 25078-84.
109. Kahl, R., S. Weinke, and H. Kappus, Production of reactive oxygen species due to metabolic activation of butylated hydroxyanisole. Toxicology, 1989. 59(2): p. 179-94.
110. Davis, M.I., et al., A Homogeneous, High-Throughput Assay for Phosphatidylinositol 5-Phosphate 4-Kinase with a Novel, Rapid Substrate Preparation. PLoS ONE, 2013. 8(1): p. e54127.
111. Sbrissa, D., O.C. Ikonomov, and A. Shisheva, Phosphatidylinositol 3-phosphateinteracting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization. J Biol Chem, 2002. 277(8): p. 6073-9.

112. Andjelković, M., et al., Role of translocation in the activation and function of protein kinase B. J Biol Chem, 1997. 272(50): p. 31515-24.
113. Stambolic, V., et al., Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell, 1998. 95(1): p. 29-39.
114. Campbell, R.B., F. Liu, and A.H. Ross, Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. J Biol Chem, 2003. 278(36): p. 33617-20.
115. Pagliarini, D.J., C.A. Worby, and J.E. Dixon, A PTEN-like phosphatase with a novel substrate specificity. J Biol Chem, 2004. 279(37): p. 38590-6.
116. Tamura, M., et al., Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science, 1998. 280(5369): p. 1614-7.
117. Leslie, N.R., et al., PtdIns(3,4,5)P3-Dependent and -Independent Roles for PTEN in the Control of Cell Migration. Current Biology. 17(2): p. 115-125.
118. Moffat, J., et al., A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell, 2006. 124(6): p. 1283-98.
119. Zufferey, R., et al., Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol, 1997. 15(9): p. 871-5.
120. Domin, J., et al., Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem J, 1997. 326 (Pt 1): p. 139-47.
121. Mellman, D.L., et al., A PtdIns4,5P2-regulated nuclear poly(A) polymerase controls expression of select mRNAs. Nature, 2008. 451(7181): p. 1013-1017.
122. Tronchère, H., et al., Production of Phosphatidylinositol 5-Phosphate by the Phosphoinositide 3-Phosphatase Myotubularin in Mammalian Cells. Journal of Biological Chemistry, 2004. 279(8): p. 7304-7312.
123. Blondeau, F., et al., Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway. Human Molecular Genetics, 2000. 9(15): p. 2223-2229.
124. Laporte, J., et al., Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. Human Molecular Genetics, 2003. 12(suppl 2): p. R285-R292.
125. Cui, X., et al., Association of SET domain and myotubularin-related proteins modulates growth control. Nat Genet, 1998. 18(4): p. 331-7.
126. Kim, S.-A., et al., Myotubularin and MTMR2, Phosphatidylinositol 3-Phosphatases Mutated in Myotubular Myopathy and Type 4B Charcot-Marie-Tooth Disease. Journal of Biological Chemistry, 2002. 277(6): p. 4526-4531.
127. Schaletzky, J., et al., Phosphatidylinositol-5-Phosphate Activation and Conserved Substrate Specificity of the Myotubularin Phosphatidylinositol 3-Phosphatases. Current Biology. 13(6): p. 504-509.

128. MacKay, C., et al., Identification of KIAA1018/FAN1, a DNA Repair Nuclease Recruited to DNA Damage by Monoubiquitinated FANCD2. Cell. 142(1): p. 65-76.
129. Kratz, K., et al., Deficiency of FANCD2-Associated Nuclease KIAA1018/FAN1 Sensitizes Cells to Interstrand Crosslinking Agents. Cell. 142(1): p. 77-88.
130. PAULUS, H. and E.P. KENNEDY, The enzymatic synthesis of inositol monophosphatide. J Biol Chem, 1960. 235: p. 1303-11.
131. Irvine, R.F., et al., The inositol tris/tetrakisphosphate pathway--demonstration of Ins(1,4,5)P3 3-kinase activity in animal tissues. Nature, 1986. 320(6063): p. 631-4.
132. Dewaste, V., et al., Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C. Biochem J, 2000. 352 Pt 2: p. 343-51.
133. Dewaste, V., et al., The three isoenzymes of human inositol-1,4,5-trisphosphate 3kinase show specific intracellular localization but comparable Ca2+ responses on transfection in COS-7 cells. Biochem J, 2003. 374(Pt 1): p. 41-9.
134. Nalaskowski, M.M., et al., Subcellular localisation of human inositol 1,4,5trisphosphate 3-kinase C: species-specific use of alternative export sites for nucleocytoplasmic shuttling indicates divergent roles of the catalytic and N-terminal domains. Biol Chem, 2006. 387(5): p. 583-93.
135. Liu, Y., et al., New aspects of formation of 1,2-cyclic phosphates by phospholipase C-delta1. Bioorg Med Chem, 2003. 11(11): p. 2471-5.
136. Michell, R.H., et al., Characteristics of rat liver phosphatidylinositol kinase and its presence in the plasma membrane. Biochim Biophys Acta, 1967. 144(3): p. 649-58.
137. Minogue, S., et al., Cloning of a Human Type II Phosphatidylinositol 4-Kinase Reveals a Novel Lipid Kinase Family. Journal of Biological Chemistry, 2001. 276(20): p. 16635-16640.
138. Barylko, B., et al., A Novel Family of Phosphatidylinositol 4-Kinases Conserved from Yeast to Humans. Journal of Biological Chemistry, 2001. 276(11): p. 7705-7708.
139. Balla, A., et al., Characterization of Type II Phosphatidylinositol 4-Kinase Isoforms Reveals Association of the Enzymes with Endosomal Vesicular Compartments. Journal of Biological Chemistry, 2002. 277(22): p. 20041-20050.
140. Wang, Y.J., et al., Phosphatidylinositol 4 Phosphate Regulates Targeting of Clathrin Adaptor AP-1 Complexes to the Golgi. Cell. 114(3): p. 299-310.
141. Wang, J., et al., PI4P Promotes the Recruitment of the GGA Adaptor Proteins to the Trans-Golgi Network and Regulates Their Recognition of the Ubiquitin Sorting Signal. Molecular Biology of the Cell, 2007. 18(7): p. 2646-2655.
142. Gu, S., et al., The Loop Position of shRNAs and Pre-miRNAs Is Critical for the Accuracy of Dicer Processing In Vivo. Cell. 151(4): p. 900-911.
143. Rong, Y., et al., Clathrin and phosphatidylinositol-4,5-bisphosphate regulate autophagic lysosome reformation. Nat Cell Biol, 2012. 14(9): p. 924-34.

144. Moreau, K., et al., Arf6 promotes autophagosome formation via effects on phosphatidylinositol 4,5-bisphosphate and phospholipase D. J Cell Biol, 2012. 196(4): p. 483-96.
145. Wang, M., et al., Genomic tagging reveals a random association of endogenous PtdIns5P 4-kinases IIalpha and IIbeta and a partial nuclear localization of the IIalpha isoform. Biochem J, 2010. 430(2): p. 215-21.
146. Richardson, J.P., et al., Genomic tagging of endogenous type IIbeta phosphatidylinositol 5-phosphate 4-kinase in DT40 cells reveals a nuclear localisation. Cell Signal, 2007. 19(6): p. 1309-14.
147. Ciruela, A., et al., Nuclear targeting of the beta isoform of type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its alpha-helix 7. Biochem J, 2000. 346 Pt 3: p. 587-91.
148. Jones, D.R., et al., Nuclear PtdIns5P as a transducer of stress signaling: an in vivo role for PIP4Kbeta. Mol Cell, 2006. 23(5): p. 685-95.
149. Bunce, M.W., I.V. Boronenkov, and R.A. Anderson, Coordinated activation of the nuclear ubiquitin ligase Cul3-SPOP by the generation of phosphatidylinositol 5phosphate. J Biol Chem, 2008. 283(13): p. 8678-86.
150. Castellino, A.M., et al., A Novel Interaction between the Juxtamembrane Region of the p55 Tumor Necrosis Factor Receptor and Phosphatidylinositol-4-phosphate 5Kinase. Journal of Biological Chemistry, 1997. 272(9): p. 5861-5870.
151. Andjelkovic, M., et al., Role of Translocation in the Activation and Function of Protein Kinase B. Journal of Biological Chemistry, 1997. 272(50): p. 31515-31524.
152. Yung, H.W., D.S. Charnock-Jones, and G.J. Burton, Regulation of AKT phosphorylation at Ser473 and Thr308 by endoplasmic reticulum stress modulates substrate specificity in a severity dependent manner. PLoS One, 2011. 6(3): p. e17894.
153. Heffetz, D., et al., The insulinomimetic agents H2O2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. J Biol Chem, 1990. 265(5): p. 2896-902.
154. Rudich, A., et al., Oxidant stress reduces insulin responsiveness in 3T3-L1 adipocytes. Am J Physiol, 1997. 272(5 Pt 1): p. E935-40.
155. Carricaburu, V., et al., The phosphatidylinositol (PI)-5-phosphate 4-kinase type II enzyme controls insulin signaling by regulating PI-3,4,5-trisphosphate degradation. Proc Natl Acad Sci U S A, 2003. 100(17): p. 9867-72.
156. Oshiro, N., et al., The Proline-rich Akt Substrate of 40 kDa (PRAS40) Is a Physiological Substrate of Mammalian Target of Rapamycin Complex 1. Journal of Biological Chemistry, 2007. 282(28): p. 20329-20339.
157. Kovacina, K.S., et al., Identification of a Proline-rich Akt Substrate as a 14-3-3 Binding Partner. Journal of Biological Chemistry, 2003. 278(12): p. 10189-10194.

158. Stokoe, D., et al., Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science, 1997. 277(5325): p. 567-70.
159. Nogueira, V., et al., Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell, 2008. 14(6): p. 458-70.
160. Rocher, G., et al., Inhibition of B56-containing Protein Phosphatase 2As by the Early Response Gene IEX-1 Leads to Control of Akt Activity. Journal of Biological Chemistry, 2007. 282(8): p. 5468-5477.
161. Kuo, Y.-C., et al., Regulation of Phosphorylation of Thr-308 of Akt, Cell Proliferation, and Survival by the B55α Regulatory Subunit Targeting of the Protein Phosphatase 2A Holoenzyme to Akt. Journal of Biological Chemistry, 2008. 283(4): p. 1882-1892.
162. Hadari, Y.R., et al., Insulin and insulinomimetic agents induce activation of phosphatidylinositol 3'-kinase upon its association with pp185 (IRS-1) in intact rat livers. J Biol Chem, 1992. 267(25): p. 17483-6.
163. Kouchi, Z., et al., Phosphatidylinositol 5-phosphate 4-kinase type II beta is required for vitamin D receptor-dependent E-cadherin expression in SW480 cells. Biochemical and Biophysical Research Communications, 2011. 408(4): p. 523-529.
164. Pendaries, C., et al., PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. EMBO J, 2006. 25(5): p. 1024-34.
165. Leslie, N.R., et al., Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J, 2003. 22(20): p. 5501-10.
166. Tibarewal, P., et al., PTEN protein phosphatase activity correlates with control of gene expression and invasion, a tumor-suppressing phenotype, but not with AKT activity. Sci Signal, 2012. 5(213): p. ra18.
167. Hopkins, B.D., et al., A secreted PTEN phosphatase that enters cells to alter signaling and survival. Science, 2013. 341(6144): p. 399-402.
168. Maccario, H., et al., Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress. J Biol Chem, 2010. 285(17): p. 12620-8.
169. Inanami, O., et al., Hydrogen peroxide-induced activation of SAPK/JNK regulated by phosphatidylinositol 3-kinase in Chinese hamster V79 cells. Antioxid Redox Signal, 1999. 1(1): p. 113-21.
170. Maejima, Y., et al., Induction of premature senescence in cardiomyocytes by doxorubicin as a novel mechanism of myocardial damage. Aging Cell, 2008. 7(2): p. 125-36.
171. Clarke, J.H. and R.F. Irvine, Evolutionarily conserved structural changes in phosphatidylinositol 5-phosphate 4-kinase (PI5P4K) isoforms are responsible for differences in enzyme activity and localization. Biochem J, 2013. 454(1): p. 49-57.

172. Keune, W.J., et al., Low PIP4K2B expression in human breast tumors correlates with reduced patient survival: A role for PIP4K2B in the regulation of E-cadherin expression. Cancer Res, 2013. 73(23): p. 6913-25.
173. Tronhere, H., et al., Production of Phosphatidylinositol 5-Phosphate by the Phosphoinositide 3-Phosphatase Myotubularin in Mammalian Cells. JBC, 2004. 279(8): p. 7304-7312
174. Roberts, H., et al., Effects of lipid kinase expression and cellular stimuli on phosphatidylinositol 5-phosphate levels in mammalian cell lines. FEBS Lett, 2005. 579(13): p. 2868-72.
175. Ikonomov, O., et al., The phosphoinositide kinase PIKfyve is vital in early embryonic development: preimplantation lethality of PIKfyve-/- embryos but normality of PIKfyve+/- mice. J Biol Chem, 2011. 286(15): p. 13404-13.
176. Bazenet, C., et al., The human erythrocyte contains two forms of phosphatidylinositol-4-phosphate 5-kinase which are differentially active toward membranes. J Biol Chem, 1990. 265(29): p. 18012-22.
177. Trotman, L., et al., Ubiquitination regulates PTEN nuclear import and tumor suppression. Cell, 2007. 128(1): p. 141-56.
178. Wang, X., et al., NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. Cell, 2007. 128(1): p. 129-39.
179. Denning, G., et al., A short N-terminal sequence of PTEN controls cytoplasmic localization and is required for suppression of cell growth. Oncogene, 2007. 26(27): p. 3930-40.
180. Rahdar, M., et al., A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. Proc Natl Acad Sci U S A, 2009. 13;106(2): p. 480-5.
181. Vazquez, F., et al., Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol, 2000. 20(14): p. 5010-8.
182. Vazquez, F., et al., Tumor suppressor PTEN acts through dynamic interaction with the plasma membrane. Proc Natl Acad Sci U S A, 2006. 103(10): p. 3633-8.
183. Suzuki, A., et al., High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol, 1998. 22;8(21): p. 1169-78.
184. Maehama, T., et al., The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem, 1998. 29;273(22): p. 13375-8.
185. Ndamukong, I., et al., Phosphatidylinositol 5-phosphate links dehydration stress to the activity of ARABIDOPSIS TRITHORAX-LIKE factor ATX. PLoS One, 2010. 13;5(10): e13396.
186. Niebuhr, K., et al., Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. EMBO J, 2002. 1;21(19): p. 506978.
187. Rankin, P., et al., Quantitative studies of inhibitors of ADP-ribosylation in vitro and in vivo. J Biol Chem, 1989. 15;264(8): p. 4312-7.

188. Bitterman, K., et al., Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem, 2002. 22;277(47): p. 45099-107.
189. Underhill, C., et al., A review of PARP inhibitors: from bench to bedside. Ann Oncol, 2011. 22(2): p. 268-79.
190. Magni, G., et al., Structure and function of nicotinamide mononucleotide adenylyltransferase. Curr Med Chem. 2004. 11(7): p. 873-85.
191. Lindahl, T., et al., Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem Sci. 1995. 20(10): p. 405-11.
192. Klaunig, J., et al., Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol. 2010. 38(1): p. 96-109.
193. Dizdaroglu, M. Oxidative damage to DNA in mammalian chromatin. Mutat Res. 1992. 275(3-6): p. 331-42.
194. Myers, M., et al., P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A. 1997. 19;94(17): p. 9052-7.
195. Myers, M., et al., The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci U S A. 1998. 10;95(23): p. 13513-8.
196. Rosivatz, E., et al., A small molecule inhibitor for phosphatase and tensin homologue deleted on chromosome 10 (PTEN). ACS Chem Biol. 2006. 15;1(12): p. 780-90.
197. Zou, J., et al., Type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase regulates stress-induced apoptosis. Proc Natl Acad Sci U S A. 2007. 23;104(43): p. 16834-9.
198. Alvarez-Venegas, R., et al., The Arabidopsis homolog of trithorax, ATX1, binds phosphatidylinositol 5-phosphate, and the two regulate a common set of target genes. Proc Natl Acad Sci U S A. 2006. 103(15): p. 6049-54.
199. Gutierrez-Uzquiza, A., et al., p38α mediates cell survival in response to oxidative stress via induction of antioxidant genes: effect on the p70S6K pathway. J Biol Chem. 2012. 287(4): p. 2632-42.
200. Raingeaud, J., et al., Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem. 1995. 270(13): p. 7420-6.
201. Schacht, J. Purification of polyphosphoinositides by chromatography on immobilized neomycin. J Lipid Res. 1978. (8): p. 1063-7.
202. Franke, T., et al ., Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science. 1997. 275(5300): p. 665-8.
203. Ungelwickell, A., et al., The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. Proc Natl Acad Sci U S A. 2005. 102(52): p. 18854-9.

204. Williamson, F., et al., Distribution of phosphatidylinositol biosynthetic activities among cell fractions from rat liver. Biochem Biophys Res Commun. 1976. 68(4): p. 1201-5.
205. Schwertassek, U., et al., Reactivation of oxidized PTP1B and PTEN by thioredoxin 1. FEBS J. 2014. 281(16):3545-58.