Targeting PFKFB4 in mitotically vulnerable ovarian cancer cells



Thesis submitted for the degree of Doctor of Philosophy

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“A smooth sea never made a skilled sailor.”

- Franklin D. Roosevelt

Memorandum

This thesis is the result of my own work except for part of section 3.2.4, in which I have reanalysed data from a high content siRNA screen that was carried out by my supervisor, Professor Ahmed Ahmed, in 2011. All other experiments were carried out by myself at the Weatherall Institute of Molecular Medicine or the Nuffield Department of Obstetrics and Gynaecology.

I hereby state that no part of this thesis has been submitted for any other degree, diploma or other qualification, at this or any other university.

This thesis is approximately 46,500 words.

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List of common abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
Acetyl-CoA	Acetyl coenzyme A
ACC	Acetyl-CoA carboxylase
ACLY	ATP citrate lyase
ADP	Adenine diphosphate
ALDA	Aldolase A
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APC/C	Anaphase-promoting complex/cyclosome
ARID1a	AT-rich interaction domain 1A
AS160	AKT substrate of 160 kDA
ASK1	Apoptosis signal-regulating kinase 1
ATR	Ataxia telangiectasia and Rad3-related protein
BAD	BCL-2-associated death promoter
BAX	BCL-2-associated X Protein
BCA	Bicinchoninic acid assay
BCL-2	B-cell lymphoma 2
BH3	BCL-2 homology domain 3
BIM	BCL-2-like protein 11
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BRCA	Breast cancer 1
BSA	Bovine serum albumin
BUB	BUB3 budding uninhibited by benzimidazoles
CAMKK	Calcium/calmodulin-dependent protein kinase kinase
CCNE1	Cyclin E gene
CDC20	Cell division cycle 20
CDC25	Cell division cycle 25
CDH1	CDC20 homologue 1
CDK	Cyclin-dependent kinases
CHUK	Conserved helix-loop-helix ubiquitous kinase
COX-2	Cyclooxygenase-2
CPT1	Carnitine palmitoyltransferase I
CREB	CAMP responsive element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CTNNB1	Catenin beta 1
DNA	Deoxynucleic acid
DNA-PK	DNA-dependent protein kinase
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
eIF2B	Eukaryotic translation initiation factor 2B

ENO1	Enolase 1
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERK	Extracellular signal–regulated kinases
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FIGO	Federation of gynecology and obstetrics
FOXO	Forkhead box-O
GAP	GTPase-activating proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT	Glucose transporter
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine 5'-triphosphate
GYS	Glycogen synthase
HER2	Human epidermal growth factor 2
HGSOC	High-grade serous ovarian cancers
HIF1-α	Hypoxia inducible factor 1-α
HK2	Hexokinase 2
HRP	Horseradish peroxidase
IL-1	Interleukin-1
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinases
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAT1	L-type amino acid transporter
LDH	Lactate dehydrogenase
LKB1	Liver kinase B1
MAD	MAX dimerisation protein
MAP	Microtubule-associated proteins
MAPK	Mitogen activated protein kinase
MCL-1	Myeloid Cell Leukemia **1**
MDM2	Mouse double minute 2 homolog
MEK	MAPK/ERK kinase
MO25	Mouse protein 25
MPS1	Monopolar spindle 1
MTOC	Microtubule organising centre
mTORC	Mammalian target of rapamycin complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NEK	Never in mitosis A-related kinases
NF-1	Neurofibromatosis type 1
ORF	Open reading frame

PARP	Poly ADP ribose polymerase
p70S6K	Ribosomal protein S6 kinase beta-1
PDK1	3-Phosphoinositide-dependent protein kinase-1
PFK1	Phosphofructokinase 1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PI	Propidium iodide
PH	Pleckstrin homology
PHLPP	Pleckstrin homology domain leucine-rich repeat protein phosphatase
PI3K	Phosphoinositide 3-kinase
PLK	Polo-like kinase
PP-1	Protein phosphatase-1
PP-2A	Protein phosphatase-2A
PRAS40	Proline-rich AKT1 substrate 40
PTEN	Phosphatase and tensin homolog
PK	Pyruvate kinase
RB1	Retinoblastoma 1
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAC	Spindle assembly checkpoint
SH2	Src homology 2
SREBP-1	Sterol regulatory element-binding transcription factor-1
STRAD	STE-related adaptor
TAE	Tris base, acetic acid and EDTA
TCA	Tricarboxylic acid
TCGA	The cancer genome atlas
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TNF-α	Tumour necrosis factor-α
TP53	Tumor protein p53
TRAIL	TNF-related apoptosis Inducing ligand
TSC	Tuberous sclerosis
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
XIAP	X-linked inhibitor of apoptosis protein
XYLB	Xylulose kinase
YAP	Yes-associated protein

Targeting PFKFB4 in mitotically vulnerable ovarian cancer cells

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Submitted in candidature for the degree of Doctor of Philosophy, Trinity Term 2016

Abstract

Taxanes represent some of the most common chemotherapeutic agents for ovarian cancer treatment. However, they are only effective in approximately 40% of patients. As such, novel therapeutic strategies are required to potentiate their effect in ovarian cancer to improve patient outcome and prevent chemotherapy resistance.

A hallmark of many cancers is the constitutive activation of the PI3K/AKT pathway, which drives cell survival and metabolism. In this thesis, I identified a potential vulnerability in ovarian cancer cell lines during paclitaxel-induced mitotic arrest, comprising of a striking decrease in AKT activity coupled with a significant reduction in glucose 6-phosphate and ATP levels. The reanalysis of a high-content siRNA screen to discover metabolic enzymes important for ovarian cancer cell survival during paclitaxel-induced mitotic arrest, identified the metabolic enzyme PFKFB4. PFKFB4 depletion followed by paclitaxel treatment resulted in a significant decrease in mitotically arrested cells. This was accompanied by a significant increase in caspase-3/7 activity and the levels of reactive oxygen species only in mitotically arrested cells, and a significant enhancement in mitotic cell death and mitotic slippage. Depletion of the related family member, PFKFB3, demonstrated a similar phenotype.

The exogenous expression of constitutively active AKT or siRNA-resistant PFKFB4 did not confer resistance to PFKFB4 depletion and paclitaxel treatment, indicating that the mechanism of mitotic cell reduction is complex. Nonetheless, the observation that some ovarian cancer cells lose AKT activity during mitotic arrest and could become vulnerable to metabolic targeting is a new concept in cancer therapy. In addition, I have identified previously unrecognised roles of PFKFB3 and PFKFB4 in mitotically arrested ovarian cancer cells. This work supports the notion that combining mitotic-targeted therapies with metabolic inhibitors may act to potentiate the effects of antimitotics in ovarian cancer cells.

Chapter 1

Introduction

1. Introduction

1.1. Ovarian cancer

Ovarian cancer is the fourth most common cause of cancer death in women in the UK, with a 5-year survival rate of 41% (Jacobs et al., 2016). Despite survival rates nearly doubling over the last 30 years, around 4,300 women still die of ovarian cancer each year in the UK (CRUK, 2016). Early diagnosis of ovarian cancer is uncommon with more than 70% of cases being diagnosed at the advanced stage (Cho and Shih Ie, 2009). Patients usually respond successfully to initial therapy, consisting of cytoreductive surgery followed by a combination of paclitaxel and carboplatin chemotherapy. However, due to the development of chemotherapy resistance, recurrence occurs in over 70% of patients who present with advanced stage ovarian cancer (Ushijima, 2010). Therefore, the development of novel treatment strategies to prevent recurrence and improve the prognosis for ovarian cancer patients is critical.

1.1.1. Clinical presentation and staging of ovarian cancer

Ovarian cancer is staged using the Federation of Gynaecology and Obstetrics (FIGO) system, which determines how widespread the cancer is (Table 1) (Prat and Oncology, 2014). Approximately 84% of ovarian cancer patients are diagnosed at stage IIIC, which is when the abdominal metastasis beyond the pelvis measures greater than 2 cm in diameter, or is present in lymph nodes in the upper abdomen or groin (Heintz, 2006). The main reason for ovarian cancer being diagnosed at such an advanced stage is the lack of specific symptoms during the early stages of the disease. Symptoms such as abdominal swelling, abdominal pain, increased urination and loss of appetite have been found to occur at a much higher frequency than usual, but due to their non-specific nature, they are not always thought to be indicative of ovarian cancer (Goff et al., 2007). This has lead to ovarian cancer being historically known as the “silent killer”.

Stage		Clinical presentation of tumour
I		**Confined to the ovaries or fallopian tube**
	IA	Restricted to one ovary
	IB	Restricted to both ovaries/fallopian tubes, no surface tumour
	IC	Restricted to one or both ovaries/fallopian tubes, with surgical spill, ascites containing malignant cells or surface tumour
II		**Involves one or both ovaries/fallopian tubes, with pelvic extension or primary peritoneal cancer**
	IIA	Extension and/or implants on uterus/fallopian tubes/ovaries
	IIB	Extension to other pelvic intraperitoneal tissues
III		**Involves one or both ovaries/fallopian tubes or primary peritoneal cancer, with pelvic extension and/or metastasis to the retro peritoneal lymph nodes**
	IIIA	Positive retroperitoneal lymph nodes, with/without microscopic extrapelvic peritoneal involvement
	IIIB	Macroscopic peritoneal metastasis beyond the pelvis ≤2 cm
	IIIC	Macroscopic peritoneal metastasis beyond the pelvis >2 cm
IV		**Distant metastasis excluding peritoneal metastasis**

Table 1.1. Staging of ovarian cancer as outlined in the Federation of Gynaecology and Obstetrics (FIGO) system.

1.1.2. Histological subtypes of ovarian cancer

Ovarian tumours are divided into five main categories according to the World Health Organisation, these are epithelial, sex cord-stromal, germ cell, metastatic and other (Serov, 1973). Epithelial tumours account for more than 90% of ovarian malignancies and can be further subdivided corresponding to the different morphologies of epithelia in the female reproductive tract, such as serous, endometrioid, mucinous, clear cell, Brenner/transitional, undifferentiated and mixed epithelial. Ovarian cancers are also graded according to their microscopic appearance; grade 1 are well differentiated and more likely to grow at a slower rate, grade 2 are moderately differentiated and grade 3 are undifferentiated or poorly differentiated and classed as high-grade cancers (CRUK, 2016). The majority of ovarian cancer patients are diagnosed with high-grade serous ovarian cancers (HGSOC) at stage IIIC (Heintz, 2006).

Epithelial tumours can also be further subdivided into type I or type II tumours, according to their pathway of tumourigenesis and genetic signatures (Shih Ie and Kurman, 2004). Type I tumours include low-grade serous, endometrioid, clear cell and mucinous carcinomas, and transitional call carcinomas. They are typically confined to one ovary (stage IA) and have a good prognosis. Type II tumours consist of high-grade serous, endometrioid, malignant mixed mesodermal tumours and undifferentiated carcinomas, which commonly present at the advanced stage (II-IV) and are highly aggressive.

1.1.3. Molecular genetics of ovarian cancer

Ovarian cancer is a heterogenous disease comprised of a number of subtypes that originate from different areas and display distinct molecular biology and clinical behaviour. Type I and type II epithelial tumours are distinguished from one another by their molecular genetics.

Type I tumours are relatively genetically stable and typically display mutations in a number of genes, with the frequency largely dependent on the type of epithelial tumour. Low-grade serous tumours often harbour mutations in *KRAS, BRAF* or less frequently *ERBB2* genes (Nakayama et al., 2006), with mutations being mutually exclusive (Shih Ie and Kurman, 2004). However, as KRAS, BRAF and ERBB2 proteins are all upstream regulators of MAPK, regardless of which gene is affected, mutation often results in the constitutive activation of the MAPK pathway and the uncontrolled proliferation of cells (Hsu et al., 2004). Mucinous tumours also possess mutations in *KRAS* (Ichikawa et al., 1994), whereas low-grade endometrioid and clear cell carcinomas commonly harbour mutations in *PTEN* (Sato et al., 2000), *ARID1A* (Wiegand et al., 2010) and *PIK3CA* (Campbell et al., 2004). Low-grade endometrioid carcinomas have been associated with mutations in *CTNNB1* (Moreno-Bueno et al., 2001). Mutations in *TP53* and BRCA inactivation are rare in all type I tumours (Singer et al., 2005).

Type II tumours are chromosomally highly unstable and harbour *TP53* mutations in more than 96% of cases (Ahmed et al., 2010). Unlike type I tumours, mutations in *KRAS, BRAF* or *ERBB2* are less common, but nonetheless can occur (Singer et al., 2003). HGSOCs frequently demonstrate deficiencies in the homologous

recombination DNA repair pathway, with approximately 22% of HGSOC possessing mutations in *BRCA1* and *BRCA2* (Cancer Genome Atlas Research, 2011; Jayson et al., 2014). Other low prevalence, but statistically significant recurrent mutations have been observed in *NF-1*, *RB1, CSMD3* and *CDK12*, while DNA copy number gains can occur in *CCNE1*, *NOTCH3*, *AKT2* and *PIK3CA* (Nakayama et al., 2007), *MAPK, RICTOR, AKT1 and AKT3* (Cheaib et al., 2015). The downregulation or loss of *PTEN* has also been observed in HGSOC (Martins et al., 2014). These genetic alterations can influence a number of key signalling pathways in HGSOC. For example, an activating mutation in *PIK3CA,* amplification of *AKT* or *PTEN* loss, could all result in the constitutive activation of the PI3K/AKT signalling pathway. This is one of the most frequently deregulated signalling pathways in ovarian cancer (Huang et al., 2011).

1.1.4. Current treatment regimes for ovarian cancer

Patients who present with stage I ovarian cancer are surgically staged and if deemed high risk they are offered adjuvant chemotherapy consisting of six-cycles of carboplatin (NICE, 2011). Patients who present with stages II-IV undergo optimal cytoreductive surgery followed, or preceded, by platinum-based chemotherapy with or without paclitaxel. Paclitaxel can also be used as a monotherapy when platinum-based chemotherapy has failed. Additional chemotherapeutics available to patients experiencing recurrent ovarian cancer include pegylated liposomal doxorubicin hydrochloride, topotecan, gemcitabine, trabectedin (NICE, 2016b) and olaparib (NICE, 2016a). These drugs are recommended according to the nature of the tumour.

Despite the successful response to first line chemotherapy (McGuire et al., 1996), recurrence is seen in more than 50% of patients who present with advanced disease and 25% of those with early-stage disease (Trimbos et al., 2003; Armstrong et al., 2006). This is due to the development of chemotherapy resistance. Resistance to chemotherapy is a major barrier to the successful treatment of ovarian cancer and new therapeutic strategies are required to improve patient prognosis. The combination of targeted therapies with tailored chemotherapy regimes has the potential to reduce the occurrence of chemotherapy resistance and improve overall survival.

Significant progress has already been made in targeted medicine, one example is the approval of the poly ADP ribose polymerase (PARP)-1 inhibitor, olaparib. Olaparib induces synthetic lethality in *BRCA*-deficient cells and thus has been effective as a third-line treatment for women who possess *BRCA1/2* mutations (Audeh et al., 2010). In addition to PARP inhibitors, antiangiogenic therapies have also shown promise in the treatment of ovarian cancer (Oliver and McGuire, 2014). One example is bevacizumab, a humanised monoclonal antibody to vascular endothelial growth factor (VEGF) that prevents the binding of VEGF to its receptor (Presta et al., 1997; Garcia and Singh, 2013). Other approaches include ERBB2 and MEK inhibitors to target the constitutive activation of MAPK pathway (Farley et al., 2013), EGFR inhibitors (Schilder et al., 2005), folate receptor antagonists (Teng et al., 2012) and inhibitors of the PI3K/AKT/mTOR pathway (Cheaib et al., 2015; Mabuchi et al., 2015).

1.2. Molecular mechanisms of paclitaxel response

1.2.1. Regulation of mitosis

Cell cycle progression is dependent on the careful regulation of specific kinases and phosphatases. Cyclin-dependent kinases (CDKs) are expressed in a specific manner throughout the cell cycle and are important in its regulation (Morgan, 1997). As their name suggests, CDKs require cyclins for their activity, forming a CDK-cyclin complex. Additional kinases critical for coordinating mitosis include the Aurora kinases (Glover et al., 1995; Carmena and Earnshaw, 2003), Polo-like kinases (PLKs) (Sunkel and Glover, 1988; Barr et al., 2004), spindle assembly checkpoint (SAC) kinases (Hoyt et al., 1991; Li and Murray, 1991; Musacchio and Salmon, 2007) and Never in mitosis A-related kinases (NEK) (Fry et al., 2012). Global phosphoproteomic studies have identified thousands of phosphorylation events that occur during mitosis (Daub et al., 2008; Olsen et al., 2010; Pagliuca et al., 2011). Both substrate selectivity and spatial separation are crucial in coordinating the activity of mitotic kinases.

There are five main stages of mitosis, these are prophase, prometaphase, metaphase, anaphase and telophase (Table 2) (Flemming, 1882; Paweletz, 2001). Telophase is followed by division of the cytoplasm into two daughter cells, known as cytokinesis.

Stage of mitosis	Key cellular events
Prophase	Chromosomes start to condense Spindle assembly begins Microtubules begin to polymerise
Prometaphase	Nuclear envelope breakdown Microtubules continue to polymerise Kinetochores are connected by microtubules
Metaphase	Chromosomes fully condensed Centromeres of chromosomes line up SAC checkpoint
Anaphase	Separation of sister chromatids Chromosomes move towards spindle poles/segregation Spindle poles separate
Telophase	Chromosomes reach the poles Nuclear membrane reforms Chromosomes begin to decondense

Table 1.2. Main stages of mitosis and the key cellular changes that take place.

Mitotic entry into prophase is largely governed by the nuclear activity of the cyclin B-CDK1 complex, also known as M-phase promoting factor (Hunt, 1989). Together with other kinases, such as Aurora kinases and PLKs, cyclin B-CDK1 induces extensive protein phosphorylation (Dephoure et al., 2008; Van Horn et al., 2010; Hegemann et al., 2011), which serves to orchestrate the structural changes during the early stages of mitosis. These include centrosome maturation, chromatin condensation, nuclear envelope breakdown and spindle assembly (Guttinger et al., 2009; Lindqvist et al., 2009; Smith et al., 2011). The Aurora kinase substrate, histone H3, is phosphorylated on S10 and S28 during this period and can be used to distinguish mitotic cells (Hsu et al., 2000; Crosio et al., 2002; Goto et al., 2002; Hirota et al., 2005). CDK1 activity is inhibited by Wee1- or Myt1- dependent phosphorylation (Parker and Piwnica-Worms, 1992; Booher et al., 1997) and activated by CDC25-mediated dephosphorylation (Russell and Nurse, 1987; Gautier et al., 1991). Cyclin B-CDK1 can promote its own activity through direct phosphorylation and activation of CDC25, (Hoffmann

et al., 1993) and inactivation of Wee1 (Watanabe et al., 2004), which enables rapid amplification of cyclin B-CDK1 activity.

Progression through mitosis is dependent on the correct spatiotemporal control of events, which are tightly coordinated by the cell. During metaphase, the SAC monitors the attachment of microtubules to kinetochores and inhibits anaphase until chromosomes are correctly aligned on the metaphase plate to ensure accurate segregation of sister chromatids (Musacchio and Salmon, 2007; Cheeseman and Desai, 2008). Key SAC kinases include BUB1, BUB3, BUBR1, MAD1, MAD2 (Hoyt et al., 1991; Li and Murray, 1991), CDC20 (Sudakin et al., 2001), MPS1 (Stucke et al., 2002), and Aurora B (Hauf et al., 2003). The SAC proteins prevent entry into anaphase by inhibiting the activity of the anaphase-promoting complex/cyclosome (APC/C) and hinder the proteasomal degradation of securin and cyclin B (Clute and Pines, 1999; Hagting et al., 2002). When proper chromosome-spindle attachment is achieved and there is adequate tension between the sister chromatids, the SAC is inactivated and the APC/C is phosphorylated by CDKs, which stimulates its association with CDC20, enhancing its activity (Castro et al., 2005; Nakayama and Nakayama, 2006). This results in the degradation of cyclin B and securin, which initiates the cleavage of sister chromatids through the activation of separase (Hagting et al., 2002; Lara-Gonzalez et al., 2012).

The completion of mitosis relies on the inactivation of cyclin B-CDK1, which occurs through ubiquitin-dependent proteolysis of cyclin B by the APC/C-CDC20 complex (Harper et al., 2002; Peters, 2002). Decreased CDK1 activity stimulates the association of APC/C with CDC20 homologue 1 (CDH1) (Peters, 2006). APC/C-CDH1 activity can promote the degradation of many cell cycle progression activators, such as PLK1 (Lindon and Pines, 2004) and Aurora kinases (Stewart and Fang, 2005), as well as activators of APC/C, such as CDC20 (Pfleger and Kirschner, 2000). It is not yet clear whether CDH1 activity is essential for mitotic exit (Sivakumar and Gorbsky, 2015). Mitotic exit also requires the reversal of cyclin B-CDK1-mediated mitotic phosphorylation via the activity of phosphatases, such as PP-1 and PP-2A (Wurzenberger and Gerlich, 2011). A major regulator of PP-2A activity is the Greatwall kinase (Vigneron et al., 2009). Greatwall is activated by cyclin B-CDK1 at the onset of mitosis and acts to inhibit PP-2A

activity. Therefore when cyclin B-CDK1 activity declines at the end of mitosis, PP-2A becomes active and induces global dephosphorylation. Cytokinesis then ensues and two daughter cells are formed.

1.2.2. Microtubules and paclitaxel

Paclitaxel was originally isolated from the bark of the Pacific Yew tree (Wani et al., 1971) and was found to be cytotoxic through its stabilisation of microtubules (Schiff et al., 1979). Microtubules are a critical component of the cytoskeleton that orchestrate key cellular functions, such as mitosis, intracellular transport, secretion and maintenance of cell shape (Desai and Mitchison, 1997; Oakley, 2000; Sharp et al., 2000). Microtubules are highly dynamic tubular polymers composed of α- and β-tubulin heterodimers (Weisenberg et al., 1968; Bryan and Wilson, 1971; Amos and Klug, 1974) (Figure 1.1). The polarisation of microtubules arises from the head to tail arrangement of the αβ heterodimers, which causes the ends of microtubules to have different polymerisation rates (Wade and Hyman, 1997). The αβ heterodimers are orientated with the β-tubulin pointing towards the faster growing end (plus end), while the α-tubulin is exposed to the slower growing end (minus end). In cells, cytosolic microtubules normally radiate outwards from the nucleus, with their minus ends associated with the microtubule organising centre (MTOC) (Brinkley, 1985). The MTOC is a centrosome; a lattice of microtubule-associated proteins that sometimes, but not always, contains a pair of centrioles.

In each αβ heterodimer, one molecule of GTP binds to each tubulin present (Desai and Mitchison, 1997). GTP binds to α-tubulin in an irreversible manner at the non-exchangeable site (N-site) and is not hydrolysed (Spiegelman et al., 1977), whereas GTP binding to β-tubulin is reversible and occurs at the exchangeable site (E-site) (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976). Microtubule growth requires β-tubulin to be charged with GTP (Weisenberg et al., 1976), leading to the presence of a GTP cap at the plus end, which stabilises the microtubule structure (Mitchison and Kirschner, 1984). GTP is hydrolysed to GDP upon the addition of each αβ heterodimer to the elongating microtubule. After hydrolysis, the guanine nucleotide becomes non-exchangeable, thus microtubules are predominantly composed of GTP bound α-

tubulin and GDP bound β-tubulin (Nogales et al., 1998). A third tubulin isoform known as γ-tubulin, is located at the MTOC and functions as a template for microtubule nucleation through its interaction with α-tubulin (Oakley and Oakley, 1989; Oakley, 2000). Microtubule dynamics are regulated by a number of proteins, some of the best characterised are the microtubule-associated proteins (MAPs), including Tau, MAP1, MAP2 and MAP4 (Olmsted, 1986). Other proteins, such as stathmin, bind to two αβ heterodimers, preventing their incorporation into microtubules and promoting microtubule depolymerisation (Curmi et al., 1997; Jourdain et al., 1997).

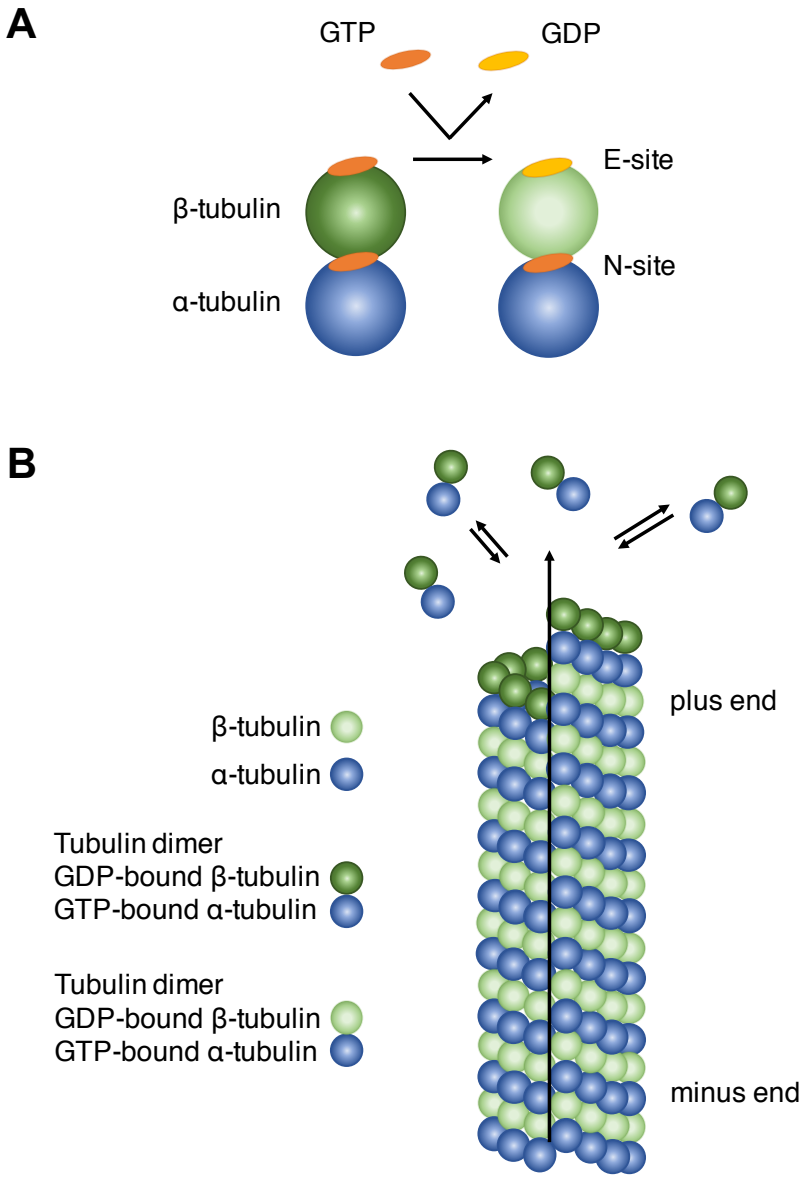


Figure 1.1. Microtubule components and structure.
(A) αβ tubulin heterodimer with α-tubulin containing the non-exchangeable N-site and β-tubulin with an exchangeable E-site. (B) Microtubule structure with the plus and minus ends depicted.

Unlike other antimitotic drugs that bind preferentially to tubulin dimers and prevent microtubule assembly (Jordan and Wilson, 2004), paclitaxel binds to the β-subunit in microtubule polymers (Rao et al., 1992; Rao et al., 1994; Rao et al., 1995). The paclitaxel-binding site is present only on polymerised tubulin and not on tubulin dimers (Nogales et al., 1995; Nogales, 2001). Paclitaxel binding enhances polymerisation of tubulin, by reducing the concentrations of αβ heterodimers, MAPs and GTP that are required for tubulin polymerisation (Schiff et al., 1979; Kumar, 1981; Schiff and Horwitz, 1981). Binding of paclitaxel to the β-subunit is thought to stabilise microtubules by strengthening the lateral contacts between protofilaments, via a conformational change in the M-loop of β-tubulin (Nogales, 2000). The region of α-tubulin that corresponds to the paclitaxel-binding pocket of β-tubulin is occupied by an S loop, which consists of eight amino acids (Nogales et al., 1998; Nogales et al., 1999; Downing, 2000; Nogales, 2000). The S-loop of α-tubulin is thought to promote the lateral association between protofilaments, thus acting as an endogenous microtubule-stabilising factor.

The stabilisation of microtubules after paclitaxel treatment makes them more resistant to depolymerisation by calcium, cold temperature and dilution (Schiff et al., 1979). This stabilisation also decreases microtubule dynamics and prevents the normal formation of mitotic spindles (Jordan and Wilson, 2004). Paclitaxel allows the attachment of chromosomes to stabilised microtubules, however, due to the reduction in microtubule dynamics (Jordan et al., 1993), the tension across sister chromatids is lost (Waters et al., 1998; Kelling et al., 2003), which constitutively activates the SAC and arrests cells at prometaphase/metaphase (Jordan and Wilson, 1998).

Mitotically arrested cells cannot remain viable indefinitely and usually follow one of three fates; mitotic cell death, mitotic slippage or aberrant mitosis (Figure 1.2). Mitotic slippage occurs when cells ‘slip’ into the next interphase without chromosome segregation or cytokinesis, often resulting in a tetraploid cell (Rieder and Maiato, 2004; Brito and Rieder, 2006). Tetraploid cells usually undergo cell death or become senescent, however can undertake subsequent aberrant divisions (Vitale et al., 2011). Cells that undergo aberrant mitosis often die or

become arrested in the subsequent mitotic phase where they are prone to mitotic cell death.

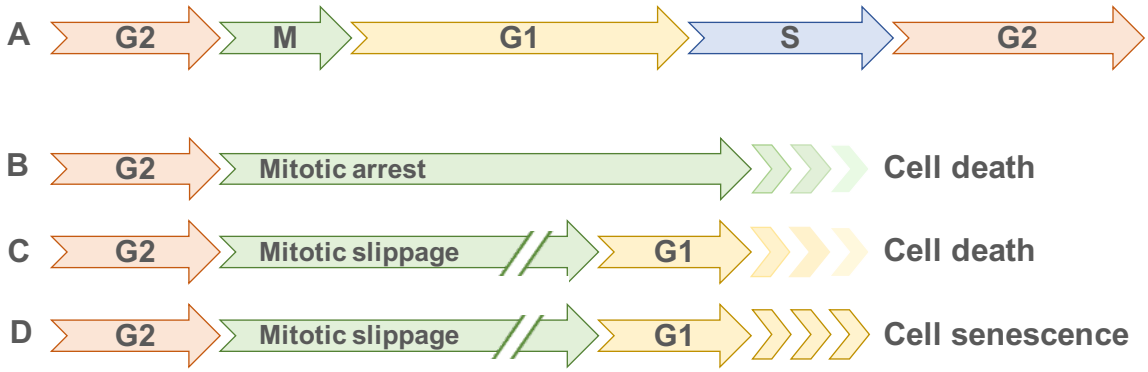


Figure 1.2. Schematic displaying cell fate following mitotic arrest.
(A) Cells progressing through a normal cell cycle without mitotic arrest. (B) Following mitotic arrest, cells can undergo cell death in mitosis. (C) Alternatively, mitotically arrested cells can enter G1 known as 'mitotic slippage' and then undergo cell death. (D) Mitotic slippage can be followed by cellular senescence.

1.2.3. Paclitaxel induced cellular alterations

The cellular effects of paclitaxel are concentration dependent. At low concentrations of paclitaxel, cell death is thought to occur after an aberrant mitosis and an aneuploid G1 cell population (Demidenko et al., 2008). Whereas at higher concentrations (> 9 nM), mitotic arrest results primarily in cell death (Torres and Horwitz, 1998; Chen and Horwitz, 2002). Thus, paclitaxel does not elicit a uniform response in cells, with its cytotoxic, anti-proliferative and apoptotic effects varying between different cancer cell lines and different tissues (Weaver, 2014). In addition to its physical effects on microtubules, paclitaxel can elicit a number of responses in cancer cells, such as transcriptional changes, inhibition or activation of signalling pathways, and further alterations to key cell cycle regulators.

Paclitaxel treatment can elicit many transcriptional changes in cells (Moos and Fitzpatrick, 1998a; Bani et al., 2004). These include downregulation of the antiapoptotic protein, B-cell lymphoma 2 (BCL-2) (Ferlini et al., 2003), and increased expression of inflammatory genes, such as *CRG-2, COX-2, TNF-α, IL-1, CHUK* (Moos and Fitzpatrick, 1998a; Sprowl et al., 2012). The increase in inflammatory cytokines is thought to contribute to the fatigue and flu-like

symptoms experienced by patients (Pusztai et al., 2004) and drive the cell towards cell death (Beyaert and Fiers, 1994).

As well as modulating the transcriptional activity of the cell, paclitaxel can directly alter the activity of proteins. For example, paclitaxel can phosphorylate and inhibit the antiapoptotic activities of BCL-2 (Haldar et al., 1996), as well as directly bind to BCL-2 resulting in its involvement in a proapoptotic response (Ferlini et al., 2009). Paclitaxel treatment can also result in the activation of a number of signalling pathways, including ERK (Torres and Horwitz, 1998; Okano et al., 2007), MAPK (McDaid and Horwitz, 2001) and JNK (Wang and Wieder, 2004), all of which have been shown to promote paclitaxel-induced apoptosis (Bacus et al., 2001; Brichese et al., 2004). However, JNK activation can also promote cell survival (Yu et al., 2004). Paclitaxel promotes the expression of forkhead box O 3A (FOXO3A) (Sunters et al., 2006) and its translocation to the nucleus, where it can induce transcription of its target genes (Sunters et al., 2003; Bonezzi et al., 2012). FOXO3A translocation is dependent on JNK1/2 activity and the subsequent dephosphorylation of AKT (Sunters et al., 2006). Enhanced FOXO3A activity can contribute to paclitaxel-mediated apoptosis through the enhanced expression of the proapoptotic protein, BIM (Sunters et al., 2003). The activity of caspase-3 (Moos and Fitzpatrick, 1998b) and caspase-8 (Oyaizu et al., 1999) are increased in paclitaxel treated cells and thought to be important for paclitaxel-induced apoptosis.

Paclitaxel can also manipulate the aberrant activation of cyclin-dependent kinases commonly observed in cancer cells. At low concentrations, it can downregulate CDK4 to induce an additional cell cycle block at G1-S phase, which promotes paclitaxel-induced apoptosis (Yoo et al., 1998; Zhang et al., 2013). While at higher concentrations paclitaxel can increase the expression of CDK1 that promotes paclitaxel-induced apoptosis through the activation and stabilisation of cyclin B-CDK1 complexes and further activation of the SAC (Rudner and Murray, 1996; Chadebech et al., 2000).

Paclitaxel can also increase the levels of reactive oxygen species (ROS) in cancer cells, including superoxide, hydrogen peroxide and nitric oxide (Ramanathan et al., 2005; Alexandre et al., 2006). An increase in ROS can

promote cell death by increasing cytochrome c release from the mitochondria, which results in the enhanced activity of caspase-3 and 9 (Andre et al., 2000; Varbiro et al., 2001; Galindo et al., 2003). Increased ROS can also facilitate calcium-dependent mitochondrial permeability transition, which can lead to ATP depletion and necrosis, or caspase-dependent apoptosis (Orrenius, 2007).

In addition, paclitaxel can prevent the formation of new blood vessels from preexisting vasculature. The possible mechanisms for its antiangiogenic activity involve the downregulation of important survival factors for endothelial cells, such as VEGF (Lau et al., 1999) and angiopoietin 1 (Hata et al., 2004), along with an increase in the secretion of thrombospondin-1 in the tumour microenvironment, which is a potent and endothelial-specific inhibitor of angiogenesis (Bocci et al., 2003).

1.2.4. Paclitaxel resistance

Chemotherapy resistance is a major barrier to successful therapy. Paclitaxel resistance can be present prior to treatment, known as innate or primary resistance, or it can develop after exposure to the drug, known as acquired resistance (Fojo and Menefee, 2007). The major mechanisms shown to confer paclitaxel resistance are discussed below.

Overexpression of drug efflux pumps

Drug efflux proteins export potential toxins, such as paclitaxel, from cells in both normal and cancerous tissues. The most widely studied group of drug efflux proteins are those belonging to the ATP-binding cassette (ABC) superfamily, which includes P-glycoprotein (MDR/ABCB1) (Juliano and Ling, 1976), the multidrug resistance-associated protein (MRP-1/ABCC1) (Cole et al., 1992) and the breast cancer resistance protein (BCRP/ABCG2) (Doyle et al., 1998). However, MRP-1 and BCRP are less efficient at transporting paclitaxel than P-glycoprotein (Borst et al., 2000; Leslie et al., 2005) and have not been as strongly associated with paclitaxel resistance.

P-glycoprotein overexpression can arise from gene amplification, as well as increased transcription and translation (Chen et al., 1986; Shen et al., 1986; Xu et

al., 2001; Schondorf et al., 2003; Hille et al., 2006). Increased expression of P-glycoprotein has been shown to confer resistance to paclitaxel in numerous cancer cell lines, including those derived from ovarian cancers (Parekh et al., 1997; Mechetner et al., 1998). In addition, the screening of 60 cell lines demonstrated that low P-glycoprotein expression is correlated with increased paclitaxel sensitivity (Alvarez et al., 1995). Paclitaxel sensitivity can also be restored with the use of P-glycoprotein inhibitors (Burns et al., 2001). However, there have been conflicting findings from clinical studies combining P-glycoprotein inhibitors with paclitaxel treatment (Fracasso et al., 2001). Thus, the extent of the contribution of p-glycoprotein to paclitaxel resistance remains unclear.

Alterations in microtubule dynamics

Resistance to paclitaxel can also arise through alterations to tubulin and/or microtubules, which disrupt the ability of paclitaxel to bind and stabilise microtubules. One potential mechanism is through enhanced microtubule dynamics. Given that paclitaxel preferentially binds to microtubule polymers and not dimers, a polymer with increased dynamics could be advantageous to a cell exposed to paclitaxel. It has been suggested that paclitaxel-resistant cell lines favour the formation of tubulin dimers over microtubule polymers (Cabral and Barlow, 1989; Minotti et al., 1991) and increasing microtubule stability can enhance paclitaxel sensitivity (Ahmed et al., 2007; Ahmed et al., 2011). Moreover, alteration in tubulin isotype expression and incorporation can influence microtubule dynamics. Six different β-tubulin isotypes have been identified in humans (Sullivan, 1988) and have been found to effect the stability of microtubules. For example, microtubules composed of βIII tubulin are more unstable than other isoforms (Panda et al., 1994) and have been shown to be more resistant to paclitaxel treatment (Derry et al., 1997). In addition, increased mRNA and protein expression of βIII-tubulin in 41 ovarian cancer patients correlated with enhanced paclitaxel resistance (Mozzetti et al., 2005). High expression of βIII-tubulin has also been associated with poor prognosis in advanced ovarian cancer (Ferrandina et al., 2006). Changes in microtubule dynamics can also arise from mutations in β-tubulin, leading to a reduction in their polymerisation and subsequent decrease in paclitaxel sensitivity

(Giannakakou et al., 1997; Monzo et al., 1999). Mutations occurring in sites critical for the activity of β-tubulin could reduce microtubule polymerisation, for example, if mutations occur in the binding site of MAPs (Orr et al., 2003).

Post-translational modifications to α- and β-tubulin can also regulate microtubule dynamics, however there is mixed evidence for their role in paclitaxel resistance. An increase in acetylated tubulin has been identified in paclitaxel-resistant small lung cell carcinoma when compared to wild-type cells (Ohta et al., 1994). However, the mechanism for resistance is unclear as the acetylation of α-tubulin after paclitaxel treatment has also been shown to promote the stabilisation of microtubules and not promote microtubule dynamics (Piperno et al., 1987). Enhanced tyrosination of α-tubulin has also been found in paclitaxel-resistant breast cancer cell lines (Banerjee, 2002). Changes in post-translational modifications and expression of microtubule regulatory proteins has also been implicated in paclitaxel resistance. As mentioned previously, stathmin and MAP4 are regulators of microtubules dynamics. Unphosphorylated stathmin promotes microtubule disassembly and enhances microtubule dynamics (Marklund et al., 1996; Horwitz et al., 1997). High expression of stathmin correlates with a reduction in disease-free progression and overall survival in ovarian cancer patients (Su et al., 2009). In contrast to stathmin, unphosphorylated MAP4 acts to stabilise microtubules and its phosphorylation inhibits this activity (Chapin et al., 1995; Chang et al., 2001). An increase in MAP4 expression can act synergistically with paclitaxel to stabilise microtubules, making cells more sensitive to paclitaxel (Zhang et al., 1998).

Changes in protein expression and activity

Alterations in protein activity in response to paclitaxel can confer significant growth and survival advantages to cancer cells. The BCL-2 family have been shown to contribute to paclitaxel resistance. Overexpression of the anti-apoptotic member BCL-XL has been associated with increased paclitaxel resistance (Ibrado et al., 1997), while the overexpression of the pro-apoptotic members, BIM, BAX and BAD, can increase paclitaxel sensitivity (Strobel et al., 1996; Craik et al., 2010). Contradictory evidence has been presented regarding BCL-2, with low levels correlating with resistance to paclitaxel (Ogretmen and Safa, 1996;

Ferlini et al., 2003). However, this observation could be due to the inability of paclitaxel to bind BCL-2 and promote apoptosis (Ferlini et al., 2009). In addition, the low expression of the functional tumour-suppressor, p53, has been associated with increased paclitaxel sensitivity (Goff et al., 1998). This could arise from an increase in G2/M arrest and apoptosis (Wahl et al., 1996). p53 loss can also upregulate MAP4, which as previously discussed can increase microtubule stability and sensitivity to paclitaxel (Zhang et al., 1998).

Activation of the PI3K/AKT pathway has also been strongly implicated in promoting paclitaxel resistance. AKT activity can support the altered metabolism of cancer cells and maintain the glycolytic rate of paclitaxel treated cells (VanderWeele et al., 2004), while also promoting their survival through the phosphorylation and inactivation of BAD and RAF-1 (Mabuchi et al., 2002). Overexpression of the p110 catalytic subunit of PI3K has been shown to confer paclitaxel resistance in ovarian cancer cells by promoting cell survival (Hu et al., 2002). Furthermore, paclitaxel sensitivity could be restored using a PI3K inhibitor. Overexpression of the epidermal growth factor receptor (EGFR) variant known as EGFRvIII has been shown to activate both the PI3K/AKT pathways and ERK pathway and confer resistance to paclitaxel (Montgomery et al., 1995). Moreover, human epidermal growth factor receptor 2 (HER2) overexpression can also lead to enhanced PI3K/AKT activity and paclitaxel resistance (Knuefermann et al., 2003). HER2 can also inhibit CDK1 activity, which prevents apoptosis in paclitaxel treated cells (Yu et al., 1998). Due to its role in cell survival and metabolism, the activity of the PI3K/AKT pathway clearly has an important role in the development of chemotherapy resistance. The PI3K/AKT pathway will be discussed in more detail in the next section.

1.3. The PI3K/AKT pathway

1.3.1. Regulation of the PI3K/AKT signalling pathway

The PI3K/AKT signalling pathway has been intensely studied since the identification of AKT as an oncogene (Staal, 1987; Bellacosa et al., 1991). There are three main classes of PI3K complexes, which are based on their structural and biochemical properties (Domin and Waterfield, 1997). Class I PI3Ks are heterodimeric complexes consisting of a catalytic subunit and regulatory subunit,

and can be further broken down into class IA and class IB depending on which two subunits they possess. Class IA PI3Ks contain a p50α/p55α/p55γ/p85α/p85β regulatory subunit paired with a p110α/β/δ catalytic subunit, whereas class IB PI3Ks consist of a p101 regulatory subunit and a p110γ catalytic subunit (Vanhaesebroeck et al., 1997). Unlike class I, class II PI3Ks are monomeric catalytic enzymes, while class III currently only have one member, Vps34.

Class IA PI3Ks have been the most widely studied of the three classes due to their enhanced activity in cancer cells (Yuan and Cantley, 2008). Class IA PI3Ks are receptor-stimulated kinases that are commonly activated by receptor tyrosine kinase (RTK) signalling (Figure 1.3) (Whitman et al., 1987). However, there is evidence that class IA heterodimers containing the p110β subunit can also be activated by G protein-coupled receptors (Kurosu et al., 1997; Roche et al., 1998). PI3K activation by RTKs is mediated by the Src-homology 2 (SH2) domain of the p85 regulatory subunit (Rameh et al., 1995). The SH2 domain can bind phosphotyrosine residues on the activated RTK, whilst the SH3 domain on the adaptor molecule, GRB2, can bind to proline-rich motifs on p85 (Wang et al., 1995b). Interaction with GRB2 or phosphotyrosine acts to recruit the p85-p110 heterodimer to the plasma membrane and relieves the inhibition of p110 by p85 (Yu et al., 1998; Liu et al., 2014b). The active and catalytic p110 subunit can then phosphorylate phosphotidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to produce $PI(3,4,5)P_3$.

$PI(3,4,5)P_3$ can be dephosphorylated by SH2 domain-containing inositol phosphatase 1 (SHIP1) (Damen et al., 1996) and SHIP2 to form $PI(3,4)P_2$ (Pesesse et al., 1997). Proteins harbouring a PH domain can directly bind $PI(3,4,5)P_3$ and $PI(3,4)P_2$, which stimulates their recruitment to the plasma membrane. The PH domain of AKT binds $PI(3,4,5)P_3$ and $PI(3,4)P_2$ with similar affinities (Franke et al., 1997). This results in AKT being at the plasma membrane for a longer duration than proteins that preferentially bind $PI(3,4,5)P_3$ because the accumulation of $PI(3,4)P_2$ is delayed and more sustained.

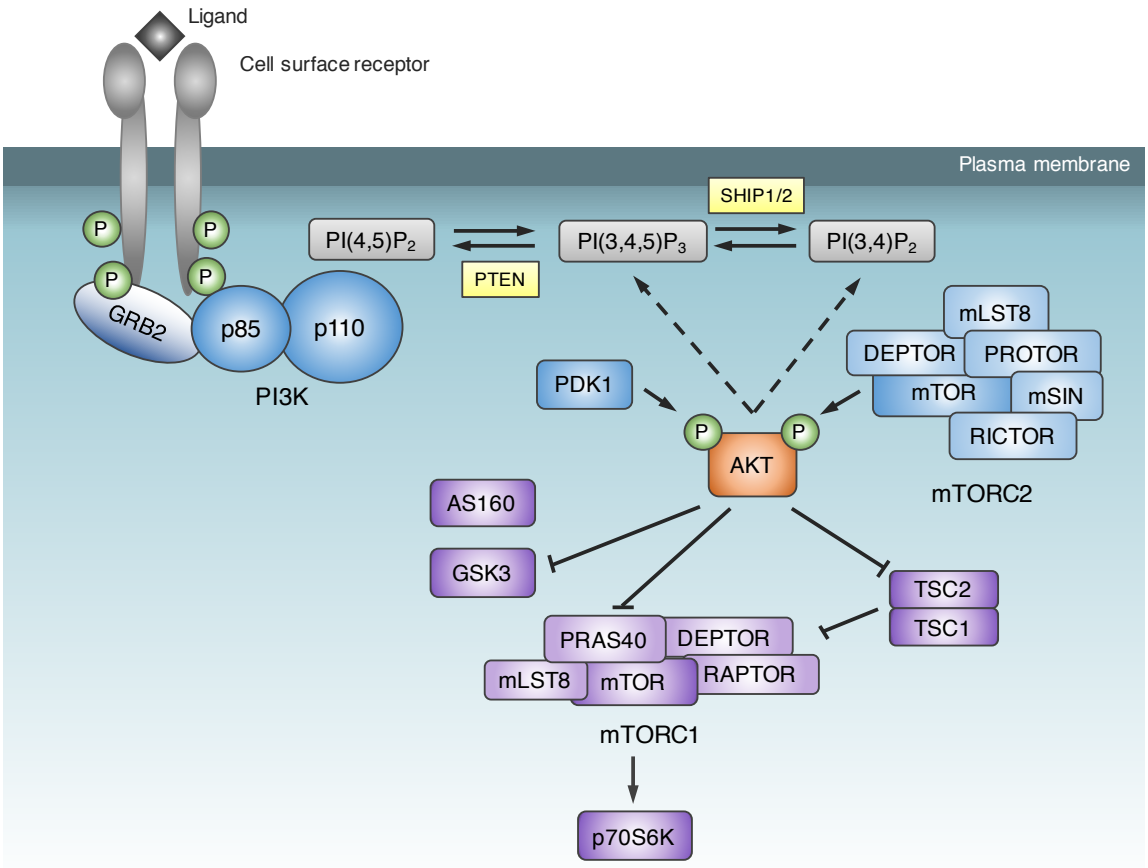


Figure 1.3. Schematic of the PI3K/AKT/mTOR signalling pathway.
PI3K is activated downstream of growth factor receptors and phosphorylates phosphatidylinositol (PI) lipids at the cell membrane. $PI(3,4,5)P_3$ and $PI(3,4)P_2$ recruit AKT to the cell membrane where it is phosphorylated by PDK1 at T308 and mTORC2 at S473. Phosphorylated and activated AKT can phosphorylate a plethora of downstream targets to influence cell metabolism, survival, proliferation and growth. This pathway can be negative regulated by PTEN and SHIP1/2, which dephosphorylate specific PI lipids.

Once recruited to the plasma membrane, AKT is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at T308 in the activation loop, leading to partial activation of the protein (Alessi et al., 1997). The additional phosphorylation of AKT at S473 in the carboxy-terminal hydrophobic motif is required for full activation of the protein (Alessi et al., 1996a). The mammalian target of rapamycin complex 2 (mTORC2) is widely accepted as the major regulator of S473 phosphorylation (Sarbassov et al., 2005), with the depletion of any of the mTORC2 components preventing phosphorylation at S473 (Frias et al., 2006; Guertin et al., 2006). DNA-dependent protein kinase (DNA-PK) has also been shown to associate with, and phosphorylate, AKT at S473 during the double-strand break response (Bozulic et al., 2008). However, the contribution of DNA-PK to AKT activity during normal cellular conditions is not clear. Recent evidence suggests that AKT phosphorylation at S473 could be governed by the

phosphorylation of the nearby residues S477 and T479, which is mediated by cyclin A-CDK2 or mTORC2 (Liu et al., 2014a). Phosphorylation at these two sites has been shown to promote, or partially compensate for, S473 phosphorylation. Therefore, S477 and T479 phosphorylation provide a novel link between aberrant cell cycle progression and the constitutive activation of AKT.

AKT is also phosphorylated by mTORC2 at T450 in the turn motif of the protein (Bellacosa et al., 1998). T450 phosphorylation has been shown to promote AKT stability and kinase activity (Facchinetti et al., 2008; Xiao et al., 2010). However, the exact effect of T450 phosphorylation on AKT activity is not clear as mutating T450 to D or A has been shown to have a little effect on AKT signalling (Liao and Hung, 2010; Hart and Vogt, 2011). A number of additional residues of AKT have been identified as potential phosphorylation sites (Guo et al., 2014), but the extent of their contribution to AKT activity has not yet been determined.

Activation of the PI3K/AKT pathway is tightly controlled. As I have previously mentioned, $PI(3,4,5)P_3$ can be dephosphorylated by SHIP1 and SHIP2 producing $PI(3,4)P_2$, which can still stimulate downstream signalling. In addition, PTEN can dephosphorylate $PI(3,4,5)P_3$ to produce $PI(4,5)P_2$ (Stambolic et al., 1998), which terminates PI3K/AKT signalling. Residues phosphorylated on AKT are subject to the action of a number of other phosphatases, including PH domain leucine-rich repeat protein phosphatases (PHLPPs) that dephosphorylate AKT at S473 (Gao et al., 2005; Brognard et al., 2007), protein phosphatase 2A (PP-2A), which dephosphorylates T308 (Andjelkovic et al., 1996; Kuo et al., 2008) and PP-1 that targets T450 (Xiao et al., 2010). There are also important negative feedback loops that are triggered downstream of AKT activation. The first is a consequence of mTORC1 activation that leads to the inhibitory phosphorylation and transcriptional repression of insulin response substrate-1 (IRS-1) (Harrington et al., 2005). IRS-1 is an adaptor protein that is important for PI3K activation and therefore its inhibition results in a reduction in PI3K/AKT signalling (Araki et al., 1994). A second mechanism involves the inhibitory phosphorylation of FOXO by AKT, which prevents FOXO from stimulating the transcription of the insulin receptor, resulting in the downregulation of PI3K/AKT activity (Puig and Tjian, 2005).

1.3.2. Cellular processes downstream of AKT

AKT has three evolutionary related isoforms, AKT 1/2/3 (also known as PKB α/β/γ respectively), which are encoded by three separate genes (Coffer and Woodgett, 1991; Jones et al., 1991; Nakatani et al., 1999). They are all serine/threonine kinases belonging to the AGC kinase family and contain a highly conserved domain structure, which includes an N-terminal PH domain, a central catalytic kinase domain and a regulatory hydrophobic C-terminus (Nicholson and Anderson, 2002). Once phosphorylated and activated, AKT can phosphorylate a large number of proteins to regulate their activity. The minimal recognition motif of AKT has been identified as R-X-R-X-X-S/T-B, where X represents any amino acid and B represents bulky hydrophobic residues (Alessi et al., 1996b). AKT activity can influence a vast number of cellular processes, including autophagy, angiogenesis, survival, growth, proliferation and metabolism (Figure 1.4).

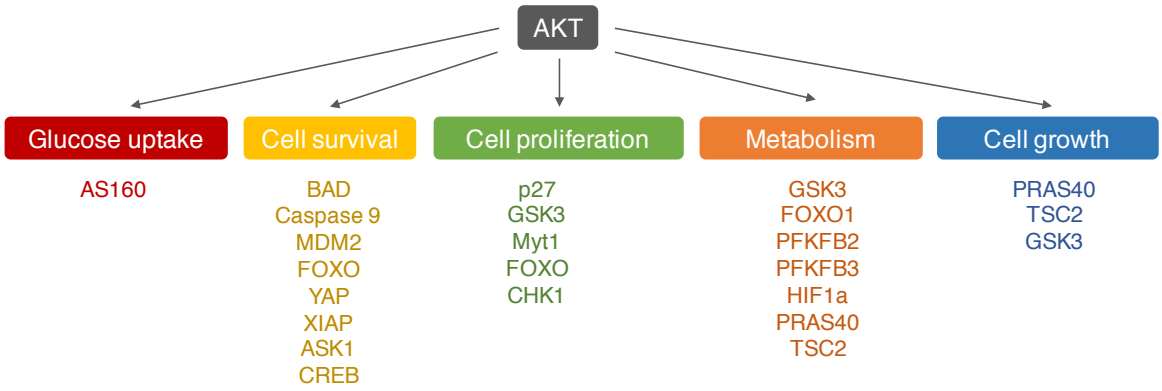


Figure 1.4. Cellular processes that are influenced by AKT activity.
Once activated, AKT can phosphorylate a number of proteins, which alters their function and effects a number of cellular processes.

Cell survival

AKT activity inhibits apoptosis through a number of mechanisms. A common route is through the association with 14-3-3 proteins, whereby the residue phosphorylated by active AKT acts as a docking site for 14-3-3 (Fu et al., 2000). This can inhibit the proapoptotic functions of a protein either via its translocation to the cytoplasm, or through the sequestration away from its substrates. One example is the phosphorylation of FOXO1, FOXO3A and FOXO4 by AKT (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999). Phosphorylation induces the binding of FOXO proteins to 14-3-3, which promotes their cytoplasmic localisation (Brunet et al., 2002) and blocks their transcription of proapoptotic genes, such as the BCL-2 homology domain 3 (BH3)-only protein *BIM* (Gilley et al., 2003), Fas Ligand (*FasL*) (Brunet et al., 1999) and *TRAIL* (Modur et al., 2002). AKT-mediated phosphorylation and cytoplasmic sequestration of FOXO proteins also downregulates their activity by promoting their ubiquitinylation and degradation (Matsuzaki et al., 2003). AKT can also phosphorylate the Yes-associated protein (YAP), which promotes its association with 14-3-3 and its sequestration in the cytoplasm (Basu et al., 2003). This impairs the ability of YAP to induce the transcription of proapoptotic genes, such as *p73*, which promote *BAX* expression and promotes apoptosis following DNA damage. In addition, AKT can directly phosphorylate the proapoptotic protein, BAD, which promotes its association with 14-3-3 proteins in the cytoplasm (Datta et al., 1997). The sequestration of BAD prevents it from initiating the release of cytochrome c from the mitochondria into the cytoplasm, thus increasing cell survival.

Another substrate of AKT is the ubiquitin ligase murine double minute 2 (MDM2). Once phosphorylated by AKT, MDM2 targets p53 for ubiquitination by promoting its translocation to the cytoplasm and its subsequent proteasomal degradation (Ogawara et al., 2002). This prevents p53 from promoting apoptosis through its transcriptional regulation of genes mediating apoptosis (Gottlieb and Oren, 1998). AKT can also phosphorylate the X-linked inhibitor of apoptosis proteins (XIAP), which is a direct inhibitor of caspase-3 and caspase-9 and thus a modulator of the BAX/cytochrome c pathway (Deveraux et al., 1998). AKT-mediated phosphorylation of XIAP prevents its ubiquitination and degradation, which

promotes cell survival (Dan et al., 2004). AKT can also directly phosphorylate and inactivate caspase-9 (Cardone et al., 1998).

AKT can phosphorylate and inactivate the apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase kinase kinase that mediates stress and cytokine-induced cell death (Kim et al., 2001a). Moreover, AKT phosphorylates and activates the cyclic AMP-response element-binding protein (CREB) (Du and Montminy, 1998), which increases the transcription of anti-apoptotic genes, such as *BCL-2* (Pugazhenthi et al., 2000) and *MCL-1* (Wang et al., 1999). MCL-1 expression can also be controlled through AKT-mediated phosphorylation and inhibition of GSK3, which prevents GSK3 from phosphorylating MCL-1 to promote its degradation, facilitating its anti-apoptotic function (Maurer et al., 2006).

Cell growth

As well as supporting cell growth through its numerous effects on nutrient uptake and metabolism, AKT activity can directly promote an increase in cell mass. One way it does this is via the downstream activation of mTORC1, which has a critical role in translation initiation and ribosome biogenesis (Jastrzebski et al., 2007; Thoreen et al., 2012). AKT activity promotes the activation of mTORC1 through the direct phosphorylation of the tumour suppressor complex 2 (TSC2) (Inoki et al., 2002; Manning et al., 2002) and proline-rich AKT substrate of 40 kDa (PRAS40) (Kovacina et al., 2003). Together with TSC1, TSC2 acts as a GTPase-activating protein (GAP) for the Ras-related small G protein, Rheb (Inoki et al., 2003). The activity of TSC2 is inhibited by AKT phosphorylation, which enables Rheb to become guanosine triphosphate (GTP) loaded and strongly activate mTORC1 (Huang and Manning, 2009). In addition, AKT phosphorylation of PRAS40 promotes its binding to 14-3-3, which relieves its inhibition of mTORC1 (Vander Haar et al., 2007).

Activation of mTORC1 enables it to phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and p70 S6 kinase (p70S6K) (Fingar et al., 2002). The phosphorylation of 4E-BP1 inhibits its ability to bind to the cap-binding protein eIF4E, which enables it to participate in the

formation of the eIF4F complex that is required for the initiation of cap-dependent translation (Gingras et al., 1999). Whereas mTORC1 phosphorylation and activation of p70S6K results in an increase in mRNA biogenesis, as well as translational initiation and elongation (Ma and Blenis, 2009). AKT induced phosphorylation and inhibition of GSK3 can also stimulate translation initiation events through its ability to relieve GSK3s inhibition of eIF2B (Singh and Wahba, 1996; Welsh et al., 1997). eIF2B is the guanine nucleotide exchange factor (GEF) for eIF2 during mRNA translation initiation that enables eIF2 to become GTP loaded and bind the initiator methionyl-transfer RNA.

Cell proliferation

AKT activity plays an important role in the G1 to S phase transition. PI3K/AKT activity regulates the levels of c-Myc and cyclin D, both of which promote G1 to S phase progression (Resnitzky et al., 1994; Beier et al., 2000; Blagosklonny and Pardee, 2002; Gartel and Shchors, 2003). AKT prevents the proteolytic degradation of c-Myc and cyclin D via its phosphorylation and inhibition of GSK3, which leads to their stabilisation and prolonged activity (Liang and Slingerland, 2003). AKT also promotes the transcription of *cyclin D* and *c-Myc* genes (Muise-Helmericks et al., 1998; Zhu et al., 2008).

AKT directly phosphorylates $p27^{Kip1}$, which leads to its cytoplasmic sequestration via 14-3-3 binding, attenuating its inhibitory effects on the cell cycle (Sekimoto et al., 2004). A reduction in $p27^{Kip1}$ activity is also achieved through the AKT-mediated phosphorylation and inhibition of the FOXO transcription factors, which act to inhibit $p27^{Kip1}$ expression (Medema et al., 2000). Moreover, AKT can phosphorylate $p21^{Cip1}$ inducing its cytoplasmic localisation, which alleviates its inhibitory effects on the cell cycle (Zhou et al., 2001a). $p21^{Cip1}$ expression can also be inhibited via AKT-mediated phosphorylation and activation of MDM2, which downregulates p53.

AKT activity negatively regulates Myt1 (Okumura et al., 2002), a member of the Wee1 family that is important in maintaining low CDK1 activity and preventing mitotic entry (Mueller et al., 1995). A decrease in Myt1 would lead to enhanced CDK1 activity enabling G2/M phase transition. In support of this, it has been reported that PI3K inhibition prevents the expression of cyclin B and reduces

CDK15C phosphatase activity; both of which would contribute to delayed G2/M transition (Dangi et al., 2003). Levels of cyclin B and PLKs are also kept low via the negative regulation of FOXO proteins by AKT, which promote mitotic entry (Alvarez et al., 2001). Moreover, AKT directly phosphorylates the DNA damage checkpoint kinase, CHK1 (King et al., 2004). Phosphorylation of CHK1 stimulates its translocation to the cytosol, where it is sequestered from the DNA damage-sensing kinases ATM and ATR, which hinders the checkpoint function and promotes cell cycle progression (Puc et al., 2005).

Glucose uptake and metabolism

AKT can regulate nutrient uptake and metabolism in a variety of ways. Firstly, AKT activity can influence glucose transport into the cell. AKT activity is essential for insulin-stimulated translocation of GLUT4 to the plasma membrane (Sano et al., 2003). The Rab GTPase-activating protein, AS160, is a direct substrate of AKT that links insulin signalling and GLUT4 trafficking (Kane et al., 2002; Miinea et al., 2005). Once phosphorylated at T642, the GTPase activity of AS160 is inhibited, allowing Rab proteins to become GTP loaded and stimulate GLUT4 vesicular exocytosis. AKT signalling can also stimulate the expression of the main glucose transporter, *GLUT1* (Barthel et al., 1999).

Secondly, AKT plays an important role in driving glycolysis. AKT can increase the levels of glucose 6-phosphate by increasing mitochondrial-associated hexokinase activity (Gottlob et al., 2001; Majewski et al., 2004). This association also acts to block apoptosis by preventing cytochrome c release. Through its activation of HIF1α, AKT can promote the expression of many glycolytic enzymes, such as aldolase A, phosphoglycerate kinase 1, and pyruvate kinase M (Semenza et al., 1994; Jiang et al., 2001). Alternatively, AKT can influence glycolysis through the phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2) (Deprez et al., 1997) and PFKFB3 (Manes and El-Maghrabi, 2005). Both PFKFB2 and PFKFB3 catalyse the synthesis of fructose 2,6-bisphosphate (Van Schaftingen and Hers, 1981b), which is a powerful allosteric activator of phosphofructokinase 1 (PFK1) that controls one of the critical rate-limiting steps of glycolysis (Van Schaftingen, 1987; Okar and Lange, 1999). Moreover, the inhibition of GSK3 by AKT relieves the inhibitory effects of GSK3 on pyruvate

dehydrogenase (Hoshi et al., 1996), type-II regulatory subunit of cyclic-AMP-dependent protein kinase (Hemmings et al., 1982) and IRS-1 (Eldar-Finkelman and Krebs, 1997); all of which help to facilitate AKT in driving glycolysis.

As I have mentioned previously, AKT can directly phosphorylate FOXO proteins. In addition to promoting cell survival, the phosphorylation of FOXO1 by AKT has been shown to negatively regulate gluconeogenesis (Nakae et al., 2001). AKT phosphorylation of FOXO1 sequesters it in the cytoplasm, which prevents the FOXO1-mediated induction of gluconeogenic genes, such as glucose 6-phosphatase and phosphoenolpyruvate carboxykinase (Hall et al., 2000; Schmoll et al., 2000). This reduces the amount of glucose being generated from non-carbohydrate carbon substrates.

Furthermore, AKT can stimulate glycogen synthesis and lipogenesis. The phosphorylation and inactivation of GSK3 by AKT prevents GSK3 from inhibiting glycogen synthase and promotes glycogen synthesis (Parker et al., 1983; Cross et al., 1995). Inhibition of GSK3 activity could also prevent the degradation of the sterol regulatory element-binding protein 1 (SREBP-1) (Sundqvist et al., 2005; Bengoechea-Alonso and Ericsson, 2009), which promotes the expression of genes involved in cholesterol and fatty acid biosynthesis, such as fatty acid synthase (*FASN*) (Magana and Osborne, 1996), acetyl-CoA carboxylase (*ACC*) (Magana et al., 1997) and steroyl-CoA desaturase (Bene et al., 2001; Krycer et al., 2010). An alternative pathway for the regulation of lipogenesis by AKT is via mTORC1. AKT-induced mTORC1 activity can induce the expression of SREBP-1 target genes (Porstmann et al., 2008). AKT is also thought to directly phosphorylate SREBP-1c (Yellaturu et al., 2009) and ATP citrate lyase (ACL) (Berwick et al., 2002) to promote lipogenesis.

AKT can influence the cell surface expression of transporters for a number of nutrients in an mTORC1-dependent manner (Edinger and Thompson, 2002). This includes the plasma membrane expression of the low-density lipoprotein receptor that mediates the endocytosis of cholesterol, the transferrin receptor for receptor-mediated endocytosis of iron, and the glycoprotein CD98 that forms the amino acid transporter, LAT1.

1.3.3. Targeting the PI3K/AKT pathway for cancer therapy

The PI3K/AKT pathway is frequently overactive in cancer patients, with components of the pathway commonly targeted by amplification, mutation and translocation (Altomare and Testa, 2005; Yuan and Cantley, 2008). The most common genetic alterations are deletions and mutations in the tumour suppressor *PTEN*, amplifications of the genomic regions encoding AKT, and activating mutations in *PIK3CA*; all of which have been demonstrated in ovarian cancer patients (Cheaib et al., 2015). Constitutive activation of the PI3K/AKT pathway can promote oncogenic transformation and chemotherapy resistance. Therefore, this pathway has been an attractive target for the development of novel cancer therapeutics. Many inhibitors have been developed targeting PI3K, AKT and mTORC1 and mTORC2 (Musa and Schneider 2015). However, despite encouraging observations in preclinical models, the results of clinical trials have not been as fruitful. This could be due to the complexity of the PI3K/AKT signalling pathway, or the need for carefully selecting patient populations that will benefit from such treatments.

1.4. Metabolic reprogramming in cancer cells

Advances in cancer research have furthered our understanding about the complex process of the development of cancer. The transformation of normal cells to malignant cells is now recognised as a multi-step process that evolves progressively, with each change conferring an advantage to the cell (Nowell, 1976; Greaves and Maley, 2012). In 2000, Hanahan and Weinberg put forward six acquired capabilities of cancer cells that govern their transformation (Hanahan and Weinberg, 2000). These include resistance against cell death, initiating angiogenesis, activating invasion and metastasis, acquiring replicative immortality, avoiding growth suppressors and uncontrolled proliferative signalling (Figure 1.5). Due to further advances in the field, specific characteristics that facilitate the acquisition of these hallmarks have been acknowledged, including genomic instability and inflammation (Hanahan and Weinberg, 2011). Moreover, two additional hallmarks have since been identified. One is the ability of cancer cells to avoid the immune system, which enables their continued growth and colonisation. While the second involves the capability of cancer cells to undergo

oncogene-directed metabolic programming to most efficiently support their increased growth and proliferation (Ward and Thompson, 2012). Below I have explored cellular metabolism in more detail and highlighted some of the ways in which cancer cells undergo metabolic reprogramming.

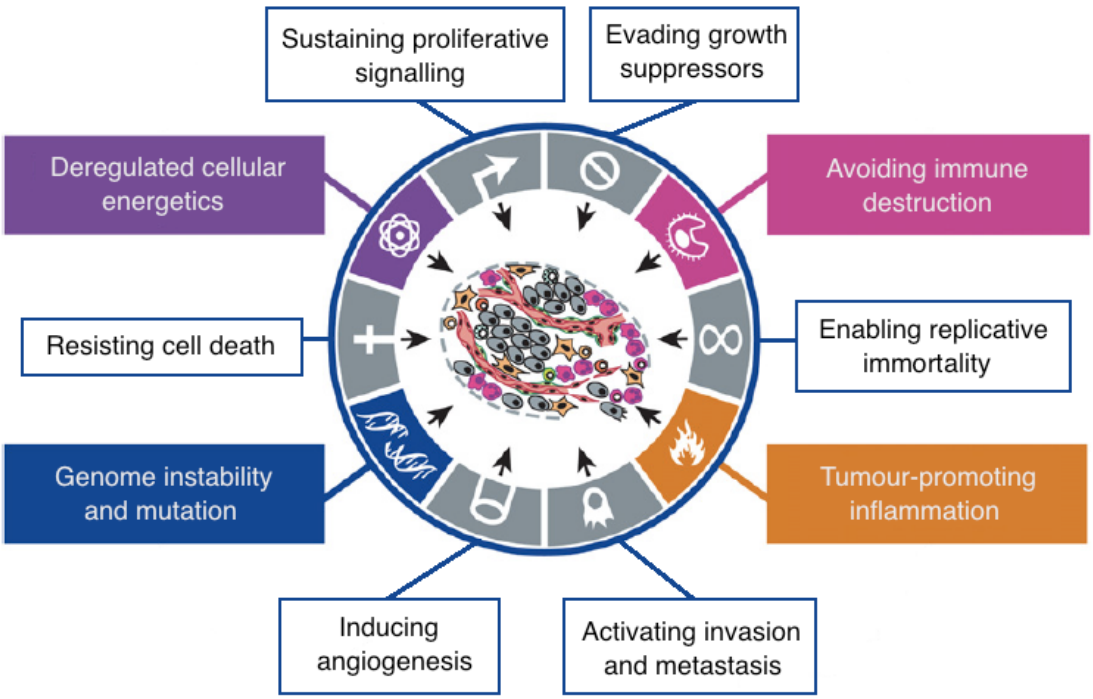


Figure 1.5. Schematic of the hallmarks of cancer cells.
In white are the six acquired capabilities of cancer cells originally identified by Hanahan and Weinberg in 2000. In pink and purple are the two new hallmarks proposed in 2011, along with the two new characteristics of cancer cells shown in orange and blue.

1.4.1. The Warburg effect

In adult mammalian cells, glucose can be metabolised to generate ATP via two pathways, aerobic glycolysis and oxidative phosphorylation; both of which initially require the catabolism of glucose to pyruvate via glycolysis (Figure 1.6). Glucose is transported into cells via glucose transporters, such as GLUT1 and GLUT4 (Pessin and Bell, 1992). It is then phosphorylated by hexokinase to form glucose 6-phosphate, which is the first committed step of glycolysis and thus is highly regulated (Berg, 2002). Glucose 6-phosphate cannot diffuse out of the cell and undergoes isomerisation to fructose 6-phosphate, which is then further phosphorylated by PFK1 to form fructose 1,6-bisphosphate (Van Schaftingen et

al., 1980a). PFK1 is a critical rate-limiting enzyme of this pathway and is thought to set the pace for glycolysis (Berg, 2002). Fructose 1,6-bisphosphate is cleaved into two three-carbon sugars that enter a series of sequential reactions to form phosphoenolpyruvate. In the last step, phosphoenolpyruvate is converted to pyruvate by pyruvate kinase (PK) (Tanaka et al., 1967; Imamura et al., 1972; Nakashima et al., 1974). PK has four isoforms derived from two distinct genes. The isoforms are known as L, R, M1 and M2. The most predominant isoform in cancer cells is M2, which is less efficient than M1 and, unlike M1, can alter its activity state according to the demands of the cell (Christofk et al., 2008). The lower efficiency of M2 results in the use of glycolytic intermediates for biosynthetic processes, but also controls the levels of ATP in the cell, which at high concentrations can inhibit glycolysis.

Pyruvate can be converted to lactate in the cytoplasm by an inefficient, but rapid, reaction producing 2 ATP per glucose molecule (Kim and Dang, 2006; Vander Heiden et al., 2009). Alternatively, pyruvate can be converted to acetyl-CoA by pyruvate dehydrogenase and enter the tricarboxylic acid (TCA), also known as the Krebs cycle. In the presence of oxygen and via oxidative phosphorylation, this can yield approximately 36 ATP from each fully oxidised glucose molecule. In non-tumourigenic cells, mitochondrial oxidative phosphorylation is the primary mechanism for ATP generation, with lactate only being produced when oxygen is limited, which is known as anaerobic glycolysis. However, a major biochemical hallmark of cancer cells is the shift in ATP generation from mitochondrial oxidative phosphorylation to anaerobic glycolysis, even in the presence of oxygen, known as aerobic glycolysis and termed “the Warburg effect” (Warburg, 1956).

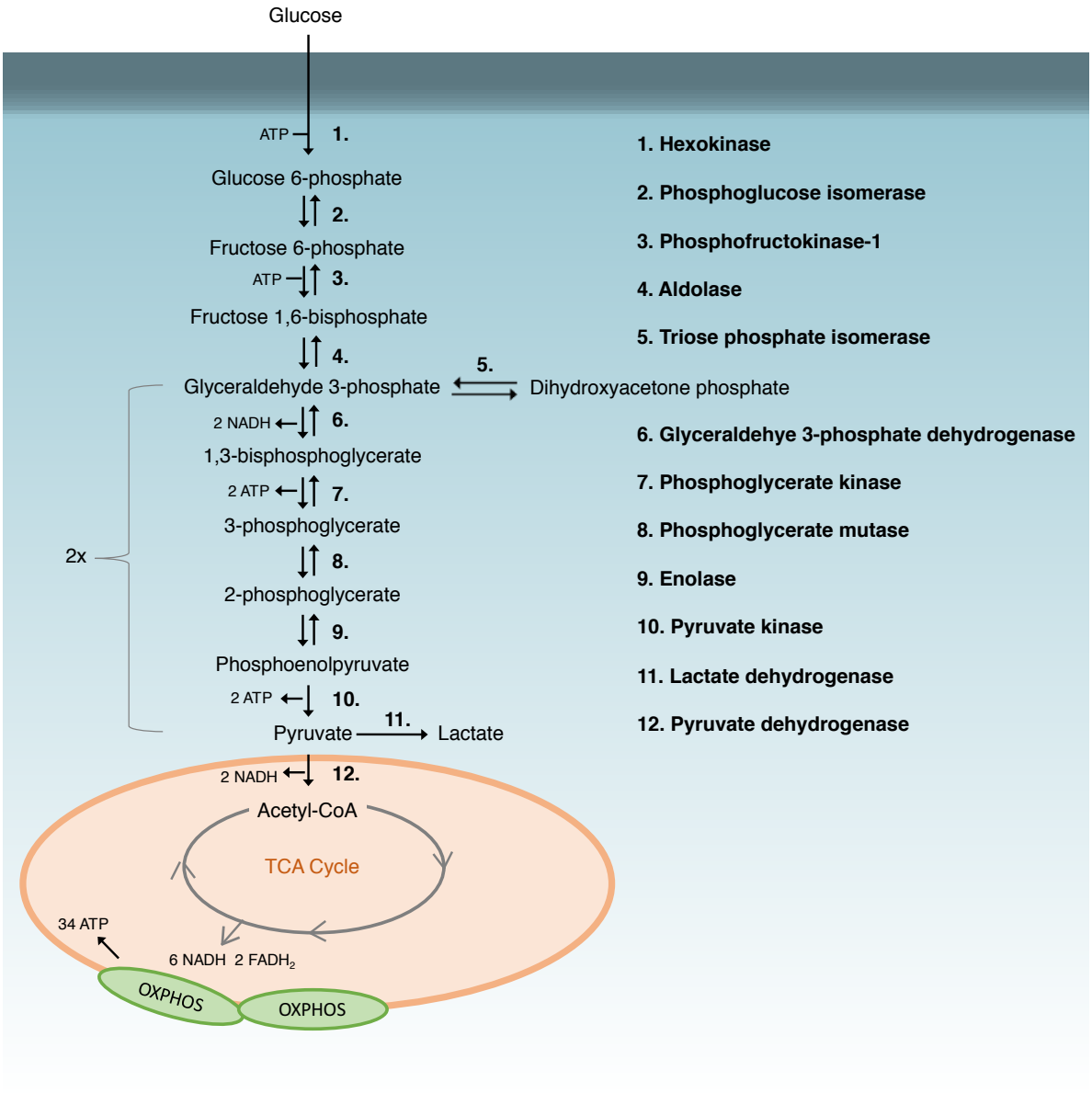


Figure 1.6. Glycolysis and ATP production.
Glucose is taken into the cell by glucose transporters and converted to glucose 6-phosphate, which is metabolised to pyruvate by glycolytic enzymes (numbered). Pyruvate can be converted to lactate, or enter the mitochondria where it is utilised in the tricarboxylic acid (TCA) cycle. The NADH and $FADH_2$ produced from the TCA cycle enter the electron transport chain that produces ATP via oxidative phosphorylation (OXPHOS).

The consumption of glucose in cancer cells is much higher than their non-tumourigenic counterparts. This is supported and fuelled by the constitutive activation of oncogenes, such as AKT, the inactivation of tumour suppressors and the activation of the HIF pathway, which are discussed at the end of this section. Increased glucose uptake and anaerobic glycolysis serves to fulfil many metabolic requirements of cancer cells that extend beyond that of ATP generation (Vander Heiden et al., 2009; Lunt and Vander Heiden, 2011). Most importantly, it allows cancer cells to meet their high biosynthetic demands by providing intermediates for anabolic reactions, such as nucleotide, amino acid and lipid biosynthesis (Ward and Thompson, 2012). It also increases the production of lactate, which acidifies the local environment and promotes invasion and metastatic spread (Kroemer and Pouyssegur, 2008). Aerobic glycolysis also creates a survival advantage to cancer cells when exposed to oxygen poor environments, such as the hypoxic conditions within a tumour. Moreover, metabolites of glucose can be redirected towards the pentose phosphate pathway, which provides ribose for nucleotide synthesis, and NADPH to support fatty acid synthesis and the antioxidant defence system.

The pentose phosphate pathway is comprised of two branches; the oxidative branch and the non-oxidative branch (Figure 1.7) (Patra and Hay, 2014). The oxidative branch consists of three irreversible reactions and results in the generation of NADPH and ribose 5-phosphate (Kruger and von Schaewen, 2003). The non-oxidative branch comprises three reversible reactions that utilise glycolytic intermediates, such as fructose 6-phosphate and glyceraldehyde 3-phosphate, to form pentose phosphates that can be used for nucleic acid synthesis (Horecker, 2002). The pentose phosphate pathway is regulated according to the demands of the cell. For example, if detoxification is more important than nucleic acid synthesis, the oxidative branch is prioritised so that transketolase and transaldolase in the non-oxidative branch are directed towards synthesising fructose 6-phosphate, which can then be converted to glucose 6-phosphate to replenish the oxidative branch.

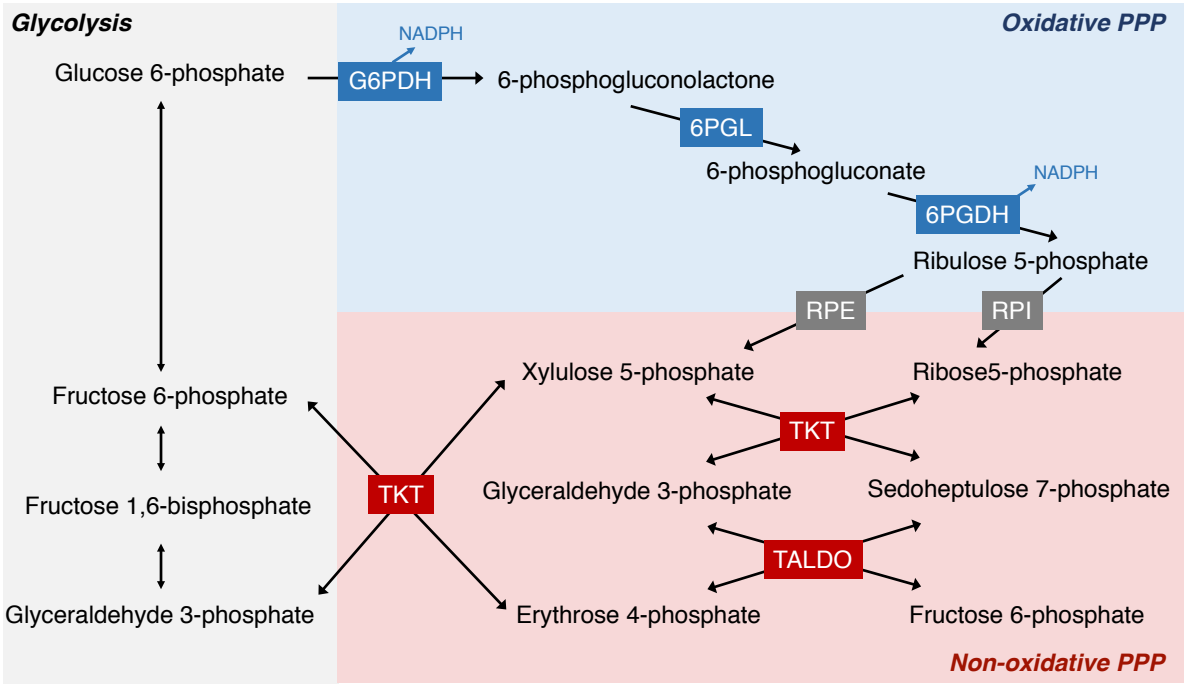


Figure 1.7. Schematic of the pentose phosphate pathway (PPP).
In blue is the oxidative arm of the PPP, in red is the non-oxidative arm of the PPP and in grey displays the interaction of the PPP with glycolysis. G6PDH, glucose 6-phosphate dehydrogenase, 6PGL, 6-phosphogluconolactonase, 6PGDH, 6-phosphogluconate dehydrogenase, RPE, ribulose 5-phosphate isomerase, RPI, ribulose 5-phosphate 3-epimerase, TKT, transketolase, TALDO, transaldolase.

Despite the broad acceptance of the Warburg effect, there has been evidence to the contrary. For example, a literature review carried out in 2004 demonstrated that the contribution of aerobic glycolysis to ATP production in normoxic cancer cells can range from 1.2 to 64%, with the remainder being derived from oxidative phosphorylation (Zu and Guppy, 2004). This indicates that the utilisation of aerobic glycolysis may vary depending on cell type and might not always be the predominant pathway for ATP generation.

1.4.2. Glycogen metabolism

Glycogen can act as a readily available source of glucose to highly proliferating cells. Glycogen can be broken down to glucose 1-phosphate through glycogenolysis, a process that requires the enzyme glycogen phosphorylase (Newgard et al., 1989). Glucose 1-phosphate can then be converted to glucose 6-phosphate, which can either enter glycolysis or the pentose phosphate pathway. The key enzyme in glycogen synthesis is glycogen synthase, which has two isoforms, GYS1 in muscle and a number of other tissues, and GYS2 in the liver

(Roach et al., 1998). Glycogen utilisation and storage is another metabolic pathway that appears to be reprogrammed in cancer cells. For example, a number of tumours, including those of the breast and ovary, display increased glycogen accumulation (Rousset et al., 1981). Increased glycogen accumulation in tumours has been inversely correlated with decreased proliferation.

1.4.3. Lipid synthesis and breakdown

Fatty acids (FAs) are important sources of energy and act as essential components in lipid membranes, as well as being critical precursors for lipid-based post-translational modifications of proteins. FAs can either be obtained from the exogenous environment or synthesised endogenously. While most normal cells preferentially use extracellular FAs for the synthesis of new structural lipids, many tumours display unusually high rates of endogenous fatty acid synthesis, irrespective of the availability of exogenous lipids (Medes et al., 1953; Ookhtens et al., 1984).

The first committed step of *de novo* FA synthesis is the conversion of acetyl-CoA to malonyl-CoA by ACC (Wakil et al., 1983). Subsequent elongation of the FA occurs through the sequential addition of two-carbon units by the multifunctional and homodimeric enzyme, fatty acid synthase (FAS). This process requires NADPH as a reducing equivalent. The conversion of glucose to lipids is dependent on the cells ability to convert mitochondrial-derived citrate into acetyl-CoA and oxaloacetate by ACLY (Srere, 1959). Oxaloacetate can be converted to pyruvate, which generates reducing power in the form of NADPH to enable lipid synthesis. The enhanced glycolytic rate of many cancer cells enables the redirection of pyruvate for utilisation in *de novo* FA synthesis. Cancer cells rewire their metabolism in a number of ways to enhance lipogenesis, these include; increased *ACLY* expression and ACLY activity (Swinnen et al., 2006), overexpression of *ACC* (Milgraum et al., 1997; Moncur et al., 1998), enhanced *FASN* expression and FAS activity, as well as increased activity of other lipogenic enzymes (Swinnen et al., 2000; Yahagi et al., 2005). Moreover, mutations in *BRCA1* can affect its ability to interact with ACCα, which prevents ACC phosphorylation and enhances its activity to drive fatty acid synthesis (Moreau et al., 2006).

Fatty acids are metabolised in the mitochondria via β-oxidation, which comprises a cyclical series of reactions that gradually shorten the FA chain, resulting in the production of acetyl-CoA, NADH and $FADH_2$ (Carracedo et al., 2013). NADH and $FADH_2$ can enter the electron transport chain to produce ATP, while acetyl-CoA can enter the TCA cycle where it is completed oxidised. In the TCA cycle, acetyl-CoA can be combined with oxaloacetate to produce citrate. Citrate can be extruded to the cytoplasm and converted to pyruvate through a series of reactions, which also produces acetyl-CoA, malonyl-CoA and cytosolic NADPH (Farfari et al., 2000). Acetyl-CoA can be utilised for FA synthesis and the generation of biomembranes, while the NADPH produced is important for the detoxification of ROS (Pike et al., 2011). When the cell is metabolically stressed, β-oxidation is important for maintaining ATP levels and NADPH production (Schafer et al., 2009; Jeon et al., 2012). Carnitine palmitoyltransferase 1C (CPT1) controls a critical step in β-oxidation; the import of FAs into the mitochondria (Kerner and Hoppel, 2000). Certain isoforms of CPT1 have been found to be critical for cell survival and tumour growth in metabolically stressed cells (Zaugg et al., 2011). In addition, the expression of genes involved in β-oxidation are increased in a number of tumours, demonstrating that lipid turnover, as well as lipid synthesis, is important for cancer cell growth (Hirsch et al., 2010).

Lipid synthesis and degradation are mutually exclusive due to their mechanisms of regulation. For example, malonyl-CoA produced by ACC acts to inhibit enzymes involved in β-oxidation, such as CPT1 (McGarry and Brown, 1997). ACC is also phosphorylated and inhibited by 5′ adenosine monophosphate-activated protein kinase (AMPK) (Davies et al., 1990). AMPK responds to a reduction in ATP/AMP ratio by inhibiting energy-consuming processes, such as lipid synthesis, and promoting energy producing pathways, such as β-oxidation (Hardie, 2008). AMPK promotes β-oxidation through its inhibitory effects on ACC (Merrill et al., 1997) and potentially through the regulation of peroxisome proliferator-activated receptors α (PPARα) signalling (Lee et al., 2006). Moreover, chronic activation of AMPK decreases the expression of *SREBP-1c*, resulting in the reduced expression of *ACC*, *FASN* and other lipogenic enzymes (Zhou et al., 2001b).

1.4.4. Glutamine metabolism

Glutamine is the most abundant amino acid in the blood and the upregulation of glutamate transporters and glutaminases has been reported in a number of cancer cells (Gao et al., 2009). Glutamine metabolism serves many functions in the cell, including the generation of ATP through its utilisation in the TCA cycle (Yang et al., 2014), NADPH production, and as an anaplerotic precursor for nucleotide biosynthesis and the synthesis of non-essential amino acids (DeBerardinis et al., 2007); all of which are important in the maintenance of mitochondrial membrane potential (Wise and Thompson, 2010). Along with glucose, glutamine is also required for hexosamine biosynthesis and subsequent glycosylation reactions (Wellen et al., 2010).

1.4.5. Drivers of metabolic reprogramming

Metabolic reprogramming is driven through vast alterations in protein activity. However, the specific activation of certain oncogenes and the inactivation of tumour suppressor genes largely effects the cells capability to undergo extensive reprogramming. One example is the acquisition of inactivating mutations in the tumour suppressor PTEN, which results in the constitutive activation of the PI3K/AKT signalling pathway (Keniry and Parsons, 2008; Ortega-Molina and Serrano, 2013). Given the abundant number of roles that this pathway has in regulating cell metabolism, it is not surprising that it is a key player in the reprogramming of cancer cell metabolism. Below I discuss some of the other key proteins that cooperate to rewire the metabolism of cancer cells.

p53

TP53 is the most frequently mutated gene in human tumours, including HGSOC (Petitjean et al., 2007; Network, 2011). p53 is a tumour suppressor that has a key role in responding to DNA damage and cellular stress, along with possessing functions in metabolism (Vousden and Ryan, 2009). Loss of p53 function can assist cancer cells in rewiring their metabolism through a number of mechanisms. Firstly, wild-type p53 binds directly to the promoters of the glucose transporters GLUT1 and GLUT4 to inhibit their expression (Schwartzenberg-Bar-Yoseph et al., 2004), and negatively regulates the glycolytic enzyme phosphoglycerate mutase

(Kondoh et al., 2005). Wild-type p53 also stimulates the expression of TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR), which reduces the activity of PFK1, resulting in the inhibition of glycolysis (Bensaad et al., 2006). Therefore, loss of p53 function results in increased glucose transport into the cell and enhanced activity of the glycolytic enzymes; both of which assist in rewiring the glycolytic metabolism of cancer cells. p53 loss can also relieve its transcriptional activation of PTEN (Stambolic et al., 2001), resulting in enhanced activity of the PI3K/AKT pathway and stimulation of glycolysis (Liu and Feng, 2012).

Wild-type p53 can also increase the expression of the regulator of complex IV (Matoba et al., 2006) and subunit 1 of complex IV of the electron transport chain (Okamura et al., 1999). Therefore, a loss of p53 activity could act to reduce oxidative phosphorylation and promote the Warburg effect. A number of studies have also demonstrated a role for p53 mutations in supporting the flux through the pentose phosphate pathway. For example, inactivating mutations in p53 can enhance glucose 6-phosphate dehydrogenase activity and increase NADPH production (Jiang et al., 2011). p53 can also promote fatty acid synthesis through the direct induction of FAS transcription (D'Erchia et al., 2006). Therefore, p53 loss can reduce fatty acid synthesis and promote β-oxidation.

c-Myc

c-MYC is one of the most highly amplified oncogenes in cancer cells (Beroukhim et al., 2010) and it is able to respond to the activation of numerous signalling pathways. The activity of c-Myc can promote aerobic glycolysis by enhancing the expression of *GLUT1*, *hexokinase 2 (HK2), PFK1, enolase 1 (ENO1)* (Osthus et al., 2000; Kim et al., 2007a) and *lactate dehydrogenase A* (*LDHA*) (Shim et al., 1997). c-Myc also favours the *PKM2* splice variant over *PKM1* (David et al., 2010), which again directly contributes the Warburg effect. The catabolism of glutamine is also stimulated by c-Myc activity. For example, c-Myc can inhibit the expression *miR-23a* and *miR-23b*, which increases the transcription of mitochondrial *glutaminase 1* (Gao et al., 2009). It can also upregulate the transcription of glutamine transporters, which further enhance glutaminolysis (Wise et al., 2008).

HIF1α

The hypoxia inducible factor 1 (HIF1) is a transcription factor that comprises an oxygen-inducible HIF1α subunit and a constitutively expressed HIF1β subunit (Wang et al., 1995a). Under normoxic conditions, HIF1α is usually targeted for degradation via Von Hippel-Lindau (VHL)-dependent ubiquitination (Maxwell et al., 1999; Jaakkola et al., 2001). However, in cancer cells, stabilisation of HIF1α can occur in the presence of oxygen through a number of mechanisms, including the loss of p53 (Ravi et al., 2000), oncogenic signalling pathways (Laughner et al., 2001), or mutations in VHL (Kim and Kaelin, 2004).

HIF1 controls the expression of hundreds of genes, including glycolytic enzymes, glucose transporters and lipogenic enzymes, and therefore has an important role in reprogramming the metabolism of cancer cells. HIF1 can promote aerobic glycolysis through the enhanced expression of *PFKFB4* (Minchenko et al., 2004), *PFKFB3* (Minchenko et al., 2002), *PKM2* (Sun et al., 2011), *HK2* (Riddle et al., 2000), *ALDA*, *ENO1* and *LDHA* (Semenza et al., 1994). HIF1 can also increase the expression of the glucose transporters *GLUT1* (Chen et al., 2001) and *GLUT3* (Maxwell et al., 1997).

HIF1 downregulates oxidative phosphorylation in the mitochondria by activating pyruvate dehydrogenase kinase (Kim et al., 2006a) and MAX interactor 1 (Zhang et al., 2007), which reduce mitochondrial mass and oxygen consumption. HIF1 can also promote mitochondrial autophagy through the activation of BCL-2/ Adenovirus E1B 19kDa Interacting Protein 3 (Zhang et al., 2008a). Moreover, during hypoxia, glutamate can be utilised in a novel pathway; the reductive carboxylation of a-kG (Le et al., 2012). This is driven by isocitrate dehydrogenase and results in the formation of citrate for use in lipid synthesis (Wise et al., 2011). HIF1 also enhances lipogenesis through the upregulation of *FASN* (Furuta et al., 2008).

1.4.6. Targeting metabolism for cancer therapy

Cancer cell metabolism is an attractive target for therapeutic intervention due to the potential weakness from the high energetic and biosynthetic demand of cancer cells. The overactivation of key signalling pathways results in cancer cells becoming addicted to certain oncogenes, known as "oncogene addiction" (Weinstein, 2002). This phenomenon gives metabolic targeting an advantage over conventional chemotherapy because non-tumourigenic proliferating cells do not possess a high dependency on enhanced metabolic pathways and thus, it is more specific to cancer cells. Moreover, non-tumourigenic proliferating cells are not surrounded by the often hypoxic and nutrient poor environment that cancer cells are, which makes them less susceptible to metabolic targeting.

The identification of metabolic targets that support the reprogrammed metabolism of cancer cells aids in the development of novel targeted therapies and has been the focus of much investigation. In recent years, preliminary studies have indicated the potential of combining metabolic-targeted therapies with current chemotherapy regimes. For example, metformin, which has been widely used to treat type-2 diabetes and acts to suppress hepatic gluconeogenesis by activating AMPK, has been shown to potentiate the effects of paclitaxel (Hanna et al., 2012; Lengyel et al., 2015). There is also evidence to support a role for metabolic reprogramming in the development of chemotherapy resistance, including paclitaxel resistance (Zhou et al., 2010; Zhao et al., 2011a). Therefore, combining chemotherapy regimes with metabolic-targeted therapies could represent a promising strategy to overcome drug resistance in cancer treatment.

1.5. Aims of this thesis

The aim of this thesis was to identify critical cellular targets in mitotically arrested ovarian cancer cells that can be exploited to improve the response to paclitaxel. In order to achieve this aim, I pursued the following objectives:

1. Investigate AKT signalling in paclitaxel treated mitotically arrested ovarian cancer cell lines

2. Identify novel targets that have the potential to exploit any critical vulnerabilities in paclitaxel treated mitotically arrested ovarian cancer cell lines

3. Determine the validity and robustness of these targets in their ability to enhance the response of ovarian cancer cell lines to paclitaxel treatment

Chapter 2

Methods

2. Methods

2.1. Cell culture

Cells were grown in T75 or T175 vented flasks (Sarstedt) and incubated at 37°C and 5% CO_2. SKOV3, OVCAR-3 and HEK293T cell lines were obtained from the American Tissue Type Culture Collection, A2780 cells were purchased from Sigma-Aldrich, and OC 316 and UPN-251 were purchased from Stratech Scientific. Human skin fibroblast cells were a gift from Jo Poulton in the Nuffield Department of Obstetrics and Gynaecology, Oxford. SKOV3 cells were maintained in McCoy's 5A medium (Thermo Fisher Scientific), A2780 and OVCAR-3 cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium (Thermo Fisher Scientific), while HEK293T, OC 316, UPN-251 and human skin fibroblasts were all cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific). Growth medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific). SKOV3 cell lines stably expressing wild-type or mutant AKT1, PFKFB4 or PFKFB3 were grown in media containing 50 ng/L puromycin (Sigma-Aldrich). All cells were routinely subjected to mycoplasma testing using the MycoAlert Mycoplasma Detection Kit (Lonza) and MycoAlert Assay Control Set (Lonza) as per the manufacturer's instructions. To detach cells from tissue culture plates or flasks, media was removed and cells were washed with 1X phosphate buffered saline (PBS) prior to adding 0.5% trypsin containing EDTA (Thermo Fisher Scientific) and incubation at 37°C for 5 minutes. Media was added to neutralise the trypsin and cells were harvested into collection tubes prior to centrifugation at 1500 rpm (365 xg) for 5 minutes in a microcentrifuge (Thermo Fisher Scientific).

2.2. Reagents and compounds

Stock solutions of 1 mM paclitaxel (Sigma-Aldrich), 10 mM nocodazole (Sigma-Aldrich), 10 µM calyculin A (Cell Signaling) and 1mM staurosporine (Cell Signaling) were prepared with dimethyl sulphide (DMSO) (Sigma-Aldrich) and stored at -80°C. 500 mM N-acetylcysteine was freshly prepared in deionised water for each experiment.

2.3. Isolation of mitotic cells

Cells were treated with 50 nM paclitaxel for 16 hours, after which media was removed and cells were washed once with PBS to remove dead cells. Fresh PBS was then added to the flask and mitotic cells were detached using mechanical shaking. The detachment of cells was assessed by viewing the cells under the AE2000 Inverted microscope (Motic), and shaking continued until the majority of the mitotic cells had detached. Spontaneous mitotic cells were obtained using the same protocol, but cells were not treated with paclitaxel beforehand and therefore a minimum of ten T175 flasks were used to obtain the spontaneous mitotic cells. Remaining interphase cells were collected by trypsinisation as previously described. Confirmation of the successful isolation of mitotic cells was assessed by western blotting for anti-phosphohistone H3 S10.

2.4. Cell lysis and protein quantification

Cells were lysed in 100 µl lysis buffer (20 mM HEPES, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 10% glycerol, 10 mM sodium fluoride, 1 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM EDTA and 1 mM EGTA diluted in deionised water, pH 7.5) with 10 µl 1X protease inhibitor (Sigma-Aldrich) and 10 µl 1X phosphatase inhibitor cocktail (ThermoFisher Scientific) freshly added and placed on a rotator for 30 minutes at 4°C. After centrifugation at 13,500 rpm for 15 minutes, supernatants were collected and protein concentrations were determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). This is a detergent-compatible colorimetric assay that combines the biuret reaction (reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium) and detection of the cuprous cation using bicinchoninic acid (BCA). The chelation of two molecules of BCA with one cuprous ion exhibits a strong absorbance at 562 nm. In brief, nine BSA standards ranging from 25-2000 µg/ml were prepared using bovine serum albumin (BSA) and deionised water. Samples were diluted 1:10 with deionised water to ensure the sample concentrations fell within the standard curve and 10 µl of each were added to a standard clear 96-well plate (Corning). BCA working reagent was prepared by mixing 50 parts reagent A with 1 part reagent B and adding 200 µl to each well. The plate was incubated at 37°C for 30 minutes before reading the absorbance at 562 nm on the ELx800 absorbance microplate reader (BioTek).

2.5. SDS PAGE and western blotting

Samples were prepared for SDS-PAGE by mixing 30 µg of protein with 1X NuPage LDS Sample Buffer (Thermo Fisher Scientific) and deionised water before heating at 95°C for 10 minutes to ensure denaturation of the proteins. Heated samples were centrifuged at 1500 rpm (365 xg) for 1 minute. For analysis of proteins larger than 160 kDa, 1X NuPAGE Sample Reducing Agent (Thermo Fisher Scientific) was added to the samples prior to boiling. Samples were then subjected to SDS-PAGE using 1.5 mm NuPage 4-12% Bis-Tris precast polyacrylamide gels (Thermo Fisher Scientific) with 1X NuPage MOPs Running Buffer diluted in deionised water (Thermo Fisher Scientific). Alternatively, 1.5 mm NuPage 3-8% Tris-Acetate precast polyacrylamide gels (Thermo Fisher Scientific) with 1X NuPage Tris-Acetate Running Buffer diluted in deionised water (Thermo Fisher Scientific) supplemented with 500 µl NuPage Antioxidant in the inner buffer chamber were used for proteins over 160 kDA. The NuPage Antioxidant contains dithiothreitol, which prevents sample reoxidisation and maintains the proteins in a reduced state.

Proteins were transferred to a nitrocellulose membrane using the iBlot dry gel transfer device (Thermo Fisher Scientific). SeeBlue Plus2 Pre-stained protein standard (Thermo Fisher Scientific) was loaded into the first lane of each gel cassette followed by the samples, and gels were run at 160 V for approximately 50 minutes. Membranes were incubated in blocking buffer (5% skimmed milk powder (Sigma-Aldrich) diluted in TBST (1X Tris-buffered saline and 0.1% Tween-20)) at room temperature and placed on a rocker for 1 hour. Primary antibodies were diluted in blocking buffer and incubated with the membrane overnight at 4°C on a rocker (Table 2.1). The next day, membranes were washed three times for 15 minutes in TBST before incubating with either horseradish peroxidase (HRP)-conjugated secondary antibodies or LI-COR Odyssey secondary antibodies for 1 hour at room temperature. Membranes were washed three times for 15 minutes in TBST and visualised using Pierce enhanced chemiluminescence Western Blotting Substrate (ThermoFisher) for HRP-conjugated secondary antibodies or the Odyssey Imaging System CLx (LI-COR) for fluorescent secondary antibodies.

Antibody	Supplier	Dilution
GAPDH	Millipore	1:2500
pan-AKT	Abcam	1:1000
pan-AKT	Cell Signaling Technology	1:1000
pan-phospho-AKT S473	Cell Signaling Technology	1:1000
pan-phospho-AKT T308	Cell Signaling Technology	1:1000
phospho-histone H3 S10	Cell Signaling Technology	1:1000
p70S6K	Cell Signaling Technology	1:1000
phospho-p70S6K T389	Cell Signaling Technology	1:1000
GSK3b	Cell Signaling Technology	1:1000
phospho-GSK3β S9	Cell Signaling Technology	1:1000
phospho-PRAS40 T246	Cell Signaling Technology	1:1000
FOXO3A	Cell Signaling Technology	1:1000
FOXO1	Cell Signaling Technology	1:1000
mTOR	Cell Signaling Technology	1:1000
RICTOR	Cell Signaling Technology	1:1000
AMPK	Cell Signaling Technology	1:1000
phospho-AMPK T172	Cell Signaling Technology	1:1000
ACC	Cell Signaling Technology	1:1000
phospho-ACC S79	Cell Signaling Technology	1:1000
AS160	Cell Signaling Technology	1:1000
phospho-AS160 T642	Cell Signaling Technology	1:1000
PTEN	Cell Signaling Technology	1:1000
PFKFB4	Abcam	1:250
PFKFB3	Proteintech	1:1000
V5	Abcam	1:1000
Thiophosphate Ester	Abcam	1:1000
Goat anti-rabbit HRP-conjugated secondary antibody	Active Motif	1:5000
Goat anti-mouse HRP-conjugated secondary antibody	Active Motif	1:25000
Goat anti-rabbit LI-COR Odyssey secondary antibody 800CW	LI-COR	1:5000
Goat anti-mouse LI-COR Odyssey secondary antibody 680RD	LI-COR	1:10000
Alexa Fluor 488 donkey anti-rabbit	Thermo Fisher Scientific	1:1000
Alexa Fluor 568 donkey anti-mouse	Thermo Fisher Scientific	1:1000

Table 2.1. Antibody suppliers and dilution factors.

2.6. RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) including the DNase-1 treatment according to the manufacturer's protocol and quantified using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc). The A_{260}/A_{280} ratio was used to assess RNA quality and was only used for RT-PCR if the value was higher than 1.8. 1 µg of RNA was reverse transcribed using TaqMan® Reverse Transcription Kit (Applied Biosystems) (Table 2.2 for reagents and Table 2.3 for thermal cycler conditions).

RT-PCR reaction mix		
Component	**Volume added to each 100µl reaction (µl)**	**Final Concentration**
10X RT Buffer	10	1X
25 mM $MgCl_2$	22	1.75 mM
10 mM dNTP mix	20	0.5 mM each
RNase Inhibitor	2	1 U/µL
MultiScribe RT (50 U/µl)	2.5	2.5 U/µL
50 µM Oligo $d(T)_{16}$	5	2.5 µM
Template RNA diluted in DEPC-treated water	38.5	1 µg

Table 2.2. Reverse transcription PCR reagents and concentrations.

Thermal cycler conditions for RT-PCR		
Step	**Temperature (°C)**	**Time (minutes)**
Incubation to maximise RNA-template binding	25	10
Reverse Transcription	48	30
Reverse Transcriptase inactivation	95	5
Cool for storage	4	indefinitely

Table 2.3. Thermo cycler conditions for reverse transcription PCR.

Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems) in a 20 µl reaction using a MicroAmp Fast Optical 96-Well Reaction Plate according to the manufacturer's protocol on the 7500 Fast Real-Time PCR System (Applied Biosystems) (Table 2.4 and 2.5). Primer sequences are listed in Table 2.6. The specificity of the PCR products was confirmed by melting curve analysis. cDNA amplification was normalised against *GAPDH* and *ACTB.* Quantification of cDNA relative to the control sample were calculated as previously described (Livak and Schmittgen, 2001).

Quantitative PCR reaction mix		
Component	**Volume added to each 20µl reaction (µl)**	**Final Conc**
FAST SYBR-Green Master Mix (2X)	10	1X
Forward and Reverse primers (2 µM)	1	100 nM
cDNA template	2	1-100 ng
Nuclease-free water	7	-

Table 2.4. Reagents and concentrations for quantitative PCR.

Thermal cycler conditions for 7500 Fast quantitative PCR			
Step	**Temperature (°C)**	**Time (seconds)**	**Cycles**
AmpliTaq Gold DNA Polymerase activation	95	20	HOLD
Denature	95	3	40
Anneal/Extend	60	30	
Final Extension	72	10	1
Cool for storage	4	Indefinitely	Hold

Table 2.5. Thermo cycler conditions for quantitative PCR.

Primers sequences for quantitative PCR			
Gene	**Forward Sequence**	**Reverse Sequence**	**PrimerBank ID (if applicable)**
AKT 1A	5'- AGCGACGTGGCTATTGTGAAG -3'	5'- GCCATCATTCTTGAGGAGGAAGT -3'	62241012c1
AKT 1B	5'- GCTGAGATTGTGTCAGCCCT -3'	5'- TAATGTGCCCGTCCTTGTCC -3'	
AKT 2A	5'- ACCACAGTCATCGAGAGGACC -3'	5'- GGAGCCACACTTGTAGTCCA -3'	339895854c1
AKT 2B	5'- AGCATTTCATCCTGTGGGGG -3'	5'- CCAGAGGTGTTGCTACAGGG -3'	
AKT 3A	5'- TGTGGATTTACCTTATCCCCTCA -3'	5'- GTTTGGCTTTGGTCGTTCTGT -3'	332078467c1
AKT 3B	5'- TTTCTCCAAGTTGGGGGCTC -3'	5'- CCCCTCTTCTGAACCCAACC -3'	
PFKFB1	5'-TACCAGAGAACGACGGTCACT -3'	5'-CTGCAATTATGCCAGGGTCATTA -3'	
PFKFB1	5'-GCTATCTAGCTCTGGAGGAAAC -3'	5'-GAACAACTGGAAAAGCCTCGC -3'	
PFKFB2	5'-AGTCCTACGACTTCTTTCGGC -3'	5'-TCTCCTCAGTGAGATACGCCT -3'	
PFKFB2	5'-TGATGCCACCAATACAACCCG -3'	5'-CACAGACGGATTCCACAAAGA -3'	
PFKFB3	5'-AGCCCGGATTACAAAGACTGC -3'	5'-GGTAGCTGGCTTCATAGCAAC -3'	
PFKFB3	5'-GGCCGCATCGGGGGCGACTC-3'	5'-TTGCGTCTCAGCTCAGGGAC-3'	
PFKFB4	5'- GCCCAGTTCATCATCAGTGACCA -3'	5'- TCGTTGAGGACCTTCCACTG -3'	
PFKFB4	5'-ACAGTGCATGTACCTCTGGATG -3'	5'-TAATGCATCCAGTCCAGCAACT -3'	
XYLB	5'-TTCCTTGGCTCTTACTCCCCT-3'	5'-CAACTGAGCATGATGGTACTGG-3'	255760023c3
XYLB	5'-TGTTGATGCAGAGTTGAATGTCT-3'	5'-ACAAGGCTAGGACTTGAGAGAA-3'	255760023c1
GAPDH	5'- GCAAATTCCATGGCACCG-3'	5'- TCGCCCCACTTGATTTTGG -3'	
ACTB	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'	

Table 2.6. Primer sequences for target cDNA sequences in quantitative PCR.

Primer efficiencies were calculated using six serial dilutions of the cDNA from 20 ng/µl to 0.0098 ng/µl. The log of the DNA concentration and the Ct value for each sample were tabulated and the slope of the standard curves were entered into the Thermo Fisher Scientific qPCR efficiency calculator to compute the amplification factor and primer efficiency.

2.7. Intracellular ATP measurements

The CellTiter-Glo Luminescent Cell Viability assay (Promega) was used to measure ATP levels according to the manufacturer's protocol. This assay uses a thermostable luciferase, which catalyses the mono-oxygenation of luciferin in the presence of magnesium, ATP and molecular oxygen, producing light as a byproduct. CellTiter-Glo Buffer and CellTiter-Glo substrate were equilibrated to room temperature prior to use and the substrate was reconstituted using the buffer. After performing a mitotic shake off and pelleting the cells by centrifugation, cells were resuspended in media and counted using a haemocytometer (Camlab) and 0.4% trypan blue solution (Gibco). Cells were diluted to 200,000 cells/ml and 100 µl were plated in an opaque-walled 96 well plate (Corning) in triplicate. Media alone was added in triplicate to allow the exclusion of background luminescence. 100µl of the reconstituted substrate was added to each well in the dark and the plate was covered with foil and placed on a plate shaker for 2 minutes prior to incubation at room temperature for 10 minutes. Luminescence was read using a FLUOstar OPTIMA microplate reader (BMG LABTECH) at a sensitivity of 190. Triplicate values were averaged and the culture media background was subtracted from the average of each experimental sample.

2.8. Glucose 6-phosphate measurements

The Glucose 6-phosphate Assay Kit was purchased from Abcam and performed following the manufacturer's protocol. This is a colourimetric assay that oxidises glucose 6-phosphate to produce a product that converts a colourless probe to an intensely coloured product, with an absorbance at 450 nm. The glucose 6-phosphate substrate mix was dissolved in 220 µl glucose 6-phosphate assay buffer, while the glucose 6-phosphate enzyme mix was dissolved in 220 µl deionised water. Cells were homogenised in ice-cold PBS using sonication and 50 µl cells were added to a 96-well plate with 50 µl reaction mix (46 µl assay buffer, 2 µl enzyme mix, 2 µl substrate mix). To measure the background, 50 µl of the background mix (48 µl assay buffer, 2 µl substrate mix) were added to 50 µl PBS. Cells grown in glucose-free DMEM (Thermo Fisher Scientific) for 24 hours were used as a negative control. Plates were incubated at room temperature for 30 minutes in the dark prior to measuring the absorbance at 450 nm.

2.9. Glutathione measurements

Glutathione levels were measured using GSH-Glo Glutathione Assay (Promega) according to the manufacturer's instructions. This assay utilises the conversion of a luciferin-derivative into luciferin, catalysed by glutathione S-transferase in the presence of glutathione. All reagents were equilibrated to room temperature and kept in the dark prior to use. Cells were resuspended in PBS and counted as previously described and diluted to 100,000 cells/ml. 50 µl cell suspension was placed in a opaque-walled 96 well plate (Corning) in triplicate. 50 µl of freshly prepared GSH-Glo Reagent 2X Mix (10 µl luciferin-NT substrate, 10 µl glutathione S-transferase with 480 µl GSH-Glo reagent) were added to each well in the dark and placed on a plate shaker for 30 seconds prior to incubation at room temperature in the dark. After 30 minutes, 100 µl/well of reconstituted Luciferin Detection Reagent was added and placed on a plate shaker for 30 seconds prior to incubation for 15 minutes. Luminescence was then read using the FLUOstar OPTIMA microplate reader (BMG LABTECH). Triplicate values were averaged and the culture media background was subtracted from the average of each experimental sample.

2.10. Caspase-3/7 activity measurements

The Caspase-Glo 3/7 Assay (Promega) was performed following the manufacturer's protocol. This assay uses a luminogenic caspase-3/7 substrate that contains the tetrapeptide sequence, DEVD. Upon cell lysis, caspase-3 and -7 can cleave the DEVD-containing substrate releasing a substrate for luciferase that then results in the luciferase reaction and the production of light. Caspase-Glo 3/7 Buffer and Caspase-Glo 3/7 Substrate were equilibrated to room temperature prior to use. Caspase-Glo 3/7 Substrate was reconstituted by transferring the entire Caspase-Glo 3/7 Buffer into the bottle. Cell pellets were resuspended in media and counted as previously described. Cells were diluted to 100,000 cells/ml and 50 µl of cell suspension were plated in an opaque-walled 96 well plate (Corning) in triplicate. Media alone was added in triplicate to allow the exclusion of background luminescence and Staurosporine (Cell Signaling Technology) treatment was used as a positive control (0.3 µM for 16 hours). 50 µl of the reconstituted substrate was added to each well in the dark and the plate was covered in foil and placed on a plate shaker for 30 seconds prior to

incubation at room temperature for 1 hour. The plate was read using the FLUOstar OPTIMA microplate reader (BMG LABTECH). Triplicates were averaged and the culture media background readings were subtracted from the average of each experimental sample. For the measurement of caspase-3/7 activity following NAC treatment, values were normalised to cell viability.

2.11. Cell Viability measurements

The CellTiter-Blue Assay (Promega) was used to measure the number of viable cells present. This assay relies on the ability of viable cells to reduce the indicator dye, resazurin, into resorufin, which is highly fluorescent at 590 nm. Cells were counted as previously described and diluted to 100,000 cells/ml. 100 µl of cell suspension were added in triplicate to each well of a black-walled 96-well plate (Corning). CellTiter-Blue Reagent was equilibrated to room temperature and 25 µl was added to each cell sample and incubated at 37°C for 2 hours. The plate was shaken prior to reading the fluorescence at 560/590 nm.

2.12. Transfections

Transient depletion experiments with siRNA duplexes purchased from GE Healthcare were conducted using a reverse transfection method with siRNA at a final concentration of 40 nM. 4 µl siRNA specific to the gene of interest, non-targeting siRNA and DharmaFECT4 transfection reagent (Dharmacon, GE Healthcare) were diluted separately in 200 µl Opti-MEM for 5 minutes according to the manufacturers protocol. 200 µl of the DharmaFECT 4-Opti-MEM complex was combined with 200 µl siRNA-Opti-MEM complex and incubated for 20 minutes prior to aliquoting the full 400 µl Dharmafect 4-siRNA complex into each well of a 6-well plate (Corning). Cells were resuspended in media, counted as previously described and diluted to 70,000 cells/ml for SKOV3 or OC 316, or 110,000 cells/ml for A2780. 1.6 ml of the diluted cells were added on top of the DharmaFECT 4-siRNA complex and plates were left for 72 hours at 37°C and 5% CO_2 to achieve efficient protein depletion. For T25 flasks, 6 µl siRNA or DharmaFECT4 were diluted in 300 µl Opti-MEM and 2.4 ml cells were added on top of 600 µl DharmaFECT 4-siRNA complex.

Silencer select and stealth siRNA duplexes (Thermo Fisher Scientific) were used with Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. In brief, 3 µl siRNA was combined with 500 µl Opti-MEM and 5 µl Lipofectamine RNAiMAX and incubated for 15 minutes at room temperature. 500 µl RNAi duplex-Lipofectamine were added to each well prior to the addition of 2.5 ml cell suspension, which had been diluted to 60,000 cells/ml in antibiotic-free media. siRNA was used at a final concentration of 20 nM as this was found to give the same degree of protein depletion as 40 nM. Cells were transfected for 72 hours at 37°C and 5% CO_2. All siRNA duplex sequences are shown in Table 2.7.

Target mRNA	siRNA	Catalog number	Purchased from
PFKFB4	GAGCGACCAUCUUUAAUUU	MU-006764-01-0002 D-006764-1, siGENOME	Dharmacon, GE Healthcare
PFKFB4	GAAAUGACCUACGAGGAAA	MU-006764-01-0002 D-006764-2, siGENOME	Dharmacon, GE Healthcare
PFKFB4	CAUCGUAUAUUACCUCAUG	MU-006764-01-0002 D-006764-4, siGENOME	Dharmacon, GE Healthcare
PFKFB4	GGGACAGGCCUCAGAACGU	MU-006764-01-0002 D-006764-17, siGENOME	Dharmacon, GE Healthcare
PFKFB3	GCCGCAUCGACUACCUGAUGAA	HSS107860 1299001, Stealth	ThermoFisher Scientific
Non-targeting	N/A	D-001210-05-05	Dharmacon, GE Healthcare
Non-targeting	N/A	4390843	ThermoFisher Scientific
PFKFB4	GUAUAUUACCUCAUGAACATT	s10361, silencer select	ThermoFisher Scientific
XYLB	GAAGCCAUCUUGAAAGUGA	MU-006793-01-0002 D-006793-1, siGENOME	Dharmacon, GE Healthcare
XYLB	AGCAAGAGGUUAAAGAUAA	MU-006793-01-0002 D-006793-2, siGENOME	Dharmacon, GE Healthcare
XYLB	GGACCAAAGGCAUCACUUC	MU-006793-01-0002 D-006793-3, siGENOME	Dharmacon, GE Healthcare
XYLB	UGAAAUUAUUGGACGUCAU	MU-006793-01-0002 D-006793-17, siGENOME	Dharmacon, GE Healthcare

Table 2.7. Sequences of siRNA duplexes

2.13. Site-directed mutagenesis

To enable the investigation of constitutive AKT activity in Chapter 3, an AKT1 ORFEXPRESS-Shuttle Clone was obtained from GeneCopoeia (A0022). Site-directed mutagenesis was performed to remove the stop codon from the entry vector as this would enable it to be contiguous with the expression of a V5 peptide once it is subcloned into a pLX302 expression plasmid (Addgene). Primer sequences were designed using Quikchange Primer Design (Agilent

Technologies) (Table 2.8) and reactions were performed using the Quikchange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's guidelines. In brief, 50 µl PCR reactions were set up containing 50 ng of template DNA along with the reaction mix (Table 2.9) and run using the thermo cycler conditions listed in Table 2.10 in the first instance, or the conditions outlined in Table 2.11 if the PCR was unsuccessful. After completion of the PCR, 40 µl of the PCR reaction was digested with Dpn1 (New England BioLabs) for 1 hour at 37°C to remove any template DNA that had not been mutagenised.

Site-directed mutagenesis was also performed to introduce specific nucleotide changes in the AKT1 entry vector to generate AKT-T308D/S473D.

Primer sequences for site directed mutagenesis		
Mutant	**Forward primer**	**Reverse primer**
AKT1 stop codon removal	5'-AGCAGCACGGCCCTCGAGTGCGGC-3'	5'-GCCGCACTCGAGGGCCGTGCTGCT-3'
AKT1-S473D	5'-TTCCCCCAGTTCGACTACTCGGCCAGCA-3'	5'-TGCTGGCCGAGTAGTCGAACTGGGGGAA-3'
AKT1-T308D	5'-TGCCACCATGAAGGACTTTTGCGGCACA-3'	5'-TGTGCCGCAAAAGTCCTTCATGGTGGCA-3'
PFKFB4 siRNA resistant	5'-ACAAAATGTCGACATCTCAAGACCTCCAGAG-3'	5'-AGATGTCGACATTTTGTGGGCGATCGCGGTGCGTGTTCACAGCAGCCAC-3'

Table 2.8. Primer sequences for site-directed mutagenesis of AKT1.

SDM PCR reaction mix	
Reagent	**Volume added to each reaction (µl)**
Nuclease-free water	27
5x Phusion HF Buffer	10
10mM dNTPs	1
10µM Forward Primer	2.5
10µM Reverse Primer	2.5
10ng/µl Template DNA	5
DMSO	1.5
Phusion DNA Polymerase	0.5

Table 2.9. Reagents for site-directed mutagenesis PCR.

Thermo cycler conditions for 3-step SDM PCR			
Step	**Cycles**	**Temperature (°C)**	**Time**
Initial denaturation	1	98	1 minute
Denaturation Annealing Extension	30	98 63-72 depending on Tm of primers used 72	10 sec 30 sec 2.5 mins (30 s/kb)
Final extension	1	72	5 min
Hold		4	Indefinitely

Table 2.10. Thermo cycler conditions for 3-step site-directed mutagenesis PCR.

Thermo cycler conditions for 2-step SDM PCR			
Step	**Cycles**	**Temperature (°C)**	**Time**
Initial denaturation	1	98	1 minute
Denaturation Extension	25	98 72	10 sec 2.5 mins (30 s/kb)
Final extension	1	72	5 min
Hold		4	Indefinitely

Table 2.11. Thermo cycler conditions for 2-step site-directed mutagenesis PCR.

To produce a PFKFB4 coding sequence that was resistant to siRNA 17, two oligonucleotides flanking the target site of the siRNA 17 were designed to inversely amplify the whole plasmid and replace the siRNA 17 target site with synonymous codons that would make the new coding sequence resistant to siRNA 17. A unique Sal1 restriction site was near to the siRNA 17 target sequence of PFKFB4, which enabled for recircularisation of the PCR product using SalI restriction enzyme and T4 DNA ligase. The inverse PCR was performed using 1% BSA and the conditions outlined in Table 2.12. The presence of the PCR product was confirmed using a 0.7% agarose gel (0.7g agarose (Sigma) in 100 ml 1X TAE buffer). The product was treated with Dpn1 (New England BioLabs) with 1X CutSmart buffer (New England BioLabs) for 1 hour at 37°C to degrade the parental plasmid and the product was gel purified using 0.7% agarose gel. 5 µl of gel purified PCR product was then digested with 1 µl of high fidelity Sal1 restriction enzyme (New England BioLabs) in 1X CutSmart buffer (New England BioLabs) in a total volume of 10 µl and incubation at 37°C for 1 hour to produce sticky ends at both ends of the linear PCR product. The Sal1 enzyme was heat inactivated at 75°C for 20 minutes and the product was self ligated by adding 1 µl of 10 mM ATP (New England BioLabs) and 1 µl of T4

DNA ligase (New England BioLabs) into the reaction in a total volume of 20 µl and incubation at 20°C for 30 minutes. 3 µl of the ligation product was transformed into One Shot Stbl3 chemically competent *Escherichia coli (E.coli)* cells (Thermo Fisher Scientific) as described in the next section.

Thermo cycler conditions for SDM PCR for siRNA resistant PFKFB4			
Step	**Cycles**	**Temperature (°C)**	**Time**
Initial denaturation	1	98	2 minutes
Annealing	1	65	30 seconds
Polymerisation	1	72	12 minutes
Denaturation Annealing Extension	30	96 58 72	20 seconds 30 seconds 6 minutes
Final extension	1	72	10 minutes
Hold	1	4	Indefinitely

Table 2.12. Thermo cycler conditions for the site-directed mutagenesis PCR to produce siRNA resistant PFKFB4.

2.14. Transformation of competent *E.coli (Top10, Stbl3)*

50 µl One Shot TOP10 or One Shot Stbl3 Chemically Competent *E.coli* (Thermo Fisher Scientific) were thawed on ice for each transformation. 5 µl plasmid DNA was added to the competent cells and mixed by flicking the tube before storing on ice for 30 minutes. Cells were then placed at 42°C for 30 seconds to enable incorporation of the DNA and placed on ice for 2 minutes. 250 µl of Super Optimal broth with Catabolite repression (SOC) medium at room temperature was added to each vial and placed in a 37°C orbital shaker at 225 rpm for 2 hours. After incubation, 20 µl and 200 µl of each transformation was placed on a pre-warmed agar plate containing the appropriate 100 µg/ml antibiotic and spread using ColiRoller Plating Beads (Millipore). Plates were wrapped in Parafilm (ThermoFisher), inverted and placed in a 37°C incubator overnight. Plates were checked the next day for bacterial colonies and placed at 4°C until required.

2.15. DNA extraction from transformed *E.coli*

Transformed colonies were picked at random from the agar plates and grown at 37°C overnight in LB broth supplemented with the appropriate antibiotic in an orbital shaker at 225 rpm. The following day, bacteria were pelleted at 4000 rpm for 10 minutes. Supernatants were discarded and DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. DNA sequences were verified by the Sanger sequencing facility at the Weatherall Institute of Molecular Medicine using the sequencing primers shown in Table 2.13.

Primer sequences used to verify SDM and LR Clonase reactions		
Primer name	**Plasmid**	**Primer sequence**
CMV Forward	pLX302 and pCDH	5'-CGCAAATGGGCGGTAGGCGTG-3'
WPRE Reverse	pLX302	5'-CATAGCGTAAAAGGAGCAACA-3'
PFKFB4 si17 Forward	PFKFB4 pDONR221	5'-CGAGAACGGAGAGCGACCATC-3'
PFKFB4 si17 Reverse	PFKFB4 pDONR221	5'-CAGATGACCAGCACATTCTCTT-3'
PFKFB3 nested Forward	PFKFB3 pDONR223	5'-CCGGGAGGCTGTGAAGCAGTAC-3'
PFKFB3 nested Reverse	PFKFB3 pDONR223	5'-CCTGTGGGCCAAAGCTGACCAA-3'
M13pUC Forward	pDONR223, A0022	5'-CCCAGTCACGACGTTGTAAAACG-3'
M13 Reverse	pDONR223	5'-CAGGAAACAGCTATGAC-3'
T7	A0022	5'-TAATACGACTCACTATAGGG-3'
U19 Forward (pDONR221)	pDONR221	5'-GTTTTCCCAGTCACGACGT-3'
AttP2 Reverse (pDONR221)	pDONR221	5'-CAGGAAACAGCTATGAC-3'

Table 2.13. Primer sequences used to confirm DNA sequences

2.16. Gateway cloning

Lentiviral vectors were generated using Gateway Technology (Thermo Fisher Scientific) according to the manufacturer's instructions. This technology employs lambda phage-based site-specific recombination as an alternative to restriction enzymes and ligation to insert a gene of interest into an expression vector.

The AKT1 ORFEXPRESS Shuttle clone (A0022, GeneCopoeia, backbone 3625bp, ORF 1443), PFKFB3 pDONR223 vector (23668, Addgene, backbone 5005bp, ORF 1563) and PFKFB4 pDONR221 vector (HsCD00040599, DNASU, backbone 4762bp, ORF 1410) were subcloned into the pLX302 lentiviral destination vector (Addgene) using the Gateway LR Clonase II enzyme mix. In brief, 5 µl reactions containing 70 ng plasmid DNA, 150 ng pLX302, 1 µl LR Clonase II Enzyme Mix and Tris EDTA (TE) buffer were mixed by vortexing and incubated at 25°C for 1 hour. Reactions were incubated with 1 µl Proteinase K solution at 37°C for 10 minutes to terminate the reaction. 1 µl of each LR reaction was transformed into One Shot Stbl3 Chemically Competent *E.coli* (Thermo Fisher Scientific) to reduce the frequency of homologous recombination of unstable regions.

For the myristoylated AKT1 lentiviral expression plasmid (Jialiang Wang from Addgene, backbone 7384bp, ORF 1500bp), DNA was extracted from the glycerol stock by inoculating LB-antibiotic broth and following the protocol for DNA extraction in 2.15. DNA was then transformed into One Shot Stbl3 cells as previously described in 2.14 ready for the lentiviral transfection of packaging cells.

2.17. Lentiviral transfection and transduction

Lentiviral technology was used to generate SKOV3 cells lines stably expressing wild-type or mutant AKT1, PFKFB4 and PFKFB3 in Chapter 5. Lentiviral technology enables the efficient delivery and stable expression of expression constructs in mammalian systems that are hard to transfect, such as SKOV3. In the second generation system used in this work, the pLX302 lentiviral transfer plasmid, VSV-G packaging plasmid, and Gag-Pol envelope plasmid were all encoded on separate plasmids to increase the safety of the lentivirus.

Packaging cells (HEK293T) were plated in vented T25 flasks in antibiotic-free media for 24 hours to obtain 70% confluency. For each flask, the following were combined; 3 µg pLX302 vector, 0.3 µg pCMV VSV-G (Addgene) and 3 µg pCMV Gag/Pol (Addgene) (10:1:10 ratio) in a total volume of 225 µl Opti-MEM (Thermo Fisher Scientific). HEK293T were then transfected using the protocol from FuGENE with a FuGENE:DNA ratio of 3:1. In brief, 18.9 µl FUGENE was added to the DNA-OptiMEM mix and mixed by pipetting before incubation at room temperature for 7-8 minutes. 100 µl of the DNA-FUGENE-OptiMEM complex was added to the cells in 2 ml fresh media and left for 48 hours. After 48 hours, viral supernatants were harvested into a 15 ml falcon tube and centrifuged at 1500 rpm (365 xg) for 5 minutes to remove any cells before being passed through a 0.45 µm Minisart NML Syringe Filter (Sartorius). 2 ml DMEM media containing 30% (v/v) FBS (Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) were added to the HEK293T cells for a further 24 hours, after which this was centrifuged and filtered as described above. 15,000 SKOV3 cells were plated in T25 flasks to obtain 50% confluence the day before transduction. The following day, media was removed and the filtered viral supernatants were added to the SKOV3 cells. SKOV3 were incubated with the supernatants for 72 hours, adding the second filtered viral supernatant after the first 24 hours. After 72 hours, the media was changed to contain 50 ng/l Puromycin (Sigma-Aldrich) to select for cells that contain the integrated virus. HEK293T and SKOV3 cells were collected by trypsinisation for lysis and examination of protein expression by western blot to assess the efficiency of transfection and transduction.

2.18. Immunofluorescence, image collection and processing

Immunofluorescence was used to quantify the number of mitotic cells present after PFKFB4 depletion and paclitaxel treatment. It was also used to look at PFKFB4 and PFKFB3 expression in SKOV3 cells stably expressing PFKFB4 and PFKFB3.

Cells were grown to 80% confluence on cover slips in 12-well plates prior to fixation with 0.5 ml 4% methanol-free formaldehyde (ThermoFisher Scientific) diluted in PBS for 4 minutes at room temperature. Permeabilisation was achieved

using 0.5 ml 100% ice-cold ethanol and incubation at -20°C overnight. Ethanol was removed and 1 ml wash buffer (1X TBS, 0.2% TritonX100 and 0.04% SDS, Sigma-Aldrich) was added to the wells for 5 minutes. Cells were blocked in 1 ml blocking buffer (3% bovine serum albumin diluted in 1XTBS) for 1 hour at room temperature. Each cover slip was incubated with 60 µl primary antibody diluted in blocking buffer for 1 hour at room temperature. Three washes were performed with 1 ml wash buffer for 5 minutes each. Secondary antibodies (Alexa Fluor 488 donkey anti-rabbit or Alexa Fluor 568 donkey anti-mouse, Thermo Fisher Scientific) were diluted in blocking buffer and 60 µl was added to each cover slip for 1 hour at room temperature in the dark. Cells were washed as before, prior to mounting the coverslips with mounting medium containing DAPI (VECTASHIELD) on microscope slides (ThermoScientific) and sealed with nail varnish at the edges. Slides were stored at 4°C in the dark until analysis was required. Images were analysed on the Zeiss Observer Z1 Microscope using a 40X oil objective or a 20X objective. Images were processed using ImageJ.

2.19. Quantification of mitotic cells by flow cytometry

Cells were collected by trypsinisation and centrifuged at 1500 rpm for 5 minutes to pellet the cells. Supernatants were removed and pellets were resuspended thoroughly in 0.5 ml PBS and transferred to Falcon 5 ml Round Bottom flow cytometry tubes (BD Biosciences). 0.5 ml 8% methanol-free formaldehyde (ThermoFisher Scientific) was added to give a final concentration of 4% and incubated for 6 minutes at room temperature. Fixative was removed by centrifugation at 1500 rpm for 5 minutes and samples were thoroughly resuspended in 1 ml ice-cold 100% ethanol and placed at -20°C overnight to enable cell permeabilisation. Cells were centrifuged at 5000 rpm for 5 minutes and washed in 1 ml blocking buffer (3% bovine serum albumin in 1XTBS). Cells were collected by centrifugation as described above and resuspended in 1 ml blocking buffer for 1 hour at room temperature. Cells were pelleted again and incubated with rabbit anti-phospho-histone H3 S10 diluted in blocking buffer for 1 hour at room temperature. Cells were washed twice in 1 ml blocking buffer before incubation with the secondary antibody (Alexa Fluor 488 donkey anti-rabbit) diluted in blocking buffer for 1 hour at room temperature in the dark. Cells were pelleted by centrifugation at 5000 rpm for 5 minutes and washed in 1 ml blocking

buffer. Cells were finally resuspended in 0.5 ml 3 µM propidium iodide solution (PI) (Thermo Fisher Scientific) diluted in PBS and analysed on the CyAn ADP Analyser (Beckman Coulter). Alexa Fluor 488 and PI were excited by a laser tuned to 488 nm and emissions were detected at 525 nm and 617 nm respectively. Forward scatter (an estimation of cell size) and side scatter (proportional to cell granularity) were used to identify the cells, pulse width and forward scatter identified singlet populations, PI and forward scatter enabled the identification of the PI positive cells, while PI and Alexa Fluor 488 allowed the quantification of cells positive for phosphohistone H3 S10. Data from approximately 100,000 cells from each sample were analysed using FlowJo software.

2.20. Measurement of reactive oxygen species

Levels of reactive oxygen species (ROS) were measured using the cell permeable indicator CM-H_2DCFDA (ThermoFisher Scientific). The acetate groups of CM-H_2DCFDA are cleaved by intracellular esterases and subsequent oxidation within the cell yields a fluorescent product that is retained in the cell.

SKOV3 cells were transfected with PFKFB4 siRNA or PFKFB3 siRNA for 72 hours in T25 flasks as described in section 2.12. After 72 hours, 2 ml 50 nM paclitaxel or DMSO (Sigma-Aldrich) diluted in media were added to cells and incubated overnight at 37°C and 5% CO_2. The following day, mitotic cells were separated from interphase cells using mitotic shake off and placed in Falcon 5 ml Round Bottom flow cytometry tubes (BD Biosciences) prior to centrifugation at 1500 rpm (365 xg) for 5 minutes. CM-H_2DCFDA was freshly reconstituted in DMSO and cells were resuspended in 1 ml 3 µM CM-H_2DCFDA diluted in warm McCoy's 5A media and incubated for 30 minutes at 37°C and 5% CO_2. 1 ml 0.5 mM Hydrogen peroxide (H_2O_2) (Sigma-Aldrich) and 3 µM CM-H_2DCFDA diluted in warm McCoy's 5A media were added to cells as a positive control. After 30 minutes, cells were centrifuged at 1500 rpm (365 xg) for 5 minutes and resuspended in 0.5 ml 10 µg/ml propidium iodide (Thermo Fisher Scientific) diluted in ice-cold PBS. Propidium iodide is membrane impermanent so enabled the exclusion of dead cells. Samples were placed on ice and kept in the dark prior to processing using CyAn ADP Analyser (Beckman Coulter). Data from

approximately 50,000 cells from each sample were analysed using FlowJo software. The mean CM-H_2DCFDA intensity was measured in the PI negative singlet cells, while the PI intensity was measured in the population of singlets. Samples were also processed for western blot to detect the presence of anti-phosphohistone H3 S10 in the mitotic samples.

To assess the effect of the antioxidant N-Acetyl Cysteine (NAC) (Sigma-Aldrich), cells were treated overnight with 5 mM NAC and 50 nM paclitaxel or DMSO control after the 72 hour siRNA-mediated depletion of PFKFB4. Cells were also exposed to 5 mM NAC during the 30 minute incubation with 3 μM CM-H_2DCFDA.

2.21. Cell cycle analysis

Cell cycle analysis was performed using flow cytometry and PI staining. After transfection with PFKFB4 siRNA for 72 hours, cells were harvested into Falcon 5 ml Round Bottom flow cytometry tubes (BD Biosciences) prior to centrifugation at 1500 rpm (365 xg) for 5 minutes. Cells were resuspended in 1 ml PBS with 3% FBS and centrifuged again at 1500 rpm (365 xg) for 5 minutes. 1 ml ice-cold 70% ethanol (Sigma-Aldrich) was added drop-wise to each sample prior to vortexing and centrifugation at 3000 rpm for 4 minutes. Cells were washed in 1 ml PBS with 3% FBS and pelleted again by centrifugation at 3000 rpm for 4 minutes. 0.5 ml 100 μg/ml RNase A (ThermoFisher Scientific) and 40 μg/ml PI diluted in PBS with 3% FBS was added to each sample and incubated at 37°C and 5% CO_2 for 40 minutes. Samples were collected by centrifugation at 3000 rpm for 4 minutes and resuspended in 1 ml PBS with 3% FBS for analysis on CyAn ADP Analyser (Beckman Coulter). Cells were kept covered until analysis. PI was analysed on a linear scale with Area vs Peak to gate out doublets or clumps, and analysed at a low flow rate (<400 events/second). Data from approximately 30,000 cells from each sample were analysed using FlowJo software.

2.22. Colony forming assays

A2780 and SKOV3 cells were transfected with either non-targeting or PFKFB4 siRNA in 12-well plates. After 72 hours, transfected cells were harvested, counted and replated in to 6-well plates at densities of 300 cells/well (SKOV3) and 600 cells/well (A2780). Three replicates were prepared. Colony formation was

observed for the following 9-12 days. When adequate colonies had formed, culture medium was removed and cells were fixed with 0.5 ml 1% gluteraldehyde (Sigma-Aldrich) for 10 minutes at room temperature and stained with 2 ml 0.5% crystal violet diluted in deionised water (Sigma-Aldrich) overnight before washing plates five times with water and leaving plates to dry overnight.

2.23. *In vitro* kinase assay

On ice, 2.5 µl of 10 mM ATPγS (TOCRIS) was added to 47.5 µl Kinase Buffer I (Stratech Scientific) (25 mM MOPS, pH 7.2, 12.5 mM glycerol phosphate, 25 mM magnesium chloride, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT) to make the 100 µM ATPγS cocktail. 25 µl reactions were made up with 1 µl phosphatase inhibitor cocktail 3 (Sigma-Aldrich), 0.5 µg recombinant full-length human PFKFB3 or PFKFB4 (Signalchem), 0.3 µg active human AKT1 (Active Motif), 5 µl ATPγS cocktail and Kinase Buffer I up to 25 µl. Reactions were incubated at 30°C for 20 minutes before the reaction was terminated at 95°C for 5 minutes. 1.25 µl 50 mM p-nitrobenzyl mesylate (PNBM) (Abcam) was added to each reaction for 1 hour at room temperature to allow alkylation and the production of thioesters. 5 µl of 4X LDS sample buffer (Thermo Fisher Scientific) was added to each sample and boiled for 8 minutes before loading onto a 4-12% Bis-Tris 10-well gel (Thermo Fisher Scientific) and processed as described in section 2.5 for anti-thiophosphate ester western blotting.

2.24. Time-lapse Imaging

Transfections with siRNA were set up as previously described, but scaled down for use of a 12-well plate, meaning 800 µl cell suspension at the densities stated were combined with 200 µl DharmaFECT 4-siRNA complex per well. After transfection for 72 hours, cells were treated with 50 nM paclitaxel or DMSO control and visualised by time-lapse microscopy using a 20X lens on the Leica DMIRE2 Hamamatsu ORCA-ER using HCI Image acquisition software while keeping the cells at 37°C and 5% CO_2. Five fields of view in each well were imaged every 10 minutes for a minimum of 16 hours. Time-lapse images for each field of view were analysed and the fate of each mitotic cell was determined. Cell death was characterised by blebbing and dense cellular irregularities. Cells that

left the field of view during the course of the experiment were not included in the analysis.

2.25. High-throughput quantification of V5 expression

The IN Cell Analyser 1000 Instrument was used to quantify the number of V5-positive cells present in the siRNA 17-resistant PFKFB4 cell line, PFKFB4 cell line and empty vector cells after transfection with PFKFB4 siRNA 17. Transfections with PFKFB4 siRNA or non-targeting siRNA were set up in 96-well plates using the empty vector, PFKFB4 OE and PFKFB4 si17 cell lines. After transfection for 72 hours, cells were treated with 50 nM paclitaxel or DMSO overnight before fixing and staining cells for analysis using the IN Cell Analyser 1000. In brief, culture medium was removed and cells were fixed with 50 µl 4% methanol-free formaldehyde (ThermoFisher Scientific) for 5 minutes. Fixative was removed and cells were permeabilised with 50 µl ice-cold absolute ethanol per well and left overnight at -20°C. Ethanol was removed and 50 µl wash buffer (1X TBS, 0.2% Triton X100 and 0.04% SDS, Sigma-Aldrich) was added to the wells for 10 minutes. Cells were blocked in 20 µl blocking buffer (3% bovine serum albumin diluted in 1XTBS) for 1 hour at room temperature. Cells were then incubated with 20 µl primary antibodies (rabbit anti-phospho histone H3 S10 and mouse anti-V5) diluted in blocking buffer for 1 hour at room temperature. Three washes were performed with 50 µl wash buffer for 5 minutes each. Secondary antibodies (Alexa Fluor 488 donkey anti-rabbit or Alexa Fluor 568 donkey anti-mouse) were diluted in blocking buffer and 20 µl was added to each well for 1 hour at room temperature in the dark. Cells were washed three times as before and incubated with 20 µl 10 µg/ml Hoechst 33342 trihydrochloride, trihydrate (Thermo Fisher Scientific) diluted in PBS for 10 minutes at room temperature. The Hoechst stain was removed and 50 µl PBS was added to each well prior to processing using the IN Cell Analyser 1000 Imaging and Analysis system. Over 300 images were captured for each condition and data were analysed using R programming language and software. The V5 nuclear intensity and V5 cell intensity from the untreated cells were used to set the threshold for V5 positive and negative cells.

2.26. High-content siRNA kinome screen

SKOV3 cells were plated in 384-well format and treated with Dharmacon siRNA kinome library targeting 779 different genes for 48 hours. Cells were treated with 10 nM paclitaxel or vehicle control for 24 hours prior to fixation with 4% paraformaldehyde for 5 minutes. Cells were permeabilised with ice-cold absolute ethanol and left overnight at -20°C prior to adding wash buffer (Tris-buffered saline, 0.2% Triton X-100 and 0.04% SDS) for 10 minutes. Cells were blocked in blocking buffer (1.5% bovine serum albumin diluted in Tris-buffered saline) for 1 hour at room temperature. Cells were incubated with anti-phospho-histone H3 S10 antibody diluted in blocking buffer for 1 hour at room temperature. Three washes were performed with wash buffer for 5 minutes each and Alexa Fluor 488 donkey anti-rabbit was diluted in blocking buffer and added to each well for 1 hour at room temperature in the dark. Cells were washed three times as before and incubated with 10 µg/ml Hoechst 33342 (Thermo Fisher Scientific) diluted in PBS for 10 minutes at room temperature. The Hoechst stain was removed and PBS was added to each well prior to image acquisition using the In Cell Analyzer 1000 (GE Healthcare) as previously described (Ahmed et al., 2011). Data analysis was carried out using R programming language and software. Cells were assigned to specific wells and the mean pixel intensity values were calculated for Hoechst and phospho-histone H3 S10 for each cell and background values were subtracted. The percentage of mitotic cells were calculated for each well by dividing the number of cells staining positive for phospho-histone H3 S10 by the total number of cells that stained positive for Hoechst. The median of triplicate values was obtained for each condition.

2.27. Statistical methods

Graphs were generated using Graphpad Prism 6 and depending on the data, either a two-tailed student's *t*-test or a two-way analysis of variance (ANOVA) with multiple comparisons (Tukey or Dunnett) were performed. * $p \leq 0.05–0.01$, ** $p < 0.01–0.001$, *** $p < 0.001$, **** $p < 0.0001$.

Chapter 3

Investigating AKT activity during mitosis in ovarian cancer cells

3. Investigating AKT activity during mitosis in ovarian cancer cells

3.1. Introduction

There has been a surge of interest in identifying new therapeutic targets that can be used to complement current chemotherapies, or alongside other targeted therapies. The PI3K/AKT/mTOR cascade is one pathway currently under investigation to determine whether its inhibition has promise in ovarian cancer treatment (Cheaib et al., 2015; Mabuchi et al., 2015). However, to determine whether such inhibitors could be used successfully in conjunction with chemotherapy in ovarian cancer treatment, it is important to understand the activity of the PI3K/AKT pathway in ovarian cancer cells, especially after exposure to chemotherapy agents, such as paclitaxel.

3.1.1. Activity of the PI3K/AKT pathway during mitosis

As I have mentioned in the introduction (Chapter 1.3), the PI3K/AKT pathway is important in cell proliferation, with its activity known to stimulate cyclin D1 expression and promote G1 to S phase progression in interphase cells (Gille and Downward, 1999; Gao et al., 2004). During the vast cellular rearrangements that take place during mitosis, such as nuclear envelope breakdown, one would expect the PI3K/AKT pathway to be tightly regulated given its large number of downstream substrates. Previous work suggests that the PI3K/AKT pathway is required for G2/M phase transition and mitotic progression (Kandel et al., 2002; Okumura et al., 2002; Shtivelman et al., 2002; Dangi et al., 2003; Silio et al., 2012). PI3K/AKT activity has been reported to promote mitotic entry through its downstream activation of CDK1 (Roberts et al., 2002) via the phosphorylation and inhibition of WEE1 (Katayama et al., 2005) and MYT1 (Okumura et al., 2002), and by its prevention of PLK1 degradation (Shtivelman, 2003). Moreover, a recent study found that AKT phosphorylation at S473 mirrors cyclin A2 expression throughout the cell cycle (Liu et al., 2014a) and attributed this to the direct phosphorylation and activation of AKT by cyclin A-CDK2. Given that cyclin A-CDK2 activity is important for mitotic entry (Hein and Nilsson, 2016), this again indicates that AKT is active during the G2/M phase transition. Additionally, AKT-mediated phosphorylation and inactivation of GSK3β during mitosis is thought to

be important for mitotic spindle dynamics, implicating AKT activity in mitotic progression (Wakefield et al., 2003). Furthermore, the inhibition of AKT activity can induce mitotic arrest through the downregulation of Aurora A expression (Liu et al., 2008), which is important for mitotic spindle formation and progression through mitosis (Liu and Ruderman, 2006). These findings demonstrate that PI3K/AKT activity is important for mitotic entry as well as mitotic progression, which is not surprising given the global phosphorylation that takes place during the early stages of mitosis.

Whilst there is much evidence to suggest that AKT activity is important for mitotic entry and progression, two studies demonstrate that PI3K/AKT signalling or AKT itself become inactivated during late mitosis (Chaussepied and Ginsberg, 2004; Liu et al., 2014a). These observations suggest that the inactivation of AKT may be required for mitotic exit, which is in line with the widespread dephosphorylation that occurs in the late stages of mitosis. There are several important factors to consider when looking at these studies; a) the cell model used, b) the technique employed to obtain the mitotic population, and c) whether the population is distinct from interphase cells or simply enriched for mitotic cells. For example, the majority of studies have used double thymidine block in HeLa or NIH 3T3 cells (Table 3.1), which are unlikely to be representative of spontaneous mitosis, or of other cell lines. To my knowledge, no studies have analysed spontaneous mitosis without preceding treatment, and separated the mitotic population from the interphase cells. Moreover, no investigations have been carried out into the activity of the PI3K/AKT during mitosis in human ovarian cancer cells.

Reference	Cell line	Method to achieve mitotic population
Okumura et al., 2002	Starfish oocytes	1-MeAde to activate cyclin B-CDK1 for mitotic arrest
Shtivelman, 2003	Cervical cancer cells; HeLa Mouse fibroblast cells; NIH 3T3 Kidney epithelial cells; MDCK	Double thymidine block
Dangi et al., 2003	Cervical cancer cells; HeLa	Double thymidine block
Roberts et al., 2002	Cervical cancer cells; HeLa Mouse fibroblast cells; NIH 3T3	Double thymidine block
Chaussepied and Ginsberg, 2004	Osteosarcoma cells; U2OS Chinese hamster ovary cells; CHO	Double thymidine block
Wakefield et al., 2003	Cervical cancer cells; HeLa	Double thymidine block or nocodazole
Liu et al., 2014a	Cervical cancer cells; HeLa Osteosarcoma cells; U2OS Glioblastoma cells; T98G	T98G and U20S with double thymidine block HeLa with nocodazole

Table 3.1. Studies investigating the phosphorylation status of AKT in cell lines synchronised in mitosis.

3.1.2. Activity of the PI3K/AKT pathway during paclitaxel-induced mitotic arrest

Given the importance of AKT signalling in promoting cell survival and its apparent role in conferring paclitaxel resistance, it would be expected that paclitaxel treatment would increase PI3K/AKT activity. An increase in AKT phosphorylation has been observed in a number of cell lines treated with paclitaxel (Heliez et al., 2003; Van Schaeybroeck et al., 2006; Zhou et al., 2006; Parsons et al., 2010). However, other studies have reported no change in AKT phosphorylation (Parsons et al., 2010), a decrease in AKT phosphorylation (Le et al., 2003), or an initial increase followed by a reduction in AKT phosphorylation (Mabuchi et al., 2002; Kim et al., 2007b). The range of responses could be attributed to the use of different cell lines (Table 3.2). Investigations studying ovarian cancer cell lines have observed an initial increase in AKT phosphorylation and activity, followed by a reduction (Mabuchi et al., 2002; Kim et al., 2007b). However, there is a distinct lack of literature investigating the downstream effects of paclitaxel-mediated AKT activity in cancer cells, including ovarian cancer cells.

Reference	Cell line
Parsons et al., 2010	Pancreatic carcinoma cell lines MiaPaCa-2, Panc-1, AsPC-1
Van Schaeybroeck et al., 2006	Non-small-cell lung carcinoma cell lines H23, H157, H460, H727, H838, and H441
Zhou et al., 2006	Pancreatic cancer cell line L3.6
Heliez et al., 2003	Nasal tumour cell line RPMI-2650
Mabuchi et al., 2002	Paclitaxel-resistant ovarian cancer cell line SW626
Le et al., 2003	Breast cancer cells SKBR3
Kim et al., 2007b	Ovarian cancer cell lines SKOV3 and PA-1

Table 3.2. Studies investigating the phosphorylation status of AKT in cell lines treated with paclitaxel.

3.1.3. Metabolic changes during mitosis

Mitosis involves extensive membrane expansion and reorganisation (Ellenberg et al., 1997; Anderson et al., 2007), therefore it is of no surprise that alterations in FA metabolism take place during this phase of the cell cycle. As well as the recycling of internalised plasma membrane to the cell surface during mitosis (Boucrot and Kirchhausen, 2007), *de novo* synthesis and degradation of phospholipids has also been documented (Bosmann and Winston, 1970; Kobayashi and Pagano, 1989; Manguikian and Barbour, 2004; Lin and Arthur, 2007) and shown to be important for mitotic exit (Scaglia et al., 2014). This could be partly attributed to the activity of AMPK, which has been found to peak at the beginning of mitosis before declining in the later stages. The decline in AMPK activity results in the dephosphorylation and induction of ACC1 activity, which increases FA synthesis (Davies et al., 1990). Due to the alterations in lipid metabolism during mitosis, it is likely that lipid-mediated signalling plays an important role during this phase (Mall et al., 2012).

Protein translation and degradation are under strict temporal control throughout the cell cycle. The majority of research into mRNA translation during mitosis has largely demonstrated a global downregulation of protein synthesis (Pyronnet et al., 2001; Tanenbaum et al., 2015). However, despite this observation, some mRNA transcripts have been identified as being actively transcribed during mitosis, such as *CCNB1* (Malureanu et al., 2010), *CDK1* and *CDC20* (Liang et al., 2012). Moreover, key complexes involved in promoting cell growth and protein

synthesis are still active. Phosphorylation of the mTORC1 downstream targets, p70S6K and 4E-BP1, are increased during G2/M and an increase in p70S6K activity during mitosis has also been observed (Boyer et al., 2008). p70S6K and 4E-BP1 are both known to control protein synthesis (Brown and Schreiber, 1996).

3.1.4. Metabolic changes during paclitaxel-induced mitotic arrest

As I have previously discussed, glycolysis is a critical source of energy for many cancer cells and PFK1 is an important rate-limiting enzyme in this process (Sola-Penna et al., 2002). Paclitaxel treatment can negatively effect glycolysis in a number of ways, such as reducing glucose 1,6-bisphosphate levels, which is an allosteric activator of PFK1 (Glass-Marmor and Beitner, 1999) and results in a reduction of fructose 1,6 bisphosphate. A decrease in fructose 1,6 bisphosphate diminishes its ability to allosterically activate pyruvate kinase (Jurica et al., 1998), which can cause a decrease in ATP in paclitaxel treated cells (Untch et al., 1994; Glass-Marmor and Beitner, 1999). Moreover, paclitaxel has been shown to cause the detachment of PFK1 from the cytoskeleton in melanoma cells. Binding of glycolytic enzymes to the cytoskeleton is critical for providing local ATP production (Beitner, 1993) and cross-linking actin-containing filaments into ordered structures (Clarke and Masters, 1975). The majority of glycolytic enzymes can bind to the cytoskeleton, except for hexokinase, which binds reversibly to the mitochondria (Arnold and Pette, 1968; Gots et al., 1972). In addition, glycolytic enzymes have been reported to bind to tubulin and microtubules, as well as actin filaments (Walsh et al., 1989). Thus any changes that paclitaxel exerts on microtubule dynamics may well affect the function of glycolytic enzymes.

Paclitaxel treated lung and liver tumours from mice have also been found to possess higher levels of TCA enzyme activity when compared to untreated tumours (Senthilnathan et al., 2006). An increase in the activities of electron transport chain complex enzymes II-V were also observed after paclitaxel treatment. This suggests that paclitaxel also acts to enhance aerobic respiration and increase ATP production.

Paclitaxel can enhance the phosphorylation of p70S6K at T421 and S424 during mitotic arrest, which promotes its inactivation (Le et al., 2003). This indicates that protein synthesis could also be affected by paclitaxel treatment. The specific effects of paclitaxel on other areas of metabolism, such as glutaminolysis and fatty acid synthesis, have not yet been determined. It is also likely that the concentration and duration of paclitaxel treatment, as well as the cell line or tissue type treated, largely influences the metabolic response to paclitaxel treatment.

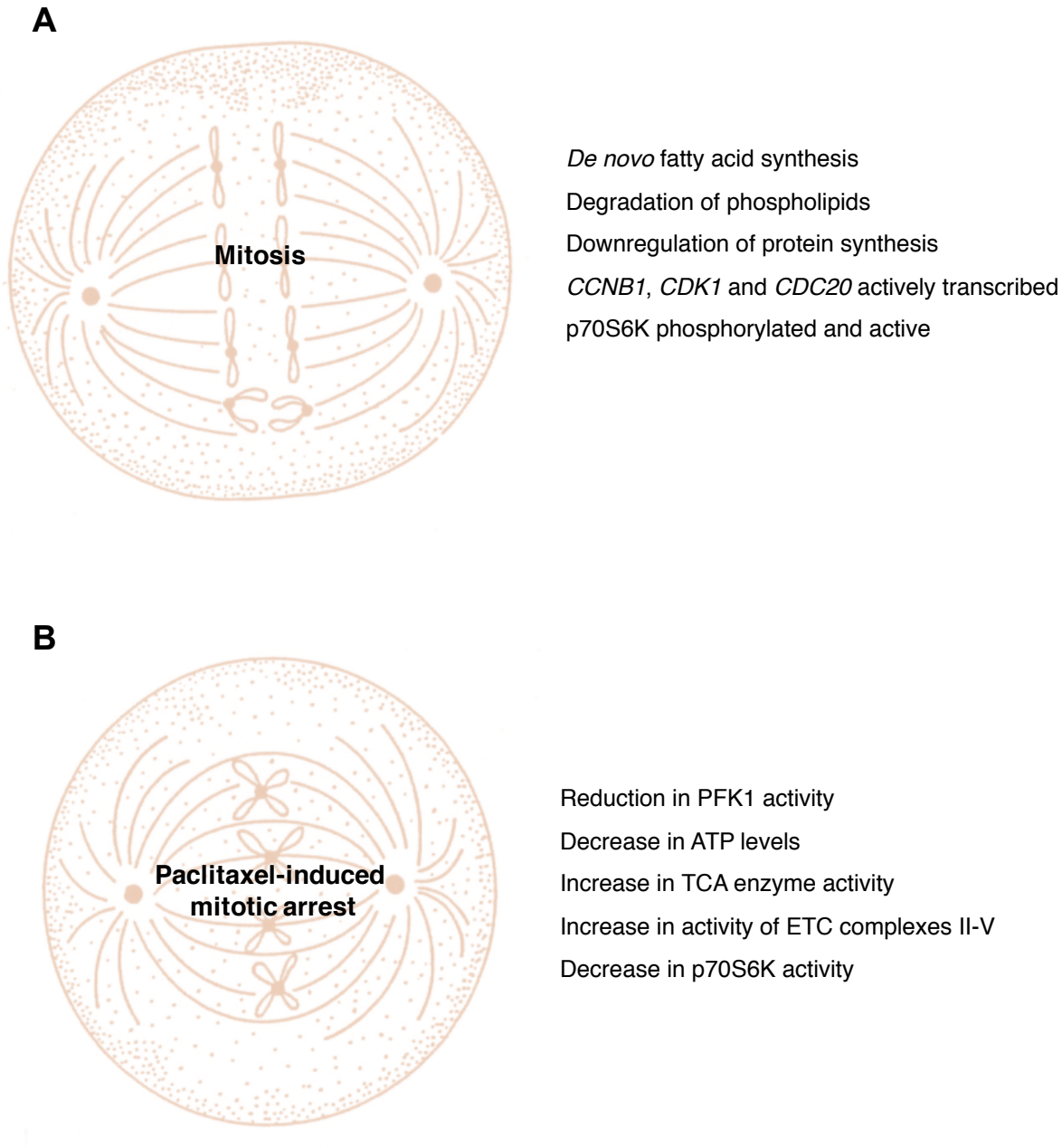


Figure 3.1. Metabolic activity during mitosis and mitotic arrest.
(A) Hypothesised cellular metabolic activities that occur during spontaneous mitosis and (B) during paclitaxel-induced mitotic arrest.

3.1.5. AMPK regulation

AMPK is an important energy sensor in the cell and when active can enhance catabolic pathways to generate ATP and inhibit biosynthetic pathways that consume ATP. AMPK activity can be stimulated by canonical means, such as metabolic stresses or drugs that induce changes in the levels of AMP, ADP (Oakhill et al., 2011; Xiao et al., 2011) and calcium (Fogarty et al., 2010), or through non-canonical pathways, such as oxidative stress or genotoxic exposure. An increase in ROS has been shown to directly stimulate AMPK (Alexander et al., 2010; Zmijewski et al., 2010) as well as being secondary to canonical pathway stimulation, such as inhibition of mitochondrial ATP synthesis (Hawley et al., 2010). Phosphorylation at T172 by LKB1-STRAD-MO25 complex is required for AMPK activation (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004; Suter et al., 2006). More recently, Ca^{2+}/calmodulin-activated protein kinase kinases (CAMKK2) has been shown to phosphorylate T172 (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005), leading to an increase in AMPK activity (Suter et al., 2006). Once activated, AMPK can enhance a number of downstream pathways including glucose uptake, PFKFB3 activity, FA oxidation and mitochondrial biogenesis (Hardie et al., 2012). AMPK activity is inhibited once energy homeostasis is restored. For example, in response to high concentrations of glucose or lipids, glycogen accumulation as well as certain amino acids (Viollet et al., 2010). Direct phosphorylation of AMPK at S485 by AKT (Hahn-Windgassen et al., 2005; Horman et al., 2006), PKA (Hurley et al., 2006) and GSK3β (Suzuki et al., 2013), as well as the dephosphorylation of T172 by PP-2A and PP-2C (Davies et al., 1995; Kudo et al., 1996; Ravnskjaer et al., 2006), have all been shown to reduce AMPK activity.

AMPK phosphorylation at T172 is high in early mitosis and decreases with mitotic progression (Vazquez-Martin et al., 2009; Banko et al., 2011). AMPKα can phosphorylate myosin regulatory light chain (MLC2) (Lee et al., 2007) and protein phosphatase 1 regulatory subunit 12C (PPP-1R12C/P85) (Banko et al., 2011); both of which are important regulators of actin function and mitotic progression (Tan et al., 2001; Jiang et al., 2014). An increase in AMPK activity has been shown to cause aberrant mitoses and an increase in multinucleate cells (Banko et al., 2011), while AMPK depletion results in a mitotic delay and disorientated

spindles (Thaiparambil et al., 2012). These observations suggest that AMPK activity needs to be tightly controlled during mitosis. Conversely, AMPK activity has been found to increase following RO-3306, paclitaxel and pro-TAME (APC/C inhibitor) treatment in HeLa and MDA-MB-231 cells (Domenech et al., 2015). Thus, indicating a potential requirement of AMPK activity in mitotically arrested cells.

3.1.6. Chapter aims

This chapter aims to:

- Investigate the activity of AKT during spontaneous mitosis and paclitaxel-induced mitotic arrest in ovarian cancer cell lines

- Elucidate the downstream effects of any alterations in AKT activity during paclitaxel-induced mitotic arrest

- Identify any potential vulnerabilities during paclitaxel-induced mitotic arrest that could be exploited to enhance the cellular response to paclitaxel

3.2. Results

3.2.1. AKT activity decreases in ovarian cancer cells during mitosis

In order to study PI3K/AKT signalling alterations in ovarian cancer cells during mitosis, the phosphorylation status of AKT was investigated by western blotting. Mitotic cells were isolated using mitotic shake-off and the population was confirmed as mitotic through the presence of phosphorylation of histone H3 at S10. There was a substantial decrease in AKT phosphorylation at T308 and S473 during both spontaneous mitosis and paclitaxel-induced mitotic arrest, despite AKT protein levels remaining unchanged in SKOV3 cells (Figure 3.2A). An analysis of *AKT* mRNA levels in mitotic cells displayed no change in *AKT1*, a slight decrease in *AKT2* and a 50% increase in *AKT3* compared to interphase cells (Figure 3.2B). However, the pan phosphospecific antibodies used in the western blot analysis strongly suggest that the apparent increase in *AKT3* mRNA does not result in an increase in protein expression and is not accompanied by a significant increase in AKT phosphorylation or activity. To determine whether this was a cell line specific phenomenon, the phosphorylation of AKT during mitotic arrest was determined in a panel of ovarian cancer cell lines (Figure 3.2C). All but one (A2780 cells) of the ovarian cancer cell lines displayed a reduction of AKT phosphorylation at S473 during mitotic arrest, suggesting that this may be a common occurrence in ovarian cancer cell lines and thus, have a functional importance. For the subsequent experiments, A2780 cells were used as a negative control due to this cell line maintaining AKT phosphorylation at S473 during mitotic arrest.

To determine the phosphorylation status of AKT in non-tumourgenic cells during mitotic arrest, mitotic cells were isolated from human skin fibroblasts following paclitaxel treatment (Figure 3.2D). AKT phosphorylation at S473 was not observed in either the interphase or mitotic fibroblast cells, suggesting that these cells possess low levels of AKT phosphorylation compared to SKOV3 cells.

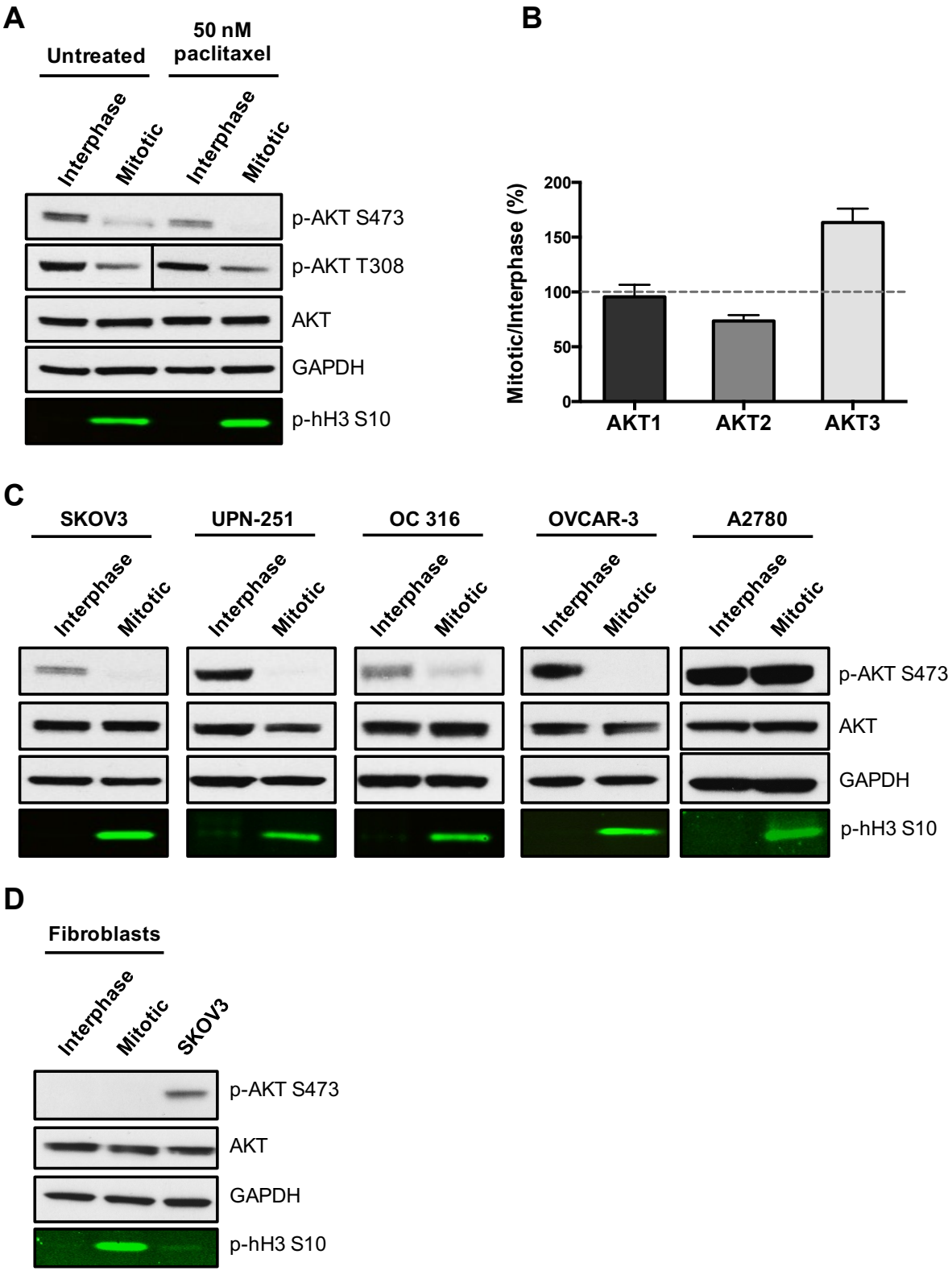


Figure 3.2. AKT expression and phosphorylation in ovarian cancer cell lines and fibroblast cells. (A, *n*=3) AKT protein expression and phosphorylation measured by western blotting in interphase and mitotic SKOV3 cells separated by mitotic shake-off after DMSO or paclitaxel treatment. (B, *n*=3) mRNA levels of AKT 1, 2 and 3 measured using quantitative PCR and displayed as the percentage of mitotically arrested cells compared to interphase SKOV3 cells. Data are represented as the mean + standard error of the mean for three independent experiments. (C, *n*=3) AKT protein expression and phosphorylation measured by western blotting after paclitaxel treatment and mitotic shake-off in ovarian cancer cell lines and (D, *n*=3) human skin fibroblast cells.

To assess whether the reduction of AKT phosphorylation in mitotically arrested cells was accompanied by a decrease in AKT activity, the phosphorylation of several downstream targets of AKT, such as GSK3β, PRAS40 and p70S6K were investigated. The phosphorylation of the direct substrates of AKT, GSK3β at S9 and PRAS40 at T246 were reduced during mitotic arrest in SKOV3 cells, as was the phosphorylation of the indirect downstream target p70S6K at T389 (Figure 3.3A). These data suggest that the decrease in AKT phosphorylation during mitotic arrest corresponds to a reduction in signalling downstream of AKT. The expression of FOXO1 and FOXO3A were also assessed as these are two well-known direct substrates of AKT (Biggs et al., 1999; Brunet et al., 1999). However, the antibodies against the phosphorylation sites S253 in FOXO3A and S256 in FOXO1 produced a negative western blot (data not shown). Despite this, a significant band shift in total FOXO3A was observed in mitotically arrested cells indicating a potential increase in its phosphorylation and its possible inactivation due to binding 14-3-3 (Brunet et al., 1999; Takaishi et al., 1999). However, due to nuclear envelope breakdown occurring during mitosis, the function of FOXO proteins is uncertain. In addition, FOXO1 was absent in mitotically arrested SKOV3 cells compared to interphase cells. Both observations suggest a reduced capacity to respond to cellular stresses, such as oxidative stress (Brunet et al., 2004; Hori et al., 2013). The ovarian cancer cell line, A2780, which displayed no change in AKT phosphorylation between interphase and mitosis (Figure 3.2C), exhibited no change in AKT signalling during mitotic arrest, with the phosphorylation of GSK3β, PRAS40 and p70S6K being maintained in the mitotically arrested population (Figure 3.3A). A band shift was also observed for FOXO3A in the mitotically arrested A2780 cells. However, unlike SKOV3 cells, FOXO1 protein levels remained similar in the mitotically arrested and interphase A2780 populations.

To investigate the possible cause of the reduction in AKT activity during mitotic arrest, the protein levels of mTOR, RICTOR and PTEN were assessed in SKOV3 and A2780 cells. A substantial reduction in the protein levels of mTOR and RICTOR were observed in mitotically arrested SKOV3 cells (Figure 3.3B), both of which are known essential components of the mTORC2 complex (Loewith et al., 2002; Sarbassov et al., 2004). This may explain, at least in part, the loss of AKT phosphorylation during mitosis as it has previously been shown that a reduction

in either RICTOR or mTORC2 proteins can prevent phosphorylation of AKT at S473 (Sarbassov et al., 2005). In addition to this, the protein levels of the unphosphorylated and active form of the tumour suppressor PTEN remained unchanged between interphase and mitotically arrested SKOV3 cells. It is therefore unlikely that the activity of PTEN is contributing to the observed decrease in AKT activity in mitotically arrested SKOV3 cells. Contrary to SKOV3, the protein expression of the mTORC2 complex components, mTOR and RICTOR, displayed a modest increase in mitotically arrested A2780 cells when compared to interphase cells (Figure 3.3B). Additionally, protein levels of PTEN were decreased in mitotically arrested cells compared to interphase. Therefore, it could be that the increase in mTORC2 activity coupled with a decrease in active PTEN is responsible for the maintenance of AKT signalling during mitotic arrest in A2780 cells.

Treatment of mitotically arrested SKOV3 cells with the phosphatase inhibitor, calyculin A, reestablished AKT phosphorylation at S473 (Figure 3.3C). Calyculin A inhibits the activity of PP-2A and PP-1 (Resjo et al., 1999), but not PHLPP, which is known to dephosphorylate AKT at S473 (Andjelkovic et al., 1996; Gao et al., 2005). However, calyculin A could be inactivating PHLPP indirectly (Pozuelo-Rubio et al., 2010), or it could act to maintain the phosphorylation and subsequent inhibition of PTEN, which would result in a reduction of AKT phosphorylation at S473.

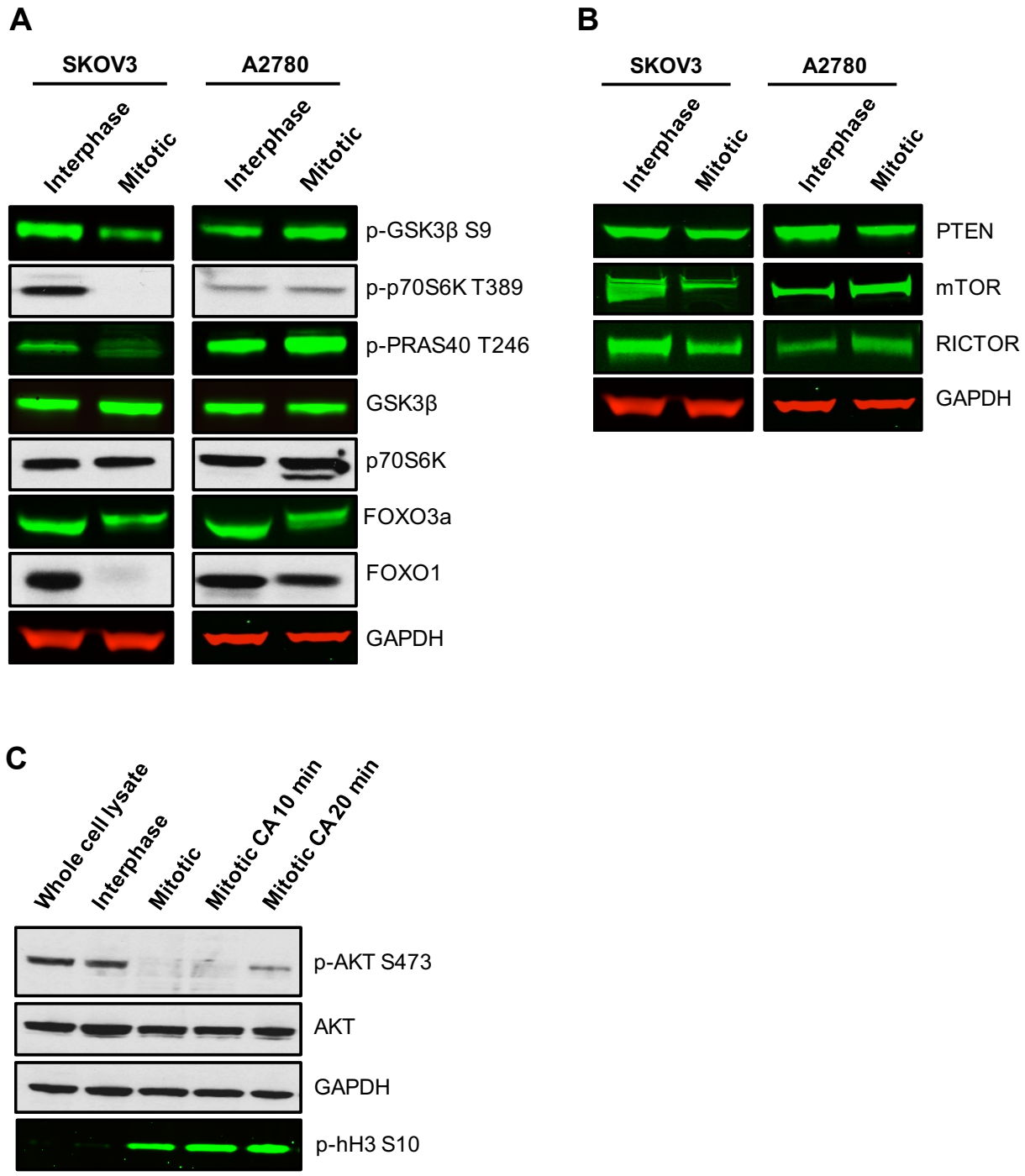


Figure 3.3. AKT activity in ovarian cancer cell lines following paclitaxel treatment.
(A, *n*=3) Protein expression and phosphorylation status of downstream targets of AKT and (B, *n*=3) upstream regulators of AKT measured by western blotting in interphase and mitotic cells separated by mitotic shake-off after paclitaxel treatment. (C, *n*=3) Phosphorylation of AKT measured by western blotting after paclitaxel treatment and mitotic shake-off in SKOV3 cells and treatment with DMSO as a vehicle control or calyculin A (CA). GAPDH was used as a loading control and phospho-histone H3 S10 as a mitotic cell marker.

The loss of AKT activity in the majority of ovarian cancer cell lines during mitosis was provocative for two reasons; the first being that the PI3K/AKT pathway is constitutively activate in approximately 70% of ovarian cancers (Li et al., 2014) and the second being the key role that AKT has in driving cancer cell metabolism, survival and proliferation (Vivanco and Sawyers, 2002; Whiteman et al., 2002). The ovarian cancer cell lines SKOV3, OC 316, A2780 (Domcke et al., 2013), UPN-251 (Huang et al., 2011) and OVCAR-3 (Shayesteh et al., 1999) are all known to possess mutations or copy number gain of *PIK3CA,* which leads to the constitutive activation of AKT. However, the presence of a *PIK3CA* mutation in A2780 has recently been challenged (Ince et al., 2015). Therefore, I predicted that the temporary withdrawal of AKT activity during mitosis might render cancer cells metabolically vulnerable and treatment with paclitaxel could extend this therapeutic window. As such, my following work focused on investigating the metabolic changes during prolonged mitosis as a result of decreased AKT signalling, and looked at how this could be exploited for therapeutic benefit.

3.2.2. Investigating glucose uptake in mitotically arrested ovarian cancer cells

AKT activity is important for insulin-stimulated translocation of GLUT4 to the plasma membrane (Miinea et al., 2005) (chapter 1.3.2). Given that the activity of AKT is decreased during mitotic arrest in SKOV3 cells, I tested whether this influenced the phosphorylation status of the Rab GAP, AS160. Phosphorylation of AS160 at T642 was decreased in mitotically arrested SKOV3 cells, but increased in mitotically arrested A2780 cells (Figure 3.4A). The increase in T642 phosphorylation in A2780 cells could be explained by the decrease in PTEN in the mitotically arrested population and a possible upregulation of AKT signalling. Furthermore, the reduction of AS160 phosphorylation in mitotically arrested SKOV3 cells indicates a decrease in GLUT4 translocation to the plasma membrane and a potential reduction in glucose uptake. It has been demonstrated that the overexpression of wild-type or constitutively active forms of AKT can increase levels of GLUT4 at the cell surface in the absence of insulin, providing evidence that AKT activity can directly influence GLUT4 trafficking (Cong et al., 1997). Moreover, AKT inhibitors have been shown to decrease glucose uptake and glycolysis in cervical cancer (Rashmi et al., 2014).

AKT activity can also stimulate *GLUT1* mRNA expression (Barthel et al., 1999), so I investigated the expression of *GLUT1* mRNA in mitotically arrested cells. *GLUT1* mRNA levels remained unchanged between mitotically arrested and interphase populations in SKOV3 cells (Figure 3.4B), while *GLUT1* mRNA levels were significantly decreased in mitotically arrested A2780 cells when compared to interphase cells (Figure 3.4C, $p = 0.0102$, *t*-test). This could be explained by the fact that *GLUT1* expression is known to be reduced in cell cultures with high levels of glucose compared to those where glucose is deprived (Klip et al., 1994). Therefore, it may be that A2780 cells do not upregulate *GLUT1* expression during mitosis due to glucose being readily available, whereas SKOV3 cells need to maintain GLUT1 levels. This difference could be due to energy reserves in A2780 cells, or the fact that A2780 cells are maintained in a medium that contains higher levels of glucose (25 mM) than SKOV3 cells (18 mM). *GLUT1* expression can also be regulated by proteins other than AKT, such as SGK1 (Palmada et al., 2006) and HIF1α (Chen et al., 2001). Given the decrease in GLUT4 trafficking and the maintenance of *GLUT1* mRNA levels in SKOV3 cells, which is presumably in order to bring glucose into the cell, glucose 6-phosphate levels were measured and found to be significantly decreased in mitotically arrested SKOV3 cells compared to interphase cells (Figure 3.4D, $p = 0.0268$, *t*-test).

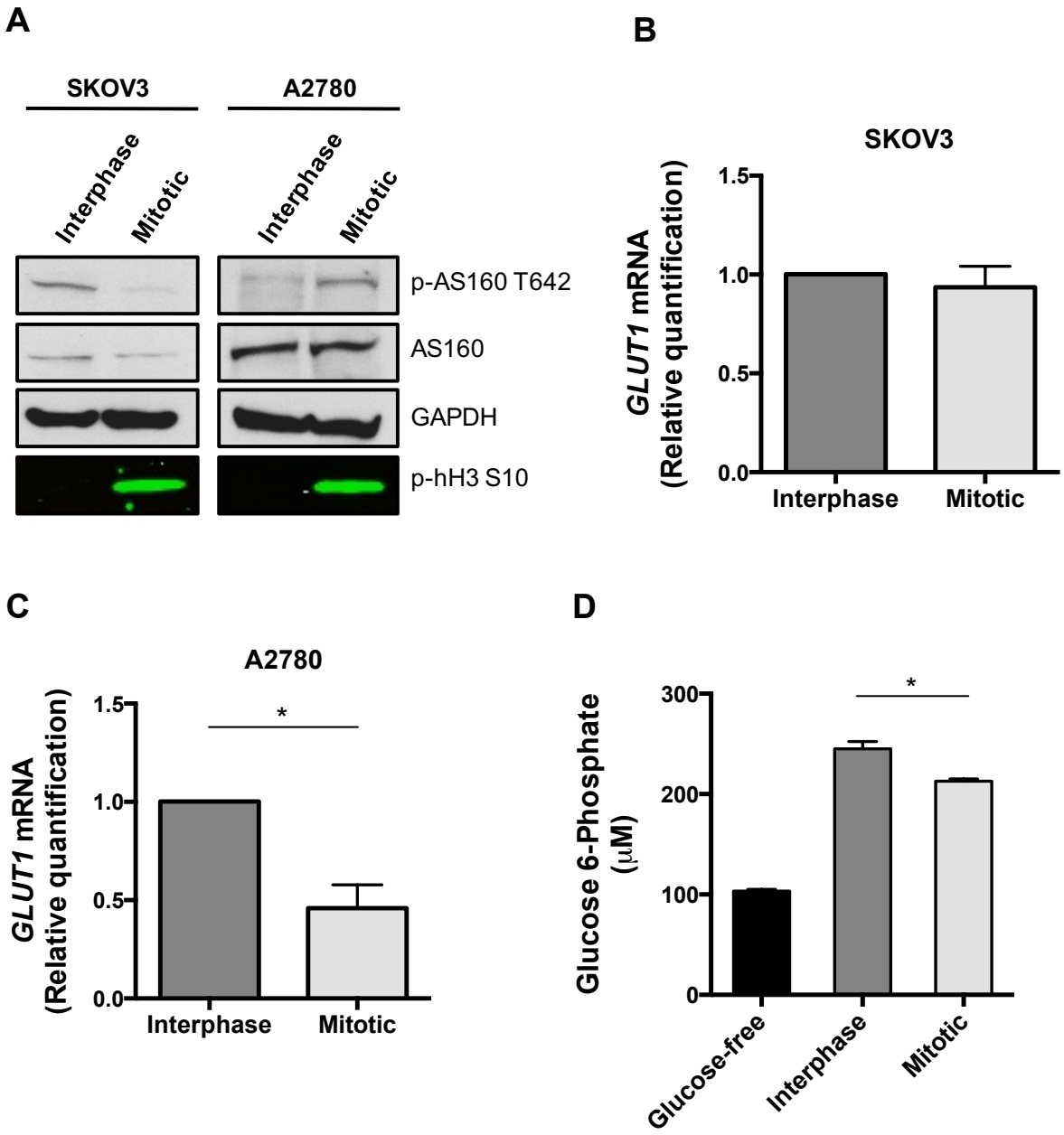


Figure 3.4. Glucose uptake in ovarian cancer cell lines following paclitaxel treatment.
(A, *n*=2) Protein expression and phosphorylation status of AS160 measured by western blotting in interphase and mitotic cells separated by mitotic shake-off after paclitaxel treatment. GAPDH was used as a loading control and phospho-histone H3 S10 as a mitotic marker. (B, *n*=3) *GLUT1* mRNA levels measured using quantitative PCR and displayed as normalised to interphase cells for SKOV3 cells and (C, *n*=3) A2780 cells. (D, *n*=3) Glucose 6-phosphate levels in SKOV3 cells following paclitaxel treatment and a mitotic shake-off. Data are represented as the mean + standard error of the mean for three independent experiments. *≤0.05–0.01.

3.2.3. ATP levels are significantly decreased in SKOV3 cells during mitosis

The decrease in glucose 6-phosphate levels in mitotically arrested SKOV3 cells could hinder it's utilisation in glycolysis and the pentose phosphate pathway. Therefore, ATP and glutathione (GSH) levels were investigated as a readout of the flux through glycolysis and the pentose phosphate pathway, respectively. ATP levels were significantly decreased in mitotically arrested SKOV3 cells (Figure 3.5A, $p = 0.005$, *t*-test), while GSH levels remained unchanged (Figure 3.5B), suggesting that the decrease in glucose 6-phosphate does not effect the flux through the pentose phosphate pathway. In A2780 cells, no statistically significant difference was identified in the levels of GSH (Figure 3.5C) and ATP (Figure 3.5D) between the mitotically arrested and interphase populations. ATP levels were also analysed in an additional cell line, OC 316, that displayed a reduction in AKT activity during mitotic arrest (Figure 3.2C). A significant reduction in ATP levels were observed in mitotically arrested cells compared to the interphase population (Figure 3.5E, $p = 0.0079$, *t*-test).

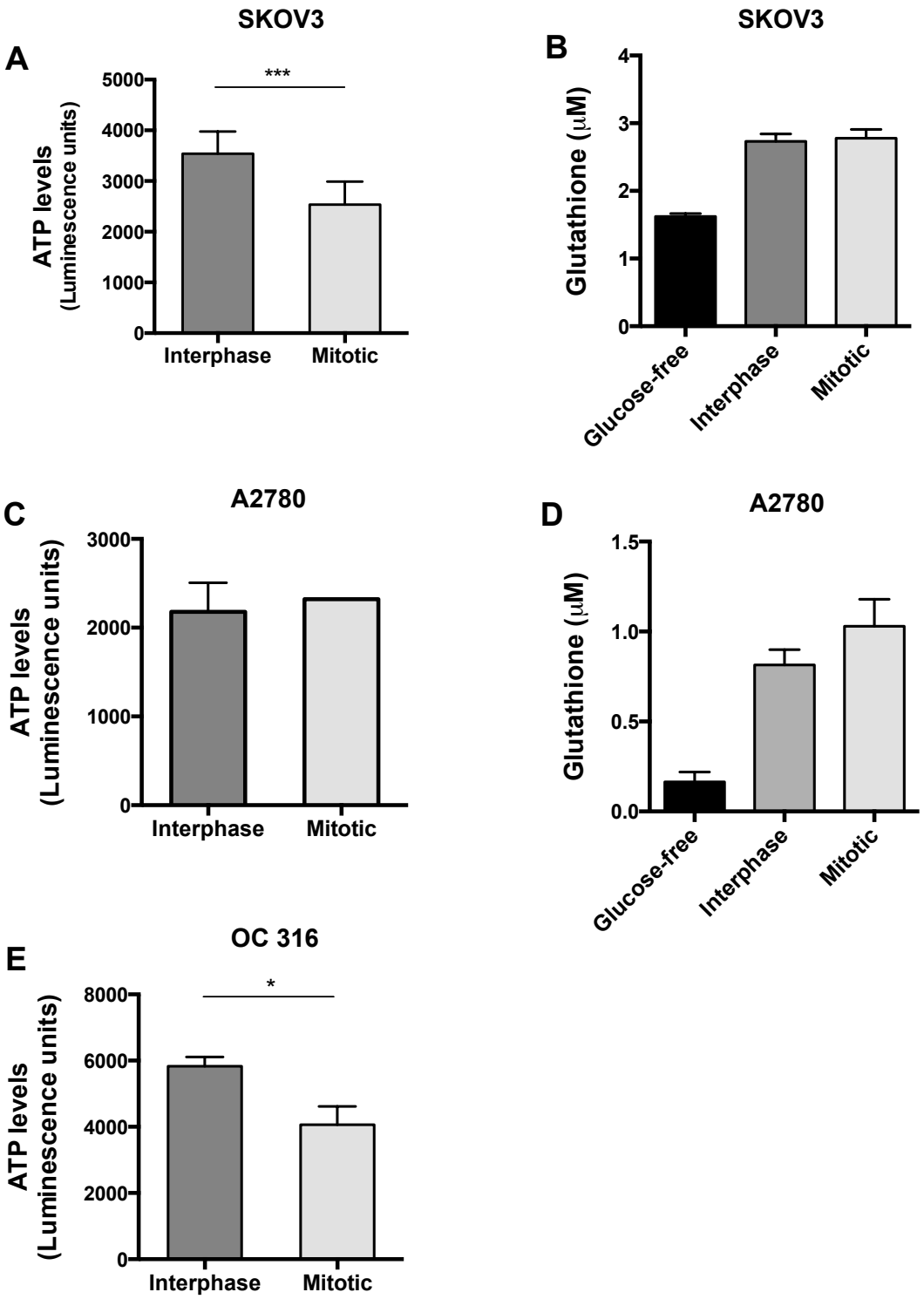


Figure 3.5. ATP and glutathione levels in ovarian cancer cell lines following paclitaxel treatment. (A, *n*=4) Levels of ATP and (B, *n*=3) glutathione were quantified following paclitaxel treatment and a mitotic shake-off in SKOV3 cells. (C, *n*=4) Levels of ATP and (D, *n*=3) glutathione were quantified following paclitaxel treatment and a mitotic shake-off in A2780 cells. (E, *n*=3) ATP levels were also quantified in OC 316 cells. Data are represented as the mean + standard error of the mean of three independent experiments. **<0.01–0.001 and ***<0.001.

To determine whether the decrease in ATP levels in SKOV3 cells induce a change in the ATP/ADP ratio and cause energetic stress, the phosphorylation status of AMPKα at T172 was examined. There was no evidence of energetic stress in mitotically arrested SKOV3 cells, with the levels of total AMPK protein and phosphorylation at T172 displaying a marked decrease in the mitotically arrested population compared to interphase cells (Figure 3.6A). In agreement with this, phosphorylation of the key enzyme in fatty acid synthesis, ACC, which is phosphorylated and inactivated by AMPK phosphorylation (Davies et al., 1992), was not increased in mitotically arrested SKOV3 compared to the interphase population (Figure 3.6B). The phosphorylation of AMPKα at T172 and ACC at S79 in paclitaxel treated A2780 cells also remained unchanged between interphase and mitosis (Figure 3.6C and D). The lack of an increase in AMPK activity in mitotically arrested cells was surprising given that AMPK activity has previously been found to increase in mitotically arrested cells (Domenech et al., 2015). However, this study did not investigate ovarian cancer cells, or arrest cells by paclitaxel treatment alone. As discussed previously in the introduction to this chapter, cells undergoing spontaneous mitosis carry out *de novo* fatty acid synthesis, indicating that they are not metabolically stressed and have low metabolic needs during this phase of the cell cycle. However, when cells are arrested in mitosis, the cell is likely to become metabolically stressed due to the effects of paclitaxel on glycolysis and ATP. Nonetheless, AMPK activation could be prevented in mitotically arrested cells in order to maintain *de novo* fatty acid synthesis and promote mitotic exit.

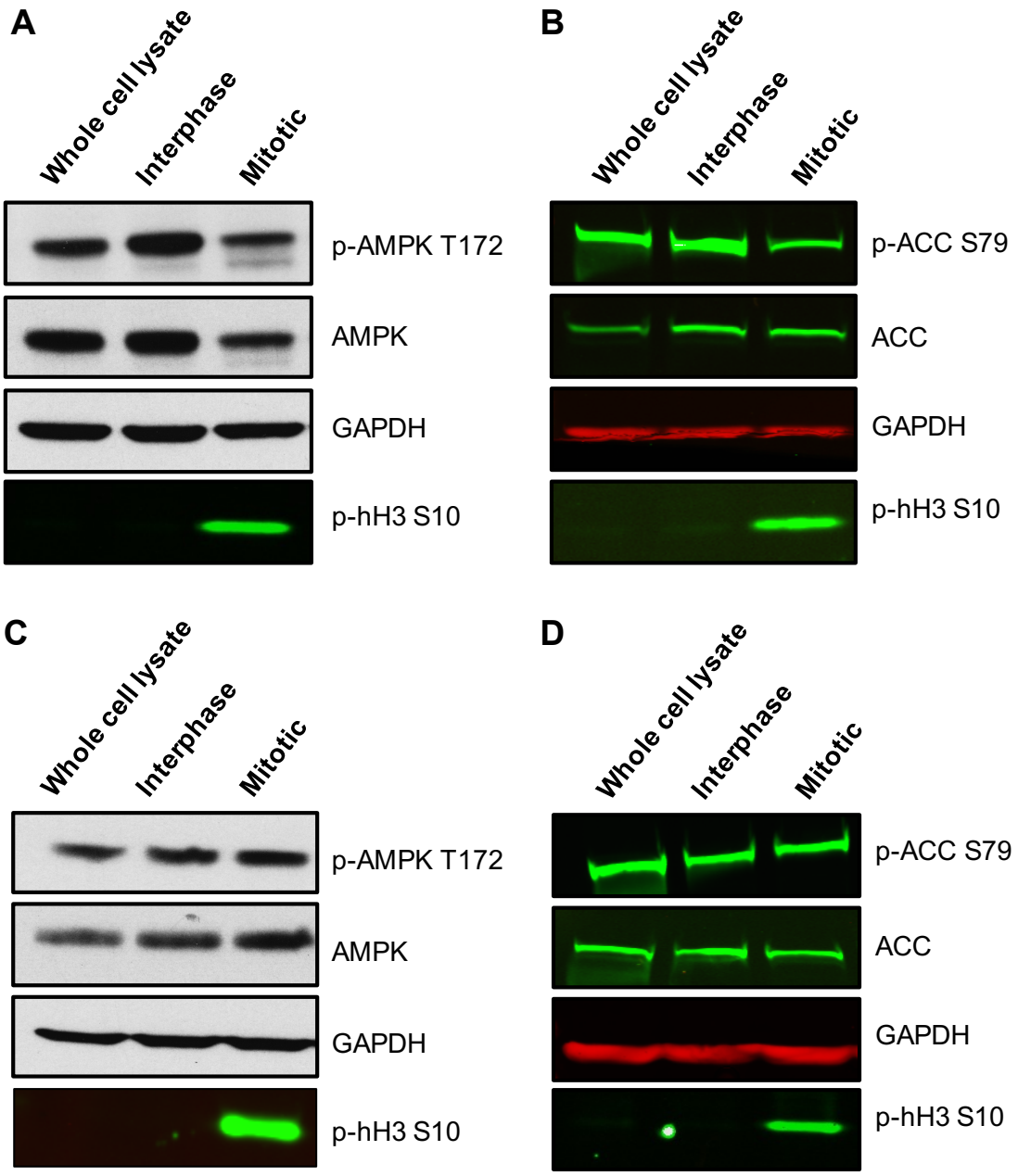


Figure 3.6. AMPK activity in ovarian cancer cells following paclitaxel treatment.
(A, *n*=3) AMPK protein expression and phosphorylation measured by western blotting in interphase and mitotic SKOV3 cells separated by mitotic shake-off after paclitaxel treatment. (B, *n*=3) ACC protein expression and phosphorylation measured by western blotting in interphase and mitotic SKOV3 cells separated by mitotic shake-off after paclitaxel treatment. (C, *n*=3) AMPK protein expression and phosphorylation measured by western blotting in interphase and mitotic A2780 cells separated by mitotic shake-off after paclitaxel treatment. (D, *n*=3) ACC protein expression and phosphorylation measured by western blotting in interphase and mitotic A2780 cells separated by mitotic shake-off after paclitaxel treatment. GAPDH was used as a loading control and phospho-histone H3 S10 as a mitotic marker.

3.2.4. A high-content siRNA screen identifies metabolic enzymes that are critical for survival in mitotically arrested ovarian cancer cells

The data so far indicate a vulnerability in SKOV3 cells and potentially other ovarian cancer cell lines, during paclitaxel-induced mitotic arrest. This vulnerability is predicted to arise from a reduction in AKT activity, FOXO1 expression, glucose 6-phosphate levels and ATP levels. The removal of a key metabolic enzyme in these cells might exploit this vulnerability and act to increase cell death during paclitaxel-induced mitotic arrest. To investigate this, a previously conducted high-content siRNA screen was reanalysed to identify potential metabolic targets that are important for cell survival following mitotic arrest by paclitaxel treatment. An overview of the screen is shown in Figure 3.7A.

The screen was performed using SKOV3 cells and a Dharmacon siRNA “kinome” library, and with 10 nM paclitaxel as this was the optimal concentration to achieve maximal mitotic cells after 24 hours without inducing significant cell death, providing a window for further mitotic cell depletion. Cells were fixed and stained for hoechst to measure DNA content and anti-phospho-histone H3 S10 to estimate mitotic cell number (Figure 3.7B). Depletion of a gene could result in low mitotic index (percentage of mitotic cells compared to all cells) by inducing cell cycle arrest prior to mitosis, interfering with the mitotic checkpoint, or inducing mitotic cell death. I reasoned that a true positive hit, one that induces cell death in mitosis, would specifically reduce the paclitaxel treated mitotic ratio (PMR), measured as the mitotic index following gene depletion in paclitaxel treated cells divided by the mitotic index following non-targeting siRNA control treatment in paclitaxel treated cells, and have limited effect on the paclitaxel untreated mitotic ratio (PUMR). Therefore, I calculated the PUMR/PMR ratio and considered a cut-off of 3-fold as a positive hit for further validation. siRNA targeting *PLK1* was used as a transfection control in the screen as if PLK1 is successfully depleted, it is known to be fatal to cells (Liu and Erikson, 2003; Strebhardt and Ullrich, 2006).

Using the above criteria, three enzymes with roles in metabolism were identified from the screen as causing a significant decrease in the number of mitotically arrested cells following paclitaxel treatment after their depletion. These were xylulokinase (XYLB) (8-fold), which functions in the glucuronate-xylulose

pathway, mevalonate diphosphate decarboxylase (MVD) (7-fold) that functions in the mevalonate pathway and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4) (3.7 fold), which is an important regulator of glycolysis (Figure 3.7C). However, it was important that the depletion of the enzyme did not cause a significant decrease in the number of cells undergoing spontaneous mitosis (more than 25% decrease), indicating that depletion was detrimental only to cells undergoing prolonged mitotic arrest. For this reason, I did not prioritise MVD for validation because it reduced the number of cells undergoing spontaneous mitosis by more than 33% (Table S3.1). A key concern with both of these hits is that after depletion of XYLB and PFKFB4 followed by paclitaxel treatment, the DNA content increased by 18% and 33%, respectively. This indicates that mitotic slippage may be partly or wholly responsible for the decrease in mitotic cells, which will be an important consideration during their validation.

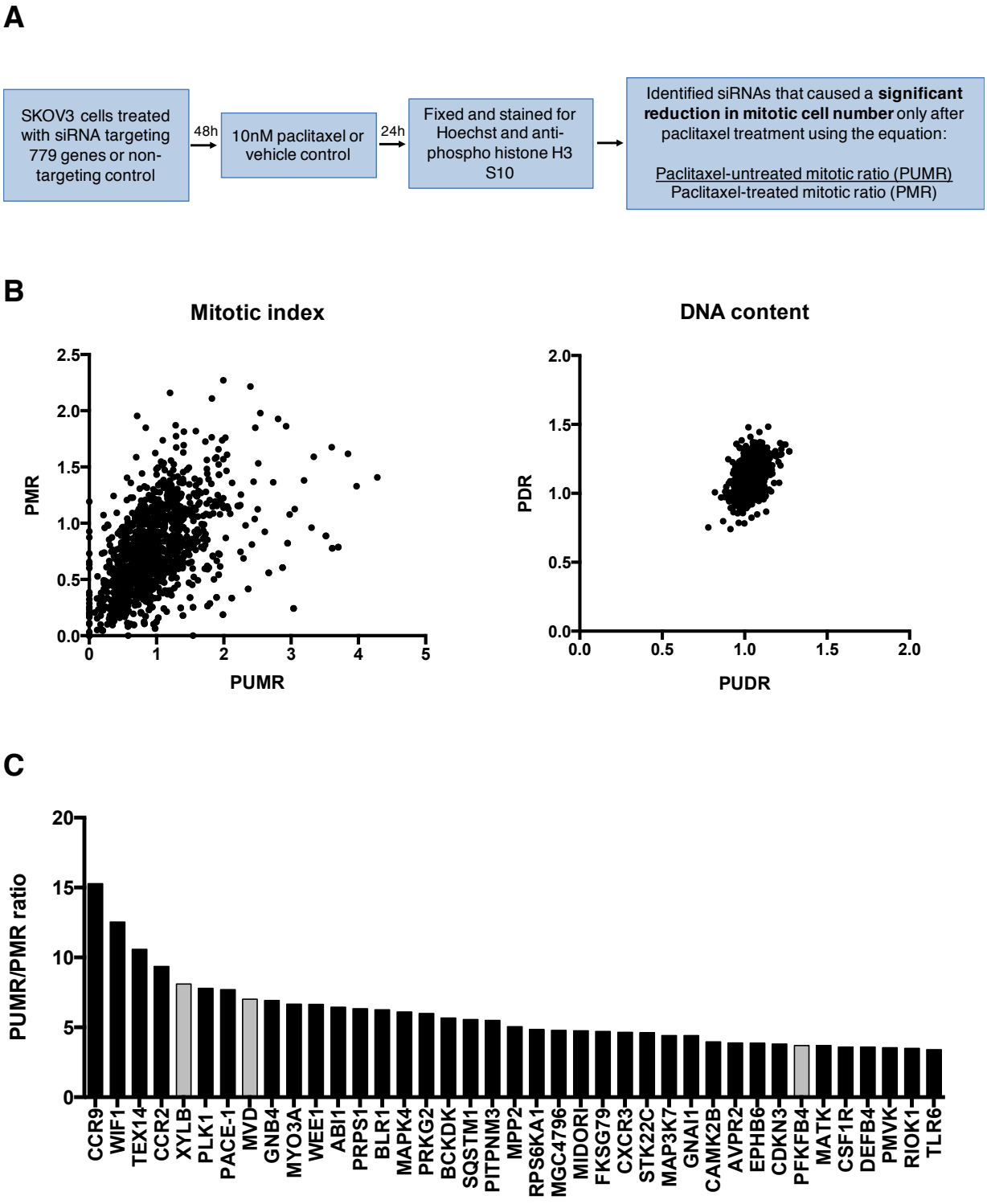


Figure 3.7. High content siRNA "kinome" screen in SKOV3 cells.
(A) Schematic outlining the screen protocol. (B) Dot plot of the mitotic index and DNA content for each siRNA duplex transfected in the screen. PDR, Paclitaxel treated DNA ratio. PUDR, paclitaxel untreated DNA ratio. (C) siRNA targets that resulted in a 3-fold or higher PUMR/PMR ratio. Grey bars represent the genes that encode proteins possessing a metabolic function.

3.3. Discussion

To my knowledge, AKT activity has not been investigated during spontaneous mitosis in cancer cells that have not been subjected to cell cycle synchronisation. Moreover, signalling downstream of the AKT pathway during paclitaxel-induced mitotic arrest in ovarian cancer cells has not been well characterised. Given the promise of targeted therapies in cancer treatment and the interest in combining paclitaxel with inhibitors of the PI3K/AKT pathway, investigations into these two areas are necessary to understand their therapeutic potential.

3.3.1. AKT phosphorylation during mitosis and mitotic arrest

This chapter investigates the phosphorylation status of AKT during spontaneous mitosis and paclitaxel-induced mitotic arrest in ovarian cancer cells. A striking decrease in AKT phosphorylation was observed in SKOV3 cells undergoing asynchronous spontaneous mitosis, which was unexpected given that they possess a *PI3KCA* mutation that is known to cause constitutive activation of the PI3K/AKT pathway (Domcke et al., 2013). Moreover, the fact that cells have acquired and maintained a *PIK3CA* mutation indicates the importance of this pathway and the potential vulnerability caused by its temporary inactivity during mitosis. This work is the first to demonstrate a decrease in AKT phosphorylation in cells that have not been subjected to any form of cell cycle synchronisation. Literature suggests that cells are unstressed during mitosis with the downregulation of translation (Pyronnet et al., 2001; Tanenbaum et al., 2015) and the presence of *de novo* fatty acid synthesis (Bosmann and Winston, 1970; Kobayashi and Pagano, 1989; Manguikian and Barbour, 2004; Lin and Arthur, 2007), indicating low metabolic needs. Therefore, AKT activity could simply not be required to drive glycolysis and survival during this phase of the cell cycle.

The loss of AKT phosphorylation during spontaneous mitosis could also be due to the requirement of AKT inactivation for mitotic progression and exit, which has been suggested by previous studies (Chaussepied and Ginsberg, 2004; Liu et al., 2014a). However, as the mitotic population are pooled in this research, meaning that AKT phosphorylation was not monitored throughout the stages of mitosis, this cannot be determined. A recent study has suggested that AKT phosphorylation at T308 and S473 could be facilitated by the phosphorylation of

S477 and T479. Therefore, the investigation of the phosphorylation status of both of these sites during mitotic arrest would be interesting as it could be that a loss of S477 and T479 phosphorylation is what is reducing S473 and T308 phosphorylation.

A reduction in AKT phosphorylation was also observed in the majority of ovarian cancer cell lines during paclitaxel-induced mitotic arrest. Given that AKT has an important role in promoting cell survival and chemotherapy resistance, along with the fact that paclitaxel treatment arrests the majority of cells in early metaphase, which is prior to the initiation of global dephosphorylation required for mitotic exit - this observation was unexpected. However, paclitaxel-induced prolonged mitosis is likely to induce disorder to the tightly regulated and spatiotemporal organisation of kinase and phosphatase activity that occurs during mitosis. Additionally, if the inactivation of AKT is required for normal mitotic exit, then the loss of AKT phosphorylation during mitotic arrest could assist cells in undergoing aberrant mitosis or mitotic slippage, enabling them to avoid cell death in mitosis. Importantly, this observation casts doubt over the use of AKT inhibitors in conjunction with paclitaxel-treatment, as it indicates that cells already possess a reduction in AKT phosphorylation. However, an analysis of AKT phosphorylation *in vivo* would need to be determined before ruling out the use of AKT inhibitors with paclitaxel in ovarian cancer treatment.

This is the only study to investigate AKT phosphorylation in paclitaxel treated ovarian cancer cells that have been isolated from the interphase population, and the first to demonstrate a complete loss of AKT phosphorylation during paclitaxel-induced mitotic arrest. Two previous studies have observed a decline in AKT phosphorylation after paclitaxel treatment, but this was not in mitotically arrested cells that had been separated from the interphase population (Mabuchi et al., 2002; Kim et al., 2007b). This may explain why the phosphorylation status of AKT was not reduced to the levels observed in this work and suggests that the separation of mitotically arrested cells from the interphase cells is important for obtaining reliable and accurate data.

Explanations for the loss of AKT phosphorylation at S473 during paclitaxel-induced mitotic arrest could be due to a combination of the following; enhanced

degradation, increased dephosphorylation, as well as inactivation of upstream signalling leading to a reduction in AKT phosphorylation. The reduction in mTOR and RICTOR protein levels during mitotic arrest indicate that there is a reduction in mTORC2 activity, which would result in a decrease of AKT phosphorylation at S473 (Sarbassov et al., 2004). However, considering the presence of AKT S473 phosphorylation in the interphase population, its degradation or dephosphorylation prior to, or at the beginning of mitotic arrest may also occur. AKT phosphorylated at S473 can be targeted for proteasomal degradation through K48-linked polyubiquitination (Xiang et al., 2008; Suizu et al., 2009; Bae et al., 2012), which is prevented by mTORC2 phosphorylation of AKT at T450 (Facchinetti et al., 2008). However, if phosphorylated AKT was being degraded, a consistent decrease in total AKT would be observed in the mitotically arrested population, which is not the case. Treatment of mitotically arrested SKOV3 cells with calyculin A was able to reestablish phosphorylation of AKT at S473, indicating that the loss of AKT phosphorylation is likely to be due to the action of phosphatases or the action of PTEN. More research is required to determine the precise mechanisms mediating AKT dephosphorylation during paclitaxel-induced mitotic arrest.

Unlike the other ovarian cancer cell lines in this work, A2780 cells did not display a reduction in AKT activity during mitotic arrest. There are a number of potential reasons for sustained AKT signalling in A2780 cells. Firstly, a 9 nucleotide deletion spanning the *PTEN* phosphatase domain has been identified in A2780 cells (Saito et al., 2000). This does not affect *PTEN* mRNA expression, but is thought to generate a hypomorphic PTEN protein (Wee et al., 2008). Secondly, the presence of a *PI3KCA* mutation in combination with lower PTEN activity (Domcke et al., 2013) could cause uncontrolled AKT activation, although the presence of a *PI3KCA* mutation in A2780 cells has been recently challenged (Ince et al., 2015).

The panel of cell lines analysed in this work represent some of the most commonly used cell lines in ovarian cancer research, for example SKOV3 cells and A2780 cells account for approximately 60% of publications related to ovarian cancer (Domcke et al., 2013). If the aim of this work is to represent HGSOC, it is important that the cell lines studied possess a *TP53* mutation, as this is a

distinguishing feature of this subtype. *TP53* mutations can also be accompanied by mutations in *BRCA1/2, PIK3CA, CCNE1* and *MYC* (Domcke et al., 2013). Despite the widespread use of SKOV3 cells and A2780 cells, a number of studies have suggested that they are not appropriate models for HGSOC due to the lack of *TP53* mutations and the presence of mutations frequently found in other subtypes, such as *ARID1A* (Anglesio et al., 2013; Domcke et al., 2013; Ince et al., 2015). Conversely, other studies have reported *TP53* mutations in SKOV3 cells (Beaufort et al., 2014; Ince et al., 2015) and *BRCA2* mutations in A2780 cells (Beaufort et al., 2014), making them more representative of HGSOC. Importantly, an in-house analysis has identified a *TP53* mutation in the SKOV3 cells I have used in this work. However, it is likely that there are cell line models more representative of HGSOC. To determine the applicability of this work to HGSOC, cell lines that more accurately represent this subtype could be used, such as those developed by Kreuzinger et al., 2015 (Kreuzinger et al., 2015). Alternatively, given the array of disadvantages of cell lines, a better approach would be to investigate whether this phenomenon occurs *in vivo*, by the use of tumour samples or primary cells. However, these pose their own technical difficulties.

3.3.2. AKT activity in mitotically arrested cells

To investigate the decrease in AKT phosphorylation further, I explored the downstream activity of AKT in paclitaxel treated A2780 and SKOV3 cells, comparing the mitotically arrested and interphase populations. This identified a reduction in the phosphorylation of both direct AKT targets (GSK3β, PRAS40) and signalling through mTORC1 (p70S6K) in the mitotically arrested SKOV3 cells, but not in the interphase population, or in A2780 cells. This disparity in AKT activity between SKOV3 and A2780 cells after paclitaxel treatment agrees with the current literature, which suggests that this observation is cell-line specific.

A decrease in GSK3β phosphorylation at S9 indicates that GSK3β is not inhibited in SKOV3 cells during paclitaxel-induced mitotic arrest. However, whether GSK3β is active is yet to be determined as the phosphorylation status of its activating site, Y216 (Hughes et al., 1993), is unknown, as well as the presence of other components for the formation of an active complex (Grimes and Jope, 2001). The

reduction in PRAS40 phosphorylation promotes its association with mTORC1, resulting in inhibition of mTORC1 and a reduction in its ability to phosphorylate p70S6K at T389. This indicates a downregulation of protein synthesis (Ma and Blenis, 2009), lipid synthesis (Li et al., 2011), glycolytic flux (Duvel et al., 2010; Laplante and Sabatini, 2012), as well as a possible increase in autophagy during paclitaxel-induced mitotic arrest (Sandilands et al., 2015). It is important to remember that p70S6K is not directly downstream of AKT and is subject to multiple phosphorylation events mediated by a range of kinases (Dufner and Thomas, 1999). In addition to mTOR, T389 can be phosphorylated by PDK1 and NEK6/7 (Avruch et al., 2001; Templeton, 2001; Volarevic and Thomas, 2001). However, despite the presence of multisite phosphorylation, the phosphorylation of T389 has been shown to closely correlate with p70S6K activity *in vivo*. In support of this observation, paclitaxel has been shown to enhance p70S6K phosphorylation at T421 and S424 in mitotically arrested cells, which promotes its inactivation (Le et al., 2003).

It is important to evaluate the robustness of GSK3β and PRAS40 phosphorylation as readouts of AKT activity. For example, during times of cellular stress, such as heat, oxidative or viral infection, PRAS40 can also be phosphorylated at T246 by another kinase, known as dual specificity tyrosine phosphorylation-regulated kinase 3 (DYRK3) (Wippich et al., 2013). Similarly, GSK3β can be phosphorylated at S9 by a number of upstream kinases, including Aurora A, p70S6K, p90RSK, PKC and PKA (Grimes and Jope, 2001). Nonetheless, considering the number of upstream signalling pathways that converge on AKT to mediate AKT-dependent GSK3β phosphorylation, AKT is likely to be a critical kinase in GSK3β regulation. Despite the presence of additional upstream kinases, investigating the phosphorylation status of these targets is still a useful indicator of signalling activity downstream of AKT.

3.3.3. Metabolic vulnerability in mitotically arrested ovarian cancer cells

AKT can regulate many aspects of glycolysis, including glucose uptake and glycolytic flux. I identified a decrease in AS160 phosphorylation, glucose 6-phosphate and ATP levels in mitotically arrested SKOV3 cells, with no change in GSH levels. A decrease in ATP levels were also observed in mitotically arrested

OC 316 cells, which also possess a reduction in AKT activity during mitotic arrest. A disruption to glycolysis and a reduction in ATP has previously been observed after paclitaxel treatment (Untch et al., 1994; Glass-Marmor and Beitner, 1999). However, the investigation of glucose uptake and the evaluation of these readouts in mitotically arrested cells isolated from the interphase population are both novel aspects of this work. Moreover, until now these observations have not been combined with a reduction in AKT activity and identified as a potential vulnerability during paclitaxel-induced mitotic arrest. An important consideration here is that metabolism is not a static process and remains constantly in flux, so obtaining a readout at one time-point may not represent the full story.

The decrease in glucose 6-phosphate and ATP were not accompanied by an increase in AMPK activity. A recent study identified an increase in AMPK activity after paclitaxel treatment, however this was in breast cancer cell lines and not ovarian cancer cell lines (Kim et al., 2015). Nonetheless, conflicting results have been demonstrated in the ovarian cancer cell line, Caov-3, with paclitaxel treatment inducing the phosphorylation and inactivation of ACC (Wu et al., 2011a). Again direct comparisons cannot be made due to differences in the technical approach, for example, cells were exposed to paclitaxel for a maximum of 2 hours and at high micromolar concentrations.

I concluded that the decrease in AKT activity coupled with a reduction in glucose 6-phosphate levels, ATP levels and AMPK activity points towards a potential vulnerability in SKOV3 cells during paclitaxel-induced mitotic arrest. To determine whether this vulnerability is exploitable, a high-content siRNA screen was reanalysed to determine whether depleting specific metabolic enzymes could increase cell death in mitotically arrested SKOV3 cells. The use of high content imaging enabled the identification of siRNA duplexes that resulted in substantial changes to cellular DNA content and form, making it possible to determine whether mitotic slippage was occurring, or whether cells become tetraploid or multinucleate. Transfection of many of the siRNA duplexes resulted in an increase in DNA content, indicating that mitotic slippage was occurring. Therefore, it will be important to address this when validating XYLB and PFKFB4.

The identification of PFKFB4 as an important enzyme for cancer cell survival is supported by two independent groups that have conducted unbiased screens for genes essential for cancer cell survival and found that PFKFB4 is required for both glioma stem-like cell (Goidts et al., 2012) and prostate cancer cell survival (Ros et al., 2012), but not for normal cell survival. However, these studies were not looking at mitotically arrested cells, but cancer cells in general. To date, XYLB has not been implicated in the survival of cancer cells or mitotically arrested cells. Given that neither of these enzymes have previously been implicated in the survival of mitotically arrested cells, this work has identified two novel therapeutic targets with the potential to improve paclitaxel response in ovarian cancer cells.

3.3.4. Conclusions

In summary, this work has made important contributions to understanding PI3K/AKT signalling during paclitaxel-induced mitotic arrest and its effect on aspects of cellular metabolism. Moreover, it has recognised a potential metabolic vulnerability during paclitaxel-induced mitotic arrest in the ovarian cancer cell line SKOV3 and identified two novel metabolic enzymes that could be important for the survival of mitotically arrested ovarian cancer cells following paclitaxel treatment. The next chapter focuses on the validation of the screen hits XYLB and PFKFB4.

Chapter 4

The effects of PFKFB4 depletion and paclitaxel on ovarian cancer cells

4. The effects of PFKFB4 depletion and paclitaxel on ovarian cancer cells

4.1. Introduction

Two proteins influencing glycolytic metabolism were identified from reanalysing a high-content siRNA screen as causing a significant decrease in the number of mitotically arrested cells following paclitaxel treatment after their depletion; these were XYLB and PFKFB4. The functions of these two enzymes are discussed below.

4.1.1. The PFKFB Family

PFKFB1-4 are a family of bifunctional enzymes that play a vital role in influencing glycolysis. The four family members are encoded by four different genes located on different chromosomes, with differential splicing giving rise to a number of distinct variants (Table 4.1). The N-terminal phosphofructokinase (PFK-2) domain catalyses the synthesis of fructose 2,6-bisphosphate from fructose 6-phosphate and ATP (Van Schaftingen and Hers, 1981b), while the C-terminal fructose 2,6-bisphosphatase domain catalyses the hydrolysis of fructose 2,6-bisphosphate to fructose 6-phosphate and inorganic orthophosphate (El-Maghrabi et al., 1982) (Figure 4.1A, B). The catalytic regions are flanked by a regulatory region at both the N- and C- terminus (Kim et al., 2006b). The PFKFB family all function as homodimers (Hasemann et al., 1996), but their kinase and phosphatase activities differ (Sakakibara et al., 1997). This variation in activity is thought to be due to small structural differences in the regulatory regions that lead to distinct post-translational modifications (Lange et al., 1991).

While PFKFB1 and PFKFB2 have been demonstrated to have marginally higher kinase activity, it has been suggested that PFKFB3 predominantly favours the formation of fructose 2,6-bisphosphate (Sakakibara et al., 1997). As a result, it is thought that PFKFB3 is responsible for setting the intracellular levels of fructose 2,6-bisphosphate (Telang et al., 2006). Conversely, PFKFB4 has been shown to possess slightly higher bisphosphatase activity (Ros et al., 2012; Strohecker et al., 2015). However, this is currently an area of debate (Chesney et al., 2014;

Chesney et al., 2015). PFKFB isoforms were initially identified in different tissues, however their expression is not restricted to specific tissues with several tissues express more than one PFKFB member (Sakakibara et al., 1999). This supports the notion that different isoforms play distinct roles under different physiological conditions.

Family member	Chromosomal location	Main splice variants, commonly referred to as	Study
PFKFB1	Xp11.21	L-PFK2, liver	Darville et al., 1989 Bruni et al., 1999
		M-PFK2, muscle	Darville et al., 1989 Darville et al., 1992 Kitamura et al., 1989 Bruni et al., 1999
		F-PFK2, foetal	Dupriez et al., 1993 Martin-Sanz et al., 1992
PFKFB2	1q31	H-PFK2, heart, 58kDa, H1, H2, H4	Tsuchya and Uyeda, 1994 Chikri and Roussea, 1995 Heine-Suñer et al., 1998 Darville et al., 1991
		H-PFK2, heart, 54kDa, H3	Vidal et al .,1993 Tsuchiya and Uyeda, 1994
PFKFB3	10p14-p15	u-PFK2, ubiquitous, placental, brain	Sakai et al., 1996 Ventura et al., 1992 Nicholl et al., 1997 Navarro-Sabaté et al., 2001
		iPFK2, inducible	Chesney et al., 1999 Navarro-Sabaté et al., 2001 Kessler and Eschrich, 2001 Watanabe et al., 1999
PFKFB4	3p21-p22	T-PFK2, testes	Sakata et al., 1991 Manzano et al., 1999

Table 4.1. PFKFB1-4 chromosomal location and splice variants.
PFKFB1-4 are encoded by four different genes located on different chromosomes, with differential splicing giving rise to a number of distinct splice variants.

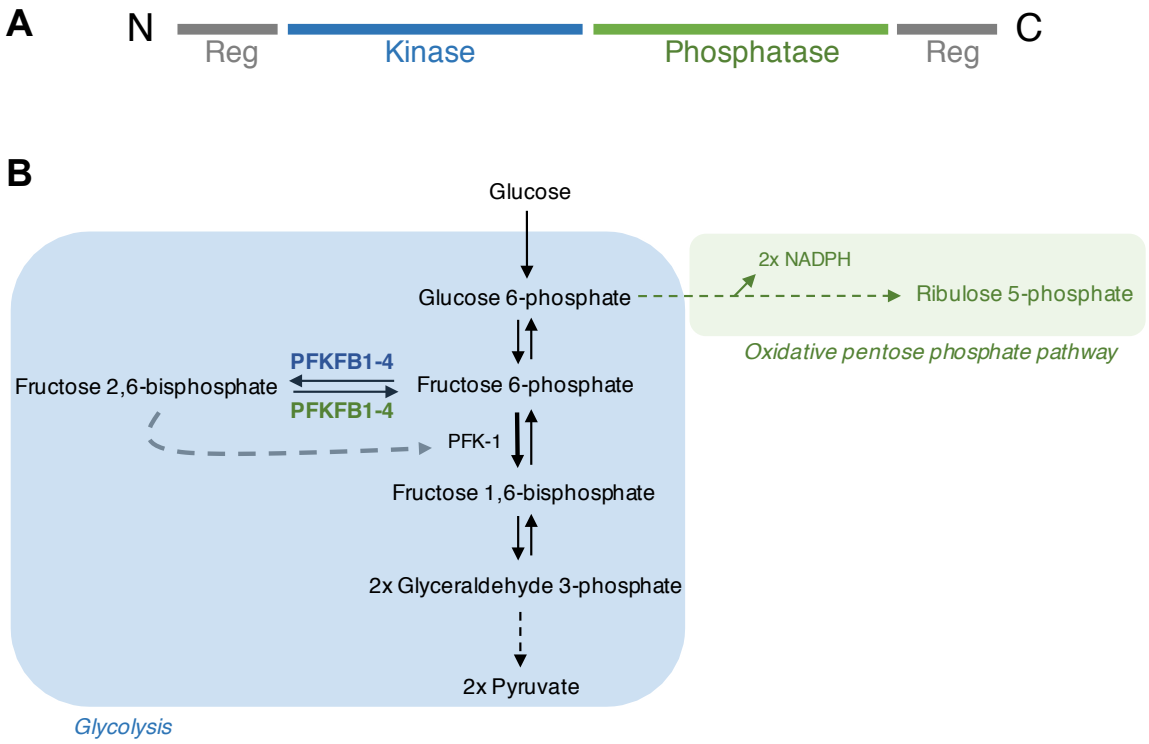


Figure 4.1. Schematic of PFKFB1-4 domain organisation and function.
(A) Domain organisation of the PFKFB1-4 family from the N-terminus to the C-terminus. (B) Schematic of where PFKFB1-4 function in relation to glycolysis and the pentose phosphate pathway. Reg, regulatory.

PFKFB enzymes in cancer

Due to their dual functionality, activity of the PFKFB family members could support cancer cell growth and survival in two main ways. First, through the production of fructose 2,6-bisphosphate, which allosterically stimulates PFK1 (Van Schaftingen and Hers, 1980; Van Schaftingen et al., 1981) and inhibits fructose 1,6-bisphosphatase (Pilkis et al., 1981; Van Schaftingen and Hers, 1981a), thus promoting aerobic glycolysis. Second, through the production of fructose 6-phosphate, which can either enter the glycolytic pathway to further enhance glycolysis, or be redirected to the pentose phosphate pathway for the production of NADPH for detoxification and lipid synthesis, and ribose 5-phosphate for nucleotide synthesis (Horecker, 2002).

All *PFKFB* mRNAs have been shown to be overexpressed in lung cancers compared to normal tissue (Minchenko et al., 2005b). PFKFB1 activity has been shown to promote growth in bladder cancer cells and this is a shared function

across the PFKFB family (Sun et al., 2016). Whereas PFKFB2 has been implicated in androgen-stimulated *de novo* lipogenesis in prostate cancer (Moon et al., 2011). While PFKFB3 and PFKFB4 have been identified as overexpressed in many cancer cell types (Atsumi et al., 2002; Minchenko et al., 2005a; Bobarykina et al., 2006; Chesney et al., 2014) and have been widely implicated in cancer cell pathogenesis (Minchenko et al., 2004; Calvo et al., 2006; Seo et al., 2011; Goidts et al., 2012; Ros et al., 2012; Yun et al., 2012; Clem et al., 2013; De Bock et al., 2013).

The role of PFKFB3 in cancer

Given the enhanced kinase activity of PFKFB3 and its potential in supporting the high glycolytic rate of transformed cells, it has been the most extensively studied isoform of the PFKFB family in cancer research. PFKFB3 activity can also be regulated by several proteins involved in tumourigenesis, for example HIF1α (Obach et al., 2004), AKT (Manes and El-Maghrabi, 2005) and PTEN (Garcia-Cao et al., 2012; Cordero-Espinoza and Hagen, 2013). PFKFB3 has two main splice variants, known as ubiquitous and inducible (Navarro-Sabate et al., 2001). The inducible isoform of *PFKFB3* is overexpressed in several human cancer cell lines compared to normal cells and is required for tumour cell growth *in vitro* and *in vivo* (Chesney et al., 1999). Moreover, the levels of PFKFB3 protein in breast, colon, lung, pancreatic, ovarian and prostate cancers are significantly higher compared to their non-tumourigenic counterparts (Atsumi et al., 2002). Not only is PFKFB3 overexpressed in cancer cells, but it is also highly phosphorylated at sites that promote its kinase activity, such as S461 (Bando et al., 2005), suggesting that it is also overactive.

Reduction of PFKFB3 activity by siRNA-mediated depletion, or the use of inhibitors, results in a reduction of fructose 2,6-bisphosphate, lactate and ATP (Calvo et al., 2006; Clem et al., 2008; Seo et al., 2011; Klarer et al., 2014), decreased cell proliferation (Xintaropoulou et al., 2015), decreased AKT phosphorylation (Huo et al., 2010; Xu et al., 2014) and a reduction in angiogenesis (De Bock et al., 2013; Schoors et al., 2014). In addition, PFKFB3 depletion increases ROS and induces autophagy (Klarer et al., 2014). PFKFB3 has also been shown to have an important role in preventing oxidative stress.

High levels of ROS can cause S-glutathionylation of PFKFB3 that reduce its kinase activity (Seo and Lee, 2014). They can also cause a reduction in PFKFB3 methylation that promotes its ubiquitination and degradation (Yamamoto et al., 2014). Both of these alterations to PFKFB3 result in increased NADPH levels, which enable cellular detoxification. Therefore, PFKFB3 could support cancer cell survival and proliferation by allowing continued energy metabolism in the presence of high ROS levels, thus reducing the risk of oxidative stress. The ability of PFKFB3 to promote the pentose phosphate pathway might also be important for the synthesis of lipids and nucleotides in cancer cells.

Unlike other PFKFB family members, PFKFB3 has a nuclear localisation signal in its C-terminus that can target it to the nucleus (Yalcin et al., 2009). Nuclear PFKFB3 can influence cell proliferation through its production of fructose 2,6-bisphosphate, which can directly increase the expression and activity of CDK1, resulting in proteasomal degradation of p27 (Yalcin et al., 2009; Yalcin et al., 2014). p27 degradation is crucial for the G1/S transition (Chu et al., 2008) and therefore a reduction in PFKFB3 can result in G1 arrest due to the maintenance of p27 (Yalcin et al., 2014). This is further supported by the presence of a KEN box motif in PFKFB3 (Herrero-Mendez et al., 2009), which can trigger its degradation via the ubiquitin ligase APC/C-CDH1 (Pfleger and Kirschner, 2000). APC/C-CDH1 is activated in late mitosis and inactivated in late G1 (Fang et al., 1998), indicating that PFKFB3 is downregulated between mitosis and G1, but able to increase in late G1 where it mediates p27 degradation. In addition, cells subjected to synchronisation display an increase in PFKFB3 levels and activity in late G1 (Tudzarova et al., 2011), again supporting the fact that in PFKFB3 has an important role in cell proliferation. Interestingly, siRNA mediated depletion of PFKFB3 results in G1/S arrest (Yalcin et al., 2014), while the inhibition of its kinase activity with the inhibitor, 3PO, induces arrest at G2 (Clem et al., 2008).

The role of PFKFB4 in cancer

The expression of *PFKFB4* mRNA has been found to be increased in a number of different cancer cell lines, including breast, colon (Minchenko et al., 2005a; Minchenko et al., 2005c), lung (Minchenko et al., 2005b; Chesney et al., 2014), gastric and pancreatic cancers (Bobarykina et al., 2006). PFKFB4 is known to be

critical for the survival of prostate cancer cells (Ros et al., 2012), hepatic cancer cells (Jeon et al., 2011) and glioma stem-like cells (Goidts et al., 2012). However, the expression and function of PFKFB4 appears to differ between cancer cell lines from different tissues. PFKFB4 depletion in glioma stem-like cells resulted in a reduction in the production of lactate and ATP, demonstrating a role for PFKFB4 in driving glycolysis (Goidts et al., 2012). However, this could be due to the low levels of PFKFB3 in glioma cells, making PFKFB4 the predominant PFKFB able to promote glycolysis, thus favouring its kinase activity. In contrast, PFKFB4 depletion in prostate cancer cells results in increased levels of fructose 2,6-bisphosphate, ROS and autophagy, and a decrease in NADPH levels and lipid synthesis (Ros et al., 2012; Strohecker et al., 2015). These data indicate the importance of the bisphosphatase activity of PFKFB4 in maintaining flux through the pentose phosphate pathway. Interestingly, the prostate cancer cell lines analysed in these studies displayed high levels of PFKFB3, which may result in PFKFB4 functioning predominantly as a bisphosphatase. However, other studies have demonstrated that PFKFB4 depletion in prostate, lung, breast and colon cancers decreased fructose 2,6-bisphosphate and ATP levels, and increased NADPH production (Chesney et al., 2014; Chesney et al., 2015). Moreover, PFKFB4 depletion has been shown to induce cell cycle arrest at G1, suggesting that it may be required for cell cycle progression.

Elucidating the precise roles of PFKFB3 and PFKFB4 enzymes in cancer is very difficult due to their bifunctional nature, apparent tissue-specific activities, and the fact that they possess the ability to adapt to a changing environment. This change could be caused by the altered expression of another family member, or a shift in the energy or redox balance of the cell.

Post-translational modifications of the PFKFB family

Only one post-translational modification has been identified on the liver isoform of PFKFB1, which is a phosphorylation at S32 by PKA that inhibits its kinase activity (Murray et al., 1984) (Figure 4.2). The 58kDa isoform of PFKFB2, but not the 54kDa variant, is subject to a number of post-translational modifications, including phosphorylation by AKT, MAPK-1, p70S6K (Deprez et al., 1997) and AMPK (Marsin et al., 2000), which enhance its kinase activity, and phosphorylation by

PKA and PKC that have not been found to alter activity (Rider and Hue, 1986; Kitamura et al., 1988; Rider et al., 1992). Both the inducible and ubiquitous isoforms of PFKFB3 are phosphorylated by AMPK (Marsin et al., 2002), PKA and PKC (Okamura and Sakakibara, 1998) at S461, which enhance the kinase activity of PFKFB3. Ubiquitous PFKFB3 can also be phosphorylated at S461 by AKT, which decreases its sensitivity to PEP inhibition, resulting in enhanced kinase activity (Manes and El-Maghrabi, 2005). PFKFB3 is also regulated via its KEN box, which enables APC/C-CDH1 to target it for degradation (Pfleger and Kirschner, 2000; Herrero-Mendez et al., 2009). Unlike the other PFKFB family members, no post-translational modifications have yet been identified for PFKFB4. Therefore, it is likely that PFKFB4 is regulated mainly by the concentrations of its substrates and products, or by the presence of kinase domain inhibitors, such as citrate and PEP (Van Schaftingen and Hers, 1981b), or phosphatase domain activators, such as GTP (Lee et al., 1994).

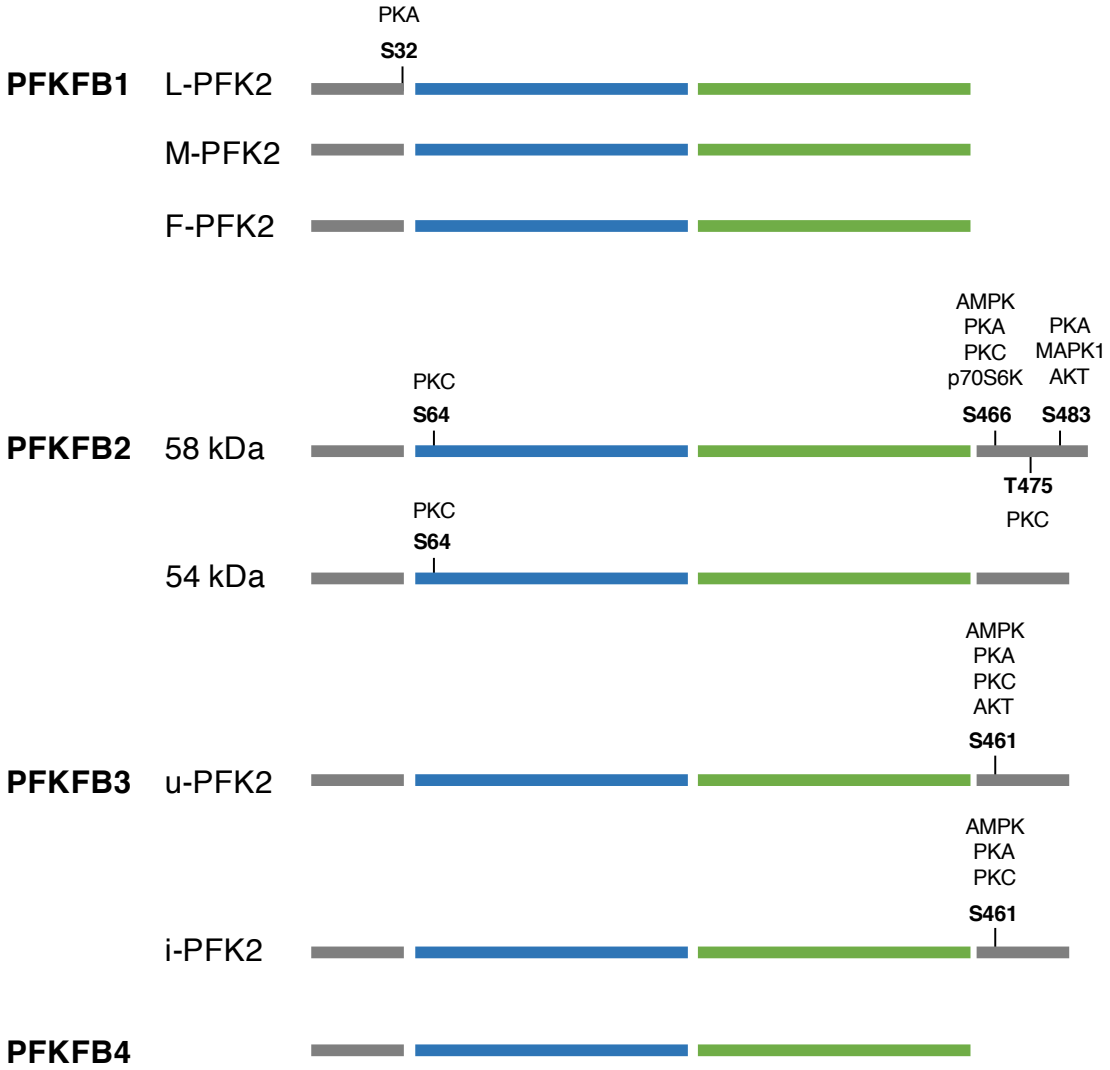


Figure 4.2. Schematic of the phosphorylation sites on PFKFB1-4.

The main splice variants for each enzyme are shown. The N-terminal phosphofructokinase domain is shown in blue, the C-terminal bisphosphatase domain in green and the regulatory domains in grey.

4.1.2. XYLB and the glucuronate-xylulose pathway

Xylulose kinase (XYLB) is the last enzyme in the glucuronate-xylulose pathway (Touster, 1960), which catalyses the ATP-dependent phosphorylation of D-xylulose to produce xylulose 5-phosphate (Figure 4.3). The glucuronate-xylulose pathway converts inositol and glucose sugars to glucuronate, and then to xylulose 5-phosphate (Touster, 1960). Xylulose 5-phosphate is an important signalling molecule that controls both acute and longterm regulation of lipogenesis and glycolysis in the liver, independently of insulin (Kabashima et al., 2003). Xylulose 5-phosphate can activate PP-2A (Nishimura and Uyeda, 1995), which causes the allosteric activation of the rate limiting enzyme of glycolysis, PFK1 (Van Schaftingen et al., 1980b). PP-2A does this through the dephosphorylation and activation of PFKFB1, resulting in increased levels of fructose 2,6-bisphosphate (Kabashima et al., 2003). Fructose 2,6-bisphosphate enhances the activity of PFK1 by increasing its affinity for fructose 6-phosphate (Van Schaftingen and Hers, 1980; Van Schaftingen et al., 1981) and diminishing the inhibitory effect of ATP (Wu et al., 2006). PP-2A activity can also upregulate the transcription of glycolytic and lipogenic pathways through carbohydrate-responsive element binding protein (ChREBP) (Kawaguchi et al., 2001). ChREBP is a transcription factor that in the presence of high glucose can upregulate *ACC*, *FAS* and liver-type *PK*, independently of insulin (Ishii et al., 2004).

Xylulose 5-phosphate can also be utilised in the non-oxidative branch of the pentose phosphate pathway. Two carbon units from xylulose 5-phosphate can be transferred to the ribose 5-phosphate generated from the oxidative arm of the pentose phosphate pathway by transketolase to produce sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate (Williams and Blackmore, 1983). These two products can be used to form fructose 6-phosphate and erythrose 4-phosphate via a three-carbon exchange reaction catalysed by transaldolase. Fructose 6-phosphate can then be utilised via glycolysis, or converted back to glucose 6-phosphate to replenish the oxidative pentose phosphate pathway. Xylulose 5-phosphate can also be converted to ribulose 5-phosphate by transketolase. Thus, xylulose 5-phosphate can affect both the pentose phosphate pathway and glycolysis.

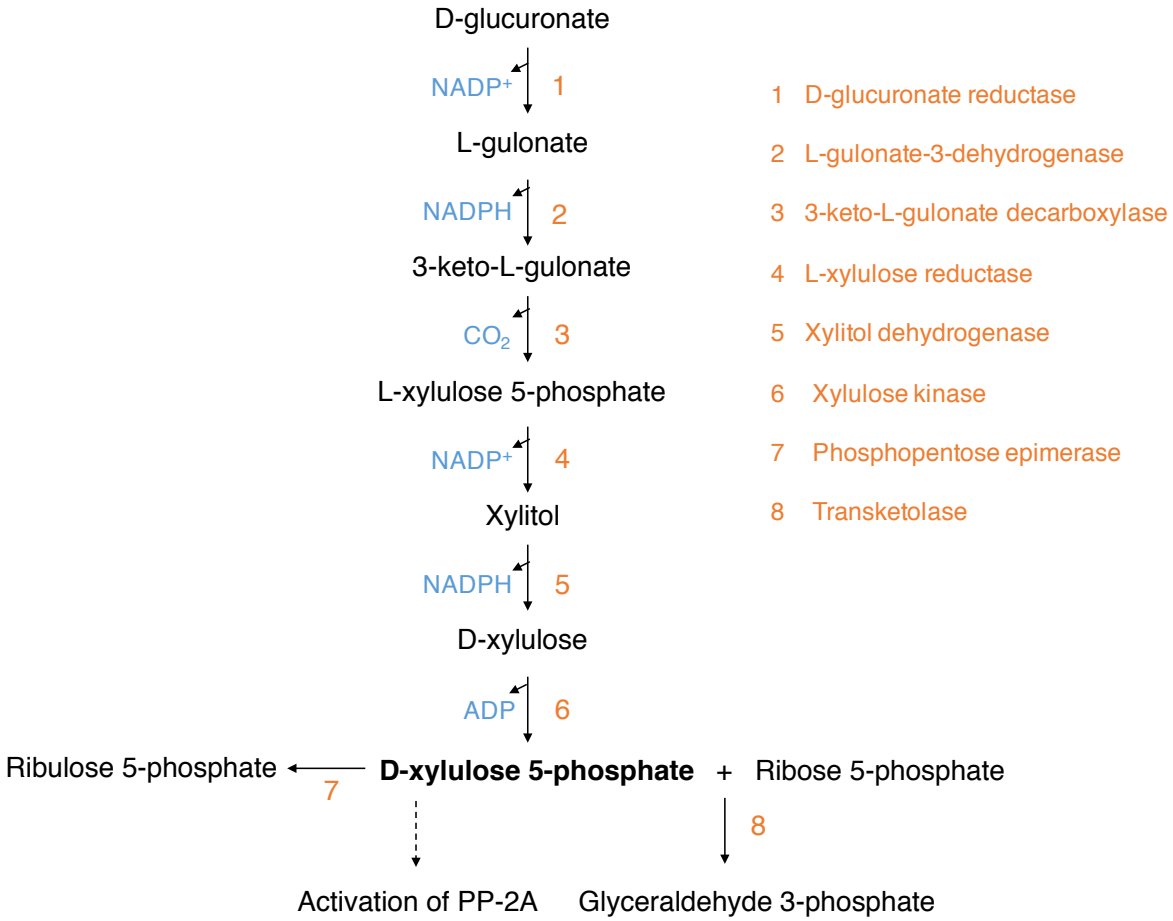


Figure 4.3. Schematic of the glucuronate-xylulose pathway.
D-xylulose 5-phosphate is the final product of the pathway, which can either activate PP-2A and subsequently PFK1, be converted to ribulose 5-phosphate, or be combined with ribose 5-phosphate to form glyceraldehyde 3-phosphate. The enzymes catalysing each reaction are labelled in orange.

4.1.3. Reactive oxygen species

Reactive oxygen species, such as superoxide anions and hydrogen peroxide, are derived from the partial reduction of oxygen and possess the ability to readily oxidise other molecules. Cellular ROS are generated endogenously from a number of sources, such as the mitochondria (Boveris and Chance, 1973), endoplasmic reticulum (Gross et al., 2006) and peroxisomes (Elliott et al., 1986; Zwacka et al., 1994), or can arise from exogenous sources, such as xenobiotic compounds. ROS have an important role in cell signalling and can influence a number of signalling pathways, including the MAPK pathway (Son et al., 2013) and the PI3K/AKT pathway. For example, ROS can enhance PI3K/AKT signalling through the oxidation and inactivation of PTEN (Lee et al., 2002; Kwon et al., 2004). Given the broad effects that ROS can have on signalling, it is important

that their abundance is tightly regulated. ROS levels are controlled by various methods, including physical compartmentalisation and the production of antioxidants. Antioxidants act to prevent ROS-mediated oxidation and are either enzymatic, such as superoxide dismutase, peroxiredoxins, glutathione peroxidases and catalase, or non-enzymatic, such as GSH and ascorbic acid (Nordberg and Arner, 2001). GSH protects cells from excessive ROS in a number of ways. For example, it is a cofactor for several detoxifying enzymes, it directly scavenges hydroxyl radicals and singlet oxygen, and it can regenerate antioxidants back to their active forms (Masella et al., 2005). If the levels of ROS become unmanageable and the cell is unable to produce an effective antioxidant response, oxidative stress can ensue. Oxidative stress is a deleterious process that causes damage to DNA, proteins and other macromolecules, and has been implicated in a number of human diseases, including cancer (Valko et al., 2006).

4.1.4. Mechanisms of mitotic cell death

Following mitotic arrest, cells often follow one of three fates; mitotic slippage, aberrant mitosis or mitotic cell death. The term mitotic catastrophe has been frequently used to describe cell death that occurs during or after aberrant mitoses (Molz et al., 1989; Roninson et al., 2001). However, there has been broader use of the term and many definitions have been suggested. In this thesis, I will use the term mitotic cell death (MCD) as referring to cell death in the mitotic phase. Currently, what determines the fate of a mitotically arrested cell is unknown. Cell fate appears to be stochastic and not genetically determined (Gascoigne and Taylor, 2008). Furthermore, the length of mitotic arrest does not correlate with MCD (Gascoigne and Taylor, 2008; Orth et al., 2008; Shi et al., 2008). MCD can occur through apoptosis, necrosis or autophagy (Vakifahmetoglu et al., 2008; Vitale et al., 2011) and appears to be modulated by the activity of a number of proteins, including PLKs (Burns et al., 2003; Spankuch et al., 2006), AKT (Lin et al., 2016), FoxM1 (Khongkow et al., 2016), survivin (Ho et al., 2009), p53 (Wahl et al., 1996; Burns et al., 2003; Kim et al., 2013) and the BCL-2 family (Puthalakath et al., 1999; Sakurikar et al., 2014). However, none are able to solely predict MCD.

Increased levels of cyclin B1 (Ling et al., 1998) and CDK1 activity (Ibrado et al., 1998) have also been implicated in MCD after paclitaxel treatment. Paclitaxel-induced CDK1 activity was found to be independent of caspase and BCL-2 activity, indicating that increased CDK1-cyclin B activity does not directly orchestrate MCD. It is likely that the maintenance of CDK1-cyclin B activity acts to prevent mitotic slippage and therefore enhances the likelihood of MCD. This is supported by Gascoigne and Taylor 2008 who suggested that cell fate is determined by the intricate relationship between cyclin B1 and caspase activity (Gascoigne and Taylor, 2008). If cyclin B1 levels fall below the mitotic exit threshold first, mitotic slippage occurs, whereas if caspase activity increases to a level that breaches the threshold for initiating cell death first, MCD prevails. This is supported by findings demonstrating a role of caspase activation in paclitaxel-induced MCD (Oyaizu et al., 1999; Lu et al., 2005). However, MCD has also been shown to be caspase-independent (Masuda et al., 2003; Mansilla et al., 2006; Vitale et al., 2011), indicating that caspase activity is not the sole instigator of MCD.

MCD has also been attributed to the energetic capacity of cells during mitotic arrest (Domenech et al., 2015). Mitotic arrest can increase mitophagy, which reduces oxidative respiration and ATP, forcing the cell to rely more heavily on glycolytic metabolism. This places a strong requirement on processes that promote glycolysis, such as AMPK-mediated PFKFB3 activation (Domenech et al., 2015). As such, if a cell is unable to adequately upregulate glycolysis during mitotic arrest, MCD can occur via mechanisms involving autophagy or apoptosis.

The mechanisms governing MCD vary tremendously, with cell fate being dependent on a number of factors, including cell-type, metabolic status, initiation of protein degradation or an increase in protein activity, as well as drug concentration and duration of exposure.

4.1.5. Chapter aims

Chapter 3 identified two enzymes that influence glycolytic metabolism, PFKFB4 and XYLB, which could be important for the survival of mitotically arrested SKOV3 cells. Confirmation of these results could lead to the discovery of novel targets that have the potential to enhance paclitaxel response in ovarian cancer. Therefore, the aims of this chapter were to:

- Validate the two high priority hits from the reanalysis of the screen data

- Investigate why the targeting of these enzymes could decrease the number of mitotically arrested ovarian cancer cells following paclitaxel treatment

4.2. Results

4.2.1. Validation of PFKFB4 and XYLB

Two high priority candidates identified from the screen were PFKFB4 and XYLB. In order to validate the screen results, the efficiency of the siRNA duplexes to mediate mRNA and protein depletion of PFKFB4 and XYLB were first investigated. Four individual siRNA duplexes against PFKFB4 and XYLB were tested with the aim of using an siRNA pool to reduce the likelihood of off-target effects. siRNA duplexes targeting XYLB provided varied results, with only siRNA 2, siRNA 17 and the siRNA pool obtaining more than a 50% reduction in XYLB mRNA expression (Figure 4.4A). Whereas with PFKFB4 targeting, siRNA 1, 2, 17 and the siRNA pool achieved more than a 50% reduction in PFKFB4 siRNA (Figure 4.4B). For both XYLB and PFKFB4, the pool of siRNA duplexes achieved approximately a 75% reduction in mRNA levels and thus were chosen for future experiments. The use of an siRNA pool also reduces off-target effects and thus the manifestation of false positive phenotypes. In SKOV3 cells, the use of pooled siRNA duplexes achieved approximately 75% reduction in XYLB mRNA and a 85% reduction in PFKFB4 mRNA (Figure 4.4C). This was accompanied by a large reduction in PFKFB4 protein levels (Figure 4.4D), but not XYLB protein (Figure 4.4E). Maintenance of XYLB protein expression may have been due to a slow turnover rate or high stability of the protein. However, extending the time of siRNA transfection to 96 h or performing subsequent transfections could not achieve sufficient depletion of XYLB protein. It is also possible that the antibody was non-specific. However, the detected protein was at the correct molecular weight and had been rigorously tested by the manufacturer.

Validation of the screen hits XYLB and PFKFB4 were carried out in SKOV3 cells,as this was the cell line used for the siRNA screen. However, in order to check whether the apparent phenotype was due to the vulnerability identified in chapter 3, the cell line A2780 was used as a negative control because, unlike other cell lines, A2780 cells did not possess the metabolic vulnerabilities identified during paclitaxel-induced mitotic arrest. siRNA-mediated depletion of *PFKFB4* and *XYLB* using pooled siRNA duplexes in A2780 cells achieved around an 80% reduction in *PFKFB4* mRNA and a 70% reduction in *XYLB* mRNA (Figure 4.4F).

However, at the protein level, PFKFB4 displayed only a 50% reduction (Figure 4.4G), while XYLB appeared to be unchanged (Figure 4.4H).

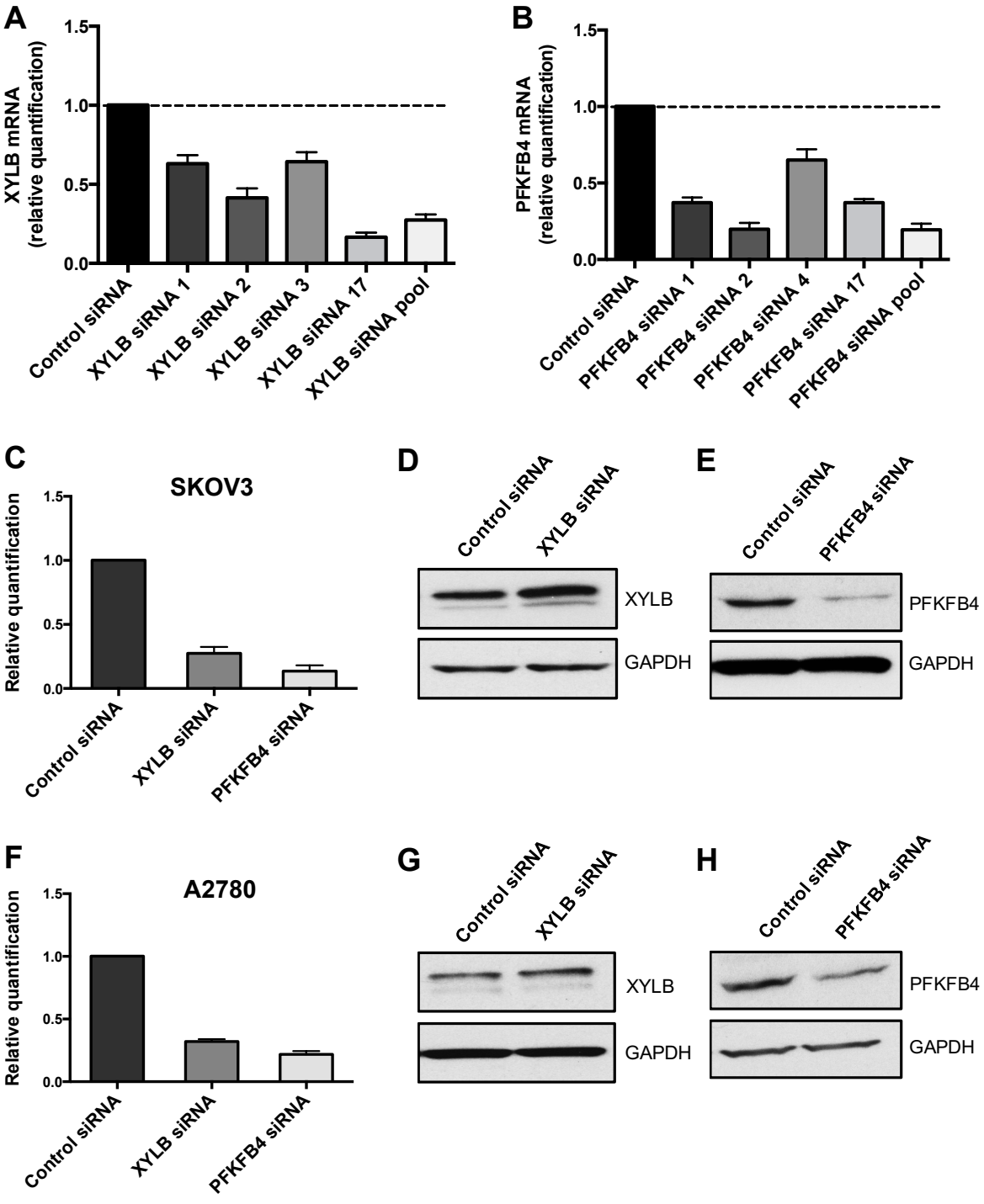


Figure 4.4. siRNA-mediated depletion of XYLB and PFKFB4.

(A, *n*=3) mRNA levels of *XYLB* and (B, *n*=3) *PFKFB4* in SKOV3 cells measured using quantitative PCR after transfection with siRNA duplexes for 72 h. (C, *n*=5) XYLB and PFKFB4 mRNA levels measured using quantitative PCR. (D, *n*=3) XYLB protein levels and (E, *n*=3) PFKFB4 protein levels quantified using western blotting after transfection with pooled siRNA duplexes in SKOV3 cells. (F, *n*=5) *XYLB* and *PFKFB4* mRNA levels measured using quantitative PCR. (G, *n*=3) XYLB protein levels and (H, *n*=3) PFKFB4 protein levels quantified using western blotting after transfection with pooled siRNA duplexes in A2780 cells. Data are represented as the mean + standard error of the mean for three independent experiments for qPCR.

Despite the inability to deplete XYLB at the protein level, the siRNA-mediated depletion of the mRNA was investigated to determine whether this had any phenotypic effects, indicating the possibility of off-target effects.

In order to replicate the screen, siRNA-mediated depletion of XYLB or PFKFB4 was performed prior to paclitaxel treatment for 16 hours and the quantification of the number of mitotic cells by immunofluorescence (Figure 4.5A). A significant decrease in the number of mitotically arrested SKOV3 cells were observed following the transfection of *XYLB* siRNA 2 ($p < 0.0001$), siRNA 3 ($p = 0.0301$), siRNA 17 ($p < 0.0001$) and the siRNA pool ($p < 0.0001$) (Figure 4.5B, two-way ANOVA with multiple comparisons). *XYLB* siRNA 1 did not result in a significant reduction in mitotically arrested cell number. *XYLB* siRNA 17 largely reduced the number of mitotic cells in the untreated population. However, this difference was not found to be statistically significant ($p = 0.7512$, two-way ANOVA with multiple comparisons). A significant reduction in the number of mitotically arrested SKOV3 cells were also observed following transfection of *PFKFB4* siRNA 1 ($p < 0.0001$), siRNA 2 ($p < 0.0001$), siRNA 4 ($p = 0.0006$), siRNA 17 ($p < 0.0001$) and the siRNA pool ($p < 0.0001$)(Figure 4.5C, two-way ANOVA with multiple comparisons). No significant differences were found between the *PFKFB4* siRNA duplexes and non-targeting control in the untreated condition. In addition, the magnitude of the depletion of mitotically arrested cells broadly correlated with the extent of mRNA depletion for both *XYLB* and *PFKFB4* siRNA duplexes. An increase in multinucleate cells were also observed by immunofluorescence following both XYLB or PFKFB4 depletion and paclitaxel treatment, which indicates a possible increase in mitotic slippage.

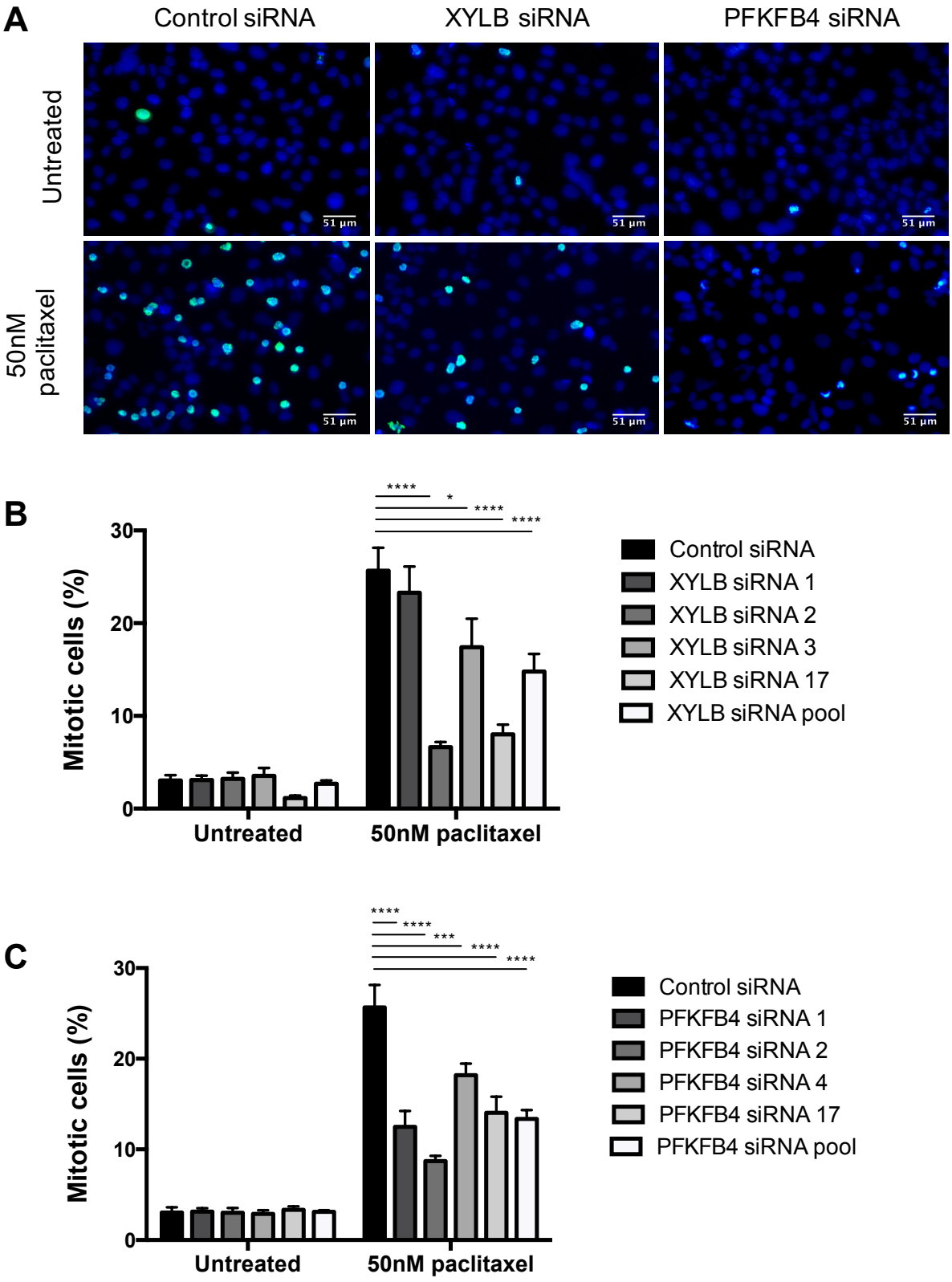


Figure 4.5. Effects of XYLB or PFKFB4 depletion on the mitotic index of SKOV3 cells following paclitaxel treatment.

(A, *n*=2) Immunofluorescence images of cells stained with DAPI (blue) and mitotic cells stained with anti-phosphohistone H3 S10 (green) after PFKFB4 or XYLB depletion using pooled siRNA duplexes followed by paclitaxel or vehicle control. (B, *n*=3) Manual quantification of the percentage of mitotic cells were quantified for XYLB and (C, *n*=3) PFKFB4 siRNA duplexes. Data are represented as the mean + standard error of the mean for three independent experiments. *≤0.05–0.01, ***<0.001. and ****<0.0001.

In order to analyse these data in a high-throughput manner, the number of mitotic cells were quantified by flow cytometry. In agreement with the immunofluorescence quantification, XYLB depletion significantly reduced the number of mitotically arrested cells ($p = 0.0013$) as did PFKFB4 depletion ($p < 0.0001$) (Figure 4.6, two-way ANOVA with multiple comparisons). However, the extent of XYLB depletion on mitotic cell number was far less than the immunofluorescence indicated. This could have been due to the small sample size of the immunofluorescence compared to the large number of cells analysed using flow cytometry. PFKFB4 depletion also caused a small reduction in the number of mitotic cells in the untreated condition, which was observed in the siRNA screen, but this was not found to be statistically significant.

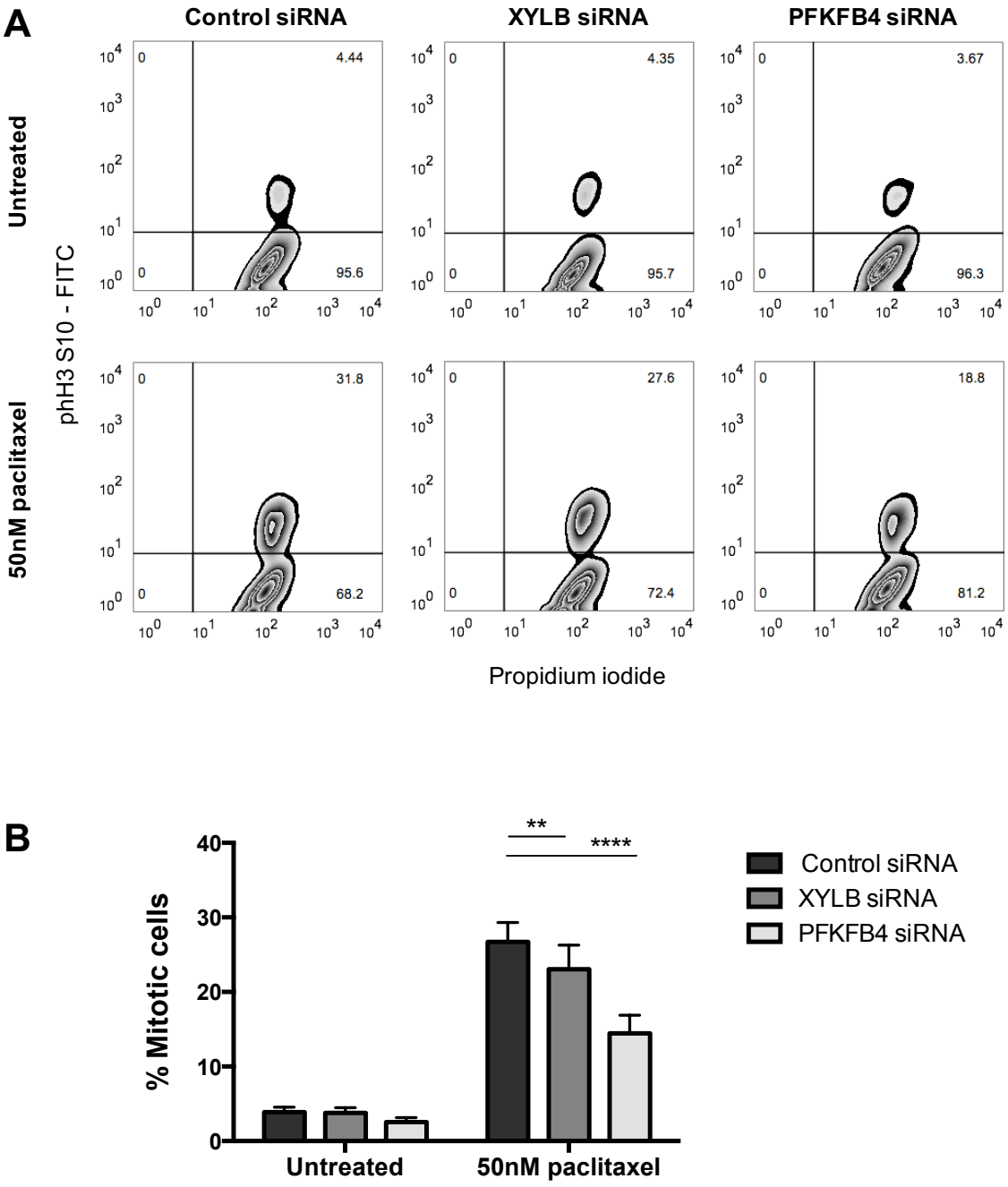


Figure 4.6. Effects of XYLB or PFKFB4 depletion on the mitotic index of SKOV3 cells following paclitaxel treatment measured by flow cytometry.

(A, *n*=5) Representative flow cytometry plots after PFKFB4 or XYLB depletion using pooled siRNA duplexes followed by treatment with paclitaxel or vehicle control. Cells were permeabilised and stained with anti-phospho histone H3 (phH3 S10) and propidium iodide. 100,000 cells were analysed for each condition. (B, *n*=5) Quantification of the percentage of mitotic cells for each condition. Data are represented as the mean + standard error of the mean for five independent experiments. **<0.01–0.001 and ****<0.0001.

These data confirm the results of the siRNA screen with siRNA treatment of XYLB or PFKFB4 causing a significant decrease in the number of mitotically arrested SKOV3 cells after paclitaxel treatment. However, siRNA targeting *XYLB* did not reduce XYLB protein levels, yet there was a reduction in the number of mitotically arrested cells after paclitaxel treatment. This suggests that the siRNA duplexes are producing off-target effects and therefore, XYLB will not be followed up as a potential target.

To determine whether the effect of PFKFB4 depletion on the number of mitotically arrested cells was due to the vulnerability that they possess during mitotic arrest, the analysis was repeated in A2780 cells, the cell line that maintained AKT signalling during mitotic arrest. There appeared to be no substantial effect of siRNA-mediated depletion of PFKFB4 on the number of mitotically arrested cells when using immunofluorescence staining (Figure 4.7). However, the immunofluorescence images could not be quantified due to the nature of the A2780 cells. A2780 cells grow in clumps, which makes it very difficult to accurately assess the number of cells. Nonetheless, analysis using flow cytometry displayed no significant change in the number of mitotically arrested cells after PFKFB4 depletion (Figure 4.8). This indicates that there are key differences between the two ovarian cancer cells lines A2780 and SKOV3, with SKOV3 cells being vulnerable to PFKFB4 depletion during mitotic arrest and A2780 cells being resistant.

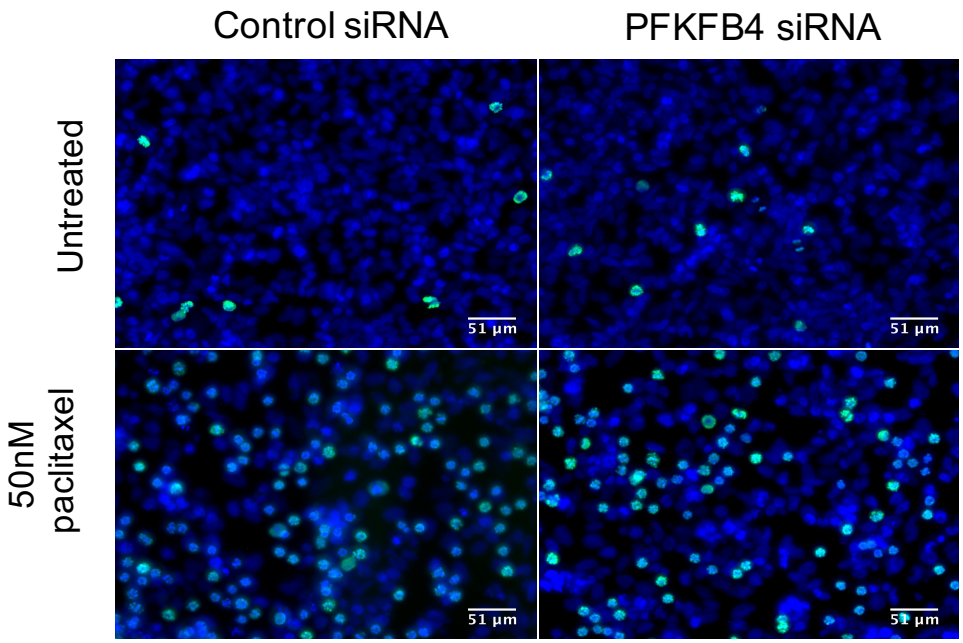


Figure 4.7. Effects of XYLB or PFKFB4 depletion on the mitotic index of A2780 cells following paclitaxel treatment.

Immunofluorescence images of cells stained with DAPI (blue) and mitotic cells stained with anti-phosphohistone H3 S10 (green) after PFKFB4 or XYLB depletion using pooled siRNA duplexes followed by paclitaxel or vehicle control. Displaying one of two independent experiments.

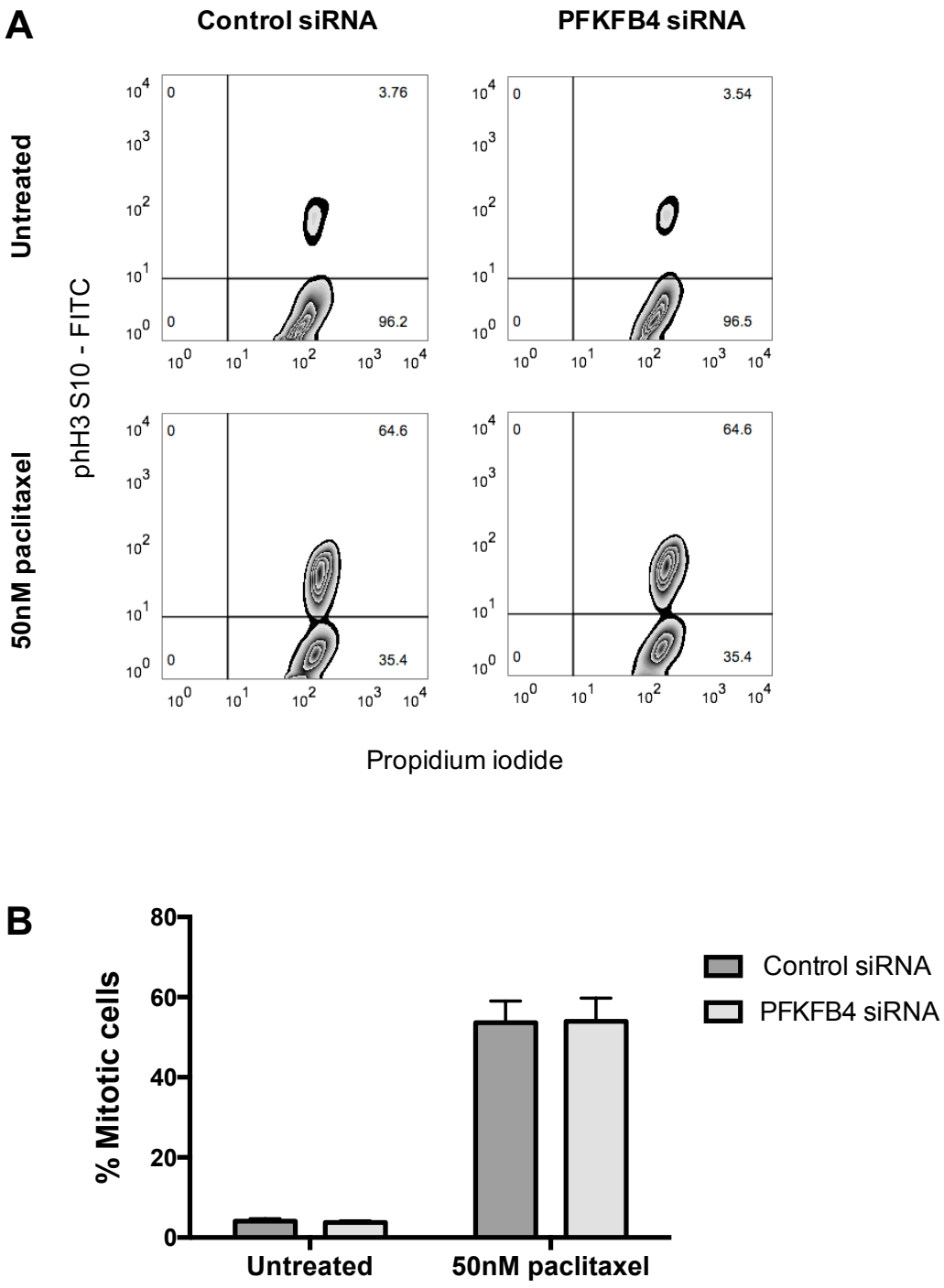


Figure 4.8. Effects of PFKFB4 depletion on the mitotic index of A2780 cells following paclitaxel treatment measured by flow cytometry.

(A, *n*=4) Representative flow cytometry plots after PFKFB4 depletion using pooled siRNA duplexes followed by treatment with paclitaxel or vehicle control. Cells were permeabilised and stained with anti-phospho histone H3 (phH3 S10) and propidium iodide. 100,000 cells were analysed for each condition. (B, *n*=4) Quantification of the percentage of mitotic cells for each condition. Data are represented as the mean + standard error of the mean for four independent experiments.

Before continuing, it was tested whether another cell line, OC 316, that was deemed vulnerable in chapter 3 due to a reduction in AKT activity and ATP levels was susceptible to PFKFB4 depletion during mitotic arrest. As such, siRNA-mediated depletion of PFKFB4 was assessed in OC 316 cells and a significant reduction in mitotically arrested cells was found after paclitaxel treatment (Figure 4.9, $p = 0.006$, two-way ANOVA with multiple comparisons).

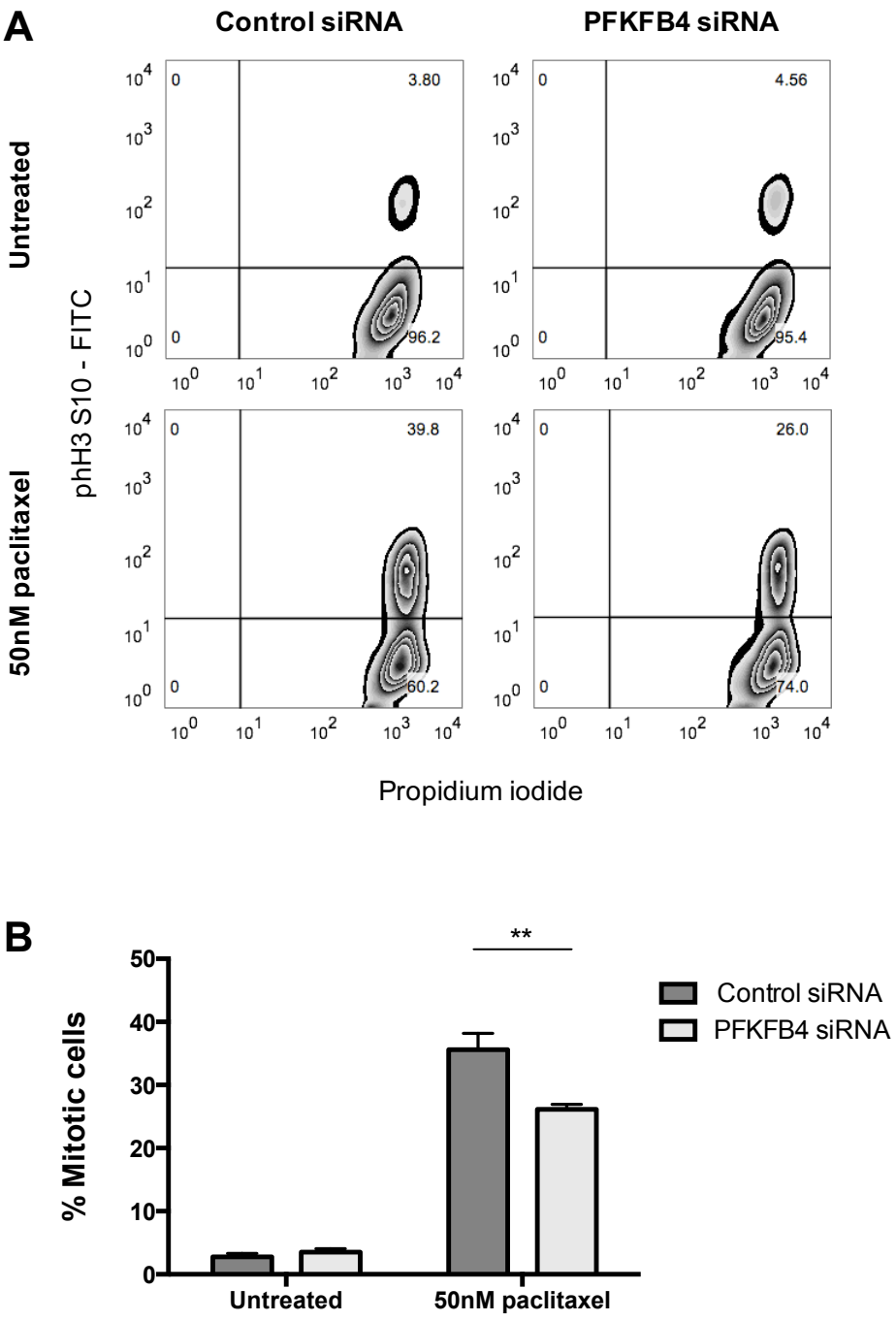


Figure 4.9. Effects of PFKFB4 depletion on the mitotic index of OC 316 cells following paclitaxel treatment measured by flow cytometry.

(A, *n*=3) Representative flow cytometry plots after PFKFB4 depletion using pooled siRNA duplexes followed by treatment with paclitaxel or vehicle control. Cells were permeabilised and stained with anti-phospho histone H3 (phH3 S10) and propidium iodide. 100,000 cells were analysed for each condition. (B, *n*=3) Quantification of the percentage of mitotic cells for each condition. Data are represented as the mean + standard error of the mean for three independent experiments. **<0.01–0.001.

4.2.2. PFKFB4 depletion can increase mitotic cell death in paclitaxel treated ovarian cancer cells

The second aim of this chapter was to investigate why siRNA targeting *PFKFB4* could result in a reduction in the number of mitotically arrested SKOV3 cells after paclitaxel treatment. I postulated that PFKFB4 depletion could achieve this reduction through three main mechanisms; inducing mitotic cell death, arresting cell cycle progression or increasing mitotic slippage, or a combination of all three. It has previously been reported that inhibition of PFKFB4 can lead to cell cycle arrest at G1 (Chesney et al., 2015), so I first sought to examine the effects of PFKFB4 depletion on cell cycle progression using propidium iodide staining and flow cytometry (Figure 4.10). A slight increase in cells in the G2/M phase was observed after PFKFB4 depletion compared to the non-targeting control (38.7 vs 35.1). This could indicate an increase in mitotic slippage as tetraploid cells would fall into the G2/M population of cells. Therefore, a cell cycle effect could also be contributing to the decrease in mitotic cell number after PFKFB4 depletion and paclitaxel treatment. However, it is unlikely that this would have been solely responsible for the 50% reduction in the number of mitotically arrested cells that are present after paclitaxel treatment. Moreover, previous studies have found that PFKFB4 inhibition or depletion can result in a G1 cell cycle arrest, which was not observed in this work. This may be due to differences in the method of siRNA-mediated depletion or the fact that the effect is cell-type specific.

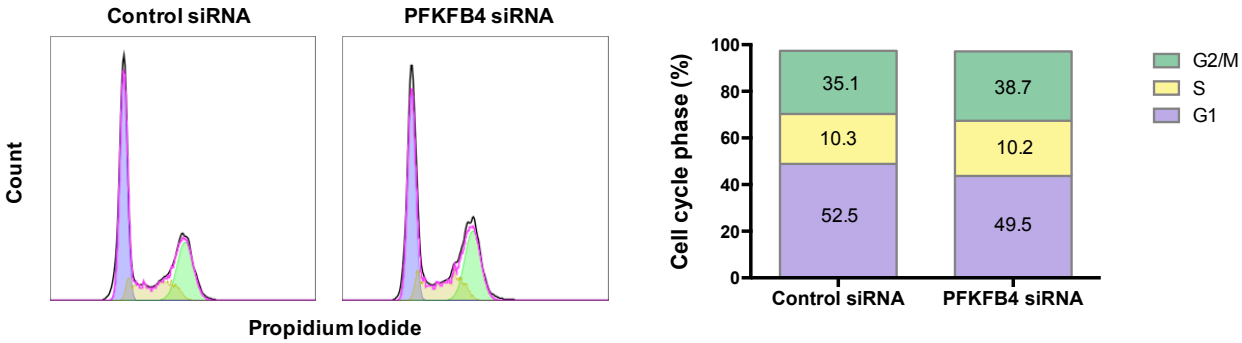


Figure 4.10. Cell cycle analysis after PFKFB4 depletion in SKOV3 cells.
Following PFKFB4 depletion using pooled siRNA duplexes, cells were stained with propidium iodide and subjected to flow cytometry to analyse the distribution of cells across the cell cycle. Flow cytometry histograms and quantification represent one of two experiments. 20,000 cells analysed for each condition.

To investigate whether the required activity of PFKFB4 is mitosis-specific, time-lapse microscopy was performed to follow the fate of mitotic SKOV3 cells for a minimum of 16 hours after the addition of paclitaxel following PFKFB4 depletion (Figure S4.1A-D). A significant increase in MCD was observed after PFKFB4 depletion compared to the non-targeting control (Figure 4.11A, $p = 0.0056$, two-way ANOVA with multiple comparisons), indicating that PFKFB4 is important for ovarian cancer cell survival during prolonged mitosis. There was also a significant increase in cells undergoing mitotic slippage (Figure 4.11B, $p = 0.0028$, two-way ANOVA with multiple comparisons), indicating that the reduction in mitotic cell number is likely to be due to the combined effects of an increase in MCD and mitotic slippage. In support of enhanced MCD, the activity of caspases 3 and 7 were significantly elevated in mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control (Figure 4.12A, $p = 0.0016$, two-way ANOVA with multiple comparisons). The increased activity of caspases 3 and 7 suggests that PFKFB4 depletion may induce apoptosis during prolonged mitosis, thus causing a decrease in the number of mitotically arrested cells (McIlwain et al., 2013). Moreover, the incorporation of PI was measured as an indicator of cell death and there was a significant increase in PI staining in mitotically arrested cells after PFKFB4 depletion when compared to the non-targeting control (Figure 4.12B, $p = 0.0081$, two-way ANOVA with multiple comparisons). This data further confirms that PFKFB4 depletion in mitotically arrested cells acts to increase MCD.

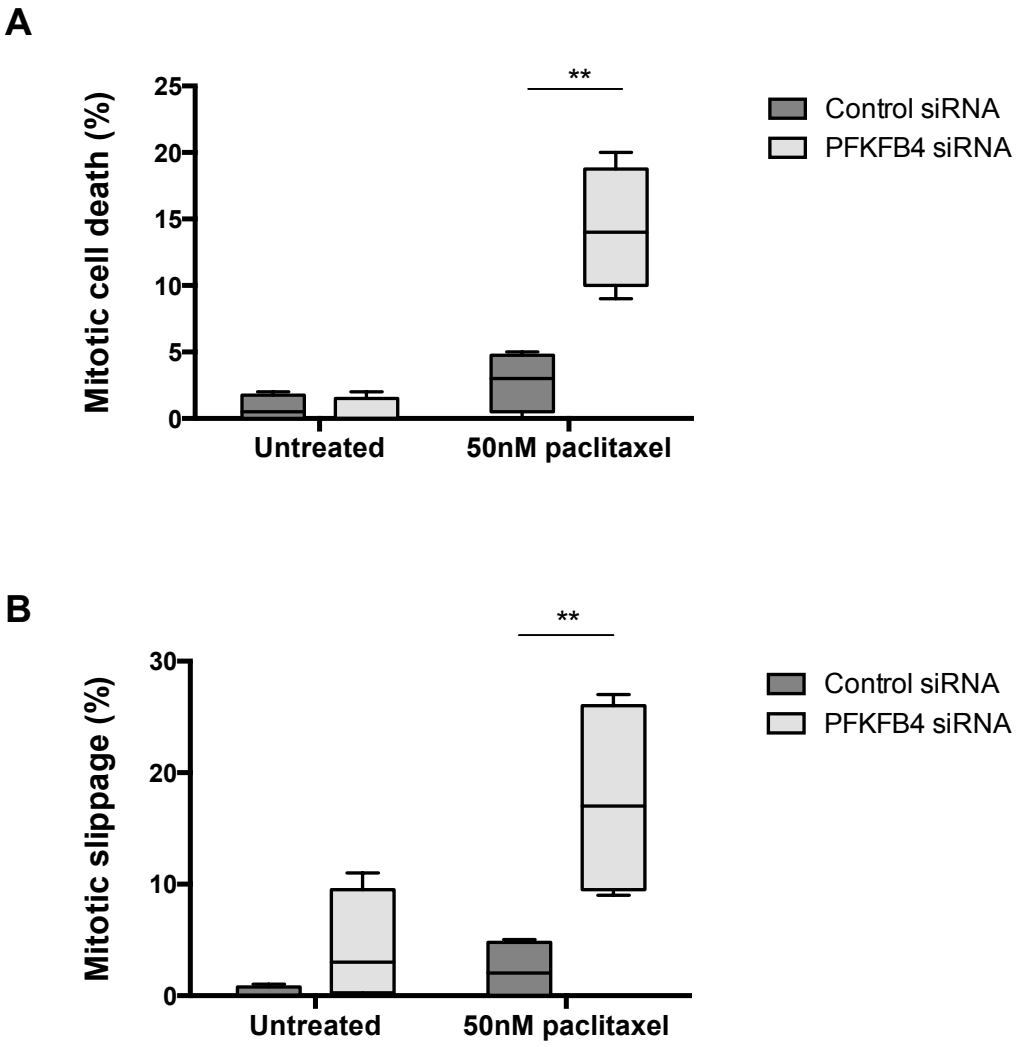


Figure 4.11. Effects of PFKFB4 depletion on mitotically arrested SKOV3 cells.

(A, *n*=3) Time-lapse microscopy following over 200 mitotically arrested cells in each condition quantified the percentage of mitotic cell death and (B, *n*=3) mitotic slippage. Data are represented as the mean + standard error of the mean for three independent experiments. **<0.01–0.001.

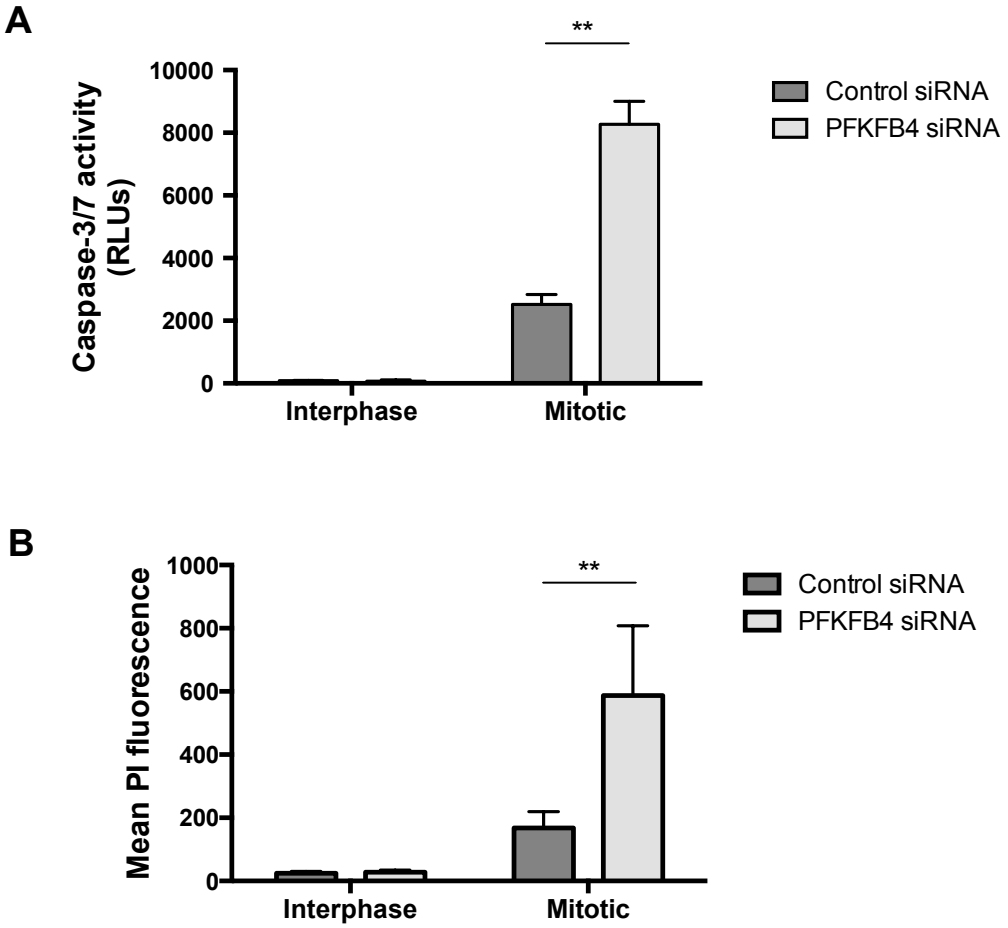


Figure 4.12. Mitotic cell death after PFKFB4 depletion and paclitaxel treatment in SKOV3 cells.

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, mitotically arrested cells were isolated by mitotic shake-off and caspase-3/7 activity was quantified using a luminescence assay. (B, *n*=3) Propidium iodide (PI) incorporation was also measured using flow cytometry. Data are represented as the mean + standard error of the mean for three independent experiments. **<0.01–0.001.

Considering the dual role of PFKFB4, siRNA-mediated depletion may principally affect the flux through glycolysis, the pentose phosphate pathway, or both. Given that a number of studies have demonstrated a decrease in fructose 2,6-bisphosphate and ATP after PFKFB4 depletion (Chesney et al., 2014; Chesney et al., 2015), it could be hypothesised that the increase in MCD after PFKFB4 depletion is due to a further decrease in ATP levels during mitotic arrest. Substantial decreases in ATP concentration have been shown to trigger apoptosis (Feldenberg et al., 1999; Izyumov et al., 2004; Nieminen et al., 2013). However, an analysis of AMPK phosphorylation at T172, an activating site within the T-loop, suggests that PFKFB4 depletion does not alter the ATP/AMP ratio and may even cause a slight decrease in AMPK activity (Figure 4.13A). Measurement of ACC phosphorylation further confirmed these findings, with no apparent increase in phosphorylation at S79 being observed in the mitotic population after PFKFB4 depletion and paclitaxel treatment (Figure 4.13B). Taken together, these data suggest that the increase in mitotic cell death after PFKFB4 depletion and paclitaxel treatment is not due to a critical decrease in ATP levels and subsequent energetic stress. The fact that mitotically arrested ovarian cancer cells do not appear to be energetically stressed after PFKFB4 depletion indicates that either glycolytic flux is not significantly affected, or that the cells rewire their metabolism to increase aerobic respiration, thus maintaining ATP levels.

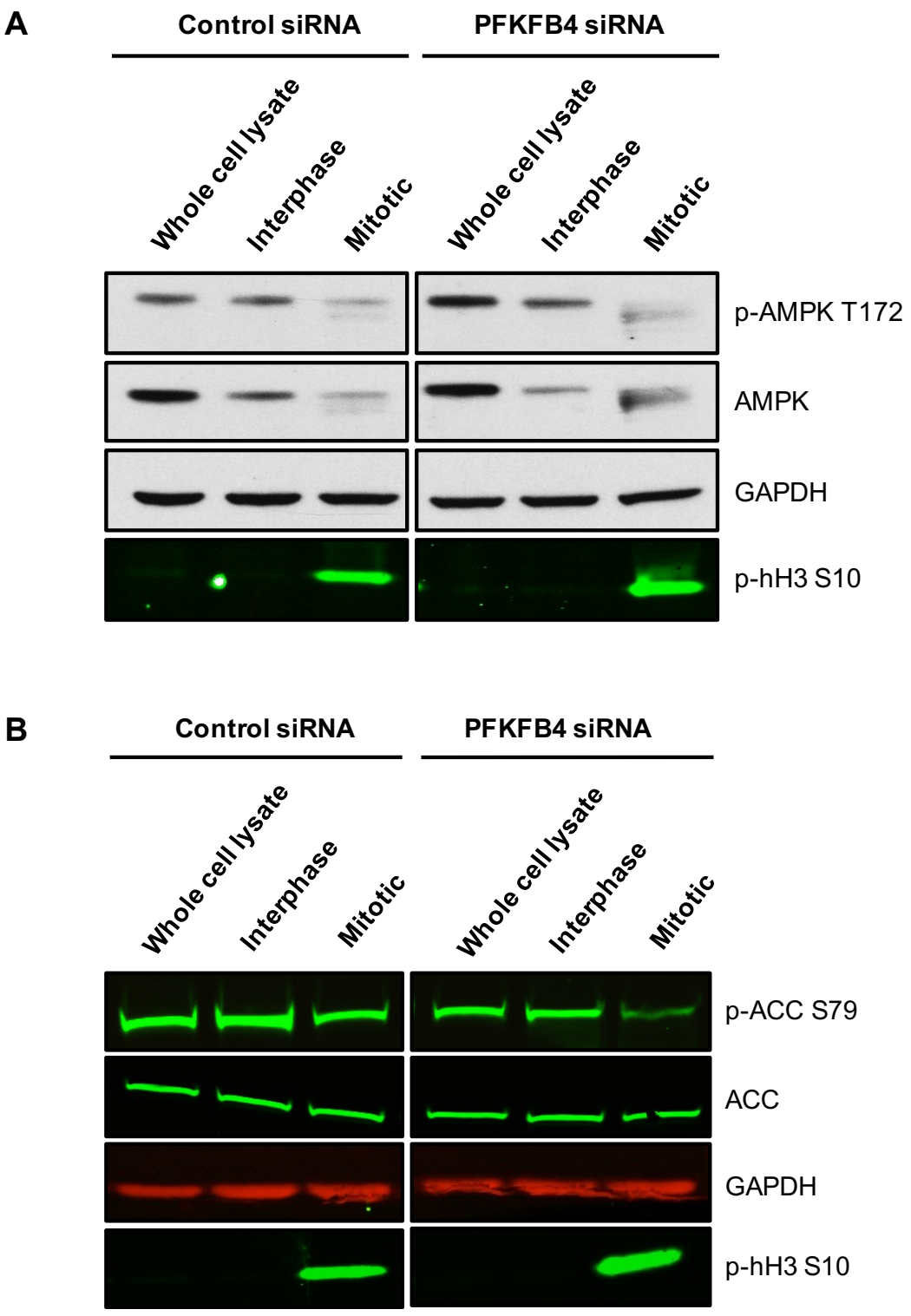


Figure 4.13. AMPK and ACC activity in PFKFB4 depleted mitotically arrested SKOV3 cells.
(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, mitotically arrested cells were isolated by mitotic shake-off and levels of AMPK protein and phosphorylated AMPK were analysed by western blotting. (B, *n*=3) Levels of ACC and phosphorylated ACC were also measured by western blotting. Images are representative of one of three experiments.

A number of studies suggest that PFKFB4 has a predominant bisphosphatase activity making it an important regulator of cellular redox reactions and lipid synthesis (Ros et al., 2012; Strohecker et al., 2015). Given these data, I hypothesised that the levels of ROS may be increased after PFKFB4 depletion due to an increase in aerobic respiration and mitochondrial leakage, or a decrease in metabolic flux into the pentose phosphate pathway resulting in a decline in NADPH levels and reduced GSH levels. Levels of ROS were measured in mitotic and interphase cells after PFKFB4 depletion followed by paclitaxel treatment. ROS were significantly increased in mitotic cells after PFKFB4 depletion compared to the non-targeting control (Figure 4.14A, $p = 0.0124$, two-way ANOVA with multiple comparisons). These data support previous studies, which demonstrate that PFKFB4 depletion can result in an increase in ROS in prostate cancer cell lines (Ros et al., 2012; Strohecker et al., 2015). However, the data presented here suggests that the increase in ROS occurs specifically during prolonged mitotic arrest and not during interphase. No significant differences in levels of ROS were found after PFKFB4 depletion alone compared to the non-targeting control (Figure 4.14B). This could be due to differences in the cell lines analysed or may identify a novel role of PFKFB4 during mitotic arrest.

An increase in ROS were also observed in mitotic cells in the non-targeting control. Paclitaxel treatment is known to increase ROS (Alexandre et al., 2006; Domenech et al., 2015). However, this increase has not been identified to be specifically in the mitotically arrested cells and not the interphase population. An increase in ROS can activate AMPK in a calcium-dependent manner (Mungai et al., 2011), but I have just demonstrated that the phosphorylation of AMPK was not increased in the mitotically arrested cells after PFKFB4 depletion in this study. This could be due to ROS levels not reaching the required threshold for AMPK activation, or due to a mechanism preventing its activation during paclitaxel-induced mitotic arrest.

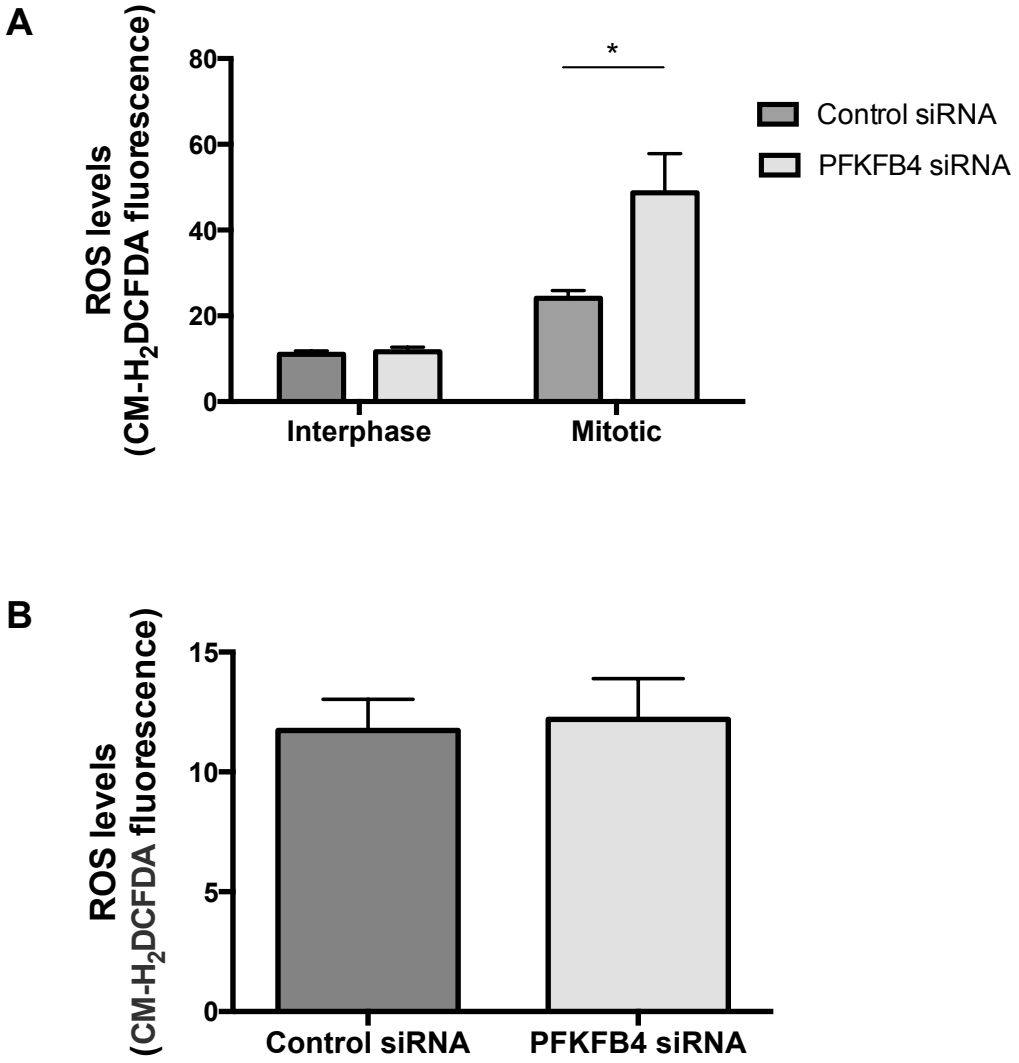


Figure 4.14. Levels of reactive oxygen species (ROS) after PFKFB4 depletion in SKOV3 cells.

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, mitotically arrested cells were isolated by mitotic shake-off and ROS were measured using flow cytometry. 50,000 cells were analysed for each condition. (B, *n*=3) ROS were also measured following PFKFB4 depletion alone, without paclitaxel treatment. 100,000 cells were analysed for each condition. Data are represented as the mean + standard error of the mean for three independent experiments. *≤0.05–0.01.

It is unclear whether the increase in ROS in mitotically arrested cells after PFKFB4 depletion contributes to the increase in cell death as it could not be rescued with N-acetylcysteine (NAC). NAC is a scavenger of ROS (Dodd et al., 2008) and has previously been shown to reduce levels of ROS following PFKFB4 depletion (Strohecker et al., 2015). Literature searches and optimisation experiments confirmed that 5 mM was an appropriate concentration of NAC to use, so cell medium was supplemented with 5 mM NAC during siRNA transfection. Levels of ROS in mitotically arrested cells could be returned to baseline after PFKFB4 depletion using 5 mM NAC (Figure 4.15A). However, this did not prevent the decrease in the number of mitotically arrested cells compared to the non-targeting control (Figure 4.15B, $p < 0.0001$, two-way ANOVA with multiple comparisons), nor did it rescue the increase in caspase-3 and 7 activity in mitotically arrested cells (Figure 4.15C, $p = 0.0008$, two-way ANOVA with multiple comparisons). Therefore, it could be that the increase in ROS is simply a byproduct of cell death and not the causative factor. However, NAC has been shown to induce cell cycle arrest at G1 (Kim et al., 2001b), thus decreasing the number of cells in G2/M, and therefore may act to potentiate the effects of paclitaxel treatment. Interestingly, a decrease in the number of mitotic cells was observed after PFKFB4 depletion in the untreated sample without a corresponding increase in caspase activity. The decrease in mitotic cell number was not found to be statistically significant, but does indicate that the depletion of PFKFB4 could be introducing cell cycle defects and affecting spontaneous mitosis.

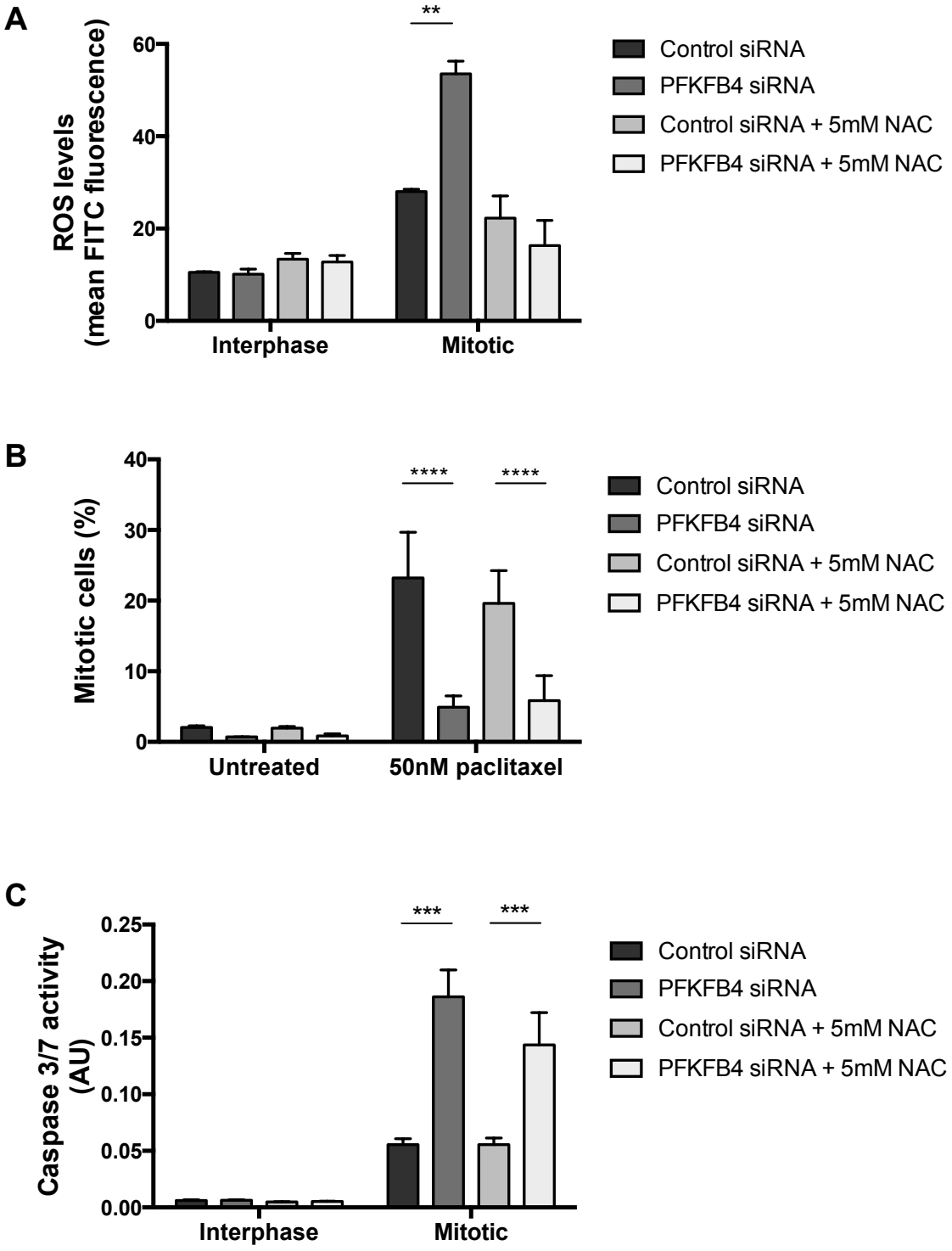


Figure 4.15. NAC treatment after PFKFB4 depletion and paclitaxel treatment in SKOV3 cells.

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, with or without N-acetylcysteine (NAC), mitotically arrested cells were isolated by a mitotic shake-off and ROS were measured using flow cytometry. 50,000 cells were analysed for each condition. (B, *n*=3) The percentage of mitotic cells were quantified using anti-phospho histone H3 S10 staining and flow cytometry. 100,000 cells were analysed for each condition. (C, *n*=3) Caspase-3/7 activity was quantified in mitotically arrested cells using a luminescence assay and normalised to cell viability readings. Data are represented as the mean + standard error of the mean for three independent experiments. *0.01–0.001, **<0.01–0.001, ***<0.001 and ****<0.0001.

4.2.3. Expression of PFKFB1-4 in ovarian cancer cells

Given that PFKFB4 depletion resulted in a reduction in the number of mitotically arrested cells after paclitaxel treatment, I investigated whether PFKFB4 was the predominant family member in ovarian cancer cells. Therefore, a quantitative PCR was performed to assess the mRNA levels of *PFKFB1-4* in a panel of five ovarian cancer cell lines. Results of the quantitative PCR demonstrated little difference between the expression of the *PFKFB* family members within each ovarian cancer cell line (Figure 4.16A). SKOV3 cells were the only cell line to possess a large reduction in *PFKFB1* mRNA compared to *PFKFB2-4* mRNA levels. However, this is unlikely to sensitise SKOV3 to PFKFB4 depletion as OC 316 do not possess this reduction in *PFKFB1* and are also sensitive to *PFKFB4* depletion. In addition to this analysis, *PFKFB1-4* gene expression were analysed from 420 ovarian cancers in The Cancer Genome Atlas (TCGA), which suggested that *PFKFB3* is the most abundant at the mRNA level, with *PFKFB4* the next most abundant (Figure 4.16B). Considering that *PFKFB3* appears to be more highly expressed than *PFKFB4* in the large scale analysis carried out by the TCGA, it begged the question as to whether PFKFB3 is also important for the survival of mitotically arrested cells and if so, why it was not identified in the high content siRNA screen.

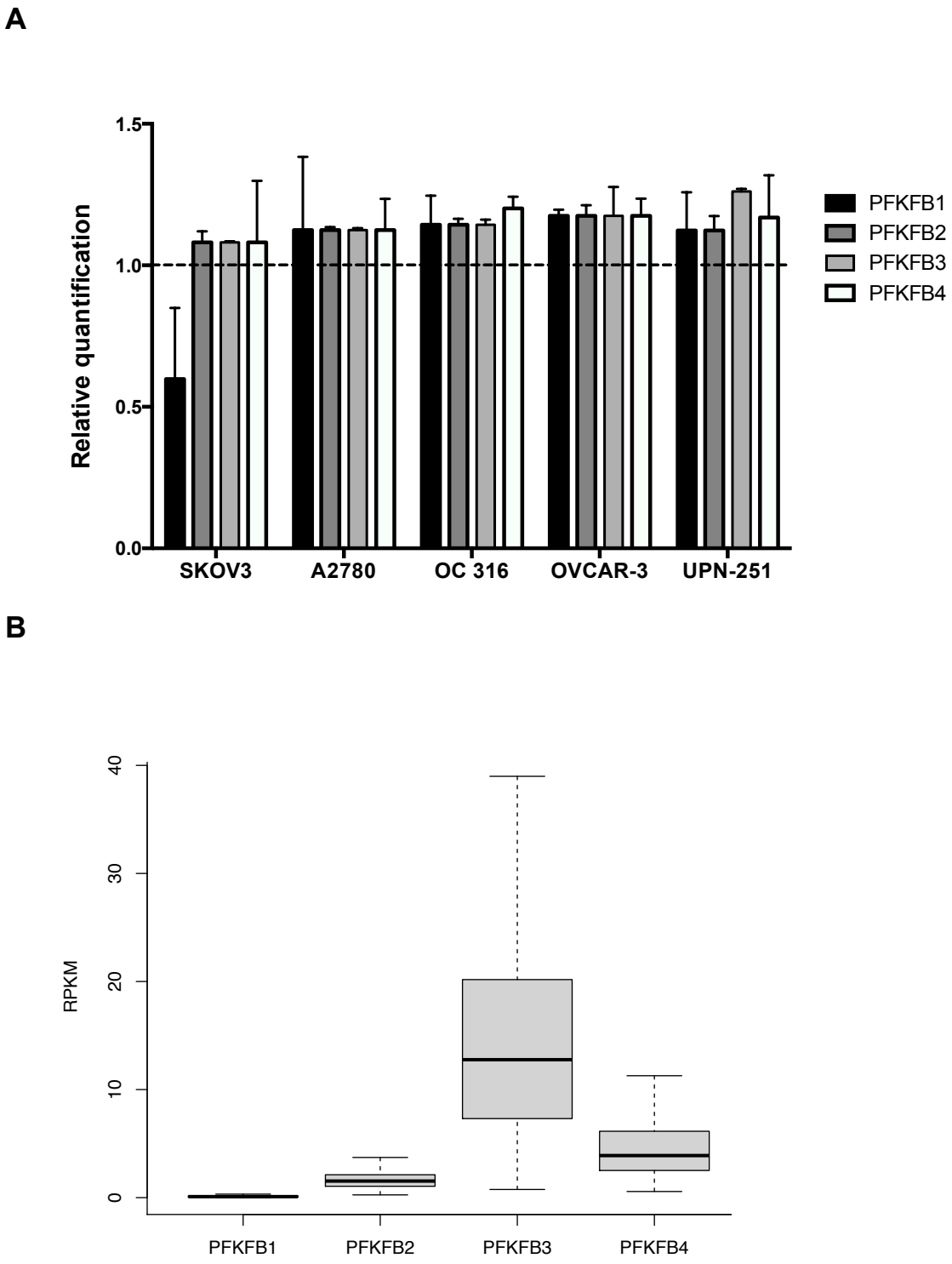


Figure 4.16. Expression of *PFKFB1-4* in ovarian cancer cells.

(A, *n*=3) Relative *PFKFB1-4* mRNA levels normalised to GAPDH measured using quantitative PCR. Data are represented as the mean + standard error of the mean for three independent experiments. (B) Analysis of RNASeq data from TCGA to determine the gene expression of *PFKFB1-4* in 420 ovarian cancers. RPKM, reads per kilobase per million. Data are represented as the median, the upper and lower quartiles, and the maximum and minimum values.

4.2.4. PFKFB3 is important for mitotically arrested ovarian cancer cell survival

Of all the PFKFB family members, PFKFB3 has been the most intensively studied and is known to be highly expressed in many human cancers, including ovarian cancer (Atsumi et al., 2002). To investigate the importance of PFKFB3 during mitotic arrest, I examined the effects of PFKFB3 depletion on paclitaxel treated SKOV3 cells. siRNA-mediated depletion *PFKFB3* resulted in a 70% reduction in mRNA (Figure 4.17A) and a large reduction in PFKFB3 protein (Figure 4.17B). *PFKFB3* depletion was achieved using stealth siRNA duplexes transfected with RNAiMAX, whereas in the siRNA screen, Dharmacon siRNA duplexes were used and transfected with DharmaFECT4 reagent. The latter approach has not been employed by any other studies depleting PFKFB3 using siRNA duplexes (Ros et al., 2012; Klarer et al., 2014; Yalcin et al., 2014; Domenech et al., 2015), which suggests that this technique may not be effective at depleting *PFKFB3* and would explain why PFKFB3 was not identified in the screen.

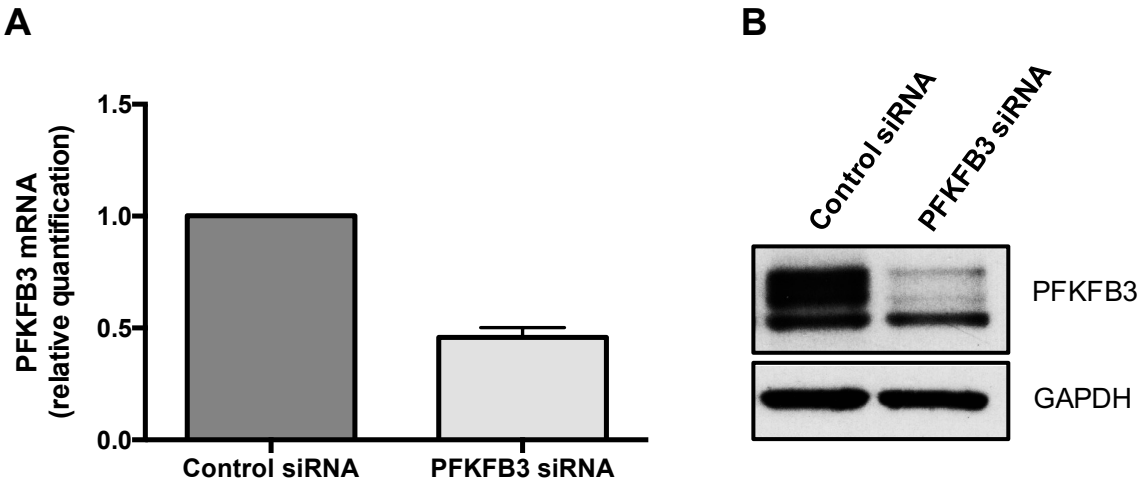


Figure 4.17. siRNA-mediated depletion of PFKFB3 in SKOV3 cells.

(A, *n*=3) Relative *PFKFB3* mRNA levels normalised to the control siRNA measured using quantitative PCR. Data are represented as the mean + standard error of the mean for three independent experiments. (B, *n*=3) PFKFB3 protein levels measured by western blotting after siRNA-mediated depletion. GAPDH was used as a loading control.

Similar to PFKFB4 targeting, depletion of PFKFB3 caused a significant decrease in mitotic cell number after paclitaxel treatment compared to the non-targeting control (Figure 4.18A, $p = 0.006$, two-way ANOVA with multiple comparisons). The decrease in mitotic index was accompanied by a significant increase in caspase-3 and -7 activity in mitotically arrested cells depleted of PFKFB3 (Figure 4.18B, $p = 0.0165$, two-way ANOVA with multiple comparisons). There was also a significant increase in the level of PI incorporation in mitotically arrested cells after PFKFB3 targeting compared to the non-targeting control (Figure 4.18C, $p = 0.0022$, two-way ANOVA with multiple comparisons). The increase in caspase-3 and -7 activity, and the greater incorporation of PI both suggest that there is an increase in cell death when mitotically arrested cells are depleted of PFKFB3.

ROS levels were significantly increased in mitotically arrested cells depleted of PFKFB3 (Figure 4.19A, $p = 0.0051$, two-way ANOVA with multiple comparisons), but not with PFKFB3 depletion alone (Figure 4.19B). An increase in ROS after PFKFB3 depletion has also been noted in other studies (Guo et al., 2010; Huo et al., 2010; Klarer et al., 2014). However, unlike these studies, I have shown an increase in ROS specifically during mitotic arrest. This may demonstrate tissue-specific regulation of these enzymes or functional alterations dependent on the presence of post-translational modifications.

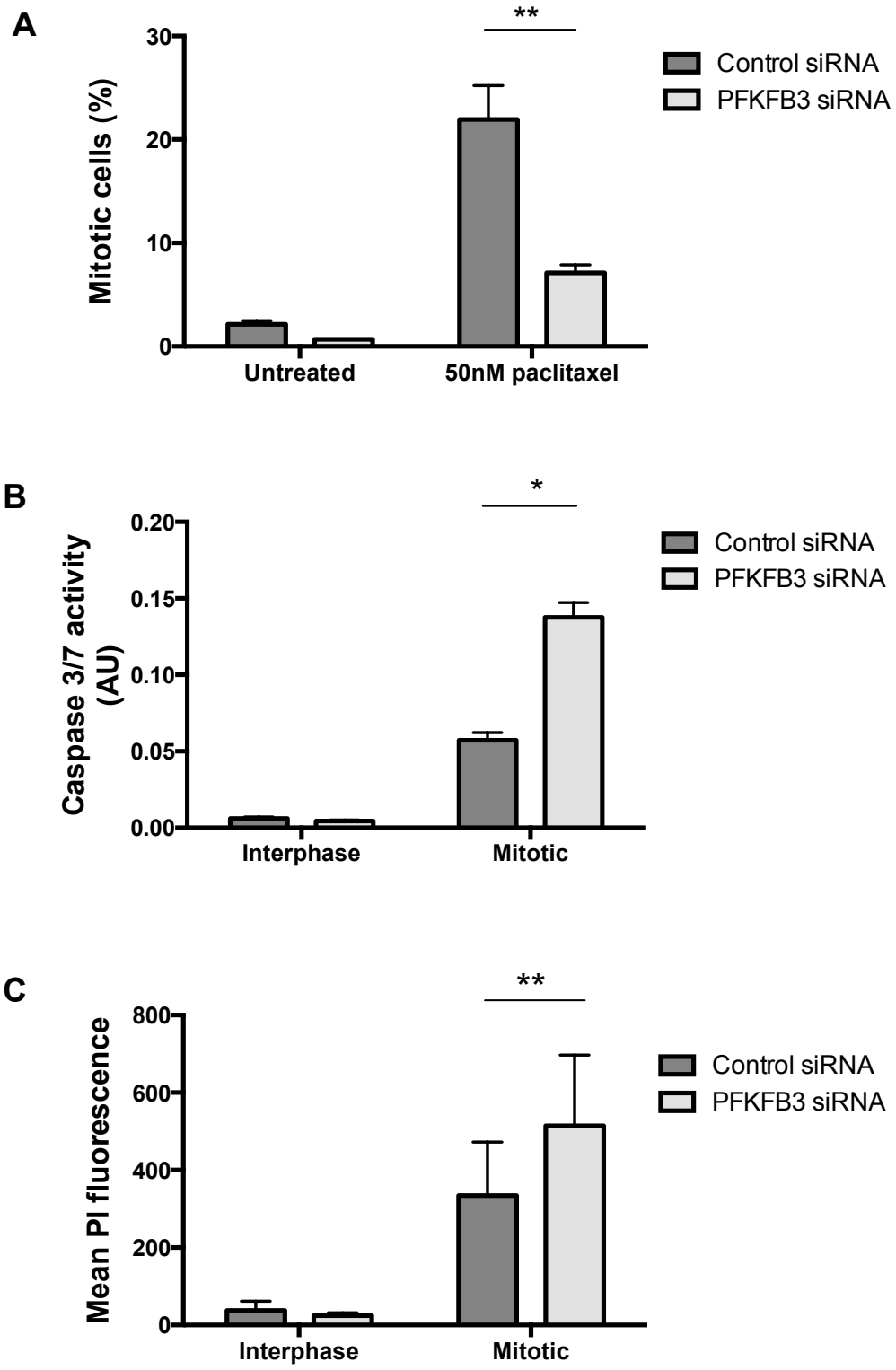


Figure 4.18. Effects of PFKFB3 depletion in SKOV3 cells.

(A, *n*=3) The percentage of mitotic cells were quantified using flow cytometry after PFKFB3 depletion and paclitaxel treatment or vehicle control. Cells were permeabilised and stained with anti-phospho histone H3 (phH3 S10) and propidium iodide. 100,000 cells were analysed for each condition. (B, *n*=3) Measurement of caspase-3/7 activity using a luminescence assay in interphase and mitotically arrested cells after PFKFB3 depletion. (C, *n*=3) Propidium iodide incorporation measured by flow cytometry in interphase and mitotically arrested cells after PFKFB3 depletion. Data are represented as the mean + standard error of the mean for three independent experiments. *≤0.05–0.01, **<0.01–0.001.

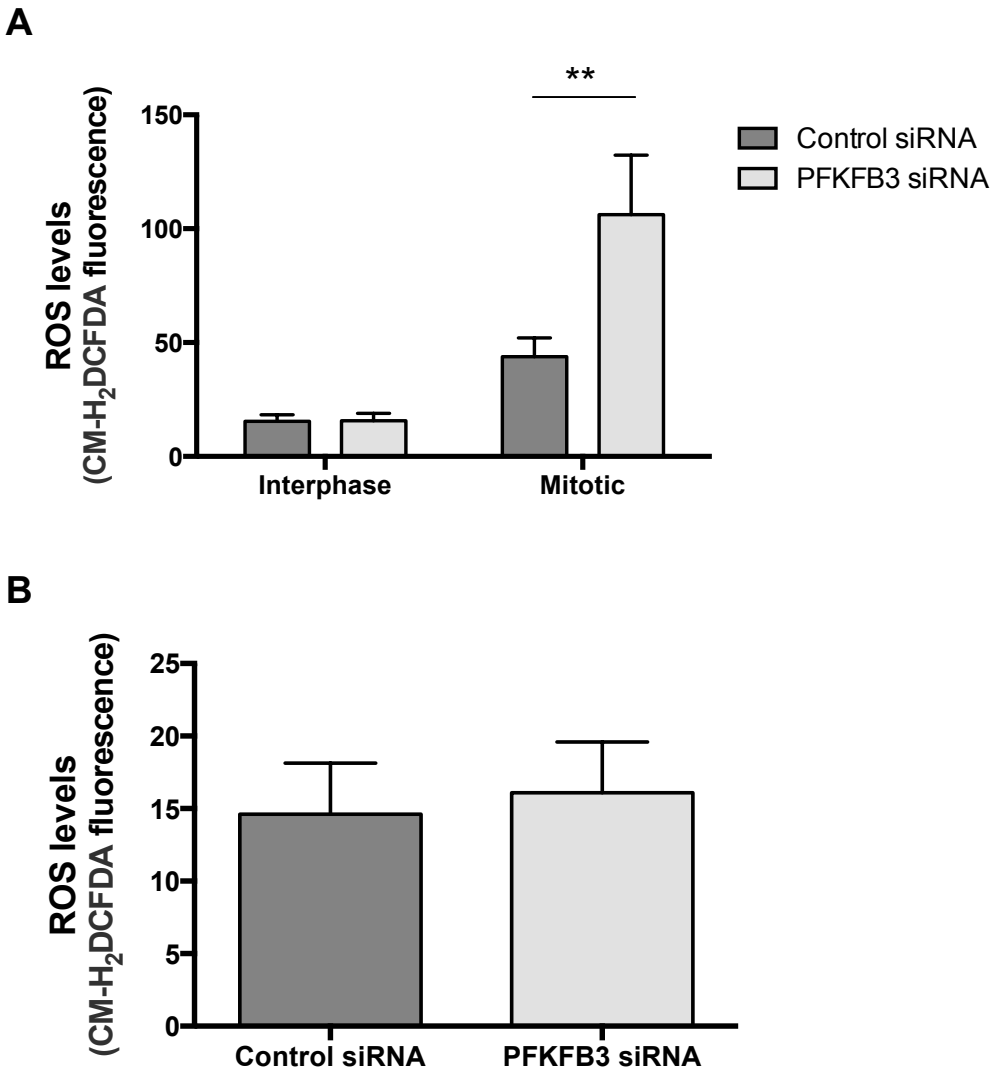


Figure 4.19. Levels of reactive oxygen species (ROS) after PFKFB3 depletion in SKOV3 cells.
(A, *n*=3) Following siRNA-mediated PFKFB3 depletion and paclitaxel treatment, mitotically arrested cells were isolated by mitotic shake-off and ROS were measured using flow cytometry. 50,000 cells were analysed for each condition. (B, *n*=3) ROS were also measured following PFKFB3 depletion alone, without paclitaxel treatment. 100,000 cells were analysed for both conditions. Data are represented as the mean + standard error of the mean for three independent experiments. **<0.01–0.001.

Given that PFKFB3 depletion displayed a comparable effect on the survival of mitotically arrested cells as PFKFB4 depletion, and that these two proteins appear to be the most abundant members of the PFKFB family in ovarian cancer cells; it was necessary to investigate whether the siRNA duplexes being used were cross-reacting with other members of the PFKFB family. In order to compare the effects of siRNA-mediated depletion on the mRNA expression of *PFKFB3* and *PFKFB4*, the exact primer efficiencies were calculated for each of the primer pairs. The primer efficiencies were comparable with the PFKFB3 primer pair displaying a slope of -3.670, which gives an amplification factor of 1.87 and an efficiency of 87.27%, while the PFKFB4 primer pair resulted in a slope of -3.631, which corresponds to an amplification factor of 1.89 and an efficiency of 88.54% (Figure 4.20A). siRNA-mediated depletion of PFKFB3 appeared to cause an increase of approximately 50% in PFKFB4 mRNA (Figure 4.20B), which may be a survival response to compensate for the decrease in PFKFB3 function. However, siRNA-mediated depletion of PFKFB4 resulted in a reduction of approximately 25% in PFKFB3 mRNA levels (Figure 4.20C). Therefore, it could be that the observed effects from PFKFB4 depletion are due to a combination of reducing the function of both PFKFB4 and PFKFB3.

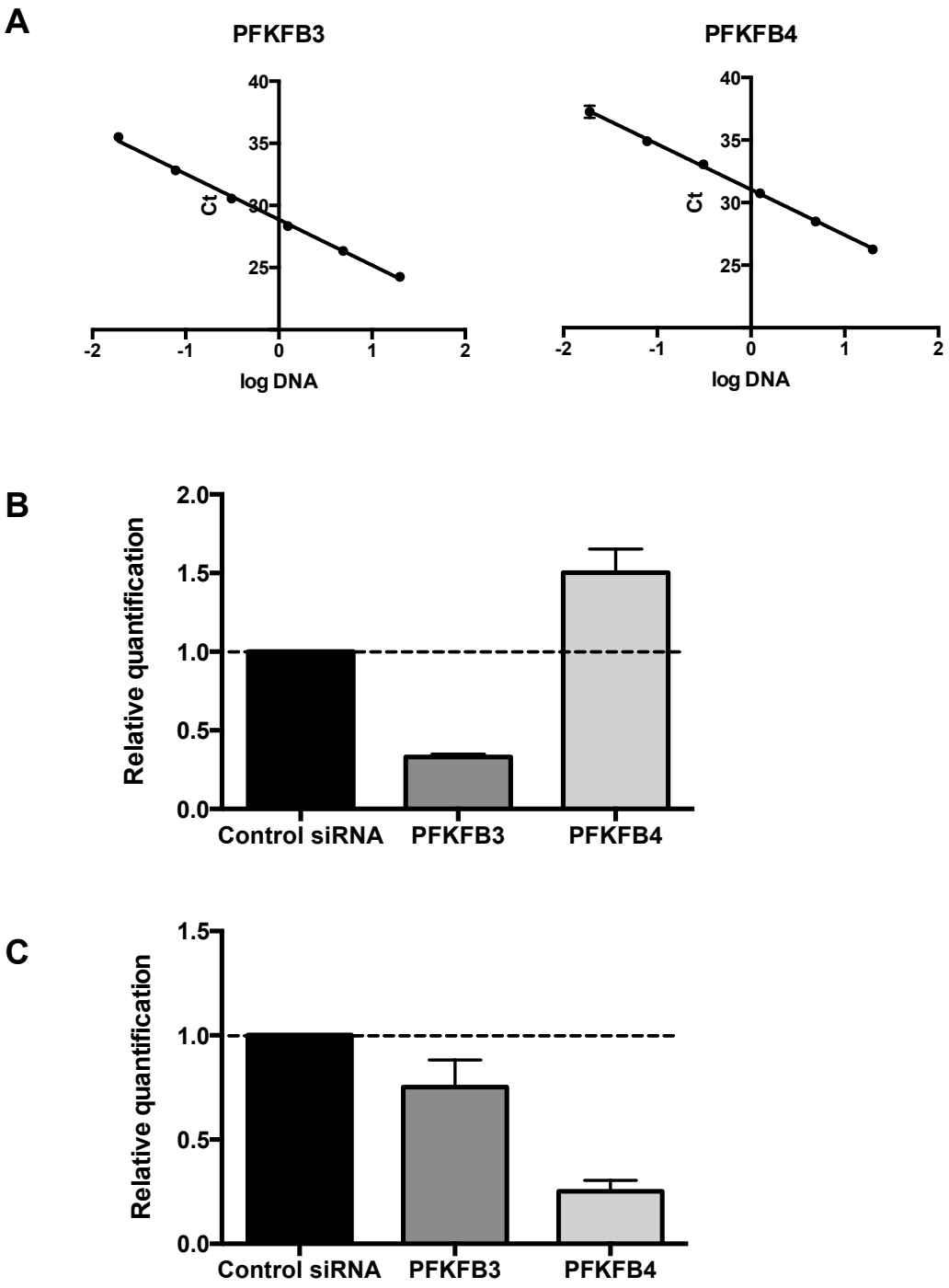


Figure 4.20. Cross-reactivity of the PFKFB3 and PFKFB4 siRNA duplexes in SKOV3 cells.

(A, *n*=1) Primer efficiencies were calculated for PFKFB3 and PFKFB4 primer pairs using quantitative PCR. (B, *n*=3) PFKFB3 and PFKFB4 mRNA levels were measured using quantitative PCR after siRNA-mediated PFKFB3 depletion. (C, *n*=3) PFKFB3 and PFKFB4 mRNA levels were measured using quantitative PCR after siRNA-mediated PFKFB4 depletion. Data are represented as the mean + standard error of the mean for three independent experiments.

4.3. Discussion

In this chapter I have thoroughly investigated the screen hits XYLB and PFKFB4, with their depletion significantly reducing the number of mitotically arrested SKOV3 and OC 316 cells after paclitaxel treatment. However, due to the inability to deplete XYLB at the protein level, this target was not followed up. Unlike SKOV3 and OC 316 cells, A2780 cells were not susceptible to PFKFB4 depletion, which confirms the predictions made in chapter 3 regarding the vulnerability of ovarian cancer cells to metabolic enzymes. Both SKOV3 and OC 316 cells lose AKT activity during mitotic arrest and display a reduction in ATP, whereas A2780 maintain AKT signalling and ATP levels remain unchanged, suggesting a possible link between AKT activity and PFKFB4 susceptibility. An interaction between AKT signalling and PFKFB4 has recently been identified by Pegoraro et al., 2015, where PFKFB4 depletion was found to reduce AKT phosphorylation at S473, however no direct molecular link between the two proteins was established (Pegoraro et al., 2015). Similarly, I found no direct link when performing kinase assays with recombinant protein (Figure S4.2).

The resistance of A2780 cells to PFKFB4 targeting could be explained by a number of reasons. Firstly, A2780 cells are thought to possess a deletion in PTEN (Saito et al., 2000) resulting in a hypomorphic protein (Wee et al., 2008). A reduction in PTEN activity enhances AKT signalling (Stambolic et al., 1998), which in turn can phosphorylate and activate PFKFB3 (Manes and El-Maghrabi, 2005) and PFKFB2 (Moon et al., 2011). A decline in PTEN function could also enhance PFKFB3 protein expression by downregulating APC/C-Cdh1 activity (Song et al., 2011), which would normally target PFKFB3 for proteasomal degradation (Herrero-Mendez et al., 2009). Therefore, a reduction in PTEN activity could increase PFKFB3 protein levels, and increase PFKFB3 and PFKFB2 activity via AKT. An increase in PFKFB3 and PFKFB2 activity in A2780 cells could compensate for the loss of PFKFB4 activity, making cells less sensitive to PFKFB4 targeting. It is possible that increasing the activity of PTEN, or more directly AKT, in “vulnerable” cells could render them resistant to PFKFB4 targeting. Secondly, it has been suggested that, unlike other ovarian cancer cell lines, A2780 cells do not possess a *TP53* mutation (Domcke et al., 2013; Beaufort et al., 2014). Active p53 can increase the expression of TIGAR

(Bensaad et al., 2006), which has a similar function to PFKFB4 (Ros et al., 2012; Ros and Schulze, 2013) and as a result could compensate for a loss of PFKFB4 activity. Therefore, if A2780 cells possess functional p53, they are likely be more resistant to PFKFB4-targeting. It has been confirmed that SKOV3 cells possess a *TP53* mutation, however the *TP53* status of OC 316 cells is unknown so the relationship between p53 activity and susceptibility to PFKFB4 targeting in these cells is yet to be established.

4.3.1. Effects of PFKFB3 or PFKFB4 depletion in mitotically arrested cells

Results from the cell cycle analysis indicate that PFKFB4 depletion could have cell cycle effects and promote mitotic slippage. This was supported by time-lapse imaging, however it did not appear substantial enough to be solely responsible for the decrease in mitotically arrested cells. The time-lapse imaging, caspase-3/7 activity assays and propidium iodide incorporation all indicate that MCD is also an important factor after PFKFB4 depletion and paclitaxel treatment. Therefore, it is most likely that the decrease in mitotically arrested cells is due to both MCD and mitotic slippage. Interestingly, MCD does not appear to be linked to either energetic stress or oxidative stress. It is possible that the interplay between caspase activity and cyclin B1 expression is an important determinant of cell fate in these cells. As suggested by Gascoigne and Taylor, 2009 (Gascoigne and Taylor, 2009), cell death could occur as a consequence of enhanced caspase activation, which precedes cyclin B degradation. Alternatively, slippage could arise from a delay in caspase activation and/or enhanced cyclin B degradation.

It has not been determined how PFKFB4 depletion could enhance caspase activity. One hypothesis is that it could be linked to a reduction of glucose utilisation in the pentose phosphatase pathway and a subsequent reduction in NADPH available for lipid synthesis. Given the importance of *de novo* fatty acid synthesis during mitosis and the apparent “lipogenic checkpoint” (Scaglia et al., 2014), its inhibition could further induce G2/M arrest and initiate apoptosis. A reduction in fatty acid synthesis has been shown to increase caspase activity and promote cell death in cancer cells (Chuang et al., 2011; Ventura et al., 2015; Yang et al., 2015). This could be a novel role of PFKFB4 during mitosis or mitotic arrest. It would be interesting to investigate the effects of caspase inhibitors to

determine whether MCD is caspase-dependent and to investigate fatty acid synthesis in mitotically arrested cells before and after PFKFB4 depletion. The requirement for *de novo* fatty acid synthesis could also explain the decrease in AMPK phosphorylation, in order to promote ACC activity and upregulate lipogenesis.

Cells arrested in mitosis after paclitaxel treatment are known to have an increase in ROS (Ramanathan et al., 2005; Alexandre et al., 2006). However, the further increase in ROS observed in mitotically arrested cells after PFKFB4 depletion could be explained by a reduction in NADPH due to the loss of PFKFB4 bisphosphatase activity. If the *PFKFB4* siRNA is also targeting *PFKFB3*, then the increase in ROS could also be attributed to the role of PFKFB3 in cellular detoxification. In the presence of ROS, PFKFB3 undergoes S-glutathionylation (Seo and Lee, 2014) and demethylation (Yamamoto et al., 2014), which function to increases NADPH levels in order to maintain a redox balance. When the levels of ROS increase in paclitaxel treated mitotically arrested cells, the detoxification function of PFKFB3 might become important. Therefore, PFKFB3 depletion would result in a reduced capacity for detoxification in mitotically arrested cells and a subsequent increase in ROS. Importantly, the increase in ROS did not appear to contribute to cell death.

Considering the fact that PFKFB3 is thought to possess very high kinase activity (Sakakibara et al., 1997; Telang et al., 2006), while PFKFB4 has been suggested to have a slightly higher bisphosphatase function (Ros and Schulze, 2013; Strohecker et al., 2015), it is surprising that their depletion results in the same phenotype. This could be due to the cross-reactivity of the siRNA, for example, if *PFKFB4* siRNA is also targeting *PFKFB3*, the observed phenotype would be due to depletion of both PFKFB4 and PFKFB3. These results could also demonstrate the ability of the PFKFB enzymes to adapt to a changing environment and compensate for a decline in activity of other family members. For example, siRNA targeting *PFKFB3* caused an upregulation of *PFKFB4* mRNA expression. This could be due to PFKFB4 attempting to fulfil the role of PFKFB3 and as a result give the phenotype of PFKFB4 depletion due to it switching to enhance its kinase activity. In addition, PFKFB4 is thought to be regulated by the abundance of its

substrates and products, demonstrating its potential adaptability. Alternatively, these data could indicate an unrecognised role for PFKFB4 in cell proliferation, or demonstrate a novel and important role of PFKFB3 and PFKFB4 in mitotically arrested cells. This is supported by studies that have found PFKFB3 protein levels to be high in mitotically arrested cells (Cieslar-Pobuda et al., 2015; Domenech et al., 2015), despite levels usually declining during spontaneous mitosis due to the activation of APC/C-CDH1 (Fang et al., 1998) and the subsequent degradation of PFKFB3 (Pfleger and Kirschner, 2000; Herrero-Mendez et al., 2009).

PFKFB3 depletion has previously been implicated in MCD of HeLa cells after mitotic arrest by nocodazole due to the reliance of mitotic cells on PFKFB3 kinase activity for the stimulation of glycolysis and maintenance of ATP levels (Domenech et al., 2015). However, in this work, AMPK was not phosphorylated after PFKFB3 depletion in mitotically arrested cells, which suggests that they are not energetically stressed and therefore would not rely on PFKFB3 for that reason.

4.3.2. Limitations

The majority of this work has used paclitaxel to induce mitotic cell arrest, which although is clinically relevant due to the broad use of paclitaxel in the treatment of ovarian cancer, it does not enable the determination of whether PFKFB4 and PFKFB3 are important for cell survival during paclitaxel-induced mitotic arrest or more generally during prolonged mitosis. Preliminary experiments with nocodazole, a microtubule destabilising antimitotic drug, have produced similar results to paclitaxel treatment (Figure S4.3), suggesting that this may be a common response following treatment with antimitotics. However, more work is required to confirm whether this is the case, and whether these observations extend beyond that of microtubule-targeted therapies.

A common assumption is that paclitaxel elicits cell death through mitotic arrest, however studies using tumour models have suggested that it enhances cell death in interphase (Komlodi-Pasztor et al., 2011; Mitchison, 2012), possibly through its effects on cell signalling and microtubule-mediated transport (Herbst and Khuri,

2003; Komlodi-Pasztor et al., 2011). However, in cell culture, clinically relevant levels of paclitaxel have not been found to induce interphase cell death without effecting a prior mitosis in the presence of the drug (Janssen et al., 2013; Zasadil et al., 2014). These data demonstrate the impact of the microenvironment on the cellular response to paclitaxel and suggest that what is observed in cell culture is not always clinically relevant. Therefore, it would be important to see whether these results hold true *in vivo*. It is also important to determine the effect of PFKFB4 depletion and paclitaxel treatment on normal ovarian cells, such as fallopian tube epithelial cells. However, given that non-tumourigenic cells proliferate at a slower rate than cancer cells, fewer cells would be arrested in mitosis and therefore less would be vulnerable to targeting. In addition, these cells will not possess the same reliance on enhanced AKT activity as cancer cells, so would not be expected to have any vulnerability to metabolic targeting during mitotic arrest.

The ability to specifically target *PFKFB4* and not other members of the *PFKFB* family using siRNA duplexes was problematic in this work. I had hoped to overcome this issue with the use of siRNA duplexes targeting the 3' untranslated region of *PFKFB4*, however I could not achieve efficient depletion of PFKFB4 at the mRNA or protein level using two different siRNA duplexes, so this could not be determined. Moreover, the more newly developed genome editing technology, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9, could not be used to knockout PFKFB4 due to preliminary colony forming assays demonstrating a profound decrease in cell growth after PFKFB4 depletion (Figure S4.4). An alternative could be an inducible knockout system that is only functional during mitosis. Another difficulty was knowing whether the observed phenotype was due to the result of the depletion or the cells ability to compensate for it. It appears that the PFKFB family can adapt very quickly to their environment and predominantly act to maintain a balance in the cell. This makes it challenging to determine the exact role of individual family members.

4.3.3. Conclusions

In summary, this chapter has identified a novel role for PFKFB4 in a subset of paclitaxel treated ovarian cancer cells. Investigations have discovered a

previously unrecognised importance of PFKFB4 and PFKFB3 in mitotically arrested ovarian cancer cell survival. It has also indicated a possible link between the AKT status of a cell during mitotic arrest and its susceptibility to PFKFB4 targeting. Investigating the validity of this relationship will be the focus of the next chapter.

Chapter 5

Attempts to rescue the effects of PFKFB4 depletion in mitotically arrested SKOV3 cells

5. Attempts to rescue the effects on PFKFB4 depletion on mitotically arrested SKOV3 cells

5.1. Introduction

In chapter 3 I identified a potential vulnerability in mitotically arrested ovarian cancer cells, including SKOV3 cells, characterised by a reduction in AKT signalling, glucose 6-phosphate, ATP levels and AMPK activity. The cell line A2780 were the only cell line analysed that did not possess this apparent vulnerability. The potential utility of this vulnerability in SKOV3 cells was explored by data mining of a previously conducted siRNA kinome screen, which identified two metabolic enzymes important for cell survival during paclitaxel-induced mitotic arrest. Chapter 4 demonstrated that siRNA-mediated depletion of PFKFB4 followed by paclitaxel treatment significantly decreased the mitotic index of SKOV3 and OC 316 cells, but not A2780 cells. This indicated a potential link between AKT activity during mitotic arrest and the sensitivity of cells to PFKFB4 depletion. To determine whether the effects of PFKFB4 depletion are exploiting the vulnerability created by the loss of AKT activity during paclitaxel-induced mitotic arrest in SKOV3 cells, I sought to maintain AKT activity during mitotic arrest. Below I discuss the main approaches to enhancing AKT activity in cell culture.

5.1.1. Methods to enhance AKT activity *in vitro*

Deregulation of the PI3K/AKT pathway commonly occurs through the overexpression of AKT (Sun et al., 2001), the persistent activation of receptor tyrosine kinases, activating mutations in PI3K, or PTEN inactivation or deletion (Carnero et al., 2008). These alterations cause the constitutive activation of AKT through its recruitment to the plasma membrane and its subsequent phosphorylation by PDK1 and mTORC2. Taking this into consideration, AKT activity could be enhanced by the overexpression of wild-type AKT, promoting the recruitment of AKT to the plasma membrane, or by mimicking AKT phosphorylation through phosphomimetic substitutions.

The simplest method of enhancing AKT activity is to overexpress wild-type AKT. Systems overexpressing wild-type AKT have demonstrated an increase in AKT

kinase activity and cellular transforming ability than empty vector controls. However, the kinase activity of wild-type AKT is far lower than that of constitutively active AKT mutants (Aoki et al., 1998; Hajduch et al., 1998). Exogenous AKT expression can also enhance the translocation of GLUT4 to the plasma membrane in the absence of insulin, however this is to a level equal to what insulin can elicit alone (Cong et al., 1997; Hajduch et al., 1998). Exogenously expressed wild-type AKT is also more dependent on endogenous PI3K activity for its activation than those systems involving AKT mutants and as a result, is more sensitive to PI3K inhibition (Kane et al., 1999) and serum withdrawal (Kennedy et al., 1997). This may have the advantage of making it more physiological, but with the caveat of AKT activation not being constitutive. Therefore, the majority of studies requiring enhanced AKT activity utilise phosphomimetic substitutions or membrane tethering.

Phosphomimetic substitutions

Phosphomimetic mutations are amino acid substitutions at a phosphoacceptor site that mimic a phosphorylated protein, resulting in altered protein activity (Tarrant and Cole, 2009). The addition of a phosphate moiety to a protein can be somewhat recapitulated by the incorporation of acidic amino acids with negatively charged side chains. For example, aspartic acid (D) is chemically similar to phosphoserine (Thorsness and Koshland, 1987), whereas glutamic acid (E) more closely resembles phosphothreonine, although they can be used interchangeably. D and E are isosteric with phosphoserine and phosphothreonine (Pearlman et al., 2011). Substitutions can also be phosphodeficient, such as a serine to alanine mutation, because alanine cannot be phosphorylated due to it lacking an hydroxy group.

A number of phosphorylation sites have been identified on AKT. However, two sites that have been shown to be critical for full activity of AKT are S473 (Sarbassov et al., 2005) and T308 (Stephens et al., 1998). T308 is situated in the activation loop of AKT and as a result, phosphorylation of this site is important for stabilisation of the active site and the catalytic function of AKT (Yang et al., 2002). S473 is present in the C-terminal hydrophobic motif of AKT and its phosphorylation has been found to stabilise the N-lobe of the kinase, enhancing

the catalytic activity of AKT up to 10-fold (Alessi et al., 1996a; Yang et al., 2002). Phosphorylation at S473 is also thought to broaden the downstream activity of AKT to include targets such as PRAS40, FOXO1 and FOXO3A (Guertin et al., 2006).

A single substitution of T308 for D or E has been infrequently used to mimic AKT phosphorylation and has achieved mixed success. Some studies have shown T308D to negatively effect AKT activity (Hart and Vogt, 2011), while others have found T308D to possess enhanced kinase activity (Alessi et al., 1996b). These inconclusive results could be due to the observation that phosphorylation at S473 precedes T308 phosphorylation and is required for effective T308 phosphorylation (Scheid et al., 2002). Thus, T308D on its own will have little effect on AKT activity as it relies on additional phosphorylation of S473. In addition, it has been suggested that T308D constructs may protect endogenous AKT from dephosphorylation by PP-2A rather than directly participating in downstream signalling (Hart and Vogt, 2011). A single mutation of S473 to D has been shown to mimic S473 phosphorylation (Alessi et al., 1996a) and significantly increase the transforming ability of AKT (Hart and Vogt, 2011). It has been suggested that S473D is a better substrate for PDK1 than wild-type AKT (Biondi et al., 2001), which would enhance its phosphorylation at T308. In contrast to these observations, one study has found the S473D substitution to increase oncogenicity, but not AKT activity (Hart and Vogt, 2011), indicating that S473D may stimulate the activation of additional pathways.

Due to the importance of T308 and S473 phosphorylation in achieving full AKT activity, numerous studies requiring constitutively active AKT have substituted both T308 and S473 for D, producing a double T308D/S473D mutant. T308D/S473D is largely independent of PI3K activity and has been shown to be constitutively active, resistant to PI3K/AKT inhibition and possess increased kinase activity compared to the overexpression of wild-type AKT, S473D or T308D (Alessi et al., 1996a; Scheid and Woodgett, 2003; Berndt et al., 2010). Moreover, T308D/S473D can directly regulate GLUT4 translocation to the plasma membrane (Foran et al., 1999) and maintain GLUT1 membrane localisation (Wieman et al., 2007). However, there is evidence suggesting that the increased kinase activity of T308D/S473D does not necessarily confer enhanced

oncogenicity (Aoki et al., 1998) and that the T308D/S473D mutant is not constitutively active in all systems (Mochizuki et al., 2002).

An additional phosphorylation site on AKT is T450 in the turn site (Alessi et al., 1996a). T450 is phosphorylated by mTORC2 and has been implicated in AKT stability and kinase activity (Facchinetti et al., 2008; Ikenoue et al., 2008; Xiao et al., 2010). However, mutation of T450 to D has been shown to have a minimal effect on AKT signalling and oncogenesis, with only a slight increase in p70S6K phosphorylation being observed (Hart and Vogt, 2011). Recent evidence has also suggested that AKT phosphorylation at S473 and T308 might be governed by S477 and T479 phosphorylation (Liu et al., 2014a). However, the effects of phosphomimetic substitutions at these residues on glucose uptake and metabolism remains unknown.

Membrane tethering

An alternative mechanism to enhance AKT activity is to promote the recruitment of AKT to the plasma membrane, which acts to enhance its phosphorylation by upstream kinases, such as PDK1 and mTORC2. PDK1 is frequently localised to the plasma membrane of cells (Chun et al., 2005; Scheid et al., 2005), so increasing the association of AKT with the plasma membrane will ultimately enhance it's interaction with PDK1. Whereas mTORC2 can associate with membranes of the endoplasmic reticulum, mitochondria, plasma membrane and is also found in the nucleus (Betz and Hall, 2013).

Membrane localisation can be facilitated by Gag-fusion, mutation of the PH domain, or fatty acylation, such as myristoylation or palmitylation (Resh, 1999). Myristoylation has been well characterised and is commonly used to promote membrane targeting and binding. Many proteins in the cell are subject to N-myristoylation, which occurs as a cotranslational process while the nascent polypeptide is still attached to the ribosome (Wilcox et al., 1987; Deichaite et al., 1988). Myristoylation involves the addition of a 14-carbon saturated fatty acid to the N-terminus of the protein via an amide bond and can help stabilise protein conformation and enhance membrane binding. Myristoylated proteins preferentially bind to the plasma membrane rather than other intracellular

membranes (Resh, 1999). This could be due to a slight enrichment of acidic phospholipids compared to other intracellular membranes (van Meer et al., 2008), or the increased likelihood of protein–protein interactions at the plasma membrane, which enhance membrane binding. N-myristoylated proteins require additional factors, such as protein-protein interactions, to achieve stable membrane binding (Resh, 1999).

Myristoylation of AKT (Myr-AKT) has been routinely used to achieve constitutive activation. Myr-AKT can strongly enhance AKT activity, resulting in increased phosphorylation of direct targets, such as GSK3β, and signalling through mTORC1, such as p70S6K (Andjelkovic et al., 1997; Aoki et al., 1998; Dufner et al., 1999; Mochizuki et al., 2002; Hart and Vogt, 2011). Myr-AKT also results in constitutive glucose uptake that is associated with an increase in *GLUT1* expression (Wieman et al., 2007), as well as an increase in GLUT4 translocation to the plasma membrane, without an increase in expression (Kohn et al., 1996; Cong et al., 1997). Moreover, an S473D substitution in addition to myristoylation does not enhance AKT activity further, suggesting that Myr-AKT is maximally activated (Hart and Vogt, 2011). Myr-AKT is also partially PI3K-independent, with its activation not reliant on insulin or growth factor stimulation, however it does retain some dependence on the recruitment of upstream kinases to the plasma membrane (Andjelkovic et al., 1997; Kennedy et al., 1997).

In conclusion, there are two main mechanisms for activating AKT activity *in vitro;* myristoylation, or phosphomimetic substitution to create a double T308D/S473D mutant (Figure 5.1). These two alterations are very different in their mechanisms of action and enable the elucidation of different aspects of AKT activation. For example, myristoylation allows the delineation of the role of PH domain binding and membrane localisation in AKT activation, but due to the membrane tethering may forgo important cytoplasmic or nuclear signalling functions. Whereas, T308D/S473D enables AKT to move freely about the cell enabling the importance of these two phosphorylation sites to be determined. The majority of studies investigating the constitutive activation of AKT have used AKT1, instead of AKT2 and AKT3. This is likely due to the fact that AKT1 is ubiquitously expressed at high levels (Coffer and Woodgett, 1991; Jones et al., 1991; Bellacosa et al.,

1993), compared to AKT2 and AKT3, which are expressed in a more tissue-specific manner (Konishi et al., 1994; Nakatani et al., 1999).

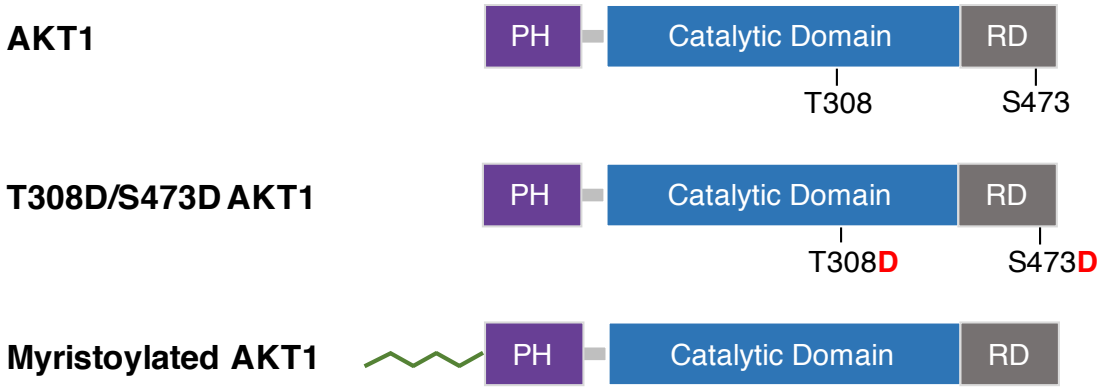


Figure 5.1. Schematic of AKT1 domain organisation displaying alterations that can produce constitutively active AKT.
AKT1 possesses a PH domain (PH) for membrane binding, a catalytic domain that contains the proteins active site and a regulatory domain (RD), which houses the hydrophobic motif. T308D/S473D contains two amino acid substitutions, while myristoylated AKT1 possess a myristoyl moiety on its N-terminus (shown in green).

5.1.2. Chapter aims

In this thesis, I have demonstrated that SKOV3 cells possess a decrease in AKT activity during paclitaxel-induced mitotic arrest and that they are susceptible to PFKFB4 depletion. Conversely, A2780 cells maintain AKT activity during paclitaxel-induced mitotic arrest and remain unaffected by PFKFB4 depletion. However, it is has not been determined whether PFKFB4 depletion is exploiting the vulnerability created by the loss of AKT activity during paclitaxel-induced mitotic arrest in SKOV3 cells, or whether the depletion of PFKFB4 is causing this phenotype and it is not due to off-target effects of the siRNA. These observations have led me to investigate three questions in this chapter:

- Is it possible to maintain AKT signalling during mitotic arrest? If so, does the maintenance of AKT signalling during paclitaxel-induced mitotic arrest confer resistance to PFKFB4 depletion?

- Can the overexpression of wild-type PFKFB4 or siRNA-resistant PFKFB4 confer resistance to siRNA-mediated PFKFB4 depletion?

- Can the overexpression of wild-type PFKFB3 confer resistance to PFKFB4 depletion?

5.2. Results

5.2.1. Enhancing AKT activity in mitotically arrested SKOV3 cells does not confer resistance to PFKFB4 depletion

In order to address the aims of this chapter, I used SKOV3 cell lines stably expressing either wild-type AKT1 (AKT WT), constitutively active AKT1 through the addition of two phosphomimetic mutations T308D and S473D (T308D/S473D), or a constitutively active N-terminal myristoylated AKT1 (Myr-AKT). Both of the AKT1 mutants have previously been shown to cause constitutive activation of AKT (Alessi et al., 1996a; Andjelkovic et al., 1997) and to directly stimulate glucose uptake and metabolism (Kohn et al., 1996). To investigate whether AKT activity was restored during mitotic arrest in these cell lines, the phosphorylation of AKT and its downstream targets were assessed by western blotting (Figure 5.2A). The phosphorylation of two direct substrates of AKT, PRAS40 and GSK3β, were increased in the mitotically arrested cells in the T308D/S473D and Myr-AKT cell lines compared to the empty vector and AKT WT. The AKT WT cell line also displayed an increase in GSK3β phosphorylation in mitotically arrested cells compared to the empty vector, suggesting that some AKT activity has been restored. However, AKT phosphorylation at S473 was not restored during mitotic arrest in AKT WT cells, indicating that this cell line is not a good model for constitutive AKT activation. The maintenance of AKT phosphorylation at S473 in mitotically arrested cells was observed in the Myr-AKT cells, but could not be determined in T308D/S473D cells due to the nature of the mutations.

Intriguingly, the phosphorylation of p70S6K was not rescued in any of cell lines stably expressing AKT, which suggests that either AKT activity is not fully restored during mitotic arrest, or p70S6K is not a direct substrate of AKT and that additional components required for its phosphorylation are not functional. Previous studies have shown that phosphorylation of p70S6K can be increased by the myristoylation of AKT (Hart and Vogt, 2011), so this effect might be cell-type specific. However, in previous studies, the phosphorylation of p70S6K was being increased from a basal level and not being established from the complete absence of phosphorylation, like what I am trying to achieve here.

Protein levels of mTOR and RICTOR were restored to comparable levels to interphase cells in both the T308D/S473D and Myr-AKT cell lines (Figure 5.2B), suggesting that there may be a positive feedback loop upstream causing further activation of the pathway. Overexpression of RICTOR has been associated with increased AKT phosphorylation at S473 and subsequent activation of AKT (Kim et al., 2011; Laugier et al., 2015). Additionally, the increased levels of mTOR would facilitate the formation of both the mTORC1 and mTORC2 complexes involved in the PI3K/AKT pathway. However, given that these two complexes are mutually exclusive, it is unclear how an increase in mTOR availability would affect AKT signalling.

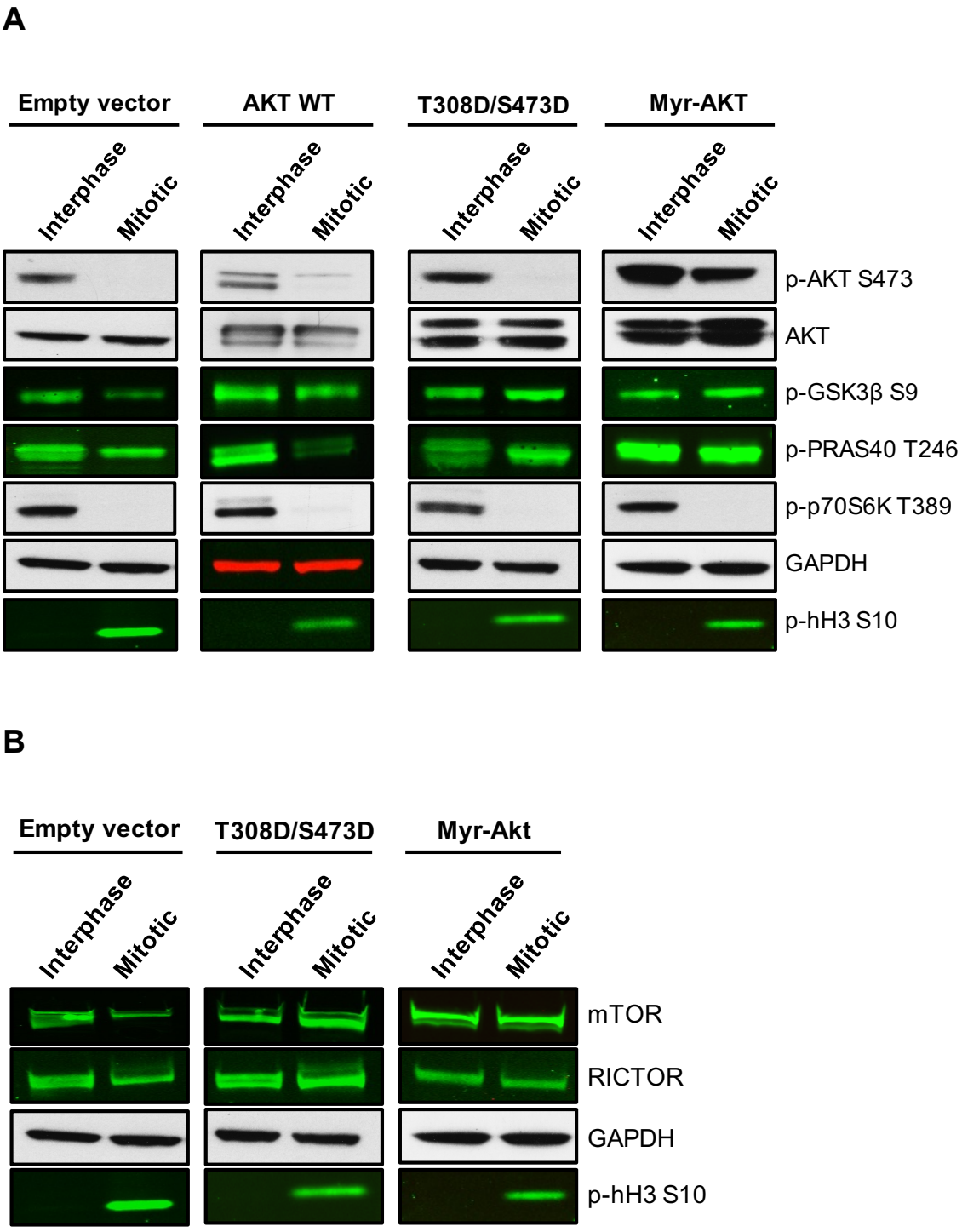


Figure 5.2. AKT activity in mitotically arrested SKOV3 cell lines stably expressing constitutively active AKT.
(A, *n*=2) Protein expression and phosphorylation status of AKT and its downstream targets and (B, *n*=2) upstream regulators of AKT measured by western blotting in interphase and mitotic cells separated by mitotic shake-off after paclitaxel treatment. GAPDH was used as a loading control and phospho-histone H3 S10 as a mitotic marker.

Given the observed increase in activity of the AKT pathway directly upstream and downstream in the T308D/S473D and Myr-AKT cell lines, I investigated whether the number of mitotically arrested cells still declined after PFKFB4 depletion. Cell lines expressing constitutively active AKT were compared to the empty vector cells due to the AKT WT cells showing evidence of increased AKT activity during mitotic arrest. There was still a minimum of a 50% reduction in the number of mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control in both the T308D/S473D (p = 0.0002, two-way ANOVA multiple comparisons) and Myr-AKT ($p < 0.0001$, two-way ANOVA multiple comparisons) cell lines (Figure 5.4A). This was accompanied by a significant increase in PI incorporation in mitotically arrested cells depleted of PFKFB4 in T308D/S473D (p = 0.0076, two-way ANOVA multiple comparisons) and Myr-AKT (p = 0.0006, two-way ANOVA multiple comparisons) (Figure 5.4B), suggesting that there was an increase in MCD. Levels of ROS in both T308D/S473D (p = 0.0420, two-way ANOVA multiple comparisons) and Myr-AKT (p = 0.0034, two-way ANOVA multiple comparisons) were also significantly increased in mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control (Figure 5.4C). The effect of individual siRNA duplexes were tested to check for off-target effects and all of them displayed a significant decrease in mitotically arrested cells after PFKFB4 depletion (Figure S5.1).

These data indicate that partial restoration of AKT signalling during paclitaxel-induced mitotic arrest does not protect cells from the detrimental effects of PFKFB4 depletion. It could be that the full spectrum of AKT activity is required to see any resistance to PFKFB4 depletion, or that PFKFB4 depletion is not exploiting this specific vulnerability, but something else. Alternatively, it could be that the overexpression of AKT enhances the frequency of mitotic slippage. Nonetheless, the decrease in mitotically arrested cell number after PFKFB4 depletion appears to be attributed to a number of different factors.

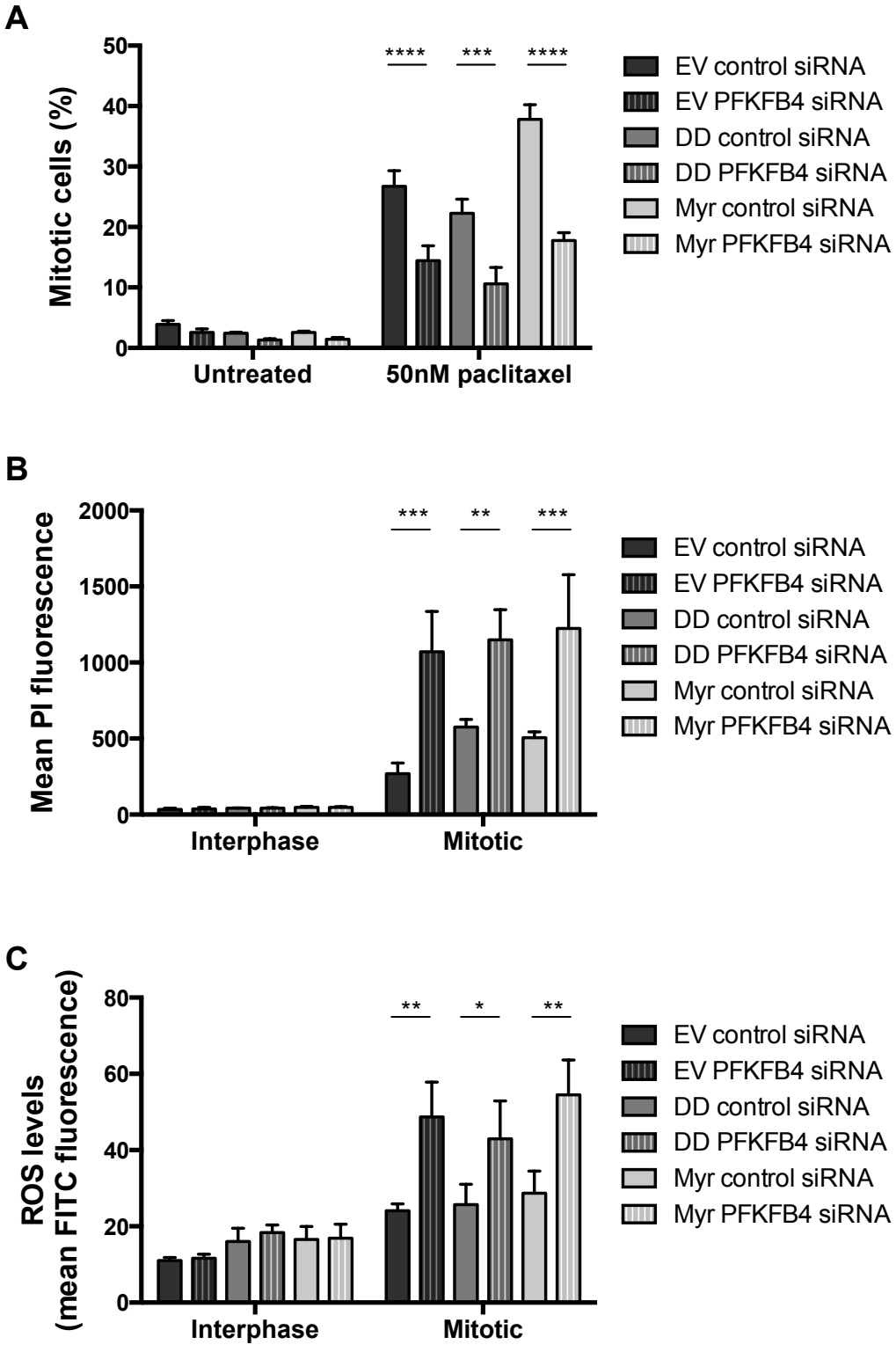


Figure 5.3. Effects of PFKFB4 depletion and paclitaxel treatment in SKOV3 cell lines stably expressing constitutively active AKT.

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, the percentage of mitotic cells were quantified using anti-phospho histone H3 S10 staining and flow cytometry. 100,000 cells were analysed for each condition. (B, *n*=3) Mitotically arrested cells were isolated by a mitotic shake-off and PI incorporation and (C, *n*=3) levels of reactive oxygen species were measured using flow cytometry. 50,000 cells were analysed for each condition. Data are represented as the mean + standard error of the mean for three independent experiments. *0.01–0.001, **<0.01–0.001, ***<0.001 and ****<0.0001.

5.2.2. PFKFB4 overexpression does not rescue the decrease in mitotic cell number after PFKFB4 depletion

To investigate whether the decrease in mitotically arrested cells could be rescued in a more direct manner, I produced SKOV3 cell lines stably overexpressing wild-type PFKFB4 (PFKFB4 OE) (Figure 5.4A) or siRNA resistant PFKFB4 (PFKFB4 si17) (Figure 5.4B), both of which were coexpressed with a V5 peptide. The V5 peptide is a 14-amino acid peptide derived from simian parainfluenza virus type 5 (Southern et al., 1991). The coexpression of the V5 peptide enables the differentiation of the endogenous and exogenous PFKFB4. The PFKFB4 siRNA resistant cell line was created against siRNA duplex 17 because it achieved a more efficient depletion of *PFKFB4* mRNA than siRNA duplexes 1 or 4, its target sequence had less similarity to *PFKFB3* than siRNA duplex 2, and importantly, silent mutations could easily be introduced into its target sequence.

Immunofluorescence images displayed very weak background staining of PFKFB4 in empty vector cells, however strong cytoplasmic staining was observed in both the PFKFB4 OE and PFKFB4 si17 cell lines (Figure 5.4C), which is in agreement with a role in glycolysis and previous observations (Yun et al., 2012; Pegoraro et al., 2015). The InCell imaging system was used to investigate the ability of the siRNA 17 duplex to deplete exogenous PFKFB4 in the PFKFB4 OE and PFKFB4 si17 cell lines. The percentage of V5 positive cells before and after transfection with siRNA 17 were calculated using a high-throughput method on the InCell Analyser. Transfection with the non-targeting siRNA duplex had no observable effects on the percentage of cells expressing V5, with both cell lines displaying over 80% positivity for V5 (Figure 5.4D). Following transfection with the siRNA 17 duplex, the percentage of V5 positive cells in the PFKFB4 OE cell line were reduced by 71% to 10.5%, while the percentage in the PFKFB4 si17 cell line remained at approximately 85%. Thus confirming that the PFKFB4 si17 cell line is more resistant to PFKFB4 depletion by siRNA 17.

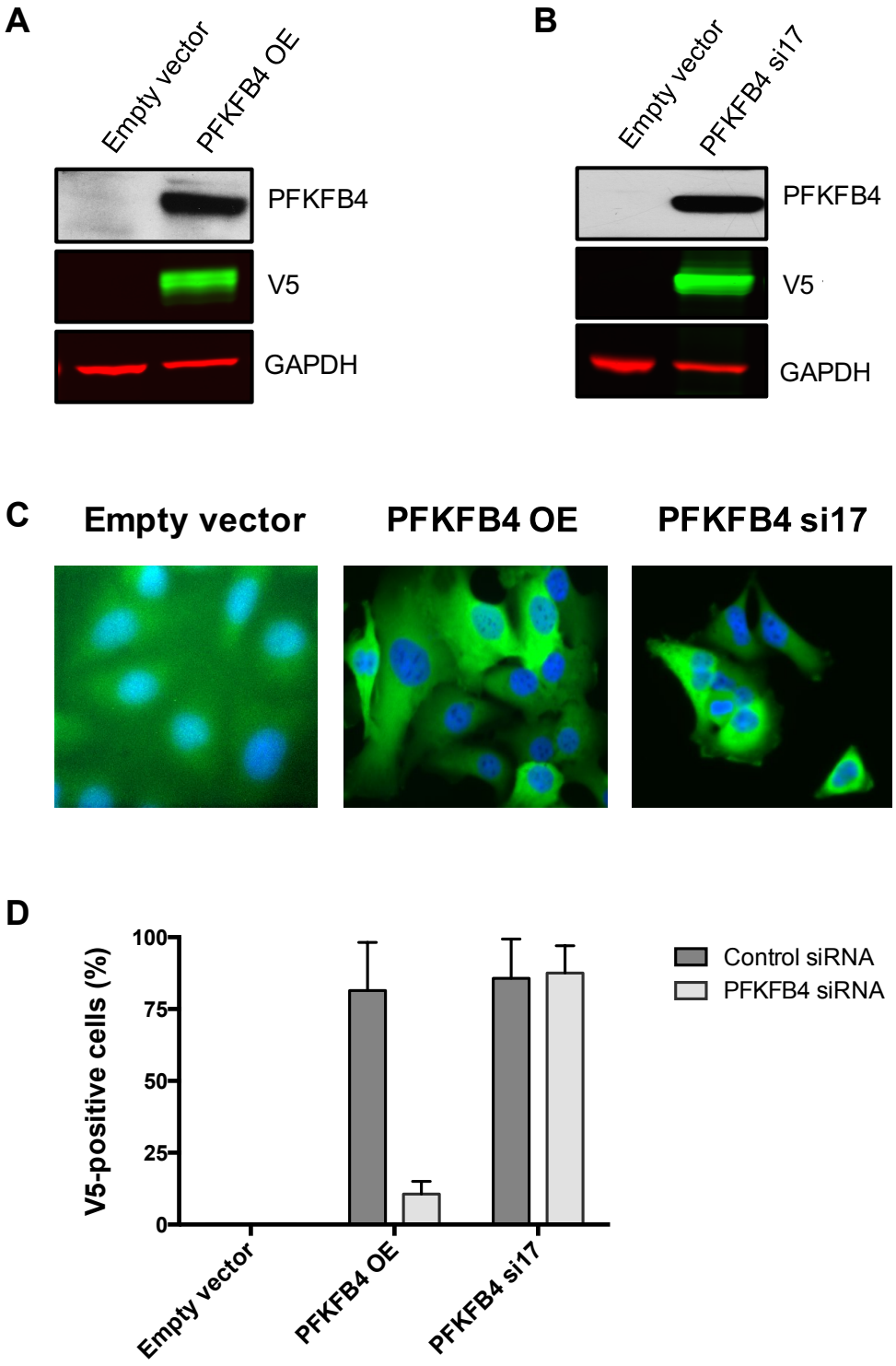


Figure 5.4. Expression of exogenous PFKFB4 in SKOV3 cells.
(A, *n*=2) PFKFB4 and V5 protein expression measured by western blotting in PFKFB4 OE cells and (B, *n*=2) PFKFB4 siRNA 17 resistant cells. GAPDH was used as a loading control. (C, *n*=2) Immunofluorescence images of cells stained with DAPI (blue) and PFKFB4 (green). (D, *n*=2) Bar plot of the results of the InCell analyser after transfection with PFKFB4 siRNA 17 and staining using an anti-V5 antibody to detect V5 positive cells. Approximately 3500 cells were analysed for each condition. Data are represented as the mean + standard error of the mean for two independent experiments.

The small increase in PFKFB4 expression in the PFKFB4 OE cell line did not protect cells from PFKFB4 depletion as transfection with siRNA 17 resulted in a significant decrease in the number of mitotically arrested cells (Figure 5.5A, $p < 0.0001$, two-way ANOVA multiple comparisons). In addition, despite the maintenance of PFKFB4 expression in the PFKFB4 si17 cell line after transfection with siRNA 17, a significant decrease (50%) in the number of mitotically arrested cells after paclitaxel treatment still occurred (Figure 5.5A, $p < 0.0001$, two-way ANOVA multiple comparisons). This was accompanied by an even greater increase in ROS in the mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control in both the PFKFB4 OE cell line (Figure 5.5B, $p = 0.0006$, two-way ANOVA multiple comparisons) and the PFKFB4 si17 cell line (Figure 5.5B, $p = 0.0014$, two-way ANOVA multiple comparisons). The further increase in ROS in the cell lines overexpressing PFKFB4 could be explained by PFKFB4 predominantly functioning as a kinase, or because of its interaction with paclitaxel. These results indicate that either the siRNA is having off-target effects, or that the exogenous PFKFB4 is not functioning like the endogenous protein and therefore is not able to rescue the effect of PFKFB4 depletion. Given that PFKFB4 is bifunctional, the results of overexpression are difficult to predict.

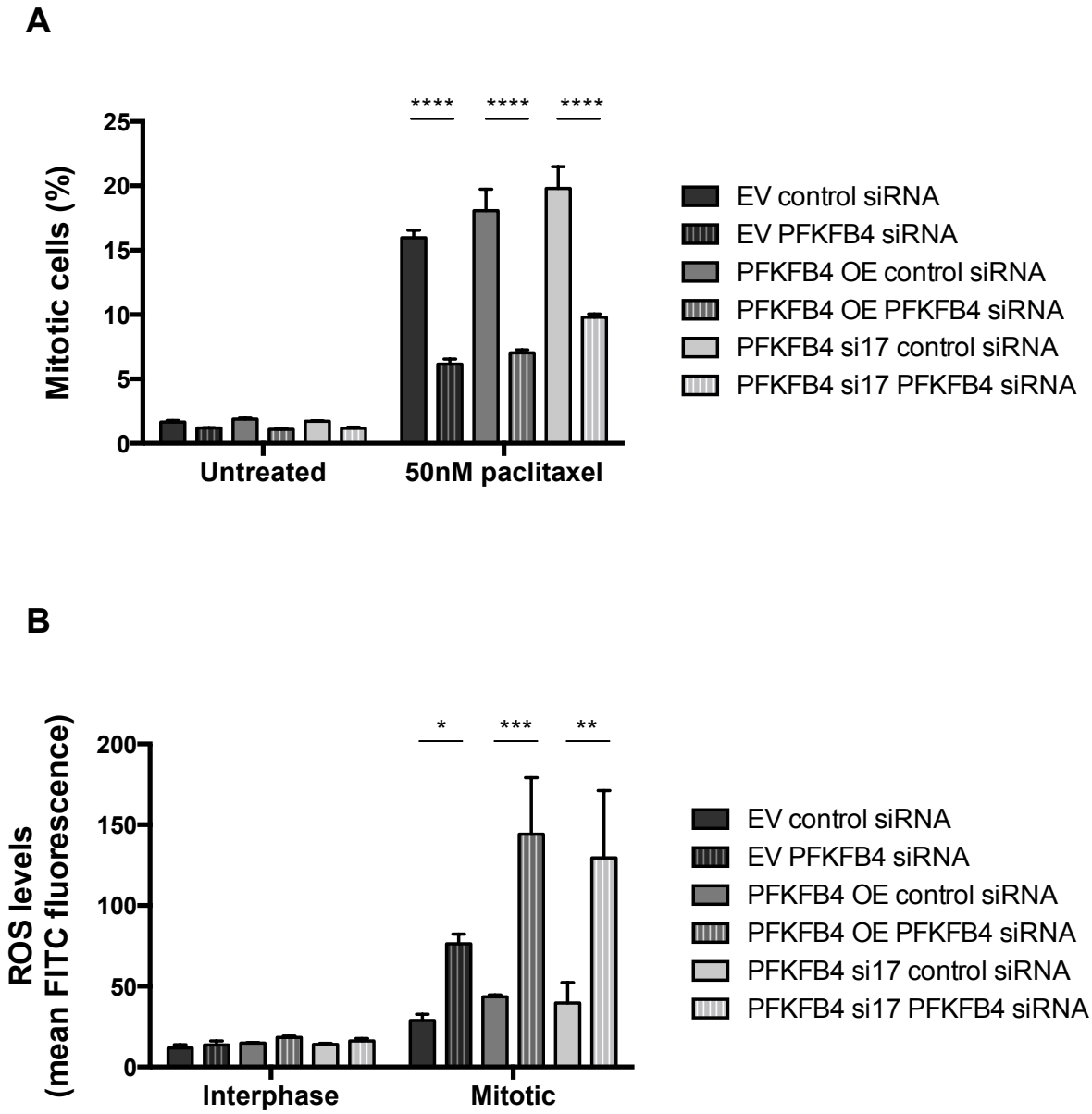


Figure 5.5. Effects of PFKFB4 depletion and paclitaxel treatment in SKOV3 cell lines stably expressing PFKFB4.

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, the percentage of mitotic cells were quantified using anti-phospho histone H3 S10 staining and flow cytometry. 100,000 cells were analysed for each condition. (B, *n*=3) Mitotically arrested cells were isolated by a mitotic shake-off and the levels of reactive oxygen species were measured using flow cytometry. 50,000 cells were analysed for each condition. Data are represented as the mean + standard error of the mean for three independent experiments. *0.01–0.001, **<0.01–0.001, ***<0.001 and ****<0.0001.

5.2.3. PFKFB3 overexpression does not rescue the decrease in mitotic cell number after PFKFB4 depletion

In order to determine whether PFKFB3 overexpression could partially rescue the decrease in mitotically arrested cells after PFKFB4 depletion, I produced a SKOV3 cell line that stably expressed inducible PFKFB3 with a V5 peptide (PFKFB3 OE) (Figure 5.6A). Endogenous PFKFB3 was predominantly nuclear with slight cytoplasmic and membrane staining, while PFKFB3 OE displayed intense nuclear staining with increased cytoplasmic staining (Figure 5.6B). Previous research has shown that PFKFB3 is predominantly nuclear in cell culture (Yalcin et al., 2009; Rodriguez-Rodriguez et al., 2012). This has been attributed to the NLS in PFKFB3 and its nuclear role in regulating cyclin-dependent kinases through the production of fructose 2,6-bisphosphate (Yalcin et al., 2009; Yalcin et al., 2014). As I am looking at mitotically arrested cells that do not possess a nucleus, the different localisations of PFKFB3 and PFKFB4 in interphase are not relevant. However, it is interesting that PFKFB3 has a unique role in cell cycle regulation, yet displays a similar effect to PFKFB4 when it is depleted in paclitaxel treated SKOV3 cells.

Despite the overexpression of PFKFB3, PFKFB4 depletion resulted in a significant reduction in the number of mitotically arrested cells compared to the non-targeting control, with no evidence of partial rescue (Figure 5.6C, $p < 0.0001$, two-way ANOVA multiple comparisons). Levels of ROS were also increased in the mitotically arrested cells after PFKFB4 depletion when compared to the non-targeting control (Figure 5.6D, $p = 0.0014$, two-way ANOVA multiple comparisons). Unlike PFKFB4, the overexpression of PFKFB3 did not increase the levels of ROS beyond that of the empty vector cells.

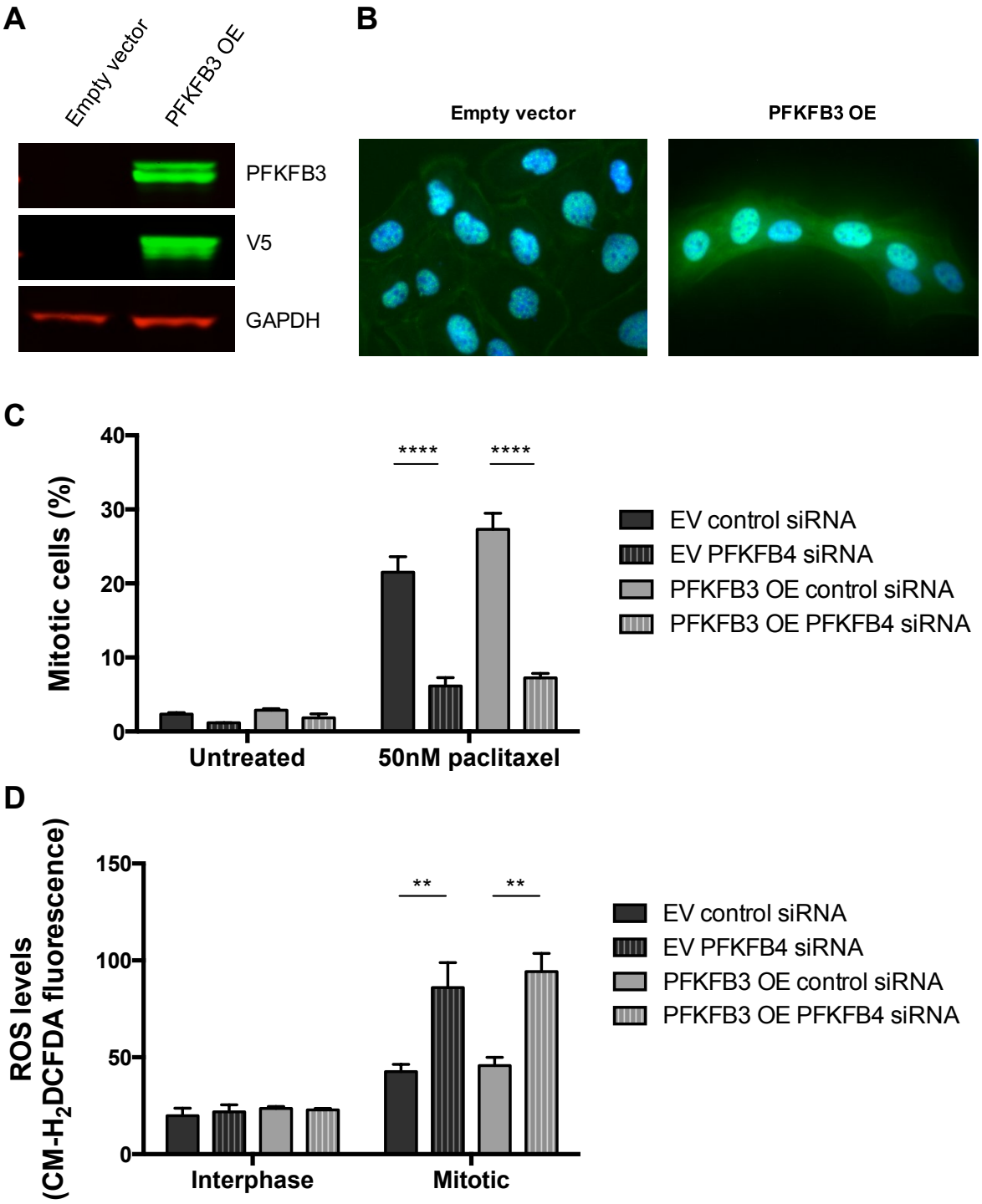


Figure 5.6. Effects of PFKFB4 depletion and paclitaxel treatment in SKOV3 cell lines stably expressing PFKFB3.

(A, *n*=2) PFKFB3 and V5 protein measured by western blotting. GAPDH loading control. (B, *n*=2) Immunofluorescence images of cells stained with DAPI (blue) and PFKFB3 (green). (C, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, the percentage of mitotic cells were quantified using anti-phospho histone H3 S10 staining and flow cytometry. 100,000 cells were analysed for each condition. (D, *n*=2) Mitotically arrested cells were isolated by a mitotic shake-off and the levels of reactive oxygen species were measured using flow cytometry. 50,000 cells were analysed for each condition. Data are represented as the mean + standard error of the mean. *0.01–0.001, **<0.01–0.001, ***<0.001 and ****<0.0001.

5.3. Discussion

This chapter aimed to investigate whether there is a causal link between the loss of AKT activity during mitotic arrest and a sensitivity to PFKFB4 depletion. To address this, I produced two SKOV3 cell lines stably expressing constitutively active AKT through the introduction of phosphomimetic substitutions, T308D and S473D (T308D/S473D), or through the use of a N-terminal myristoylated AKT (Myr-AKT). Both of these alterations have previously been shown to be able to cause constitutive activation of AKT (Alessi et al., 1996a; Andjelkovic et al., 1997) and to directly enhance various aspects of metabolism, such as glucose uptake (Kohn et al., 1996; Foran et al., 1999; Wieman et al., 2007). Additional studies have shown Myr-AKT can increase glycolysis (Elstrom et al., 2004; Govindarajan et al., 2007), glycolytic enzyme activity (Rathmell et al., 2003), ATP levels (Plas et al., 2001; Hahn-Windgassen et al., 2005), amino acid transport and protein synthesis (Hajduch et al., 1998). In order to determine whether the maintenance of AKT signalling could confer resistance to PFKFB4 depletion, I first needed to evaluate the extent to which these two systems maintained AKT signalling during mitotic arrest.

The overexpression of wild-type AKT did not restore AKT activity to levels comparable to those observed in interphase cells. Despite a small increase in GSK3β phosphorylation, the phosphorylation of AKT at S473 and PRAS40 were markedly reduced compared to interphase cells, as was p70S6K phosphorylation. These data were not surprising given the dependence of this system on the endogenous activity of kinases upstream of AKT, which are required to recruit exogenous AKT to the membrane and promote its phosphorylation and subsequent activation. Exogenous wild-type AKT could also be subject to the action of phosphatases, which could be acting to reduce AKT activity. Due to the apparent lack of constitutive AKT activation, this system was not used to assess the link between AKT activity and sensitivity to PFKFB4 depletion.

5.3.1. Evaluating the T308D/S473D SKOV3 cell line

In line with previous studies, SKOV3 cells expressing exogenous AKT with two phosphomimetic substitutions, T308D and S473D, demonstrated increased kinase activity compared to the empty vector and AKT WT cells (Alessi et al.,

1996a; Scheid and Woodgett, 2003; Berndt et al., 2010). The phosphorylation status of AKT during paclitaxel-induced mitotic arrest could not be determined due to the phosphomimetic substitutions preventing phosphoantibody recognition. However, the phosphorylation of two direct substrates of AKT, GSK3β and PRAS40, were increased in mitotically arrested cells compared to interphase, suggesting that local AKT activity had been restored. Interestingly, phosphorylation of p70S6K was not reestablished in mitotically arrested cells, suggesting that despite increased AKT-mediated phosphorylation of direct targets, signalling through mTORC1 is not maintained. A number of reasons could account for the low levels of p70S6K phosphorylation observed during mitotic arrest in T308D/S473D cells:

1). It has been suggested that p70S6K activation is dependent on membrane localisation (Dufner et al., 1999) and the T308D/S473D substitutions do not necessarily target AKT to the membrane. Moreover, additional phosphorylation sites or modifications of AKT might govern this pathway, such as the phosphorylation of T450. In support of this, mutation of T450 to D has shown a moderate increase in p70S6K phosphorylation at T389 (Hart and Vogt, 2011).

2). p70S6K is not a direct downstream substrate of AKT and therefore there is no direct link between AKT activity and p70S6K activation. mTORC1 activity is tightly regulated, with the withdrawal of glucose, amino acids, or oxygen resulting in rapid suppression of its activity (Shaw and Cantley, 2006). The TSC1/TSC2 complex regulates mTORC1 activity and integrates signals from multiple signalling pathways, such as AKT, RSK1, ERK, CDC2, MK2, and AMPK (Inoki et al., 2002; Astrinidis et al., 2003; Fingar and Blenis, 2004; Roux et al., 2004; Ma et al., 2005). Therefore alterations in any of these pathways could be effecting mTORC1 activity and p70S6K phosphorylation at T389.

3). Negative feedback inhibition. Activation of p70S6K can mediate a negative feedback loop that acts to lower AKT activity through the phosphorylation and transcriptional repression of IRS-1 (Harrington et al., 2005; Tzatsos and Kandror, 2006; Zhang et al., 2008b; Efeyan and Sabatini, 2010) and through the phosphorylation of GRB10 (Hsu et al., 2011; Yu et al., 2011). Therefore, it may be

that the presence of T308D/S473D prevents this negative feedback loop from occurring.

4). The overexpression of DEPTOR has been associated with paclitaxel exposure (Zhao et al., 2011b) and resistance in ovarian cancer cells (Foster et al., 2010). DEPTOR overexpression can suppress p70S6K phosphorylation at T389 (Peterson et al., 2009). Therefore, it could be that paclitaxel treatment is enhancing DEPTOR expression, which is inhibiting the phosphorylation of p70S6K.

5). Global downregulation of p70S6K during mitotic arrest. As previously discussed (chapter 3.1.3), a global downregulation of protein synthesis is thought to take place during mitosis and this may well be the case during paclitaxel-induced mitotic arrest. With mTORC1 and p70S6K being critical players in protein synthesis, their activity may be inhibited during this phase. This is supported by the fact that paclitaxel enhances the phosphorylation of p70S6K at T421 and S424 in mitotically arrested cells, which promotes its inactivation (Le et al., 2003).

Further work is required to investigate the mechanism of p70S6K downregulation. Nevertheless, the rescue of local AKT activity as well as RICTOR and mTOR protein levels during mitotic arrest in T308D/S473D cells did not rescue the reduction in mitotic cell number, increase in ROS or apparent MCD or slippage after PFKFB4 depletion. It is possible that the expression of T308D/S473D could not rescue the decrease in mitotically arrested cells for two reasons; either because AKT activity was not effectively maintained during mitotic arrest, or because PFKFB4 depletion is not exploiting the vulnerability created by the loss of AKT activity.

Full AKT activity may not have been restored by T308D/S473D because of the inability of D to correctly mimic phosphothreonine (pT) or phosphoserine (pS). It is important to note that there are considerable differences between the incorporated phosphate group and side chains of the non-phosphorylated amino acid mimics. Firstly, there are differences in size, with the carbon atom of the carboxylate group being smaller than the phosphate atom. Secondly, the number of oxygen atoms are dissimilar, with a phosphate group possessing three and a

carboxylate side chain containing two. Furthermore, the number of negative charges at neutral pH can differ, with the pS/pT being doubly charged and E/D being singly charged (Cooper and Hunter, 1983). pT also demonstrates a weaker resemblance to D or E, and is therefore less likely to accurately mimic phosphorylation. Phosphomimetic mutations might also change the stability of the protein, which could alter the proteins function.

Another consideration is the simplicity of the T308D/S473D system, which reduces AKT activity to be solely governed by the phosphorylation of these two sites. Although they have been shown to be critical for AKT activity, they are unlikely to influence every aspect of AKT function and other factors may well be important to promote certain downstream pathways. Similar to active endogenous phosphorylated AKT, T308D/S473D has been shown to translocate to the nucleus (Adini et al., 2003). However, an T308A/S473A mutant of AKT can also undergo nuclear trafficking, indicating that even though S473 and T308 phosphorylation correlate with AKT nuclear localisation (Wang and Brattain, 2006), they are not essential for it and additional modifications govern this role. This demonstrates that although T308D/S473D may be subject to similar spatiotemporal control as endogenous AKT, it may not be fulfilling all of the same functions.

As I have previously eluded to, AKT activity is under tight control and there are a number of negative feedback loops involved in regulating PI3K/AKT signalling, such as the negative feedback of IRS1 discussed above. Although T308D/S473D is resistant to the activity of phosphatases at these sites, other phosphorylation sites could still be affected and additional mechanisms of downregulation exist. For instance, phosphorylation of AKT at S473 had been shown to induce AKT ubiquitination and subsequent proteasomal degradation of AKT (Wu et al., 2011b). It is possible that T308D/S473D is targeted for degradation in a similar manner. However, a decrease in total AKT levels was not observed in the mitotic population, suggesting that this is unlikely.

5.3.2. Evaluating the Myr-AKT SKOV3 cell line

Membrane targeting of AKT via N-myristoylation achieved an increase in exogenous AKT phosphorylation at S473 in mitotically arrested cells. However,

this was not comparable to levels observed in interphase cells. S473 phosphorylation also appeared to be solely on exogenous AKT, but this may be due to its predominant overexpression. The phosphorylation status of endogenous AKT in the Myr-AKT cell line is unknown. Nonetheless, the phosphorylation of direct AKT substrates, such as GSK3β and PRAS40, were increased in mitotically arrested cells to comparable levels as interphase cells, or higher. However, similar to T308D/S473D, signalling through mTORC1 appeared to be prevented, with a complete lack of p70S6K phosphorylation at T389. Previous studies have suggested that p70S6K phosphorylation is a function of constitutive membrane localisation of active AKT rather than its specific activity (Dufner et al., 1999). The data I have presented does not support this observation as phosphorylation of p70S6K was not present in mitotically arrested Myr-AKT cells. However, this could be due to changes in mTORC1 regulation during mitotic arrest or due to the effects of paclitaxel treatment on p70S6K activity. An alternative explanation could be that myristoylated AKT is not fully active. This could be due to the orientation of the myristoyl moiety relative to AKT, as it is not always static (Resh, 1999). The myristoyl moiety can be sequestered in a hydrophobic pocket of the protein, or flipped out to become available to participate in membrane binding. The transition between these two states is regulated by a mechanism known as the ‘myristoyl switch’. Proteins that are subject to a myristoyl switch typically exhibit reversible membrane binding (Resh, 1999), such as AKT. A reduction in Myr-AKT activity could also be due to the targeting of AKT to internal membranes instead of the plasma membrane, however this does not appear to be the case in other studies (Andjelkovic et al., 1997). Notably, AKT can also be found at internal membranes, such as the endoplasmic reticulum, and it has been hypothesised that AKT activation can also take place here (Hresko and Mueckler, 2005; Boulbes et al., 2011). Nonetheless, the specific membranes that AKT is targeted to could influence its activity.

Moreover, myristoylation does not necessarily induce stable membrane interaction, due to the requirement of other factors, such as protein-protein interactions (Resh, 1999). It could be that these additional requirements are lacking. Once phosphorylated and active, AKT leaves the membrane and targets substrates in the cytoplasm and nucleus (Andjelkovic et al., 1997). Therefore, an additional concern is whether the myristoylation of AKT hinders its nuclear

localisation, preventing AKT from phosphorylating its nuclear targets during interphase. Myr-AKT has been shown to translocate to the nucleus (Adini et al., 2003), suggesting that it is not spatially restricted in the cell. Although the extent to which Myr-AKT is active in the nucleus is not known. However, the nuclear shuttling of Myr-AKT during mitotic arrest is not an issue due to the breakdown of the nuclear envelope. The ability of Myr-AKT to translocate to the nucleus does demonstrate that either its interaction with the plasma membrane is not stable, or that there are cellular mechanisms that can prevent, or release, membrane targeted Myr-AKT.

Although mTOR and RICTOR levels were restored in Myr-AKT, mTORC2 requires additional factors to promote its activation and phosphorylation of AKT, such as SIN1 (Facchinetti et al., 2008). Therefore, it is not known whether mTORC2 is functional in this system and this could limit the activity of Myr-AKT. However, it has been suggested that Myr-AKT can undergo autophosphorylation to phosphorylate S473 in the absence of functional mTORC2 (Facchinetti et al., 2008). The extent to which autophosphorylation governs AKT activity is not clear as a kinase inactive mutant of Myr-AKT can be highly phosphorylated at both T308 and S473 (Andjelkovic et al., 1997). The activity of Myr-AKT can also be significantly reduced by PI3K inhibitors, indicating some dependence on PI3K activity. Therefore, even though Myr-AKT does not require PI3K activity for its membrane localisation (Andjelkovic et al., 1997), it is still somewhat dependent on PI3K activity for its phosphorylation and activation. Myr-AKT is also sensitive to the action of phosphatases (Cazzolli et al., 2001) and could therefore be affected by an increase in phosphatase activity during the later stages of mitosis, or possibly during mitotic arrest.

Taking into account the recently identified link between aberrant cell cycle progression and AKT hyperphosphorylation (Liu et al., 2014a), it could be that the overexpression of AKT is inducing its own cell cycle effects. In addition, Myr-AKT can cause cells to bypass cell cycle checkpoints, leading to uncontrolled cell growth (Kandel et al., 2002). The rates of proliferation in both the T308D/S47D and Myr-AKT cell lines were increased compared to the empty vector cells, indicating that the constitutive activation of AKT is influencing the cell cycle. It would be interesting to determine the phosphorylation status of S477 and T479 in

mitotically arrested cells from the T308D/S473D and Myr-AKT cell lines as this could shed light on the possible cell cycle effects of AKT overexpression. Additionally, elucidation of the phosphorylation of S477 and T479 in the T308D/S473D and Myr-AKT cell lines would be informative.

The overexpression of AKT could also result in alterations to signalling pathways that would not normally occur. For example, the chronic activation of pathways directly upstream and downstream of AKT could sequester proteins away from carrying out their role in other pathways. Alternatively, the overexpression of AKT could cause an increase in PFKFB3 phosphorylation and activity (Manes and El-Maghrabi, 2005), which might negate the effects of AKT overexpression if the function of PFKFB3 is different from that of PFKFB4. Moreover, membrane tethering of AKT could enhance additional pathways that under physiological conditions would otherwise not be activated. For example, Myr-AKT had been found to localise to lipid rafts and possess an altered substrate preference compared with that of endogenous AKT (Adam et al., 2007).

In summary, I have achieved an increase in AKT activity in mitotically arrested SKOV3 cells with the use of T308D/S473D and Myr-AKT. However, this did not confer resistance to PFKFB4 depletion. It is probable that PFKFB4 depletion is not exploiting the decrease in AKT and that the siRNA is effecting non-specific targets or causing aberrant cell cycle effects. In addition, the overexpression of AKT is likely to have its own effects on the cell cycle, making the system very difficult to interpret. Moreover, it is unlikely that AKT activity is solely governed by phosphorylation at S473 and T308, or membrane localisation, and requires a number of additional factors for full activation. Therefore, there is doubt over whether these systems have fully restored AKT activity to those comparable to endogenous active AKT.

5.3.3. PFKFB3 and PFKFB4 overexpression studies

To further elucidate the mechanism of action and to determine whether there are off-target effects of the siRNA duplexes, I produced SKOV3 cell lines that stably overexpressed wild-type PFKFB4 (PFKFB4 OE), siRNA resistant PFKFB4 (PFKFB4 si17) or wild-type inducible PFKFB3 (PFKFB3 OE). PFKFB4

overexpression has been shown to increase fructose 2,6-phosphate, glycolysis and ATP, while decreasing NADPH in a number of different ovarian cancer cell lines (Chesney et al., 2014). Overexpression of PFKFB3 splice variant 1 has also been shown to increase the levels of fructose 2,6-bisphosphate and lactate (Seo and Lee, 2014). However, overexpression of PFKFB3 splice variant 5 can dramatically enhance the proliferation rates of cells (Yalcin et al., 2009), demonstrating its role in cell proliferation as well as metabolism. Analysis of *PFKFB4* and *PFKFB3* splice variant expression in ovarian cancers from the TCGA database identified splice variant 1 of PFKFB3 and PFKFB4 as by far the most abundantly expressed. As such, these were the splice variants overexpressed in this work. However, it would be beneficial to determine which specific splice variants are most abundantly expressed in SKOV3 cells as these may be different from that indicated in TCGA analysis and could potentially explain why the overexpression of PFKFB3 or PFKFB4 splice variant 1 did not confer any resistance.

Nuclear PFKFB3 is thought to influence cell proliferation through its production of fructose 2,6-bisphosphate in the nucleus, which can directly increase the expression and activity of CDK1 (Yalcin et al., 2009; Yalcin et al., 2014). Therefore, if the overexpression of either PFKFB3 or PFKFB4 results in increased fructose 2,6-bisphosphate, this could influence CDK1 activity and potentially mitosis. Moreover, in HeLa cells synchronised by double thymidine block, fructose 2,6-bisphosphate was found to be the lowest in G2/M and highest in G1/S (Yalcin et al., 2009), demonstrating how fructose 2,6-bisphosphate oscillates throughout the cell cycle. Thus, the overexpression of PFKFB3 and PFKFB4 could disrupt the apparent spatiotemporal control of fructose 2,6-bisphosphate and induce cell cycle effects.

The overexpression of PFKFB4 and PFKFB3 could also influence AKT activity. Both PFKFB4 and PFKFB3 depletion have been shown to reduce AKT phosphorylation at S473 (Huo et al., 2010; Xu et al., 2014; Pegoraro et al., 2015). Therefore, their overexpression could act to enhance AKT phosphorylation and activity. In addition, the ubiquitous isoform of PFKFB3 can be phosphorylated by AKT at S460 (Manes and El-Maghrabi, 2005). The overexpression of PFKFB3

could then act to sequester AKT away from its other intracellular substrates, which could reduce its activity even further.

Previous studies have observed a reduction in PFKFB3 kinase activity in response to oxidative stress, which increases NADPH and enables the cell to reduce the levels of ROS (Seo and Lee, 2014). Similarly, two studies have identified PFKFB4 as possessing a predominant phosphatase activity, which results in the redirection of metabolites to the PPP, enhancing detoxification (Ros et al., 2012; Strohecker et al., 2015). However, the overexpression of PFKFB4 or PFKFB3 did not reduce the levels of ROS induced by PFKFB4 depletion in mitotically arrested cells. This could be due to PFKFB3 and PFKFB4 possessing a different function in ovarian cancer cell lines, for example, a predominant kinase activity for PFKFB4 (Goidts et al., 2012; Chesney et al., 2014), or it could suggest an altered response of PFKFB3 to oxidative stress. In the case of PFKFB3, it could also be that the levels of ROS are not high enough to cause a switch in its activity from a kinase to phosphatase. This is supported by that fact that ROS levels do not appear to be a causative factor in mitotic cell death as demonstrated by the use of NAC treatment in chapter 4. Additional support comes from the observation that AMPK phosphorylation is not enhanced in PFKFB4 depleted mitotically arrested cells, suggesting that ROS levels may not be high enough to initiate a cellular response to oxidative stress.

Neither PFKFB4 or PFKFB3 overexpression conferred any resistance to PFKFB4 depletion and paclitaxel treatment. These results suggest that either there are additional off-target effects of the siRNA duplexes, or that the exogenous PFKFB3 and PFKFB4 are not functioning like their endogenous counterparts. One reason for a decrease in their function could be the presence of a V5-tag hindering the dimerisation of the proteins. The PFKFB family can form dimers through the interaction of their N-terminal domains (Hasemann et al., 1996). Therefore, the presence of a V5-tag at their C-terminus could act to either disrupt the formation of the dimer, or disrupt the function of the dimer once it is formed. An alternative explanation could be due to the degradation of PFKFB3 and PFKFB4 during mitotic arrest. PFKFB3 is known to be targeted for degradation by APC/C-Cdh1 via its KEN box (Tudzarova et al., 2011). However, PFKFB4 does

not contain a KEN box and has not yet been associated with ubiquitin-mediated degradation so this is unlikely.

Simply overexpressing PFKFB3 and PFKFB4 does not necessarily induce their activity. As such, another important consideration is the concentration of their substrates and products, fructose 6-phosphate and fructose 2,6-bisphosphate. This is especially important for PFKFB4 given that no post-translational modifications are currently known to regulate its activity and thus, it is largely determined by the concentration of its substrates and products. Conversely, PFKFB3 is subject to vast post-translational modifications, which could influence its activity, or lack thereof. The incorporation of phosphomimetic mutations in PFKFB3 could be a way to promote its activity, but this comes with the caveats previously noted in this discussion regarding the use of overexpression systems. The concern over whether exogenous PFKFB4 is active is an important consideration in the si17 PFKFB4 system. Given that the si17 PFKFB4 is resistant to the siRNA treatment, it was surprising that there was no observable rescue. One approach to determine whether the phenotype is the result of off-target effects of the siRNA would be to use CRISPR-Cas9 to induce silent mutations into endogenous *PFKFB4* and determine whether the siRNA duplexes result in a similar phenotype.

5.3.4. Limitations

The major limitation of this work is the use of protein overexpression, as this is not representative of endogenous protein activity and can result in a number of false positives. The lentiviral expression system has been useful in allowing the stable expression of specific proteins, however the number of viral integrations into the genome was not quantified and therefore the extent of the overexpression remains unknown. Despite the limitations, protein modifications and overexpression still remain a valuable tool in cell biology as long as one is aware of the constraints of the system.

Given the roles of AKT, PFKFB3 and PFKFB4 in metabolism, it was expected that the expression of GAPDH may be affected by the overexpression of these proteins. However, in contrast to previous observations (Govindarajan et al.,

2007), no consistent alterations were observed and therefore GAPDH was considered an appropriate loading control for western blot analysis. Nonetheless, the use of a second loading control, such as β-actin or α-tubulin may have been useful to provide confirmation of this and to rule out any inaccuracies in sample loading.

5.3.5. Conclusions

These data provide evidence of the possible complexities in cell signalling during paclitaxel-induced mitotic arrest and the extent of its regulation, which may not be able to be overcome by simply overexpressing specific proteins. The maintenance of AKT activity during mitotic arrest did not confer any resistance to PFKFB4 depletion and paclitaxel treatment. These data suggest that PFKFB4 depletion is not exploiting the vulnerability created from loss of AKT activity, or that the overexpression of AKT and/or the depletion of PFKFB4 are inducing their own cell cycle effects. However, it would be useful to characterise the metabolic phenotype of Myr-AKT and T308D/S473D cell lines in more depth in order to determine whether PFKFB4 was exploiting a metabolic vulnerability during paclitaxel-induced mitotic arrest. The inability to rescue the phenotype by the overexpression of PFKFB3 and PFKFB4 indicate that there may be off-target effects of the siRNA duplexes or that the exogenous proteins are not fully functional. Future attempts to address this phenotype should be more direct, for example, focus could be directed on the cause of mitotic cell death and attempts to rescue this, or employ more newly developed techniques that would give a more definitive answer, such as CRISPR-Cas9 technology.

Chapter 6

Summary

6. Summary

6.1. Significance

In this thesis I have discussed the problem that we currently face regarding the treatment of ovarian cancer, and the development of chemotherapy resistance as a major barrier to the success of current chemotherapy regimes. There is a vital requirement for novel therapies that enhance patient response to standard chemotherapy and improve patient outcome.

Given the importance of PI3K/AKT signalling in cancer cells and its potential utility as a therapeutic target for the treatment of ovarian cancer, I initially explored AKT signalling in ovarian cancer cell lines. To my knowledge, this is the first research exploring AKT phosphorylation during mitosis in ovarian cancer cells that have not been subject to any form of cell cycle synchronisation. This work is also novel in recognising a potential metabolic vulnerability in paclitaxel treated mitotically arrested ovarian cancer cells. This observation could open the door for the development of new therapies that target this vulnerability with the purpose of improving patient response to paclitaxel. It also provides valuable insight into the activity of AKT in paclitaxel treated mitotically arrested cells with regards to the use of AKT inhibitors in conjunction with paclitaxel treatment. If AKT activity is already reduced in mitotically arrested ovarian cancer cells, then the additional inhibition of the PI3K/AKT pathway may not further improve the cellular response to paclitaxel. However, additional work is required to confirm this hypothesis. The use of AKT inhibitors in combination with other chemotherapy regimes may still be beneficial.

The work presented in this thesis has also uncovered a novel function of the metabolic enzymes PFKFB3 and PFKFB4 in ovarian cancer cells during mitotic arrest. Whilst numerous studies have identified the importance of PFKFB3 and PFKFB4 in cancer cell survival (Ros et al., 2012; Klarer et al., 2014; Yalcin et al., 2014), I am the first to recognise the impact of their depletion in ovarian cancer cells and to identify their potential importance during paclitaxel-induced mitotic arrest. Both PFKFB3 and PFKFB4 appear to be somewhat required for ovarian cancer cell survival during mitotic arrest, and further investigation into the effects

of PFKFB4 depletion identified a potential cell cycle phenotype, characterised by an increase in mitotic slippage. These results are very provocative and require further investigation to determine the reasons why the depletion of these metabolic enzymes result in the identified phenotypes, and whether they have therapeutic potential. My observations indicate that combining paclitaxel treatment with inhibitors that target metabolic enzymes could have the potential to improve response to paclitaxel in ovarian cancer. However, the effects of PFKFB4 depletion on the cell cycle need to be addressed before this can be confirmed. Moreover, the potential importance of *de novo* lipid synthesis during mitotic arrest is a new area of investigation and could be a promising avenue for the development of targeted therapeutics.

The inability of exogenous constitutively active AKT or siRNA-resistant PFKFB4 to rescue the decrease in mitotically arrested cells following PFKFB4 depletion and paclitaxel treatment demonstrated the complexity of protein overexpression systems. It also highlighted the potential difficulties one faces when investigating siRNA-mediated depletion, protein overexpression systems, and bifunctional proteins with closely related family members. It is possible that the effects seen following PFKFB4 depletion and mitotic arrest are not due to it exploiting the vulnerability created by the loss of AKT activity, but the result of alternative detrimental pathways, or issues with the specificity of the siRNA or the activity of exogenous PFKFB4.

6.2. Key limitations

As I have acknowledged in prior discussions, there are numerous limitations regarding the use of siRNA-mediated depletion, protein overexpression systems and the use of cell lines. However, being aware of the pitfalls during the interpretation of the data enables informative conclusions to be drawn from experiments employing these techniques.

An additional and important limitation of the data that I have presented concerns the use of high glucose culture medium. The environmental conditions of cancer cells *in vivo* differ substantially from those *in vitro*. In particular, the high glucose concentrations of cell culture media, such as DMEM (4500 mg/L) and McCoy's

5A (3000 mg/L), are far higher than normal blood glucose concentrations (900 mg/L) (Freckmann et al., 2007). In addition, tumour cells frequently possess glucose concentrations that are 3- to 10-fold lower than their non-tumourigenic counterparts (Gullino et al., 1967; Hirayama et al., 2009; Urasaki et al., 2012). This is thought to be due to their poor vasculature and enhanced glucose consumption. Therefore, cancer cell lines grown in an environment where glucose is abundant may develop a strong dependence on glucose and neglect other biosynthesis and energetic pathways, and as a result become more sensitive to factors that prevent the utilisation of glucose. Moreover, the addition of FBS is regarded as non-physiological given the nature of its components and batch-to-batch variation (Gstraunthaler et al., 2013). The conditions that cells are grown in will undoubtably alter the cells metabolic phenotype and therefore is an important consideration when carrying out metabolic studies in cell culture settings.

6.3. Future directions

Future work should address the clinical relevance of these findings to determine whether the observations hold true *in vivo*. For example, elucidating the phosphorylation status of AKT in mitotic or mitotically arrested cells in tumour samples is critical to determine whether these findings have therapeutic value. I previously employed immunohistochemistry to stain for phosphorylated AKT in normal ovarian tissue as well as low- and high-grade ovarian tumour samples. However, the staining was not sufficient to enable quantification and no conclusions could be drawn. Therefore, optimisation of this staining should be undertaken. The glucose-rich environment of the cell cultures in this work should also be addressed to more accurately recapitulate the environment *in vivo*. Carrying out the experiments in cell lines maintained in 900 mg/L glucose or lower would be important to determine whether or not the observations are due to cell culture effects.

These findings could also be expanded to look at other cancer cell lines, such as breast and prostate, to determine its applicability to other cancers. In addition, it would be useful to examine normal ovarian tissue, such as fallopian tube epithelial cells. However, it is difficult to obtain such samples in high enough cell

number to be able to carry out the experiments required. Preliminary studies carried out using human skin fibroblast cells demonstrated that they do not possess phosphorylated AKT and therefore, would not have the vulnerability identified in the ovarian cancer cells in this work. In addition, treatment of these cells with paclitaxel only caused a small increase in mitotically arrested cells, which is most likely due to their slow rates of proliferation. Thus, the magnitude of the reduction of mitotically arrested fallopian tube epithelial cells after PFKFB4 depletion would be far less than what is observed when using ovarian cancer cell lines.

In order to investigate the limitations surrounding siRNA-mediated depletion, it would be informative to use the CRISPR-Cas9 system to induce silent mutations into endogenous PFKFB4 that render it resistant to siRNA duplexes. Subsequent treatment with siRNA duplexes would then enable the identification of any effects that are not specific to PFKFB4-mediated depletion. An inducible degron system could also be introduced to overcome any off-target effects arising from the use of siRNA duplexes (Holland et al., 2012; Kanemaki, 2013). This system could initiate the specific and rapid degradation of endogenous PFKFB4 after paclitaxel treatment, or specifically in mitotically arrested cells. However, the feasibility of this system depends on the stability of the protein.

The importance of these investigations are demonstrated by the large amount of research going into the development of PFKFB3 and PFKFB4 inhibitors (Clem et al., 2008; Clem et al., 2013; Pyrkov et al., 2013; Brooke et al., 2014; Chesney et al., 2015) and the undertaking of the first-in-man clinical trial for PFK158, which targets PFKFB3 (Redman et al., 2015). Investigations into whether these inhibitors can enhance the response to paclitaxel *in vivo* could be beneficial and help to elucidate whether the observations made in this thesis are attributable to the kinase or phosphatase activity of PFKFB3 and PFKFB4.

In summary, this thesis has contributed valuable observations regarding PI3K/AKT signalling in ovarian cancer cells during mitosis and paclitaxel-induced mitotic arrest. It has identified a potential therapeutic window that could be exploited to increase the cellular response to paclitaxel, and singled out two

metabolic enzymes with novel roles in mitotically arrested ovarian cancer cells. Continued research is essential to determine whether approaches using paclitaxel in combination with metabolic inhibitors have promise in ovarian cancer treatment.

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8. Appendices

8.1. Screen results

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
CCR9	122.25	132.19	98.01	6.42	15.28
WIF1	112.06	128.65	303.74	24.25	12.53
TEX14	116.07	101.37	198.67	18.78	10.58
CCR2	104.39	127.51	95.67	10.23	9.35
XYLB	101.82	118.52	99.83	12.32	8.10
PLK1	100.93	98.80	139.26	17.88	7.79
PACE-1	106.62	104.54	82.07	10.65	7.70
MVD	103.67	122.41	66.66	9.51	7.01
GNB4	99.85	100.42	57.56	8.32	6.92
MYO3A	103.58	117.68	175.94	26.41	6.66
WEE1	101.55	111.17	81.64	12.29	6.64
ABI1	121.47	136.48	102.50	15.90	6.45
PRPS1	107.19	109.87	211.93	33.44	6.34
BLR1	120.99	107.61	179.35	28.65	6.26
MAPK4	122.29	128.53	154.22	25.28	6.10
PRKG2	104.99	120.26	61.77	10.32	5.99
BCKDK	102.08	105.76	236.15	41.63	5.67
SQSTM1	114.74	117.06	189.39	34.08	5.56
PITPNM3	105.80	117.89	135.96	24.72	5.50
MPP2	106.68	98.96	56.90	11.27	5.05
RPS6KA1	98.80	110.62	83.62	17.27	4.84

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
MGC4796	112.63	117.21	266.88	55.84	4.78
MIDORI	107.17	126.74	287.25	60.51	4.75
FKSG79	110.82	118.72	370.24	78.70	4.70
CXCR3	107.99	131.34	361.37	77.70	4.65
STK22C	118.62	130.54	110.89	24.01	4.62
MAP3K7	118.63	127.22	149.88	33.92	4.42
GNAI1	102.45	111.29	20.15	4.57	4.41
CAMK2B	106.31	136.85	351.89	88.68	3.97
AVPR2	102.54	100.56	88.71	22.84	3.88
EPHB6	102.38	108.13	77.27	19.97	3.87
CDKN3	102.08	100.82	18.90	4.97	3.80
PFKFB4	97.02	133.00	76.84	20.75	3.70
MATK	95.02	135.86	93.77	25.37	3.70
CSF1R	100.57	113.92	294.74	82.19	3.59
DEFB4	127.07	130.49	34.56	9.65	3.58
PMVK	108.82	124.58	192.31	54.31	3.54
RIOK1	100.00	114.44	91.06	26.02	3.50
TLR6	111.25	110.44	91.06	26.73	3.41
EPHA7	110.44	109.50	122.28	35.97	3.40
TLR4	93.12	86.30	65.67	19.36	3.39
IHPK3	108.85	108.97	153.21	45.28	3.38
RPS6KA3	106.94	120.49	229.40	68.71	3.34
CARD10	101.20	103.37	161.62	49.29	3.28
SMG1	96.68	90.22	52.89	16.70	3.17

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
CDC2L2	108.50	123.11	194.72	61.54	3.16
DUSP4	102.36	121.50	116.20	36.90	3.15
KSR2	112.83	125.68	102.68	33.56	3.06
TJP2	105.32	109.69	428.13	140.83	3.04
DGKI	104.89	137.11	224.87	74.56	3.02
DGKE	100.42	97.56	121.18	40.26	3.01
MAP3K10	110.56	136.70	397.32	132.95	2.99
RAPGEF3	105.59	110.97	88.15	29.55	2.98
MAP2K1	123.53	135.24	241.84	81.10	2.98
KALRN	104.08	103.79	176.07	59.06	2.98
BUB1	88.81	104.79	126.62	42.49	2.98
CAMKK2	103.65	95.88	155.40	52.30	2.97
FLT4	100.71	102.26	117.74	39.92	2.95
CSNK1G3	106.80	128.91	117.90	40.09	2.94
PLK3	108.22	132.73	177.95	60.52	2.94
C6ORF199	106.00	112.28	90.98	31.28	2.91
RPS6KA4	106.09	120.51	97.10	33.40	2.91
MAP3K13	103.60	109.29	114.96	39.63	2.90
FKSG83	95.55	98.12	131.52	45.37	2.90
DGKZ	102.91	124.50	171.91	59.56	2.89
PPP2CB	105.92	119.32	109.28	37.95	2.88
CINP	108.50	123.45	54.00	18.93	2.85
EPHA4	109.71	121.71	45.30	15.88	2.85

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
PRKR	104.08	98.50	189.45	66.56	2.85
GRK4	103.49	107.14	37.12	13.09	2.83
CNR2	108.11	108.52	261.08	92.42	2.83
ADRB3	106.10	108.82	175.30	62.08	2.82
PRPS1L1	106.98	121.47	54.78	19.57	2.80
MAGI-3	104.25	115.99	298.04	107.71	2.77
PRKCB1	100.73	102.88	84.01	30.42	2.76
TRIB2	100.23	119.05	45.82	16.70	2.74
MERTK	102.88	111.61	305.54	112.62	2.71
NPR1	110.03	108.00	105.01	38.74	2.71
BTK	105.22	111.74	58.68	21.69	2.71
PXK	102.79	122.34	193.71	72.19	2.68
CXCL12	103.94	101.90	44.80	16.75	2.67
MAP3K1	101.13	110.30	73.30	27.61	2.65
AGTR2	108.96	104.61	91.72	34.75	2.64
NEK7	105.63	124.36	192.22	73.09	2.63
FGR	103.99	105.69	26.69	10.16	2.63
MAP3K8	106.79	116.12	89.93	34.44	2.61
RET	104.21	123.21	184.00	70.50	2.61
CDADC1	97.32	92.25	35.69	13.74	2.60
CX3CR1	109.20	103.25	57.88	22.37	2.59
GALK1	106.90	120.94	82.87	32.08	2.58
SMAD7	105.31	126.64	183.47	71.07	2.58
CCR6	109.44	133.95	153.68	59.73	2.57

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
SYK	105.45	113.17	129.83	51.45	2.52
FZD9	116.71	120.31	68.07	26.99	2.52
ULK2	106.65	118.47	36.61	14.62	2.50
MYLK	104.77	128.37	80.18	32.19	2.49
MAST3	103.49	140.97	87.63	35.74	2.45
SPA17	106.64	119.48	30.30	12.38	2.45
PHKG1	106.32	112.21	103.19	42.41	2.43
GIT2	104.20	115.91	84.43	34.95	2.42
GLP1R	102.17	107.07	122.45	51.03	2.40
MAP3K12	102.16	117.59	123.46	51.64	2.39
CALCA	104.91	120.61	384.71	161.69	2.38
ACK1	97.31	100.93	246.06	103.79	2.37
PTPRJ	99.64	111.90	232.41	98.04	2.37
ADRBK2	97.77	103.30	111.55	47.22	2.36
DDR2	99.87	78.26	46.97	19.97	2.35
PRKCN	102.79	109.63	80.50	34.30	2.35
MAP3K2	96.46	113.32	202.90	86.86	2.34
FPRL2	104.10	108.10	89.29	38.40	2.33
GSG2	101.49	123.35	152.01	65.61	2.32
FY	105.86	123.60	319.32	138.13	2.31
PLK2	108.49	125.37	124.09	53.71	2.31
DGKQ	102.93	128.27	138.75	60.15	2.31
ARAF1	106.37	119.50	11.84	5.15	2.30
PTK2	107.01	95.40	40.38	17.59	2.30

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
GNAI3	111.72	126.85	90.82	39.65	2.29
SNF1LK	107.60	112.32	94.45	41.25	2.29
YES1	101.47	103.59	147.84	64.66	2.29
PKN3	99.23	107.52	99.35	43.68	2.27
CHRM3	108.51	113.20	78.05	34.39	2.27
TLK1	105.76	116.67	138.99	61.32	2.27
TAO1	98.46	115.65	125.29	55.74	2.25
PDGFRA	110.93	103.34	85.82	38.19	2.25
CDC7	106.87	109.13	67.32	30.04	2.24
STK22B	113.49	128.33	250.50	112.38	2.23
TSKS	111.65	133.10	132.34	59.48	2.22
TGFBR2	99.45	113.81	107.10	48.36	2.21
CSNK1A1	97.58	103.69	137.05	62.05	2.21
JAK1	95.27	113.65	52.77	23.91	2.21
TRIM	100.35	111.52	79.36	36.04	2.20
STK6	111.27	109.92	169.81	77.48	2.19
PRPF4B	97.57	103.00	41.55	18.98	2.19
MGC26597	99.16	110.22	70.74	32.35	2.19
GCK	103.24	114.50	50.33	23.07	2.18
MST4	101.75	116.48	131.01	60.28	2.17
NME1	98.71	102.76	80.36	37.09	2.17
LYN	107.71	118.59	140.02	65.07	2.15
COL4A3BP	109.65	115.31	360.32	167.53	2.15

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
PTK9	103.62	105.01	109.14	50.82	2.15
ASK	103.90	106.23	57.92	26.98	2.15
BAI2	95.57	106.92	76.78	35.83	2.14
DRD1	108.62	127.18	172.27	80.71	2.13
NEK11	101.71	126.62	81.08	38.25	2.12
JAK2	110.90	126.93	166.52	78.91	2.11
DYRK3	110.62	116.17	130.67	61.98	2.11
MAPKAPK3	98.66	93.06	127.48	60.77	2.10
GRK1	107.58	131.28	333.77	159.15	2.10
GUCY2D	107.24	114.74	88.22	42.21	2.09
MARK2	107.18	130.15	117.71	56.33	2.09
SRC	98.48	103.45	101.41	48.66	2.08
RIPK3	93.94	102.06	158.27	76.21	2.08
LATS2	108.00	127.72	105.60	50.88	2.08
CNKSR1	101.05	122.34	148.82	71.71	2.08
CASK	106.40	111.90	76.33	36.85	2.07
MYO3B	99.88	106.30	110.46	53.38	2.07
TLR3	103.20	100.36	85.52	41.35	2.07
LYK5	106.30	116.91	175.63	85.20	2.06
ITK	121.59	127.92	34.51	16.83	2.05
STK22D	98.19	122.69	122.63	59.85	2.05
STK38L	106.87	108.72	129.13	63.04	2.05
PFKL	119.25	126.91	110.10	53.76	2.05
VRK1	101.86	116.87	138.47	68.29	2.03

Gene	PUDR	PDR	PUMR	PMR	PUMR/PMR
PKIB	100.36	106.17	146.98	73.27	2.01
RPS6KB1	105.84	117.54	273.36	136.39	2.00
FASTK	104.93	110.92	122.67	61.31	2.00

Table S3.1. Target genes that resulted in a PUMR/PMR ratio of ≥2.
Quantification of DNA content using Hoechst stain and mitotic cell number using anti-phospho histone H3 S10 staining following siRNA-mediated depletion of target genes and paclitaxel treatment. **PUMR,** The mitotic index following gene depletion in untreated cells divided by the mitotic index following non-targeting siRNA control treatment in untreated cells. **PMR**, The mitotic index following gene depletion in paclitaxel treated cells divided by the mitotic index following non-targeting siRNA control treatment in paclitaxel treated cells. **PUDR**, The DNA content following gene depletion in untreated cells divided by the mitotic index following non-targeting siRNA control treatment in untreated cells. **PDR**, The DNA content following gene depletion in paclitaxel treated cells divided by the mitotic index following non-targeting siRNA control treatment in paclitaxel treated cells

8.2. Microscopy images for time-lapse imaging

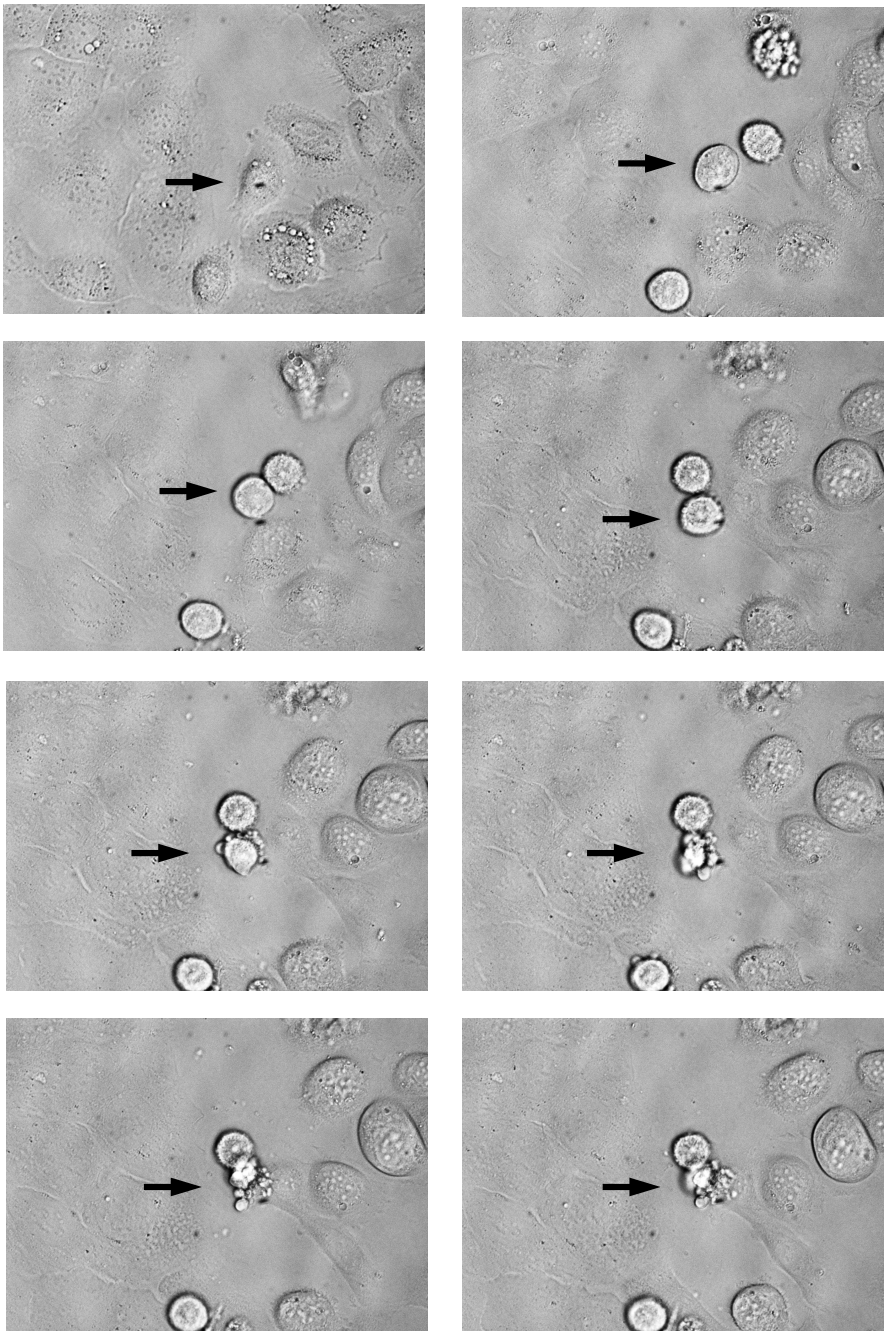


Figure S4.1A. Time-lapse images of SKOV3 cells undergoing mitotic cell death following paclitaxel treatment.
Black arrows are pointing towards mitotically arrested cells that undergo mitotic cell death.

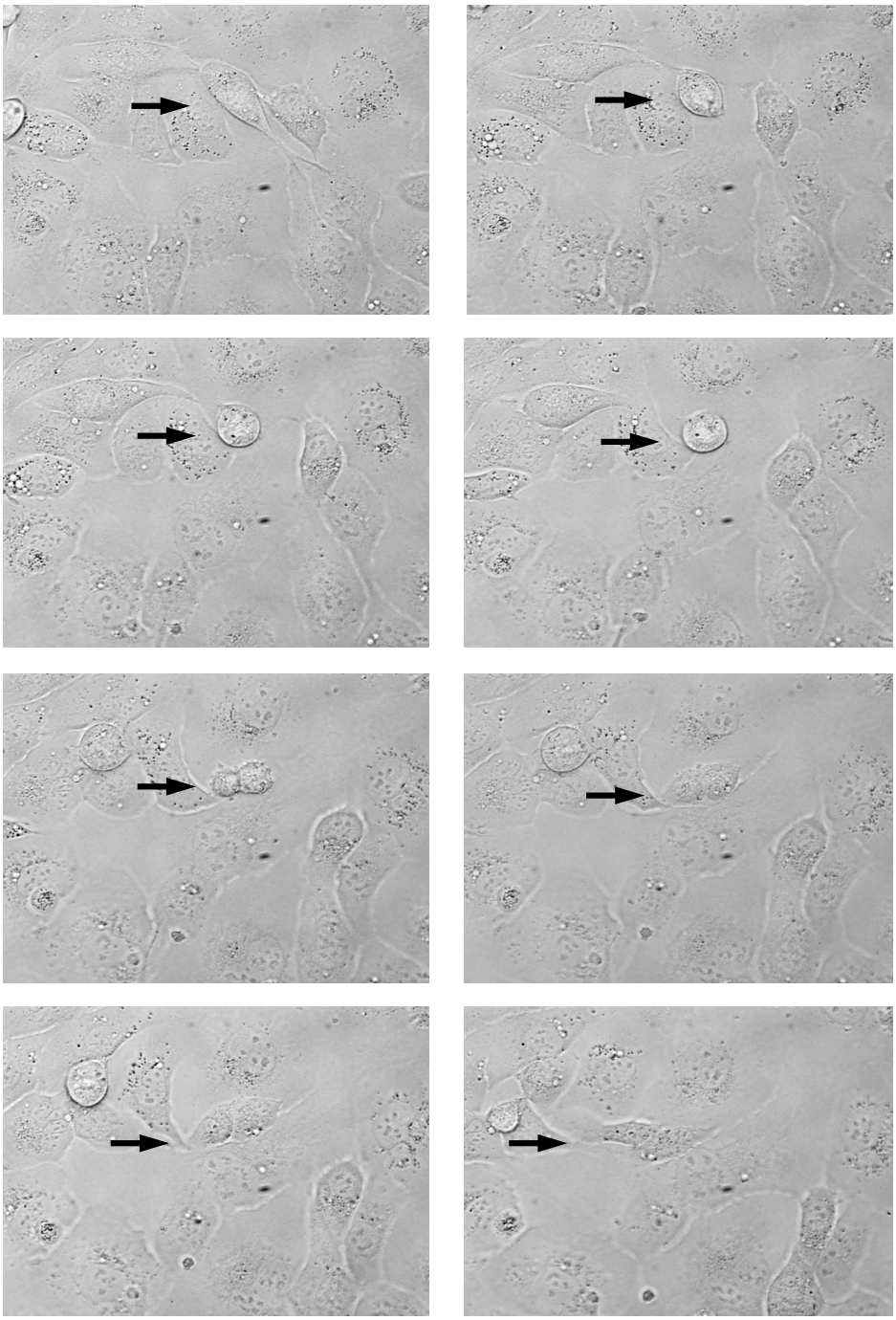


Figure S4.1B. Time-lapse images of SKOV3 cells undergoing spontaneous mitosis.
Black arrows point towards cells undergoing mitosis.

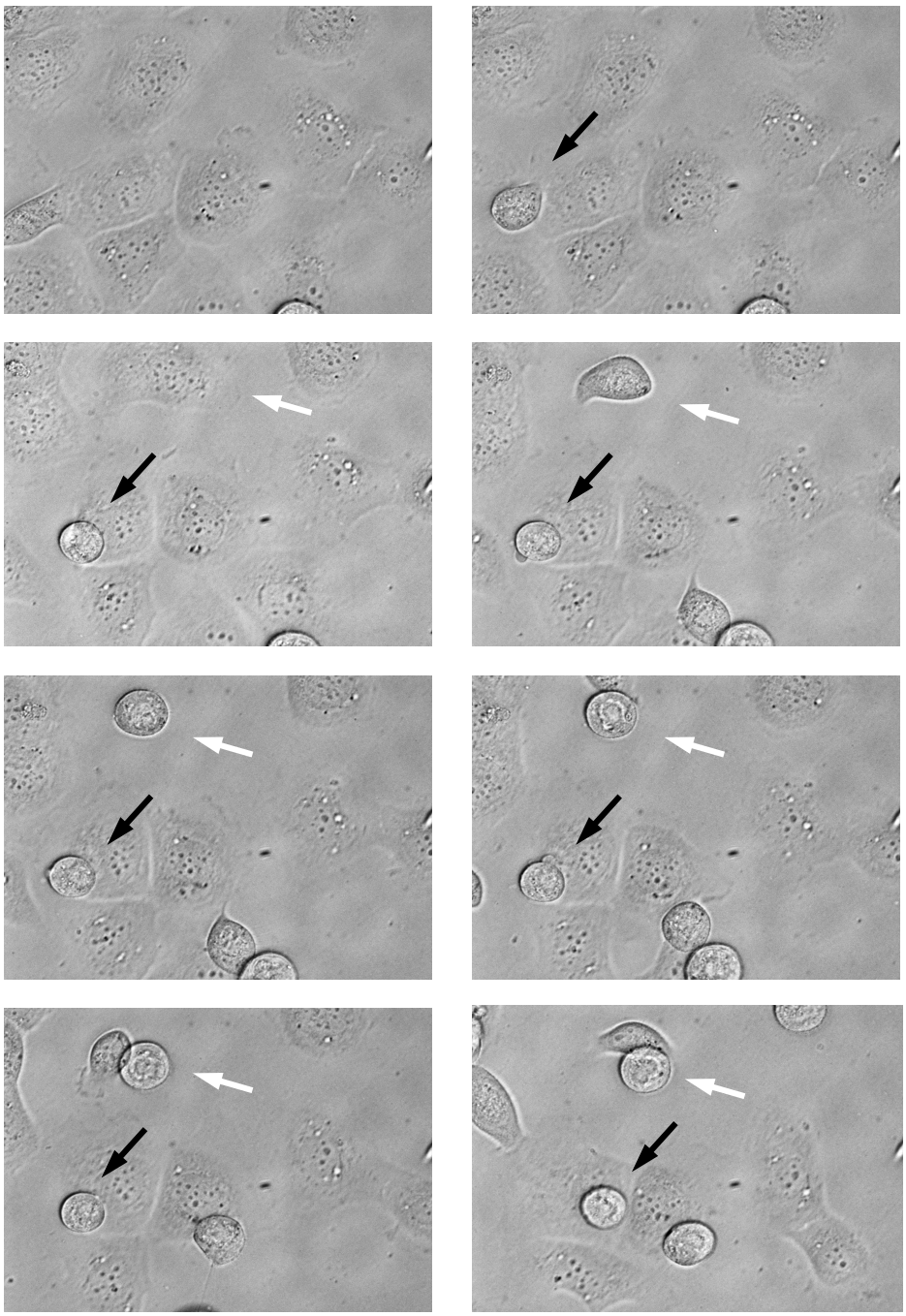


Figure S4.1C. Time-lapse images of SKOV3 cells undergoing mitotic arrest after paclitaxel treatment. Black and white arrows point towards cells arresting in mitosis.

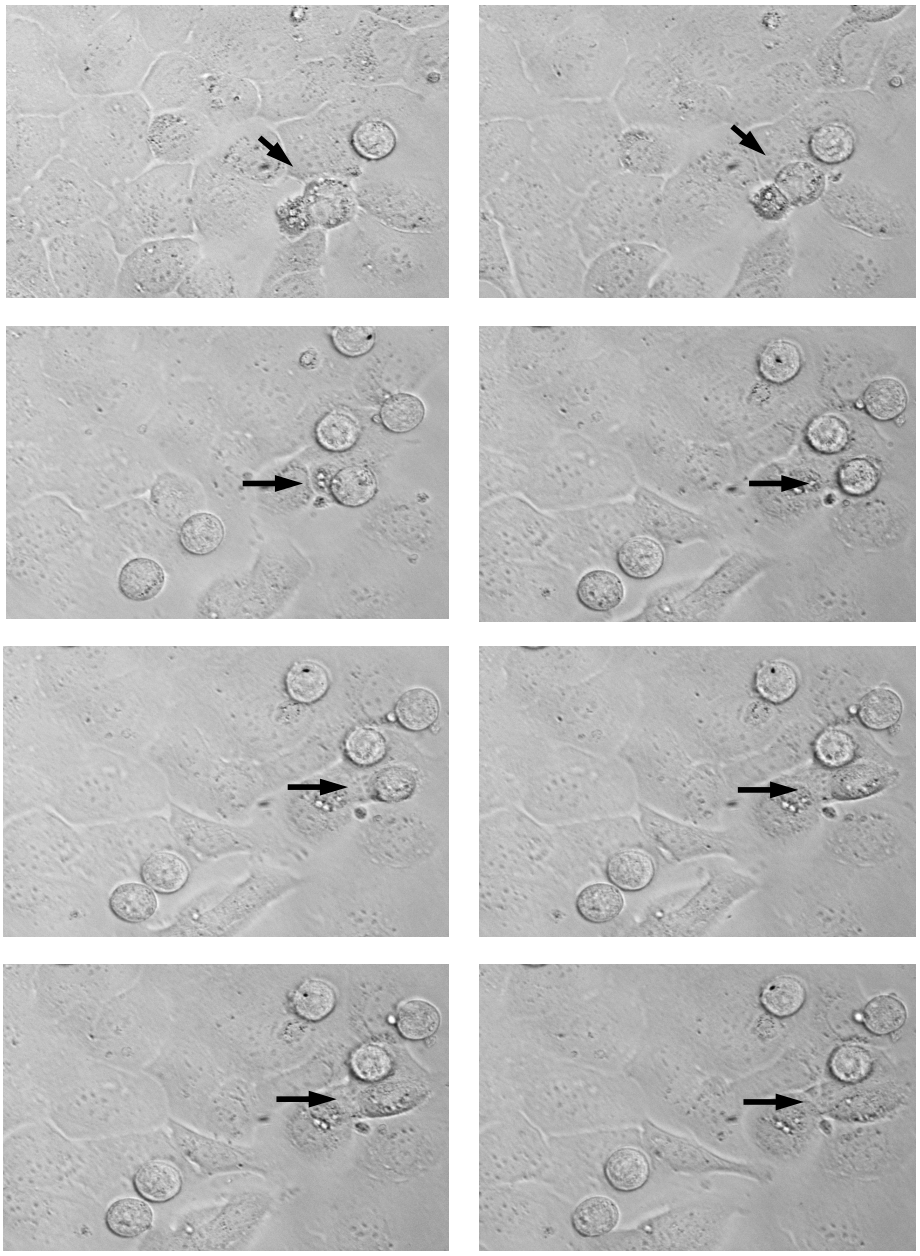


Figure S4.1D. Time-lapse images of SKOV3 cells undergoing mitotic slippage.
Black arrows follow a cell undergoing mitotic slippage.

8.3. AKT phosphorylation of PFKFB3 and PFKFB4

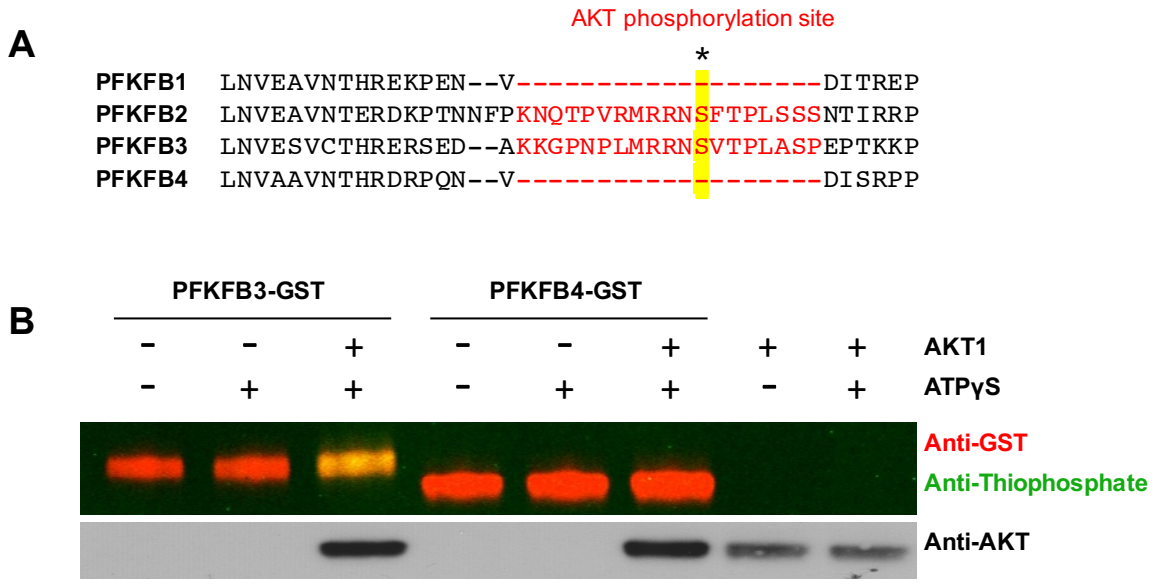


Figure S4.2. Phosphorylation of PFKFB3 and PFKFB4 by AKT.
(A) Alignment of the AKT phosphorylation site in PFKFB2 and PFKFB3 with the amino acid sequences of other PFKFB family members. (B, *n*=3) *In vitro* kinase assay performed with recombinant PFKFB3, PFKFB4 and AKT1.

8.4. PFKFB4 depletion and nocodazole treatment in SKOV3 cells

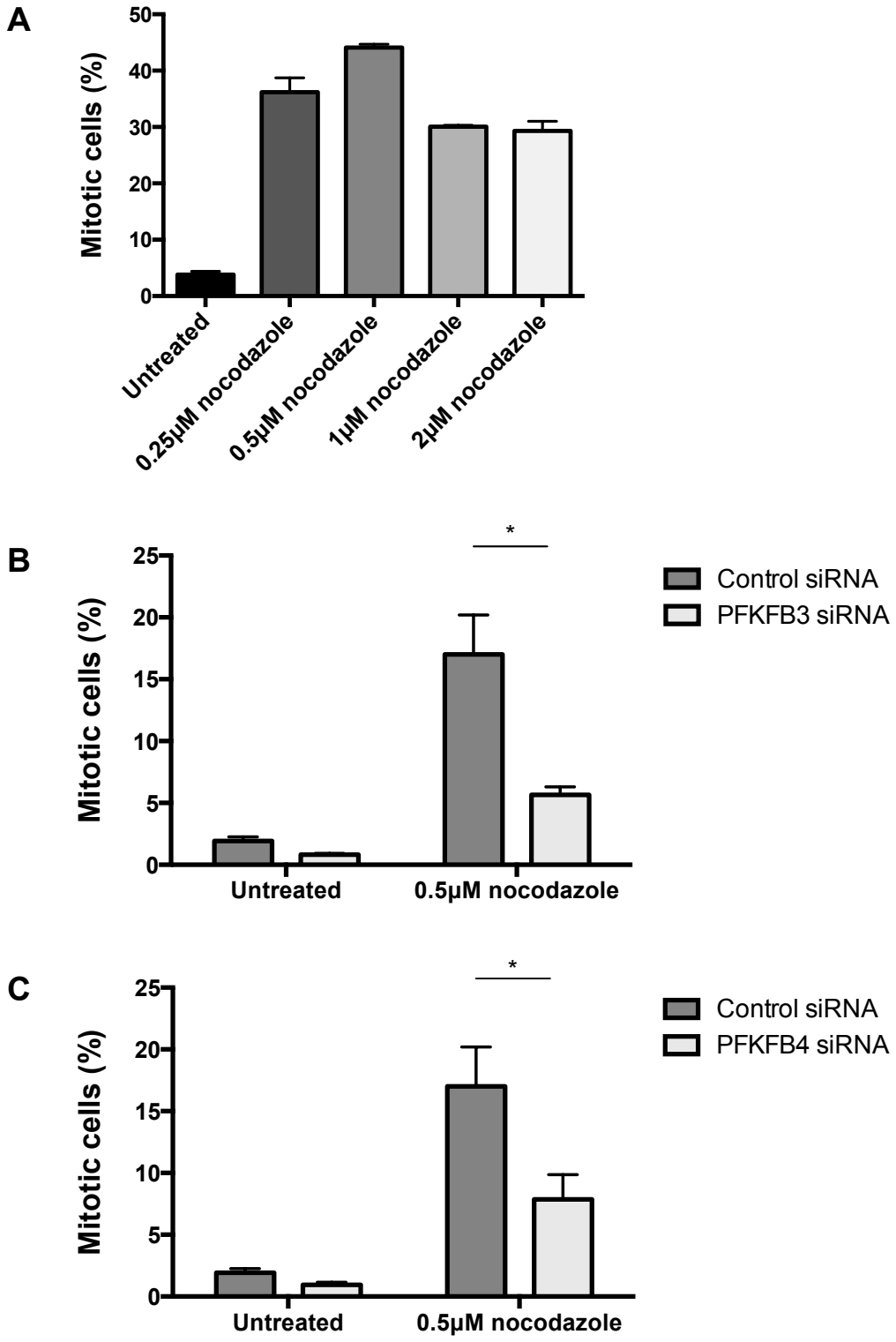


Figure S4.3. Nocodazole treatment in PFKFB3 or PFKFB4 depleted SKOV3 cells.
(A, *n*=2) Percentage of mitotic cells following nocodazole treatment of SKOV3 cells. (B, *n*=2) Percentage of mitotic cells following PFKFB3 depletion and nocodazole treatment of SKOV3 cells. (C, *n*=2) Percentage of mitotic cells following PFKFB4 depletion and nocodazole treatment of SKOV3 cells. All quantified using anti-phospho-histone H3 S10 staining and flow cytometry. Data are represented as the mean + standard error of the mean for two independent experiments. *≤0.05–0.01.

8.5. Colony forming assays following PFKFB4 depletion

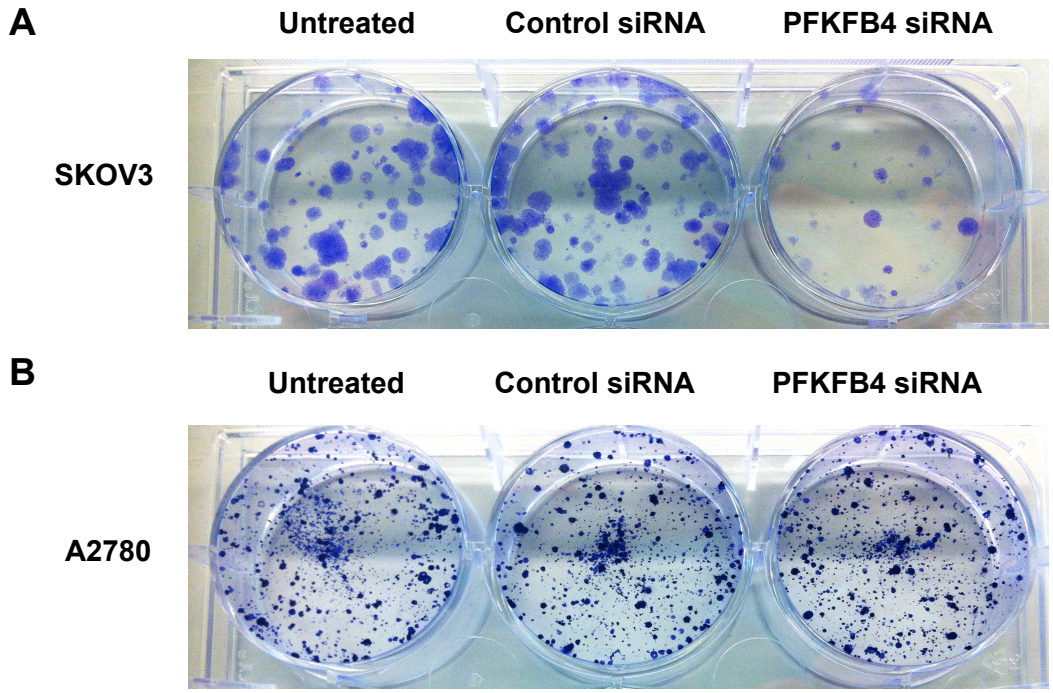


Figure S4.4. Colony formation assays following PFKFB4 depletion.
(A, *n*=2) Colony formation assays after PFKFB4 depletion followed by 12 days growth in SKOV3 cells and (B, *n*=2) A2780 cells. Samples were processed by fixing with 1% glutaraldehyde and staining using 0.5% crystal violet. Images are representative of one of two independent experiments.

8.6. PFKFB4 depletion in T308D/S473D and Myr-AKT SKOV3 cells

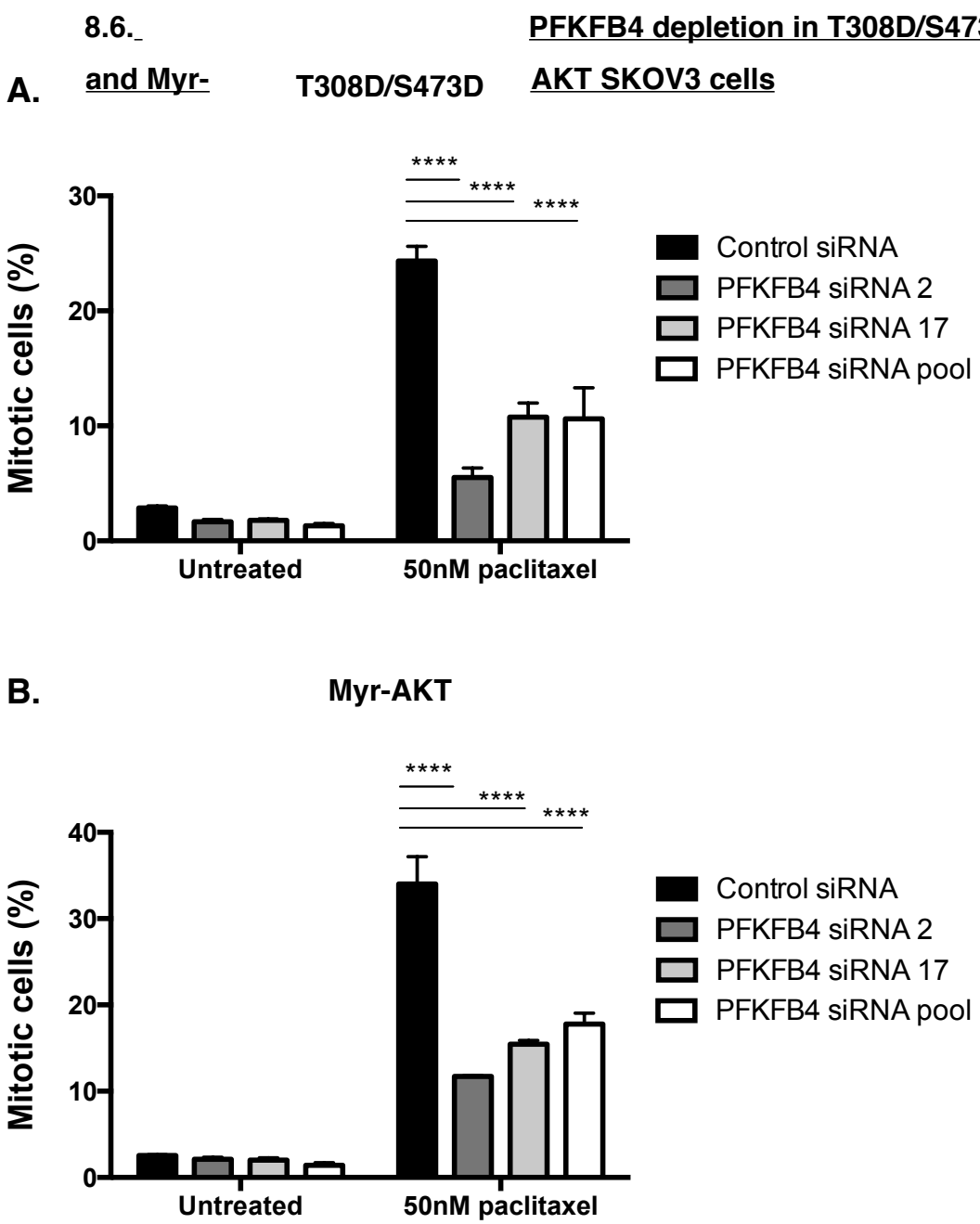


Figure S5.1. PFKFB4 depletion using individual siRNA duplexes in T308D/S473D and Myr-AKT SKOV3 cells lines

(A, *n*=3) Following siRNA-mediated PFKFB4 depletion and paclitaxel treatment, the percentage of mitotic cells were quantified using anti-phospho histone H3 S10 staining and flow cytometry in T308D/S473D (B, *n*=3) and Myr-AKT. 100,000 cells were analysed for each condition. Data are represented as the mean + standard error of the mean from three independent experiments.****<0.0001.

8.7. Manuscripts and Abstracts

Taylor, C. et al., 2017. Loss of PFKFB4 induces cell death in mitotically arrested ovarian cancer cells. *Oncotarget,* 8(11), pp. 17960-17980.

Taylor, C and Ahmed, A. 2015. Targeting PFKFB4 in mitotically vulnerable ovarian cancer cells. **Poster presentation** at Keystone symposia, Banff, Canada.

Taylor, C and Ahmed, A. 2015. Targeting mitotic vulnerability in ovarian cancer cells. **Poster presentation** at NCRI Cancer Conference, Liverpool, UK.

Taylor, C and Ahmed, A. 2015. Targeting mitotic vulnerability in ovarian cancer cells. **Oral presentation** at Nuffield Department of Obstetrics and Gynaecology Away Day, Oxford, UK.

Taylor, C and Ahmed, A. 2014. Targeting mitotic vulnerability in ovarian cancer cells. **Poster presentation** at Annual Meeting of Oxford Cancer Research Centre, Oxford, UK.

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Research Paper

Loss of PFKFB4 induces cell death in mitotically arrested ovarian cancer cells

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ABSTRACT

Taxanes represent some of the most commonly used chemotherapeutic agents for ovarian cancer treatment. However, they are only effective in approximately 40% of patients. Novel therapeutic strategies are required to potentiate their effect and improve patient outcome. A hallmark of many cancers is the constitutive activation of the PI3K/AKT pathway, which drives cell survival and metabolism. We discovered a striking decrease in AKT activity coupled with a significant reduction in glucose 6-phosphate and ATP levels during mitotic arrest in the majority of ovarian cancer cell lines tested, indicating a potential metabolic vulnerability. A high-content siRNA screen to detect novel metabolic targets in mitotically arrested ovarian cancer cells identified the glycolytic enzyme PFKFB4. PFKFB4 depletion increased caspase 3/7 activity, and levels of reactive oxygen species only in mitotically arrested cells, and significantly enhanced mitotic cell death after paclitaxel treatment. Depletion of PFKFB3 demonstrated a similar phenotype. The observation that some ovarian cancer cells lose AKT activity during mitotic arrest and become vulnerable to metabolic targeting is a new concept in cancer therapy. Thus, combining mitotic-targeted therapies with glycolytic inhibitors may act to potentiate the effects of antimitotics in ovarian cancer through mitosis-specific cell death.

INTRODUCTION

Ovarian cancer is the fifth most common cause of cancer death in women, with the five-year survival rate currently around 41% [1]. Antimitotics, such as taxanes, represent some of the most common chemotherapeutic agents used in the treatment of ovarian cancer. In spite of prolonged mitotic arrest following exposure, a significant proportion of cancer cells escape mitotic catastrophe and develop chemotherapy resistance [2]. Novel therapeutic strategies are required to potentiate the effects of current antimitotic therapies in ovarian cancer to improve patient prognosis [3, 4].

It is well recognised that many cancer cells exhibit an altered metabolism compared to non-malignant cells. While non-malignant cells use the TCA cycle and oxidative phosphorylation as the main source of ATP when oxygen is readily available, malignant cells use glycolysis as their primary energy source, even in oxygen rich conditions, which is known as aerobic glycolysis [5, 6]. This persistent utilisation of aerobic glycolysis is considered a hallmark of many cancers and is known as the Warburg effect [7]. The dependence of cancer cells on aerobic glycolysis to support their high energetic and biosynthetic demands provides a potential therapeutic opportunity, which is exploited by a number of compounds such as lonidamine, Imatinib and 2-deoxyglucose, all of which suppress aerobic glycolysis through their inhibitory effect on the glycolytic enzyme hexokinase [8]. The increased utilisation of glycolysis

in cancer cells also imposes a high demand for cellular glucose. This requirement is met by an increase in glucose uptake and breakdown, which is largely stimulated by the enhanced activity of oncogenes. One oncogene, known as AKT, is constitutively active in a large number of cancers, including ovarian cancer, and acts to promote cell survival, growth, and glycolysis [9]. AKT can enhance glucose uptake via the phosphorylation of the Rab GTPase-activating proteins TBC1 domain family 1 (TBC1D1) and AKT substrate of 160kDa (AS160/TBC1D4) [10, 11], while it also directly stimulates the glycolytic enzyme 6-phosphofructo-2-kinase [12] and promotes association of hexokinase with the mitochondria [13]. Given the influence that AKT activity has on glycolysis, investigating AKT signalling and glycolytic activity in ovarian cancer cells during mitotic arrest could lead to the identification of novel metabolic targets that are critical for ovarian cancer cell survival following treatment with mitotic-targeted therapies.

Here we identify a metabolic vulnerability in the majority of ovarian cancer cell lines during mitotic arrest, which arises from a striking decrease in AKT activity, a reduction in glucose 6-phosphate, and a significant decrease in ATP levels. In addition, we have shown that depletion of the glycolytic enzymes PFKFB3 and PFKFB4 prior to paclitaxel treatment can increase cell death in mitotically arrested cells possessing this identified vulnerability. The observation that many ovarian cancer cells lose AKT activity in mitosis and become vulnerable to metabolic targeting is a new concept in cancer therapy. Moreover, we have identified a previously unrecognised role of PFKFB3 and PFKFB4 in mitotically arrested ovarian cancer cell survival. Accordingly, the combination of antimitotics and glycolytic inhibitors may provide a means to enhance patient response to antimitotic therapies in ovarian cancer.

RESULTS

The majority of ovarian cancer cell lines exhibit reduced AKT activity and lower ATP levels during mitosis

Whilst investigating signalling alterations in ovarian cancer cells during mitotic arrest, we observed a striking loss of AKT phosphorylation at S473 in the majority of ovarian cancer cell lines tested (Figure 1A). Loss of AKT phosphorylation did not appear to be paclitaxel-dependent as it was present in both spontaneous mitosis and paclitaxel-induced mitotic arrest (Supplementary Figure 1A). Analysis of *AKT* mRNA levels in mitotic cells displayed no change in *AKT1* expression, a slight decrease in *AKT2* and a 50% increase in *AKT3* compared to interphase cells (Supplementary Figure 1B). However, the pan phospho-specific antibodies used in the western blot analysis strongly suggested that the apparent increase in *AKT3* mRNA level is not accompanied by a significant increase in AKT phosphorylation or activity. To determine the phosphorylation status of AKT in non-tumourgenic cells during mitotic arrest, mitotic cells were isolated from human skin fibroblasts following paclitaxel treatment (Figure 1B). AKT phosphorylation at S473 was not observed in either the interphase or mitotic fibroblast cells, suggesting that these cells possess very low levels of AKT phosphorylation compared to SKOV3 cells.

Loss of AKT phosphorylation at S473 in mitotically arrested ovarian cancer cells was accompanied by a significant decrease in AKT activity, since the phosphorylation of several downstream targets of AKT, such as GSK3β, PRAS40 and p70S6K, was reduced (Figure 1C). We also observed a significant reduction in the protein levels of mTOR and RICTOR in mitotically arrested cells (Figure 1D), both of which are known to be essential components of the mTORC2 complex that is important for phosphorylation and activation of AKT [14, 15]. This may explain, at least in part, the loss of AKT phosphorylation during mitosis as it has previously been shown that a reduction in either RICTOR or mTORC2 proteins can prevent phosphorylation of AKT at S473 [15]. We also found that the protein levels of the unphosphorylated and active form of the tumour suppressor PTEN remained unchanged between interphase cells and mitotically arrested cells. Given that PTEN negatively regulates AKT signaling, we concluded that PTEN is unlikely to contribute to the observed decrease in AKT activity in mitotically arrested ovarian cancer cells.

One ovarian cancer cell line, A2780, displayed no change in AKT phosphorylation between interphase and mitotic arrest. Moreover, signalling through AKT in that cell line appeared not to be affected, with the phosphorylation of GSK3β, PRAS40 and p70S6K being maintained, or slightly increased, in the mitotically arrested population (Figure 1E). Contrary to other ovarian cancer cell lines, protein expression of the mTORC2 complex components, mTOR and RICTOR, displayed a modest increase rather than a decrease during mitotic arrest when compared to interphase cells (Figure 1F). Additionally, protein levels of PTEN were decreased during mitotic arrest compared to interphase cells. Therefore, it is possible that the increase in mTORC2 activity coupled with a decrease in active PTEN could explain the maintenance, or modest increase, in AKT signalling during mitotic arrest in A2780 cells. Moreover, a 9 nucleotide deletion spanning the PTEN phosphatase domain has been identified in A2780 cells [16]. This does not affect *PTEN* mRNA expression, but is thought to generate a hypomorphic PTEN protein [17], which could also contribute to the maintenance of AKT signalling in A2780 cells.

The loss of AKT activity in the majority of ovarian cancer cell lines during mitosis was provocative because of the key role that AKT has in driving cancer cell metabolism, survival, and proliferation [18, 19]. The ovarian cancer cell

lines SKOV3, OC 316, A2780, UPN-251, and OVCAR-3 have all been shown to possess *PIK3CA* mutations or copy number gain [20–22], which cause the constitutive activation of AKT. The maintenance of PI3K/AKT activation could lead to the phenomenon known as 'oncogene addiction' [23], whereby the cancer cell becomes dependent on the enhanced activity of the PI3K/AKT pathway [24]. Therefore, we hypothesised that the observed temporary withdrawal of AKT activity during mitosis might render cancer cells metabolically vulnerable, and that treatment with paclitaxel could extend this therapeutic window. Consistent with this hypothesis was the observation that the phosphorylation of AS160, a direct substrate of AKT that links insulin signaling with the glucose transporter GLUT4 [25], was decreased in mitotically arrested SKOV3 cells, but not in A2780 cells, which instead displayed an increase in AS160 phosphorylation during mitotic arrest (Figure 1G). This implied a reduction in the translocation of GLUT4 to the membrane during mitotic arrest and a

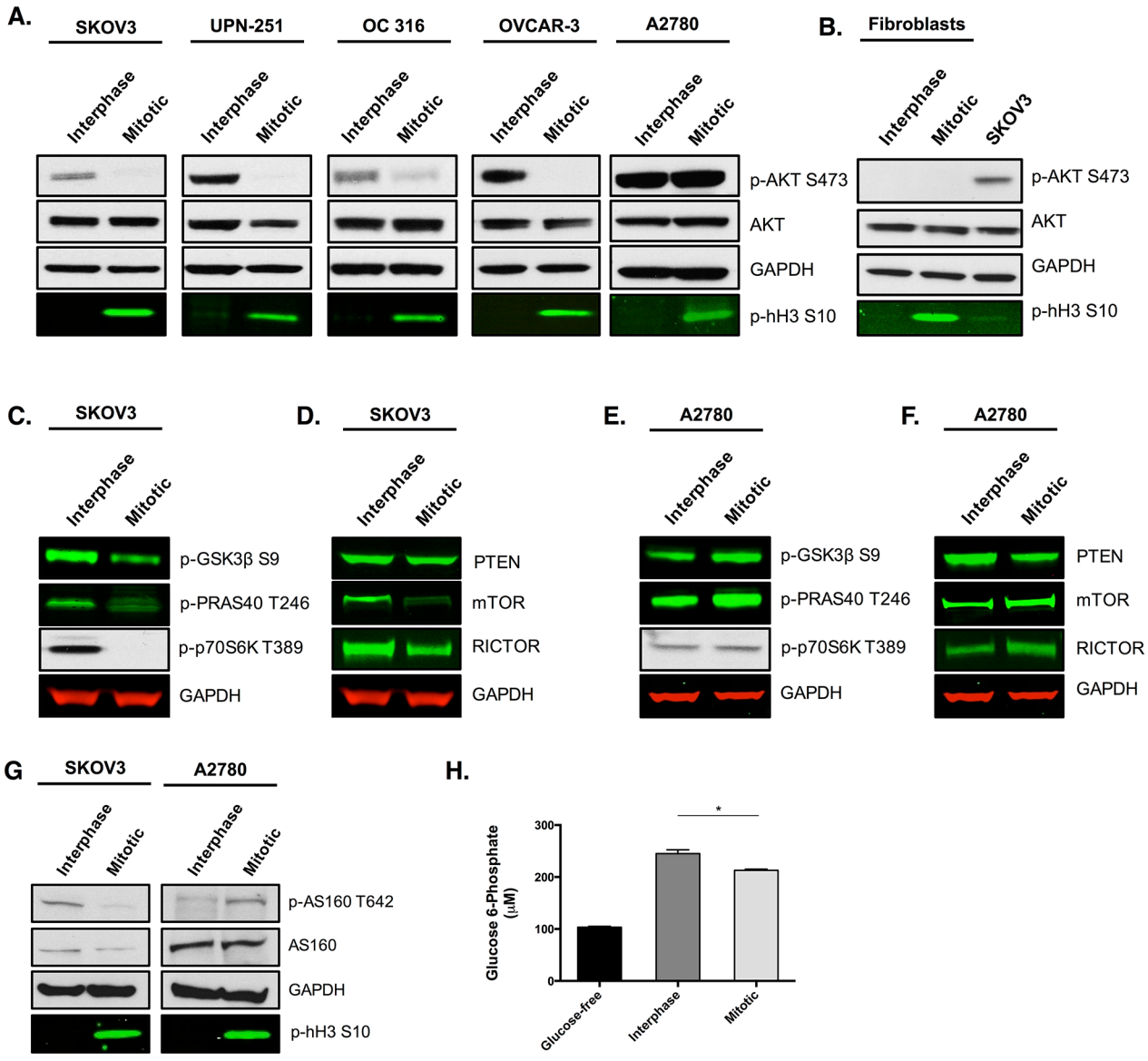


Figure 1: AKT activity is reduced in multiple ovarian cancer cell lines and skin fibroblasts during paclitaxel-induced mitotic arrest. Cells were treated with 50 nM paclitaxel for 16 h and separated by mitotic shake-off into the mitotic and interphase populations. **A.** Western blotting was used to determine total AKT expression and AKT phosphorylation at S473 in ovarian cancer cells and **B.** human skin fibroblasts. **C, D.** The phosphorylation of substrates downstream of AKT and the protein expression of key upstream components were also assessed by western-blotting in SKOV3 cells and **E, F.** A2780 cells. **G.** Analysis of AS160 protein expression and its phosphorylation at T642 was examined by western blotting. In the western blots, GAPDH is the loading control, phospho-histone H3 S10 is the mitotic marker and images represent one of three experiments. **H.** Glucose 6-phosphate levels were analysed in SKOV3 cells and presented as the mean +SEM of three experiments.

consequent reduction in glucose transport. As expected, glucose 6-phosphate levels were significantly decreased in mitotically arrested SKOV3 cells compared to interphase cells (Figure 1H, p=0.0268, t-test).

We next hypothesised that the reduction in glucose 6-phosphate would hinder its utilisation in glycolysis and the pentose phosphate pathway. Therefore, the levels of ATP and glutathione (GSH) were investigated as a readout of the flux through glycolysis and the pentose phosphate pathway, respectively. A significant decrease in ATP was observed during mitotic arrest in SKOV3 cells (Figure 2A, p=0.005, t-test) and OC 316 cells (Figure 2B, p=0.0079, t-test) compared to interphase cells, while the levels of ATP (Figure 2C) in A2780 cells remained unchanged between the mitotically arrested and interphase populations. Moreover, no significant difference was found in the GSH levels between the mitotically arrested and interphase populations in either SKOV3 or A2780 cells (Figure 2D, 2E).

A high content kinome screen identifies proteins that are critical for survival in mitotically arrested ovarian cancer cells

The data so far indicate a vulnerability in SKOV3 cells, and potentially other ovarian cancer cell lines, during paclitaxel-induced mitotic arrest. This vulnerability is predicted to arise from a reduction in AKT activity, glucose 6-phosphate, and ATP. We hypothesized that interfering with key metabolic enzymes in these cells might exploit this vulnerability and enhance cell death during paclitaxel-induced mitotic arrest. To investigate this, we conducted a high-content siRNA kinome screen to identify potential metabolic targets that are important for cell survival following mitotic arrest by paclitaxel treatment (Figure 3A). The screen was performed using SKOV3 cells, as they possessed the potential vulnerability during mitotic arrest, and with 10 nM paclitaxel, as this was the optimal concentration to achieve the maximum number of mitotic cells after 24 h without inducing significant cell death, thus providing a window for mitotic cell depletion. Cells were fixed and stained for Hoechst to measure DNA content and anti-phospho-histone H3 S10 to estimate the number of mitotic cells. Depletion of a gene could result in low mitotic index (percentage of mitotic cells compared to all cells) by inducing cell cycle arrest prior to mitosis, interfering with the mitotic checkpoint, or inducing mitotic cell death. We reasoned that a true positive hit would be one that induces cell death in mitosis with minimum interference to mitotic checkpoints or G2 to M transition. Therefore, depletion of such a protein

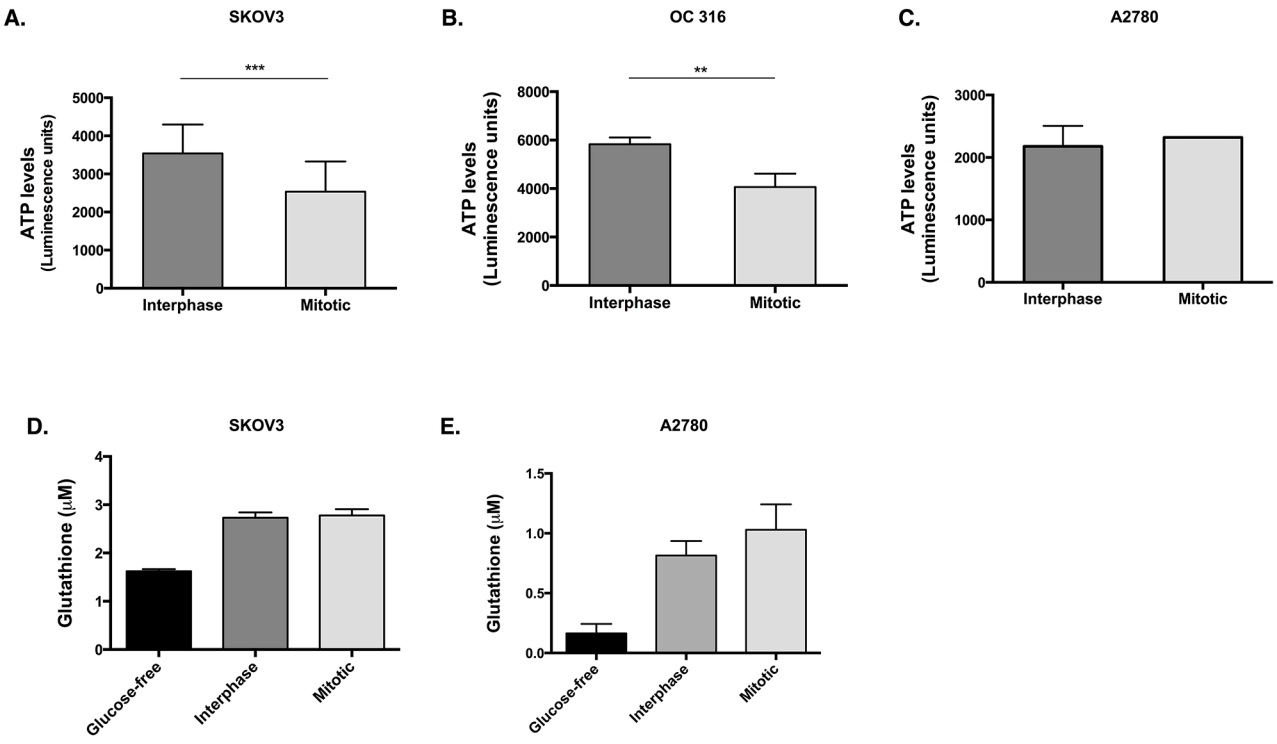


Figure 2: ATP levels are reduced and GSH levels are unchanged in cells that lose AKT activity during paclitaxel-induced mitotic arrest. Cells were treated with 50 nM paclitaxel for 16 h and separated by mitotic shake-off into the mitotic and interphase populations. **A.** After counting the cells in the presence of trypan blue and dilution to the correct concentrations, ATP levels were measured using the CellTiter-Glo assay in SKOV3 cells, **B.** OC 316 cells and **C.** A2780 cells. **D.** Glutathione (GSH) levels were assessed using the GSH-Glo Glutathione assay in SKOV3 cells and **E.** A2780 cells. Data are expressed as the mean +SEM of three experiments.

would specifically reduce the paclitaxel treated mitotic ratio (PMR), measured as the mitotic index following gene depletion in paclitaxel treated cells divided by the mitotic index following non-targeting siRNA control treatment in paclitaxel treated cells, as these cells are enriched in mitosis, but have limited effect on the paclitaxel untreated mitotic ratio (PUMR). Therefore, we computed the ratio of PUMR/PMR and considered a cut-off of 3-fold as a positive hit for further validation (Supplementary Table 1). The screen was conducted in triplicate for each condition (9 in total) and siRNA targeting *PLK1* was used as a transfection control as PLK1 depletion is known to be fatal to cells [26, 27].

Using the above mentioned criteria, a number of proteins were identified as causing a significant fold decrease in the number of mitotically arrested cells following paclitaxel treatment after their depletion (Figure 3B). These included two chemokine receptors (CCR), CCR9 and CCR2, which have been implicated in driving oncogenesis and shown to induce apoptosis when depleted in cancerous cells [28, 29], Testis expressed 14 (TEX14) that is a PLK1 regulated protein [30], as well as Wnt inhibitory factor 1 (WIF1), which antagonises Wnt signaling [31] and may therefore be important for the control of the pathway in ovarian cancer cells. In addition, Three enzymes with roles in metabolism were identified from the screen as causing a significant fold decrease in the number of mitotically arrested cells following paclitaxel treatment after their depletion, two of which were more directly linked to glycolysis; XYLB and PFKFB4. Mevalonate diphosphate decarboxylase (MVD) was also found to cause a significant decrease in the number of cells

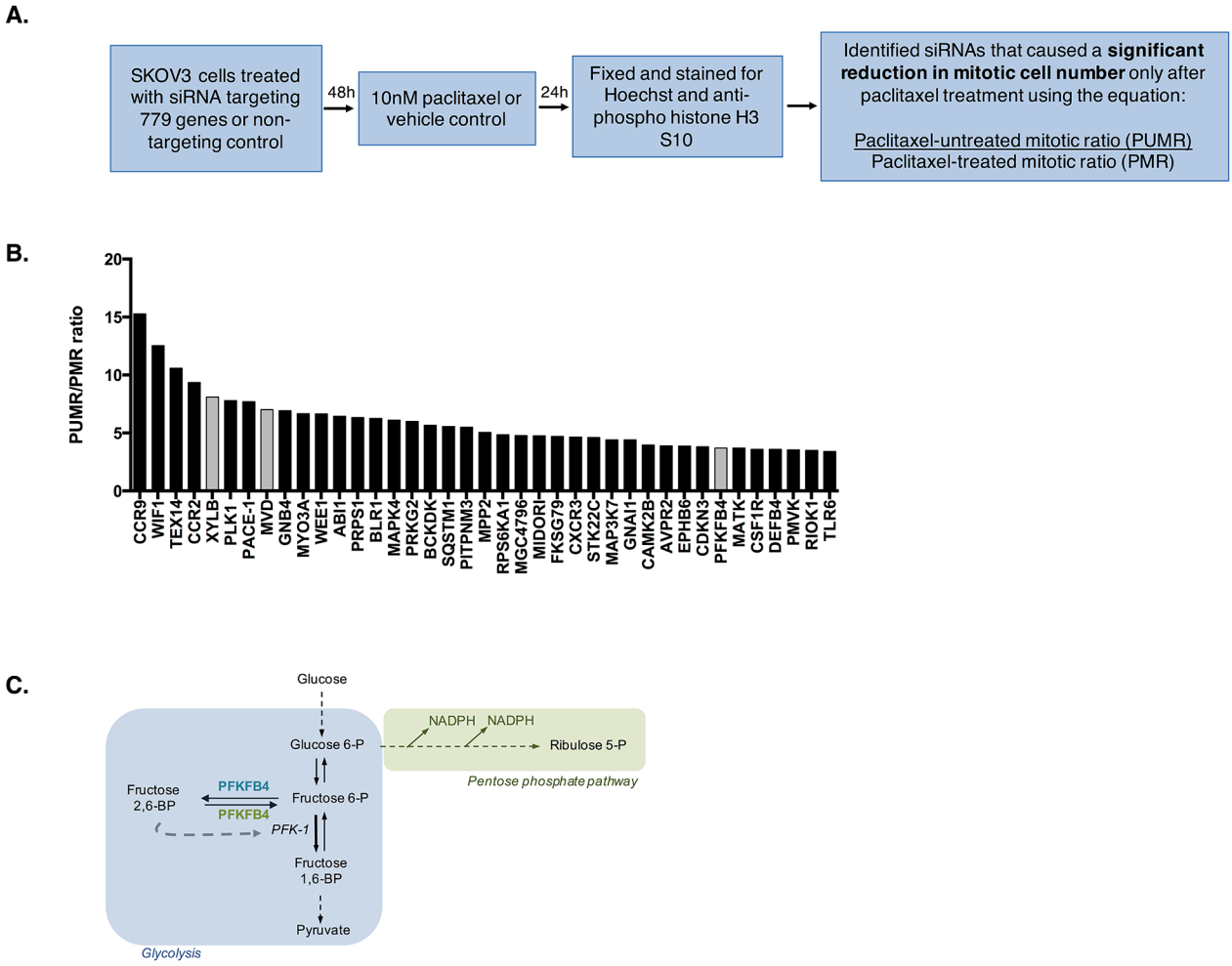


Figure 3: High-content siRNA kinome screen identifies PFKFB4 as important for ovarian cancer cell survival during paclitaxel-induced mitotic arrest. A. Schematic representation of the screen protocol. The paclitaxel treated mitotic ratio (PMR) was calculated as the mitotic index following gene depletion in paclitaxel-treated cells divided by the mitotic index following non-targeting siRNA control treatment in paclitaxel treated cells. The paclitaxel-untreated mitotic ratio (PUMR) is the mitotic index following gene depletion in untreated cells divided by the mitotic index following non-targeting siRNA control treatment in untreated cells. **B.** A bar plot showing the siRNA targets resulting in a PUMR/PMR ratio of 3 or greater, with metabolic targets highlighted in grey. **C.** Schematic demonstrating the function of PFKFB4 in glycolysis.

undergoing spontaneous mitosis, indicating that the effects of its depletion were not specific to cells undergoing prolonged mitotic arrest. For this reason, MVD was not selected for further study. Preliminary experiments investigating XYLB depletion could not validate the results of the screen, resulting in phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFKFB4) being followed up as the only high priority candidate.

PFKFB4 belongs to a family of bifunctional enzymes, known as PFKFB1-4, which have an important role in regulating glycolysis [32] (Figure 3C). The phosphofructokinase domain of PFKFB enzymes produces fructose 6-phosphate, while their phosphatase activity yields fructose 2,6-bisphosphate [33]. Fructose 2,6-bisphosphate is an allosteric activator of phosphofructokinase 1, which controls one of the critical rate-limiting steps of glycolysis [34, 35]. siRNA-mediated depletion of *PFKFB4* significantly decreased the number of mitotically arrested SKOV3 cells following paclitaxel treatment, but not during spontaneous mitosis. This suggested that PFKFB4 depletion was detrimental only to SKOV3 cells undergoing prolonged mitotic arrest. To validate the screen results we used pooled siRNA duplexes to deplete PFKFB4 prior to paclitaxel treatment (Figure 4A) and quantified the number of mitotic cells present compared to the non-targeting control. Paclitaxel treatment of PFKFB4 depleted SKOV3 cells resulted in a significant decrease in the mitotic cell index compared to the non-targeting control when analysed by both immunofluorescence (Figure 4B) $p<0.0001$, two-way ANOVA multiple comparison) and flow cytometry (Figure 4C, $p<0.0001$, two-way ANOVA multiple comparison). To exclude off-target effects, we used four individual siRNA duplexes to deplete PFKFB4 (Supplementary Figure 2A), all of which decreased the mitotic cell index when quantified by immunofluorescence (Supplementary Figure 2B). PFKFB4 depletion was also investigated in OC 316 cells, which exhibited a reduction in AKT activity and ATP levels during mitotic arrest by paclitaxel, and a significant reduction in mitotic cell number was also identified (Figure 4D, $p=0.006$, two-way ANOVA multiple comparison). In addition to paclitaxel, preliminary data investigating PFKFB4 depletion in SKOV3 cells prior to mitotic arrest using 0.5μM nocodazole decreased the number of mitotic cells to a similar extent as what is observed following paclitaxel treatment (data not shown).

To determine whether the effect of PFKFB4 depletion on the number of mitotically arrested cells is due to the vulnerability that they possess during mitotic arrest, this analysis was repeated in the A2780 cell line and siRNA-mediated depletion of *PFKFB4* was achieved (Figure 5A). There was no significant effect of PFKFB4 depletion on the number of mitotically arrested cells when using immunofluorescence staining (Figure 5B). Analysis using flow cytometry confirmed the maintenance of mitotically arrested cell number after PFKFB4 depletion (Figure 5C) two-way ANOVA multiple comparison). Therefore, we concluded that SKOV3 cells are vulnerable to PFKFB4 depletion during mitotic arrest, whereas A2780 are resistant.

PFKFB4 depletion can increase mitotic cell death in paclitaxel treated ovarian cancer cells

The effect of PFKFB4 depletion on reducing the number of mitotically arrested cells after paclitaxel treatment could occur as a result of inducing mitotic cell death, arresting cell cycle progression, or increasing mitotic slippage, or a combination of the three. Depletion of PFKFB4 did not significantly affect cell cycle progression in SKOV3 cells when using propidium iodide staining and flow cytometry (Figure 6A). Time-lapse microscopy was performed to follow the fate of mitotic cells for 24 h after PFKFB4 depletion and paclitaxel treatment as we have previously described [3]. We observed a significant increase in mitotic cell death following PFKFB4 depletion compared to the non-targeting control (Figure 6B, $p=0.0056$, two-way ANOVA multiple comparison), indicating that PFKFB4 is important for ovarian cancer cell survival during prolonged mitosis. In support of this, a significant increase in PI staining was observed in mitotically arrested cells after PFKFB4 depletion when compared to the non-targeting control (Figure 6C, $p=0.0049$, two-way ANOVA multiple comparison). Moreover, the activity of caspases 3 and 7 were significantly elevated in mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control (Figure 6D, $p=0.0016$, two-way ANOVA multiple comparison). The increased activity of caspases 3 and 7 suggests that PFKFB4 depletion may induce apoptosis during prolonged mitosis [36], thus decreasing the mitotic index.

Taking into account the critical role that PFKFB4 has in regulating glycolysis via the production of fructose 2,6-bisphosphate [35, 37, 38], and the apparent decrease in ATP levels we have observed during mitotic arrest, it could be hypothesised that the increase in mitotic cell death after PFKFB4 depletion is due to a further critical decrease in ATP levels. Substantial decreases in ATP concentration have been shown to trigger apoptosis [39–41]. However, AMPK phosphorylation at T172, an activating site within the T-loop, suggests that mitotically arrested cells are not energetically stressed after PFKFB4 depletion because AMPK phosphorylation and expression were reduced compared to interphase cells (Figure 7A). The lack of an increase in AMPK activity in mitotically arrested SKOV3 cells was surprising given that AMPK activity has previously been found to increase in mitotically arrested cells [42]. However, the study by Domenech *et al.,* did this, the or arrest cells by paclitaxel treatment alone. In support of this, the phosphorylation of the key enzyme in fatty

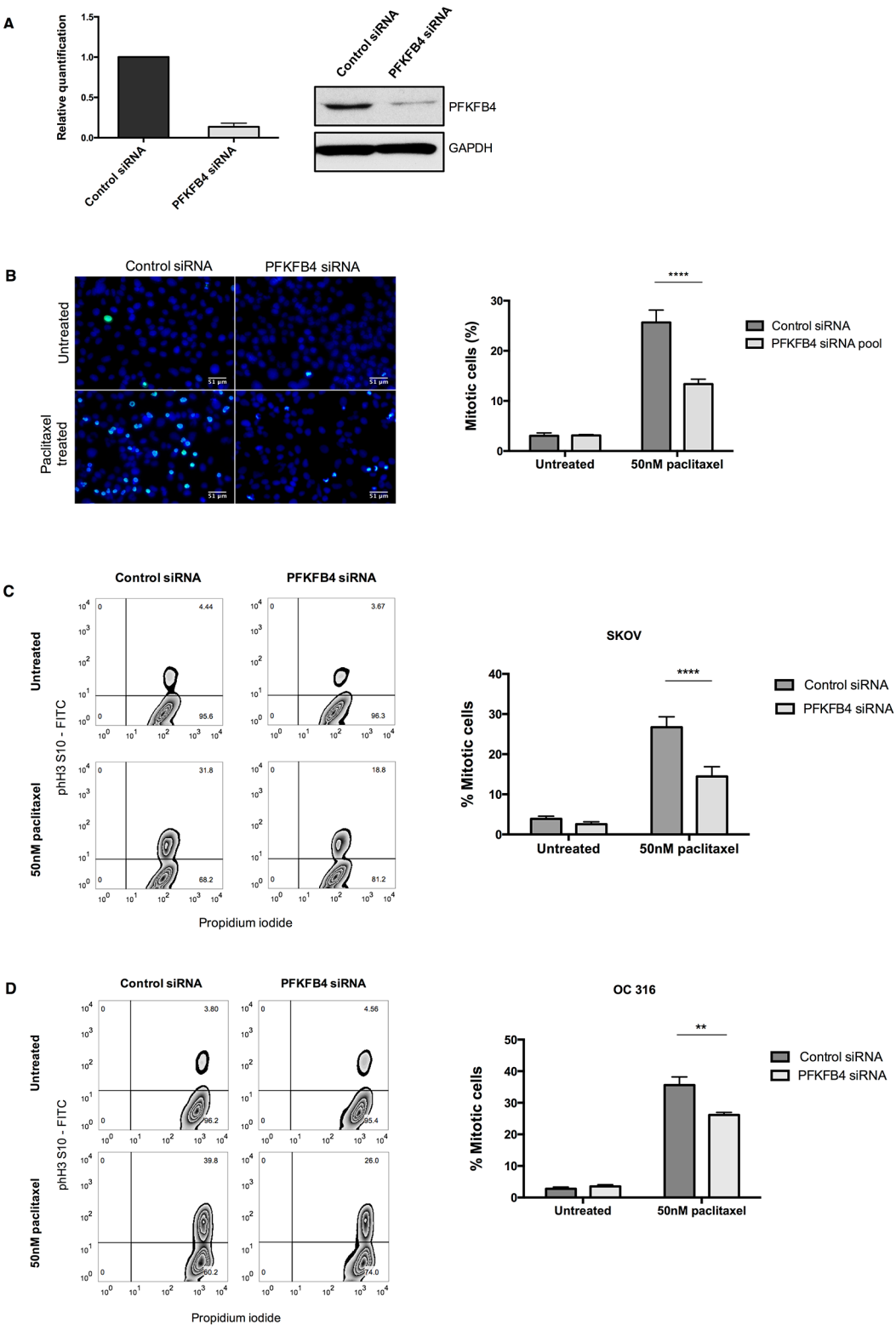


Figure 4: Validation of the screen hit PFKFB4. A. *PFKFB4* mRNA and protein levels were assessed by qPCR and western blotting after PFKFB4 depletion using pooled siRNA duplexes in SKOV3 cells for 72 h. **B.** After siRNA-mediated depletion of *PFKFB4* for 72 h followed by 50 nM paclitaxel for 16 h, SKOV3 cells were fixed and stained for immunofluorescence. Images are shown with Hoechst stain (blue) and phospho-histone H3 (S10) (green) and the percentage of mitotically arrested cells were quantified as the mean +SEM of three experiments. **C.** The percentage of mitotically arrested cells was also confirmed by flow cytometry after PFKFB4 depletion and 50 nM paclitaxel in SKOV3 cells and **D.** OC 316 cells. Data are expressed as the mean +SEM of three experiments.

acid synthesis, acetyl-CoA carboxylase (ACC), which is phosphorylated and inactivated by AMPK [43], was reduced in the mitotically arrested population after PFKFB4 depletion (Figure 7B). AMPK phosphorylation was also reduced in the mitotically arrested population treated with non-targeting siRNA, indicating that these cells are not energetically stressed, despite the reduction in ATP levels. Taken together, these data suggest that mitotically arrested cells are not energetically stressed and that the increase in mitotic cell death after PFKFB4 depletion and paclitaxel treatment is not due to a critical decrease in ATP levels and subsequent energetic stress.

As ovarian cancer cells do not appear to be energetically stressed after PFKFB4 depletion, we hypothesised that the levels of reactive oxygen species (ROS) may be increased after PFKFB4 depletion. We measured the levels of ROS in mitotic and interphase SKOV3 cells after PFKFB4 depletion followed by paclitaxel treatment and found the levels of ROS to be significantly increased in mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control (Figure 7C, p=0.0124, two-way ANOVA multiple comparison). These data suggest that the increase in ROS occurs specifically during prolonged mitotic arrest and not during interphase. In support of this, no significant difference in the levels of ROS was found after PFKFB4 depletion compared to the non-targeting control in untreated cells (Figure 7D).

The PFKFB pathway is important during mitotic arrest in ovarian cancer cells

Of all the PFKFB family members, PFKFB3 has been the most intensively studied and is overexpressed in many human cancers, including ovarian cancer [44]. To investigate the importance of the PFKFB family during

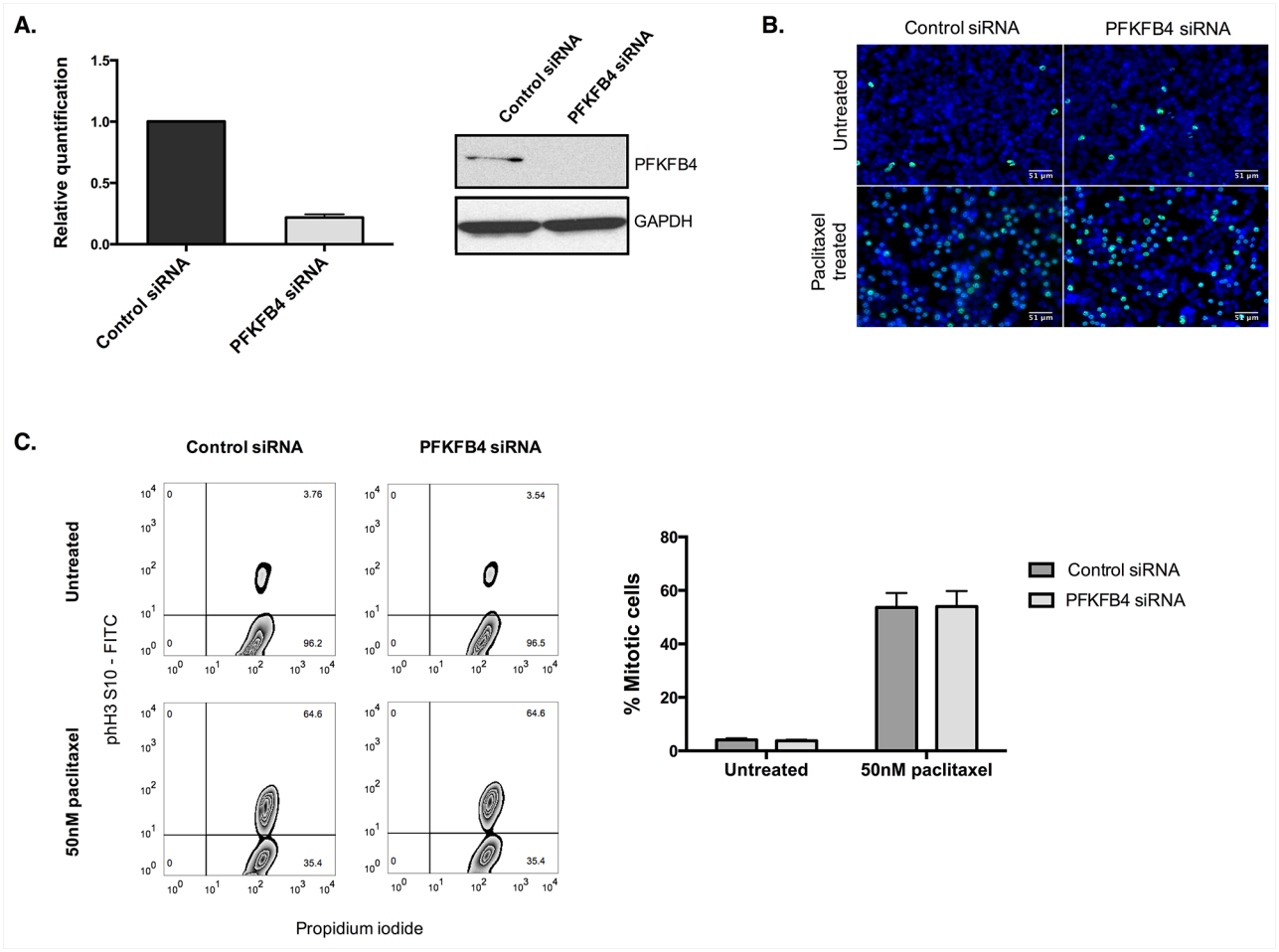


Figure 5: PFKFB4 depletion in A2780 cells has no effect on the number of mitotically arrested cells. A. *PFKFB4* mRNA and protein levels were assessed by qPCR and western blotting after PFKFB4 depletion using pooled siRNA for 72 h. **B.** After siRNA-mediated depletion of *PFKFB4* for 72 h followed by 50 nM paclitaxel for 16 h, cells were fixed and stained for immunofluorescence. Images are shown with Hoechst stain (blue) and phospho-histone H3 (S10) (green). **C.** The percentage of mitotically arrested cells was confirmed by flow cytometry after PFKFB4 depletion and 50 nM paclitaxel. Data are expressed as the mean +SEM of three experiments.

mitotic arrest, we examined the effects of siRNA-targeted depletion of *PFKFB3* on paclitaxel treated ovarian cancer cells (Figure 8A). Similar to PFKFB4 targeting, depletion of PFKFB3 caused a significant decrease in mitotic cell number after paclitaxel treatment compared to the non-targeting control (Figure 8B, p=0.006, two-way ANOVA multiple comparison). The decrease in mitotic index was accompanied by a significant increase in caspase 3 and 7 activity in mitotically arrested cells depleted of PFKFB3 (Figure 8C, p=0.0165, two-way ANOVA multiple comparison). There was also a significant increase in the levels of PI staining in mitotically arrested cells after PFKFB3 targeting compared to the non-targeting control (Figure 8D, p=0.0022, two-way ANOVA multiple comparison). The increase in caspase 3 and caspase 7 activity and the greater PI incorporation both suggest that there is an increase in cell death when mitotically arrested cells are depleted of PFKFB3. In addition to this, ROS levels displayed a significant increase specifically in mitotically arrested cells depleted of PFKFB3 (Figure 8E, p=0.0051, two-way ANOVA multiple comparison). An increase in ROS after PFKFB3 depletion has previously been noted by Klarer *et al*., 2014 [45], however this was not observed specifically in mitotically arrested cells as it is in this research. No increase in ROS was observed after siRNA-mediated depletion of PFKFB3 alone (Figure 8F). This may demonstrate tissue-specific regulation of these enzymes or highlight a previously unrecognised role during mitotic arrest.

PFKFB3 depletion was achieved using stealth siRNA duplexes transfected with RNAiMAX, whereas in the siRNA screen, Dharmacon siRNA duplexes were used and transfected with DharmaFECT4 reagent. The latter approach has not been employed by any other studies depleting PFKFB3 using siRNA duplexes [42, 45–47], indicating that this technique may not be effective at depleting *PFKFB3*, which would explain why PFKFB3 was not identified in the kinome screen.

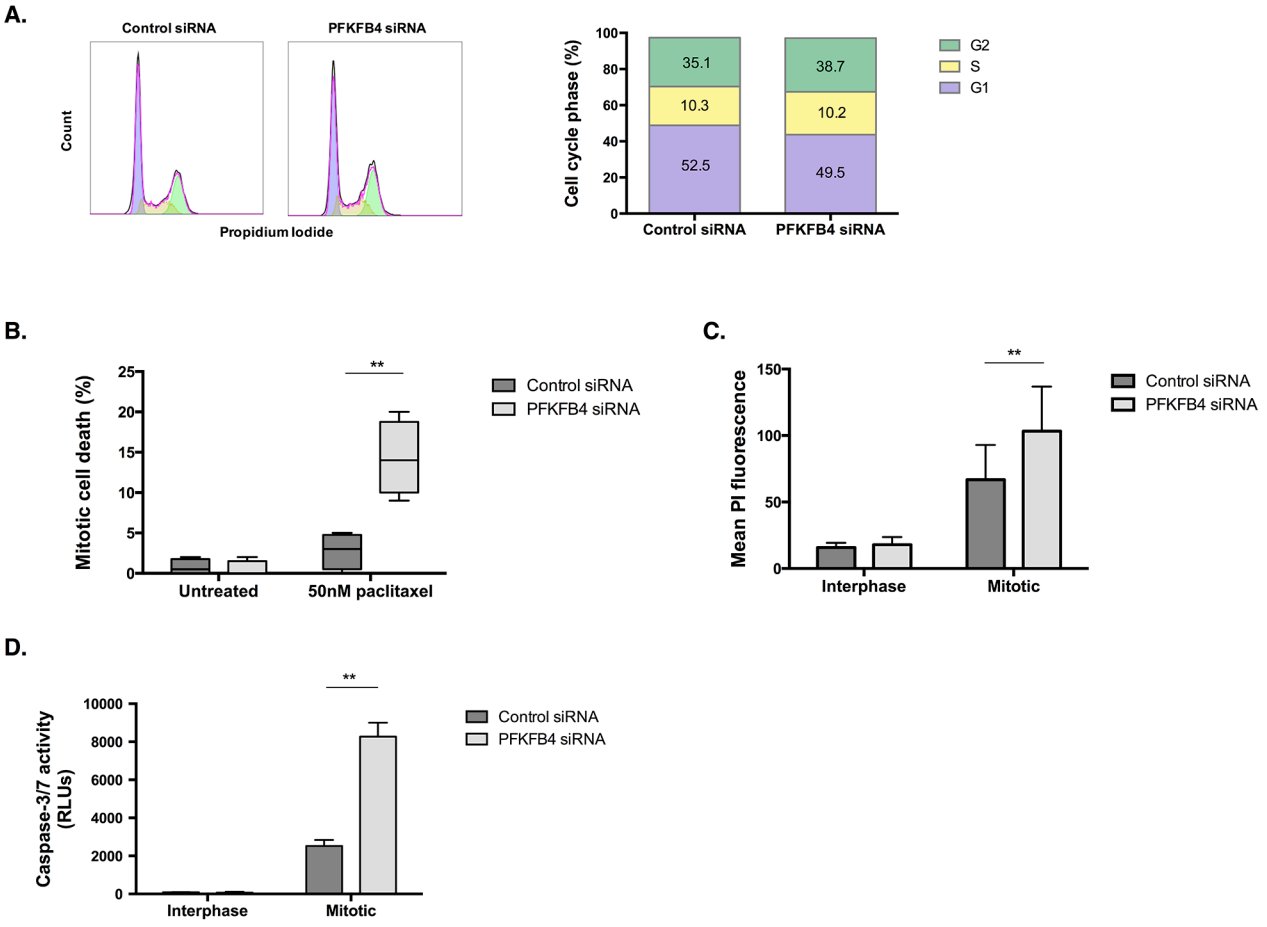


Figure 6: PFKFB4 depletion increases death of mitotically arrested SKOV3 cells. A. Cell cycle analysis after siRNA-mediated depletion of *PFKFB4* for 72 h was assessed by propidium iodide staining and flow cytometry. **B.** Mitotic cell death was assessed using time-lapse microscopy for 16 h and following the fate of over 200 mitotically arrested cells in each condition. **C.** After PFKFB4 depletion and mitotic cell isolation, the incorporation of propidium iodide was assessed by flow cytometry and **D.** the activity of caspases 3 and 7 was measured using the Caspase-Glo 3/7 assay. Data are expressed as the mean +SEM of three experiments.

Restoration of AKT activity in mitotically arrested SKOV3 cells does not confer resistance to PFKFB4 depletion

We next tested whether maintenance of AKT signalling during mitotic arrest could render cells resistant to PFKFB4 targeting. To address this, we produced SKOV3 cell lines stably expressing constitutively active AKT through two different mechanisms; either by the addition of two phosphomimetic mutations T308D and S473D or through the myristoylation of the N-terminus of AKT (Myr-AKT). Both of these alterations have previously been shown to lead to constitutive activation of AKT [48, 49] and to directly regulate GLUT4 translocation to the plasma membrane [50, 51]. To investigate whether AKT activity was restored during mitotic arrest, we first analysed the phosphorylation of AKT and its downstream targets (Figure 9A). The phosphorylation of two direct substrates of AKT, PRAS40 and GSK3β, was increased compared to the empty vector control and the wild-type AKT cell lines (AKT WT). In addition, maintenance of AKT phosphorylation at S473 in mitotically arrested cells was observed in Myr-AKT. Moreover, protein levels of mTOR and RICTOR were restored in both the T308D/S473D and Myr-AKT cell lines (Figure 9B), suggesting that there may be a positive feedback loop upstream causing further activation of the pathway. Intriguingly, the phosphorylation of p70S6K was not rescued in any of the cell lines stably expressing AKT, which suggests that either AKT activity is not fully restored during mitotic arrest, or as p70S6K is not a known direct substrate of AKT, additional components that are required for its phosphorylation are not functional in these cell lines.

Given that we observed increased activity of the AKT pathway directly upstream and downstream of AKT in the T308D/S473D and Myr-AKT cell lines, we investigated whether there was any rescue in the levels of ROS or mitotic cell index after PFKFB4 depletion. There was still at least a 50% reduction in the number of mitotically arrested cells after PFKFB4 compared to the non-targeting control

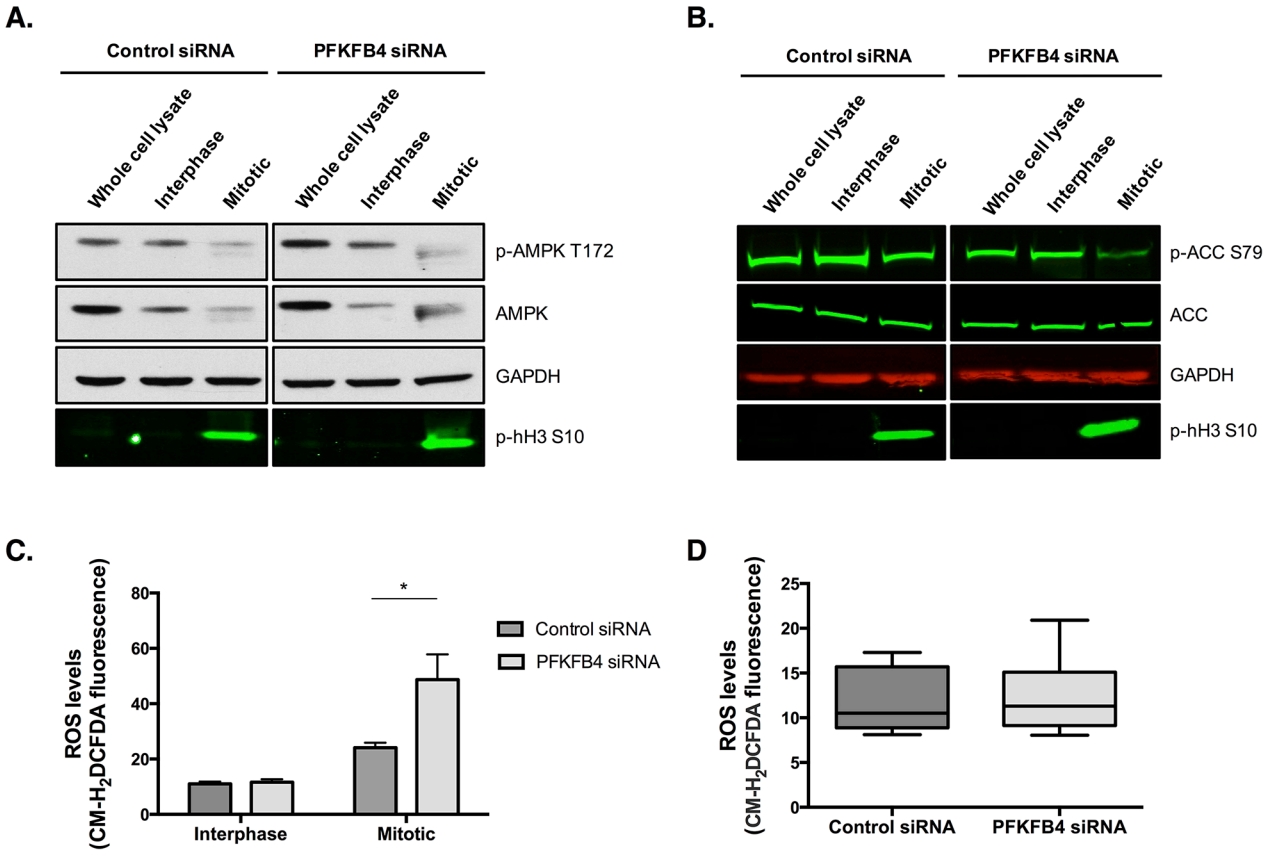


Figure 7: PFKFB4 depletion increases reactive oxygen species in mitotically arrested SKOV3 cells without increasing AMPK activity. After control siRNA or siRNA-mediated depletion of *PFKFB4* for 72 h, cells were treated with 50 nM paclitaxel for 16 h and separated by mitotic shake-off into the mitotic and interphase populations. **A.** Western blotting was used to determine the phosphorylation of AMPK at T172 and **B.** ACC at S79. **C.** Levels of ROS were determined using CM-H2DCFDA staining and flow cytometry after siRNA-mediated depletion of PFKFB4 in the interphase and mitotically arrested populations separated by mitotic shake-off and **D.** in the whole population without paclitaxel treatment. Data are expressed as the mean +SEM of three experiments.

in both the T308D/S473D (Figure 10A, $p<0.0001$, two-way ANOVA multiple comparison) and Myr-AKT (Figure 10B, $p=0.0004$, two-way ANOVA multiple comparison) cell lines. This was accompanied by a significant increase in PI incorporation into mitotically arrested cells depleted of PFKFB4 in T308D/S473D (Figure 10C, $p=0.0046$, two-way ANOVA multiple comparison) and Myr-AKT (Figure 10D, $p=0.0426$, two-way ANOVA multiple comparison), suggesting that there is an increase in cell death. Levels of ROS in both T308D/S473D (Figure 10E, $p=0.0069$, two-way ANOVA multiple comparison) and Myr-AKT (Figure 10F, $p=0.0.0003$, two-way ANOVA multiple comparison) were also significantly increased in mitotically arrested cells after PFKFB4 depletion compared to the non-targeting control. The effects of individual siRNA duplexes were tested to check for off-target effects, however all of them displayed a significant decrease in mitotically arrested cells after PFKFB4 depletion (Supplementary Figure 3A-3D). These data indicate that partial restoration of AKT signalling during mitotic arrest does not protect cells from the detrimental effects of PFKFB4 depletion. Moreover, it may indicate that PFKFB4 depletion is not directly exploiting the vulnerability created from the loss of AKT activity during mitotic arrest and is producing an effect through an alternative pathway.

DISCUSSION

This work has uncovered a novel function of the enzymes PFKFB3 and PFKFB4 in ovarian cancer cells during mitotic arrest. Depletion of PFKFB3 and PFKFB4 prior to paclitaxel treatment caused a significant

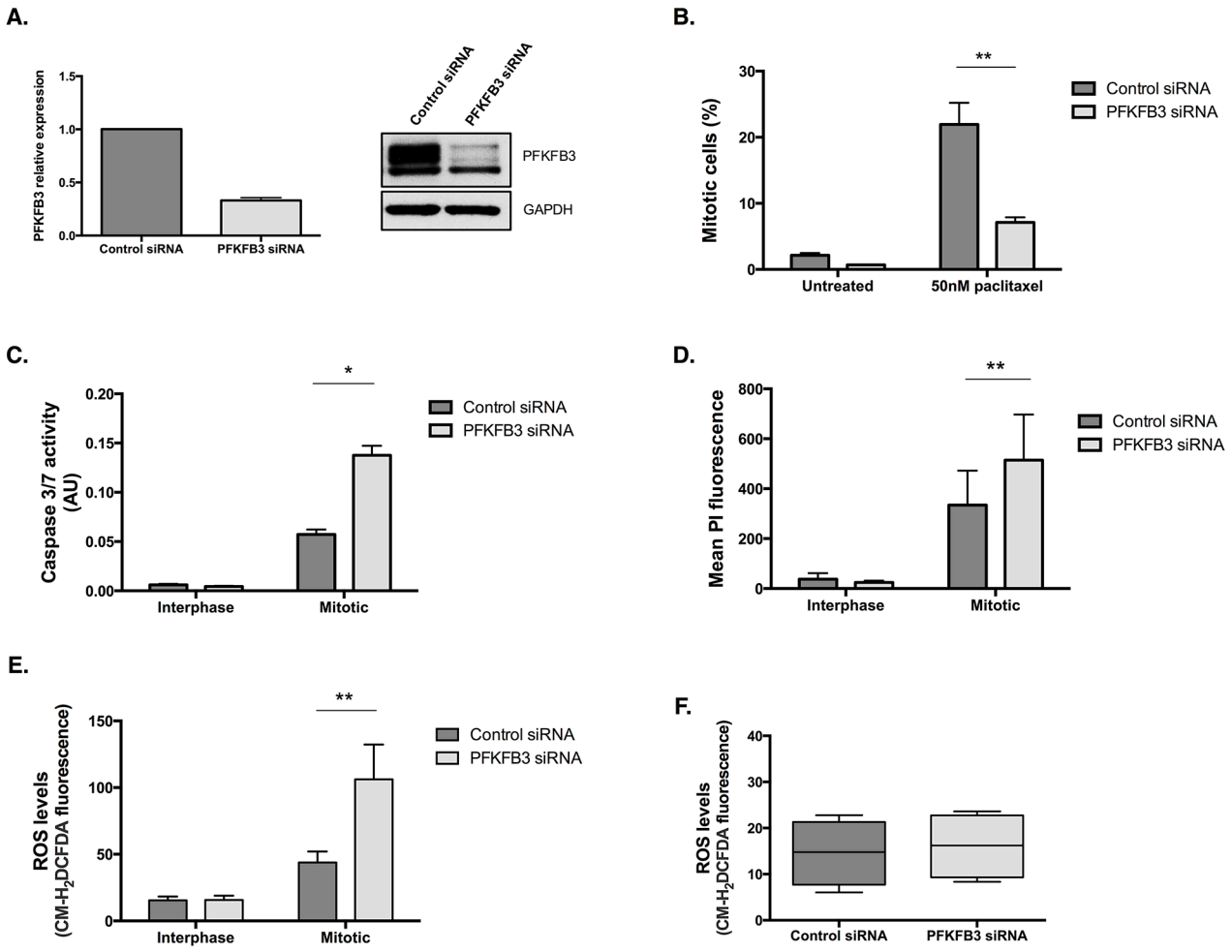


Figure 8: PFKFB3 is important for cell survival during paclitaxel-induced mitotic arrest. A. *PFKFB3* mRNA and protein expression after siRNA-mediated depletion of *PFKFB3* for 72 h was determined by qPCR and western blotting. **B.** The quantification of mitotically arrested cells by flow cytometry, **C.** caspase 3/7 activity using Caspase-Glo 3/7 assay **D.** propidium iodide incorporation by flow cytometry and E. ROS levels using CM-H2DCFDA staining and flow cytometry were assessed after siRNA-mediated depletion of *PFKFB3* for 72 h followed by 50 nM paclitaxel for 16 h and a mitotic shake-off in SKOV3 cells. **F.** The levels of ROS after PFKFB3 depletion alone, without paclitaxel treatment or mitotic cell isolation, was also analysed using CM-H2DCFDA staining and flow cytometry. Data are expressed as +SEM of three experiments.

reduction in mitotic cell number, an increase in ROS and PI incorporation, and enhanced caspase 3/7 activity in mitotically arrested cells. While numerous studies have identified the importance of PFKFB3 and PFKFB4 in cancer cell survival [45–47], we are the first to investigate the impact of their depletion in ovarian cancer cells and identify their importance during mitotic arrest.

Another important aspect of this work is that it has highlighted a targetable vulnerability in ovarian cancer cells during mitotic arrest and possibly during spontaneous mitosis, due to the reduction in AKT signalling and glucose 6-phosphate, and a significant decrease in ATP levels. This is intriguing given that the ovarian cancer cell lines used in this study possess a *PI3KCA* mutation

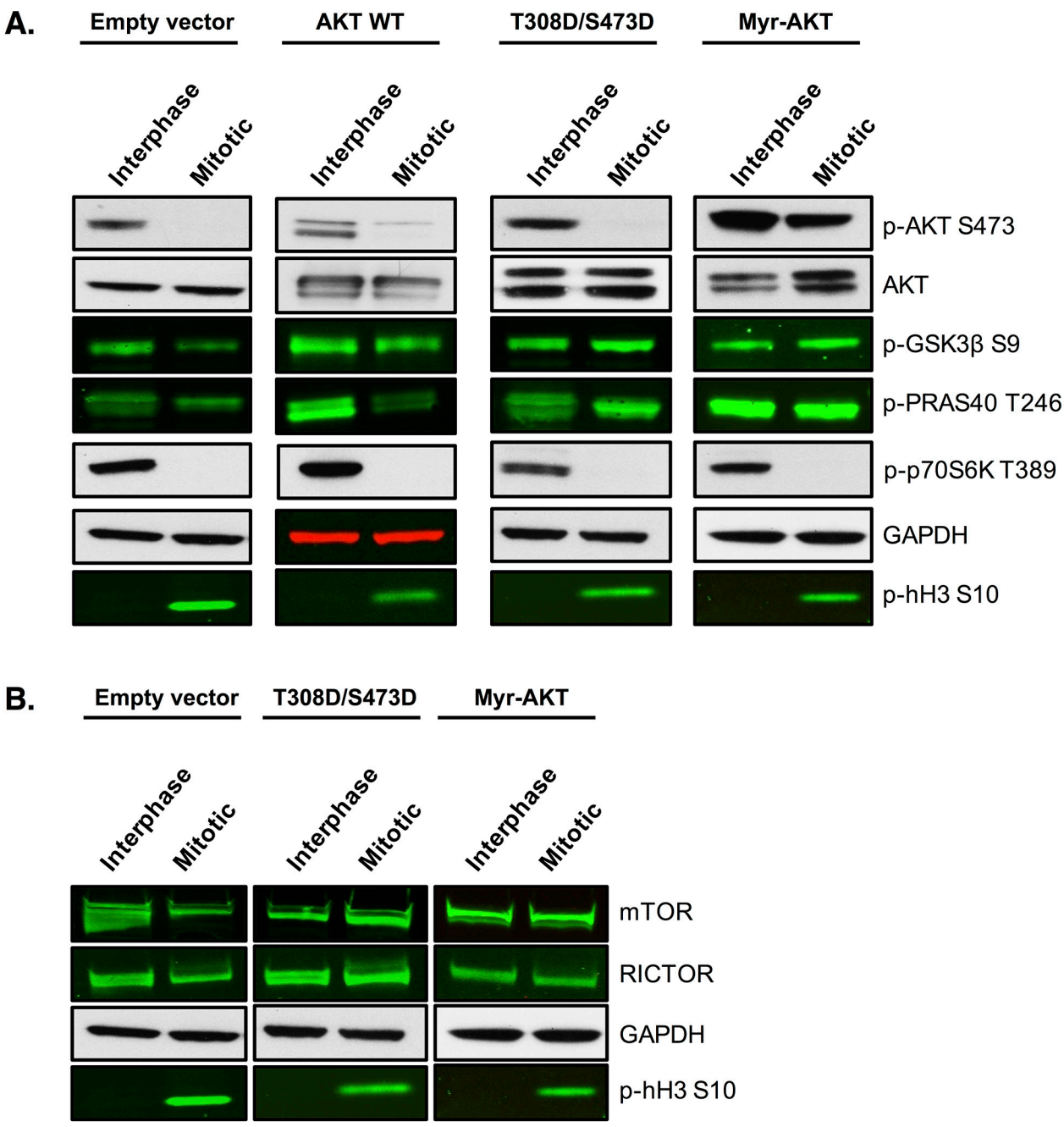


Figure 9: Overexpression of AKT restores the phosphorylation of direct AKT substrates. SKOV3 cell lines stably overexpressing wild-type AKT, T308D/S473D or Myristoylated-AKT were generated using lentiviral transfection of HEK 293T cells and transduction in SKOV3 cells. **A.** Western blotting measured the phosphorylation status of AKT and its downstream targets and **B.** the expression of proteins upstream of AKT in the empty vector, wild-type AKT, T308D/S473D and Myristoylated-AKT cell lines. In the western blots, GAPDH is the loading control, phospho-histone H3 S10 is the mitotic marker and images represent one of three experiments.

[20–22] causing constitutive activation of the PI3K/AKT pathway, which indicates the importance of this pathway and the potential vulnerability caused by its temporary inactivity during mitosis. The observation that the A2780 cell line maintains AKT activity during mitotic arrest indicates that the loss of AKT is not simply because the cells are asynchronous. It would be informative to investigate PFKFB4 depletion in *PTEN* null cells to determine whether they are also unaffected following PFKFB4 depletion during mitotic arrest. Moreover, the fact that A2780 cells remain unaffected by PFKFB4 depletion demonstrates the specificity of this vulnerability and that it is not universal across all ovarian cancer cell lines. Additionally, whether PFKFB4 depletion is exploiting this specific vulnerability is unclear as the constitutive activation of AKT did not rescue the observed increase in mitotic cell death. An interaction between AKT signalling and PFKFB4 was also recently observed by Pegoraro *et al.,* 2015 [52], where they found that PFKFB4 depletion influenced AKT phosphorylation, but no direct link was established between the two proteins. It could be that the loss of AKT phosphorylation following PFKFB4 depletion makes cells more vulnerable to AKT inhibition, however this has not been determined in this work.

Our high content siRNA screen successfully identified PFKFB4 as an important regulator of ovarian cancer cell survival following mitotic arrest by paclitaxel. These results are supported by two independent groups that have conducted unbiased screens for genes essential for cancer survival and found that PFKFB4 is required for both glioma stem-like cell [53] and prostate cancer cell survival [47], but not for normal cell survival. However, this is the first study to identify an increase in ROS specifically in mitotically arrested cells after PFKFB3

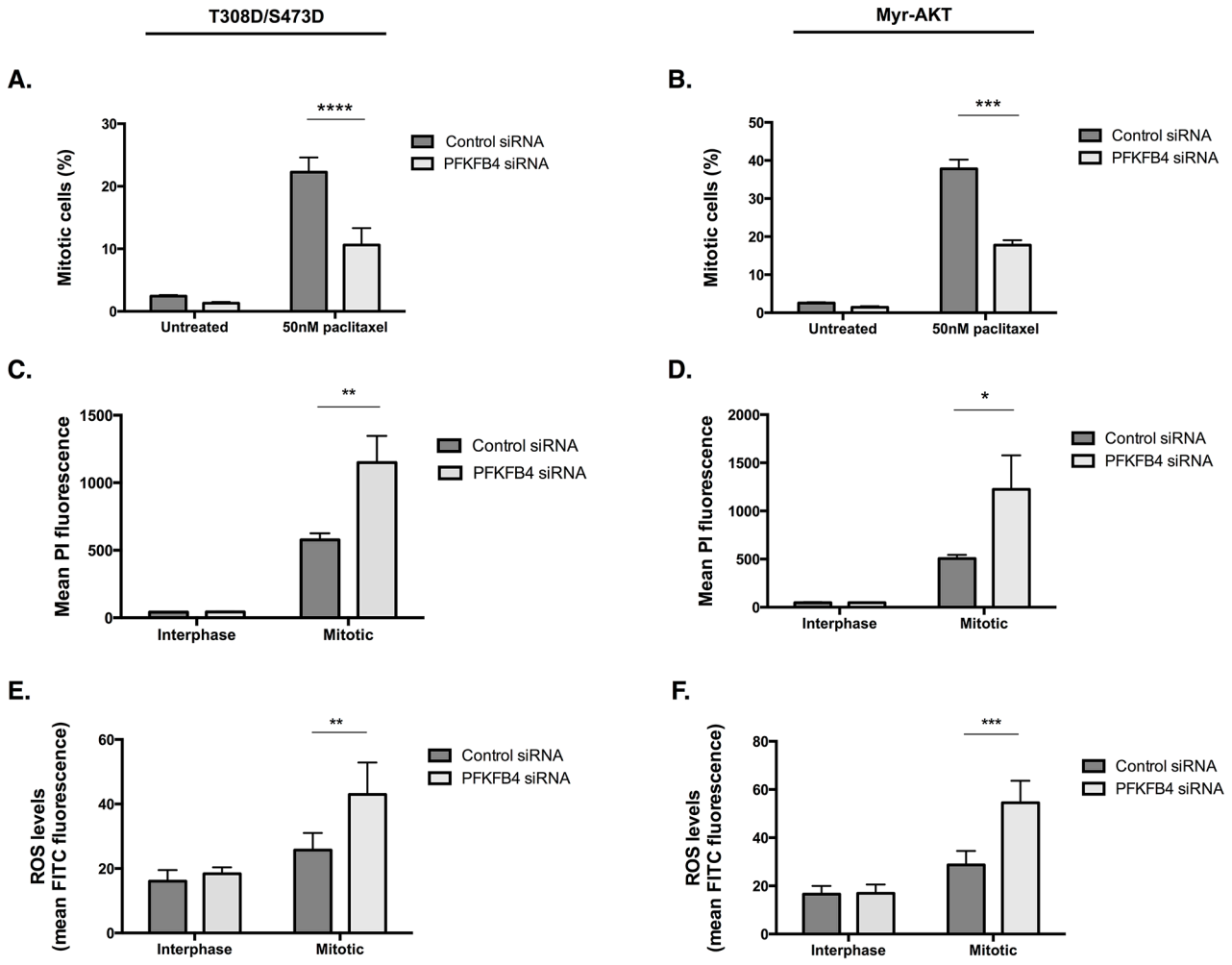


Figure 10: Overexpression of constitutively active AKT does not confer resistance to PFKFB4 depletion. A, B. After siRNA-mediated depletion of *PFKFB4* for 72 h, 50 nM paclitaxel for 16 h and mitotic cell isolation, the percentage of mitotically arrested cells was determine using flow cytometry, **C, D.** PI incorporation was measured by flow cytometry and **E, F.** levels of ROS were measured using CM-H2DCFDA staining and flow cytometry in T308D/S473D and Myr-AKT cell lines. Data are expressed as the mean +SEM of three experiments.

or PFKFB4 depletion. We predict that this specific increase in ROS only in mitotically arrested cells is due to a decrease in mitochondrial membrane potential [54] or a suppression of glycosylation [55]. An increase in ROS levels after depletion of PFKFB3 and PFKFB4 has been observed in a number of other studies [45, 47, 56], however in the work presented here an increase in ROS was observed only in the mitotically arrested cells after PFKFB3 or PFKFB4 depletion and not in interphase cells. This discrepancy might be due to PFKFB3 and PFKFB4 having distinct roles in ovarian cancer cells. The main function of PFKFB4 in cancer cells has been an area of debate, with a number of studies indicating a predominant phosphatase activity [47, 56] and others suggesting a dominant kinase activity [57, 58].

It is unlikely that the increase in mitotic cell death is attributable to oxidative stress as treatment with the antioxidant N-acetyl cysteine could not rescue the decrease in mitotic cell number despite reducing the levels of ROS (data not shown). The production of ROS may therefore be a by-product and not the causal factor in mitotic cell death following PFKFB4 depletion. Mitotic cell death has previously been attributed to the energetic capacity of cells during mitotic arrest [42]. Mitotic arrest can increase mitophagy, which reduces oxidative respiration and ATP, forcing the cell to rely more heavily on glycolytic metabolism. This places a strong requirement on processes that promote glycolysis, such as AMPK-mediated PFKFB3 activation [42]. However, in this work, AMPK was not phosphorylated after PFKFB3 depletion in mitotically arrested cells, which suggests that they are not energetically stressed and an alternative cause of mitotic cell death is most likely.

The panel of cell lines analysed in this work represent some of the most commonly used cell lines in ovarian cancer research, for example SKOV3 cells and A2780 cells, which account for approximately 60% of publications related to ovarian cancer [21]. In addition, we have confirmed that the SKOV3 cell line used in this work possess a *TP53* mutation, which is a hallmark of high grade serous ovarian cancer (HGSOC). However, to determine the applicability of this work to HGSOC and not simply epithelial ovarian cancer in general, cell lines that more accurately represent this subtype should be used, such as those developed by Kreuzinger *et al.,* 2015 [59]. A further limitation of this work is the nature and duration of mitotic arrest by paclitaxel. Cells were arrested for 16 hours in cell culture medium containing 50 nM paclitaxel, which may not accurately represent the pharmacokinetics of paclitaxel or the nature of mitotic arrest *in vivo*. Paclitaxel is known to undergo triphasic elimination in the plasma with the C_{max} being approximately 4 µmol/L following the standard three-weekly dose of 175 mg/m^2/h [60]. However, there is a lack of information regarding the exact concentration of paclitaxel that reaches the tumour, so although this work provides a useful insight into targeting PFKFB4 in mitotically arrested ovarian cancer cells, whether the nature of the mitotic arrest following paclitaxel treatment reflects that of an *in vivo* environment has yet to be established.

The main objectives of this study were to identify targetable vulnerabilities in ovarian cancer cells during mitotic arrest and to determine whether we can exploit these for therapeutic benefit. In summary, we have identified a metabolic vulnerability in the majority of ovarian cancer cells during mitotic arrest arising from a striking decrease in AKT activity, a reduction in glucose 6-phosphate, and a significant decrease in ATP levels. Moreover, we have demonstrated that the depletion of either PFKFB3 or PFKFB4 can significantly increase cell death in mitotically arrested cells. This work has identified a previously unrecognised role of PFKFB4 in mitotically arrested ovarian cancer cell survival and supports the notion that combining mitotic-targeted therapies with glycolytic inhibitors may potentiate the effects of antimitotics in ovarian cancer.

MATERIALS AND METHODS

Cell lines and cell culture

SKOV3, OVCAR-3, OC 316, UPN-251 and HEK-293T cell lines were obtained from the American Tissue Type Culture Collection (ATCC), A2780 cells were purchased from Sigma-Aldrich. SKOV3 cells were maintained in McCoy's 5A medium (Thermo Fisher Scientific), A2780 and OVCAR-3 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific), while HEK293T, OC 316, UPN-251 were cultured in DMEM (Dulbecco's modified Eagle's medium) (Thermo Fisher Scientific). Growth medium was supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) and cells were grown at 37°C and 5% CO_2. SKOV3 cell lines stably expressing AKT1 or AKT1 mutants were grown in media containing 50ng/l puromycin (Sigma-Aldrich). Stock solutions of 1 mM Paclitaxel (Sigma-Aldrich) were prepared with dimethyl sulphide (DMSO) (Sigma-Aldrich).

Mitotic cell isolation

Cells were treated with 50 nM paclitaxel for 16 h, after which media was removed and cells were washed once with PBS. Fresh PBS was added to the flask and mitotic cells were detached by gentle shaking. Detachment of cells was assessed by viewing the cells under the AE2000 Inverted microscope (Motic) and shaking continued until the majority of mitotic cells had detached. Spontaneous mitotic cells were obtained using the same protocol, however, cells were not treated with paclitaxel and therefore eight T175 flasks were used to obtain the

small number of spontaneous mitotic cells. Remaining interphase cells were collected by trypsinisation. Validation of efficient isolation of mitotic cells was always assessed by the presence of phospho-histone H3 S10 staining in the mitotic population and not the interphase cells.

SDS PAGE and western blotting

Samples were prepared for SDS-PAGE by mixing 30 µg of protein with 1x NuPage LDS Sample Buffer (Thermo Fisher Scientific) and deionised water before heating at 95°C for 10 min. For analysis of proteins larger than 200kDa, 1x NuPAGE Reducing Agent was added to the samples prior to boiling. Samples were then subjected to SDS-PAGE using 1.5mm NuPage 4-12% Bis-Tris precast polyacrylamide gels (Thermo Fisher Scientific) with 1x NuPage MOPs Running Buffer diluted in deionised water (Thermo Fisher Scientific) or 1.5mm NuPage 3-8% Tris-Acetate precast polyacrylamide gels (Thermo Fisher Scientific) with 1x NuPage Tris-Acetate Running Buffer diluted in deionised water (Thermo Fisher Scientific) supplemented with 500 µl NuPage Antioxidant in the inner buffer chamber for proteins over 200kDA. SeeBlue Plus 2 Pre-stained protein standard (Thermo Fisher Scientific) was loaded into the first lane of each gel followed by the set of samples and gels were run at 160V for approximately 50 min. Proteins were transferred to a nitrocellulose membrane using the iBlot dry gel transfer device (Thermo Fisher Scientific). Membranes were incubated in blocking buffer (5% skimmed milk powder (Sigma-Aldrich diluted in TBST (1x Tris-buffered saline and 0.1% Tween-20)) at room temperature for 1 h. Primary antibodies were diluted in blocking buffer and incubated with the membrane overnight at 4°C. Membranes were washed three times for 15 min in TBST before incubating with either horseradish peroxidase(HRP)-conjugated secondary antibodies (Goat anti-rabbit and Goat anti-Mouse, Active Motif) or LI-COR Odyssey secondary antibodies (Goat anti-Mouse IRDye 680RD and Goat anti-rabbit IRDye 800CW) for 1 h at room temperature. Membranes were washed three times for 15 min in TBST and visualised using Pierce enhanced chemiluminescence Western Blotting Substrate (Thermo Fisher) for HRP-conjugated secondary antibodies or the Odyssey Imager (LI-COR) for fluorescent secondary antibodies.

RNA extraction and quantitative PCR

Total RNA was isolated using RNeasy mini kit (Qiagen) including the DNase-1 treatment according to the manufacturer's protocol and quantified using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc). 1 µg of RNA was reverse transcribed using TaqMan® Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems) in a 20 µl reaction using a MicroAmp Fast Optical 96-Well Reaction Plate according to the manufacturer's protocol on the 7500 Fast Real-Time PCR System (Applied Biosystems). The specificity of the PCR products were confirmed by melting curve analysis. cDNA amplification was normalised against *GAPDH* and *ACTB*. Expression ratios relative to the control sample were calculated as previously described [61].

Transfections

Transient protein depletion experiments with siRNA duplexes purchased from GE Healthcare were conducted using a reverse transfection method with siRNA at a final concentration of 40 nM. 4 µl siRNA specific to the gene of interest, non-targeting siRNA or Dharmafect 4 transfection reagent (Dharmacon, GE Healthcare) were diluted separately in 200 µl Opti-MEM for 5 min according to the manufacturers protocol. 200 µl DharmaFECT 4-Opti-MEM complex was combined with 200 µl siRNA-Opti-MEM complex and incubated for 20 min prior to aliquoting the full 400 µl Dharmafect 4-siRNA complex into each well of a 6-well plate. Cells were resuspended in media and diluted to 90,000 cells/ml for SKOV3 and OC 316 cells, and 110,000 cells/ml for A2780. 1.6 ml of the diluted cells were added on top of the DharmaFECT 4-siRNA complex and plates were left for 72 h at 37°C and 5% CO_2 to achieve efficient protein depletion. For T25 flasks, 6 µl siRNA or DharmaFECT4 were diluted in 300 µl Opti-MEM and 2.4 ml cells were added on top of 600 µl DharmaFECT 4-siRNA complex.

High-content siRNA kinome screen

SKOV3 cells were plated in a 384-well format and treated with Dharmacon siRNA kinome library targeting 779 different genes for 48 h. Cells were treated with 10 nM paclitaxel or vehicle control for 24 h prior to fixation with 4% paraformaldehyde for 5 min. Cells were permeabilised with ice-cold absolute ethanol and left overnight at -20°C prior to adding wash buffer (Tris-buffered saline, 0.2% Triton X-100 and 0.04% SDS) for 10 min. Cells were blocked in blocking buffer (1.5% bovine serum albumin diluted in Tris-buffered saline) for 1 h at room temperature. Cells were incubated with anti-phospho-histone H3 S10 antibody diluted in blocking buffer for 1 h at room temperature. Three washes were performed with wash buffer for 5 min each and Alexa Fluor 488 donkey anti-rabbit was diluted in blocking buffer and added to each well for 1 h at room temperature in the dark. Cells were washed three times as before and incubated with 10 µg/ml Hoechst 33342 (Thermo Fisher Scientific) diluted in PBS for 10 min at room temperature. The Hoechst stain was removed and PBS was added to each well prior to image acquisition using the In Cell

Analyzer 1000 (GE Healthcare) as previously described [62]. Data analysis was carried out using R programming language and software. Cells were assigned to specific wells and the mean pixel intensity values were calculated for each Hoechst and phospho-histone H3 S10 cell and background values were subtracted. The percentage of mitotic cells was calculated for each well by dividing the number of cells staining positive for phospho-histone H3 S10 by the total number of cells that stained positive for Hoechst. The median of triplicate values was obtained for each condition.

Immunofluorescence

Cells were grown to 80% confluence on cover slips in 12-well plates prior to fixation with 0.5 ml 4% methanol-free formaldehyde (Thermo Fisher Scientific) for 4 min at room temperature. Permeabilisation was achieved using 0.5 ml 100% ice-cold ethanol and incubation at -20°C overnight. Ethanol was removed and 1 ml wash buffer (1x TBS, 0.2% Triton X100 and 0.04% SDS, Sigma-Aldrich was added to the wells for 5 min. Cells were blocked in 1 ml blocking buffer (3% bovine serum albumin diluted in 1xTBS) for 1 h at room temperature. Cover slips were incubated with 60 µl primary antibody diluted in blocking buffer for 1 h at room temperature. Three washes were performed with 1 ml wash buffer for 5 min each. Secondary antibodies (Alexa Fluor 488 donkey anti-rabbit or Alexa Fluor 568 donkey anti-mouse, Thermo Fisher Scientific) were diluted in blocking buffer and 60 µl was added to each cover slip for 1 h at room temperature in the dark. Cells were washed as before prior to mounting the coverslips with mounting medium containing DAPI (Vectashield) on microscope slides (ThermoScientific) sealed with nail varnish at the edges. Slides were stored at 4°C in the dark until analysis. Images were analysed on the Zeiss Observer Z1 Microscope using a 40X oil objective or a 20X objective. Images were analysed using ImageJ software.

Quantification of mitotic cells by flow cytometry

Cells were centrifuged at 1500 rpm (365 xg) for 5 min to pellet the cells. Supernatants were removed and pellets were resuspended thoroughly in 0.5 ml PBS and transferred to Falcon 5 ml Round Bottom flow cytometry tubes (Scientific Laboratory Supplies). 0.5 ml 8% methanol-free formaldehyde (Thermo Fisher Scientific) was added to give a final concentration of 4% and incubated for 6 min at room temperature. Fixative was removed by centrifugation at 1500 rpm (365 xg) for 5 min and samples were thoroughly resuspended in 1 ml ice-cold 100% ethanol and placed at -20°C overnight to allow cell permeabilisation. Cells centrifugation at 5000 rpm for 5 min and washed in 1 ml blocking buffer (3% bovine serum albumin in 1xTBS). Cells were collected by centrifugation as described above and resuspended in 1 ml blocking buffer for 1 h at room temperature. Cells were pelleted again and incubated with rabbit anti-phospho-histone H3 S10 diluted in blocking buffer for 1 h at room temperature. Cells were washed twice in 1 ml blocking buffer before incubation with the secondary antibody (Alexa Fluor 488 donkey anti-rabbit) diluted in blocking buffer for 1 h at room temperature in the dark. Cells were pelleted by centrifugation at 5000 rpm for 5 min and washed in 1 ml blocking buffer. Cells were finally resuspended in 0.5 ml 3 µM Propidium Iodide solution (PI) (Thermo Fisher Scientific) diluted in PBS to exclude dead cells as PI is membrane impermeant and analysed on the CyAn ADP Analyser (Beckman Coulter). Data from approximately 100,000 cells from each sample were analysed using FlowJo software.

Cell cycle analysis

Cell cycle analysis was performed using flow cytometry and propidium iodide (PI) staining. After incubation with PFKFB4 siRNA for 72 h, cells were harvested into Falcon 5 ml Round Bottom flow cytometry tubes (BD Falcon) prior to centrifugation at 1500 rpm (365 xg) for 5 min. Cells were resuspended in 1 ml PBS with 3% FBS and centrifuged again at 1500 rpm (365 xg) for 5 min. 1 ml ice-cold 70% ethanol (Sigma-Aldrich was added drop-wise to each sample prior to vortexing and centrifugation at 3000 rpm for 4 min. Cells were washed in 1 ml PBS with 3% FBS and pelleted again by centrifugation at 3000 rpm for 4 min. 0.5 ml 100 µg/ml RNase A (Thermo Fisher Scientific) and 40 µg/ml PI diluted in PBS with 3% FBS was added to each sample and incubated at 37°C and 5% CO_2 for 40 min. Samples were collected by centrifugation at 3000 rpm for 4 min and resuspended in 1 ml PBS with 3% FBS for analysis on CyAn ADP Analyser (Beckman Coulter). Cells were kept covered prior to analysis. PI was analysed on a linear scale with Area vs Peak to gate out doublets and analysed at a low flow rate (<400 events/second).

Detection of reactive oxygen species

Reactive oxygen species (ROS) levels were measured using the cell permeable indicator CM-H_2DCFDA (Thermo Fisher Scientific). Cells were incubated with PFKFB4 siRNA or PFKFB3 siRNA for 72 h in T25 flasks. After 72 h, 2 ml 50 nM paclitaxel or DMSO control (Sigma-Aldrich) diluted in media were added to cells and incubated overnight at 37°C and 5% CO_2. The next day, mitotic cells were separated from interphase cells and placed in Falcon 5 ml Round Bottom flow cytometry tubes (Scientific Laboratory Supplies) prior to centrifugation at 1500 rpm (365 xg) for 5 min. CM-H_2DCFDA was freshly reconstituted in DMSO and cells were resuspended in 1 ml 3 µM CM-H_2DCFDA

diluted in warm McCoy's 5A media and incubated for 30 min at 37°C and 5% CO_2. 1 ml 1 mM Hydrogen peroxide (H_20_2) (Sigma-Aldrich) and 3 μM CM-H_2DCFDA diluted in warm McCoy's 5A media were added to cells as a positive control. After 30 min, cells were centrifuged at 1500 rpm (365 xg) for 5 min and resuspended in 0.5 ml ice-cold 10 μg/ml propidium iodide (Thermo Fisher Scientific) (535nm/617nm) diluted in PBS. Samples were placed on ice and kept in the dark until analysis on CyAn ADP Analyser (Beckman Coulter). Samples were also tested by western blot for the presence of phospho-histone H3 S10 in the mitotic samples.

Caspase 3/7 measurements

The Caspase-Glo 3/7 Assay (Promega) was performed following the manufacturer's protocol. Caspase-Glo 3/7 Buffer and Caspase-Glo 3/7 Substrate were equilibrated to room temperature prior to use. Caspase-Glo 3/7 substrate was reconstituted by transferring the entire Caspase-Glo 3/7 Buffer into the bottle. Cell pellets were resuspended in media and counted as previously described. Cells were diluted to 100,000 cells/ml and 50 μl of cell suspension was plated in an opaque-walled 96-well plate in triplicate. Media alone was added in triplicate to allow the exclusion of background luminescence and Staurosporine (Cell Signaling Technology) treatment was used as a positive control (0.3 μM for 16 h). 50 μl of the reconstituted substrate was added to each well in the dark and the plate was covered in foil and placed on a plate shaker for 30 seconds prior to incubation at room temperature for 1 h. The plate was read on a luminescent plate reader. Triplicate measurements were averaged and the culture media background fluorescence was subtracted from the average of each experimental sample.

Time-lapse microscopy

Transfections with siRNA were set up as previously described, but scaled down for use of a 12-well plate, meaning 800 μl cell suspension at the densities stated were combined with 200 μl DharmaFECT 4-siRNA complex per well. After transfection for 72 h, cells were treated with 50 nM paclitaxel or DMSO control and visualised by time-lapse microscopy using a 20X objective on the Leica DMIRE2 Hamamatsu ORCA-ER using HCI Image acquisition software while keeping the cells at 37°C and 5% CO_2. Five fields of view in each condition were imaged every 10 min for a minimum of 16 h. Time-lapse images for each field of view were analysed and the fate of each mitotic cell was determined. Cell death was characterised by blebbing and dense cellular irregularities. Cells that left the field of view during the course of the experiment were not included in the analysis.

Site-directed mutagenesis

An AKT1 ORFEXPRESS-Shuttle Clone was obtained from GeneCopoeia (A0022). Site-directed mutagenesis was performed to remove the stop-codon from the entry vector as this would enable it to be contiguous with the expression of a V5 tag once it is sub cloned into a pLX302 expression plasmid. To do this, primer sequences were designed using QuikChange Primer Design (Agilent Technologies) and reactions were performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's guidelines. After completion of the PCR, 40 μl of the PCR reaction was digested with *Dpn1* (New England BioLabs) for 1 h at 37°C to remove any template DNA that had not been mutagenised. Site-directed mutagenesis was also performed to introduce specific nucleotide changes/point mutations in the AKT1 entry vector to generate AKT-T308D/S473D.

Transformation

50 μl ONE Shot TOP10 or ONE SHOT Stbl3 Chemically Competent *E.coli* (Thermo Fisher Scientific) were thawed on ice for each transformation. 5 μl plasmid DNA was added to the competent cells and mixed by flicking the tube before storing on ice for 30 min. Cells were then placed at 42°C for 30 seconds to enable incorporation of DNA and then placed on ice for 2 min. 250 μl of Super Optimal broth with Catabolite repression (SOC) medium at room temperature was added to each vial and placed in a 37°C orbital shaker for 2 h. After incubation, 20 μl and 200 μl of each transformation was placed on a pre-warmed agar plate containing the appropriate antibiotic and spread using ColiRoller Plating Beads (Millipore). Plates were wrapped in Parafilm (Thermo Fisher), inverted and placed in a 37°C incubator overnight. Plates were checked the next day for bacterial colonies and placed at 4°C until required.

DNA extraction from transformed colonies

Transformed colonies were picked at random from the agar plates and grown at 37°C overnight in LB broth supplemented with the appropriate antibiotic in an orbital shaker at 225 rpm. The following day, bacteria were pelleted at 4000 rpm for 10 min in a desktop microcentrifuge. Supernatants were discarded and DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. DNA sequences were verified with Sanger sequencing.

Gateway cloning

Lentiviral vectors were generated using Gateway Technology (Thermo Fisher Scientific) according to the manufacturer's instructions. The AKT1 ORFEXPRESS-

Shuttle Clone (A0022, GeneCopoeia was sub-cloned into the pLX302 lentiviral destination vector (Addgene) using the Gateway LR Clonase II enzyme mix. In brief, 5 µl reactions containing 70ng plasmid DNA, 150ng pLX302, 1 µl LR Clonase II Enzyme Mix and Tris EDTA (TE) buffer were mixed by vortexing and incubated at 25°C for 1 h. Reactions were incubated with 1 µl Proteinase K solution at 37°C for 10 min to terminate the reaction. 1 µl of each LR reaction was transformed into ONE SHOT Stbl3 Chemically Competent *E.coli* (Thermo Fisher Scientific) as described previously and success was checked using CMV-Forward and WPRE-Reverse primers. pCDH-puro-Myr-HA-AKT1 was a gift from Jialiang Wang (Addgene plasmid #46969), which was created as previously described [63]. DNA was extracted from the Myr-AKT1 pCDH lentiviral expression plasmid by inoculating LB-antibiotic broth with the glycerol stock and following the protocol for DNA extraction. DNA was then transformed into One Shot Stbl3 cells as previously described ready for transfection of packaging cells.

Lentiviral transfection and transduction

Packaging cells (HEK293T) were plated in vented T25 flasks for 24 h to obtain 70% confluency. For each flask, the following were combined; 3 µg pLX302 vector, 0.3 µg VSV-G and 3 µg pCMV-Gag-Pol (10:1:10 ratio) in a total volume of 225 µl Opti-MEM. HEK293T were then transfected using the protocol from FuGENE with a FuGENE:DNA ratio of 3:1. In brief, 18.9 µl FUGENE was added to the DNA-OptiMEM mix and mixed by pipetting before incubation at room temperature for 7.5 min. 100 µl of the DNA-FUGENE-OptiMEM complex was added to the cells in 2 ml fresh media and left for 48 h. After 48 h, viral supernatants were harvested into a 15 ml falcon tube and centrifuged at 1500 rpm (365 xg) for 5 min to remove any cells before being passed through a 0.45 µm Minisart NML Syringe Filter (Sartorius). 2 ml DMEM media containing 30% (v/v) fetal bovine serum (Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific) were added to the HEK293T cells for a further 24 h, after which this was centrifuged and filtered as described above. 15,000 SKOV3 cells were plated in T25 flasks to obtain 50% confluence the day before transduction. The next day, media was removed and the filtered viral supernatants were added to the SKOV3 cells. SKOV3 were incubated with the supernatants for 72 h, adding the second filtered viral supernatant after the first 24 h. After 72 h, the media of SKOV3 was changed to contain 50ng/L Puromycin (Sigma-Aldrich) to select the cells that contain the integrated virus. HEK293T and SKOV3 cells were collected by trypsinisation for lysis and examination of protein expression by western blot to assess the efficiency of transfection and transduction.

ATP assay

The CellTiter-Glo Luminescent Cell Viability assay (Promega) was used to measure ATP levels according to the manufacturer's protocol. Following mitotic cell isolation, populations of mitotic and interphase cells were counted in the presence of trypan blue and diluted to 200,000 cells/ml. 100 µl of each sample was plated in an opaque-walled 96-well plate in triplicate. Media alone was added in triplicate to allow the exclusion of background luminescence. 100 µl of the reconstituted substrate was added to each well in the dark and the plate was covered with foil and placed on a plate shaker for 2 min prior to incubation at room temperature for 10 min. The plate was read on a luminescent plate reader at a sensitivity of 190. Triplicate values were averaged and the culture media background was subtracted from the average of each experimental sample.

Glutathione assay

Glutathione levels were measured using GSH-Glo Glutathione Assay (Promega) according to the manufacturer's instructions. Cells were diluted to 100,000 cells/ml. 50 µl cell suspension was placed in an opaque-walled 96-well plate in triplicate. 50 µl of freshly prepared GSH-Glo Reagent 2X Mix (10 µl luciferin-NT substrate, 10 µl glutathione-s-transferase with 480 µl GSH-Glo reagent) was added to each well in the dark and placed on a plate shaker for 30 seconds prior to incubation at room temperature for 30 min in the dark. After this, 100 µl/well of reconstituted Luciferin Detection Reagent was added and placed on a plate shaker for 30 seconds prior to incubation for 15 min and reading the luminescence. Triplicate values were averaged and the culture media background was subtracted from the average of each experimental sample.

Glucose 6-phosphate assay

A Glucose 6-phosphate Assay Kit was purchased from Abcam and performed following the manufacturer's protocol. Cells were homogenised with ice-cold PBS and 50 µl were added to a 96-well plate with 50 µl reaction mix (46 µl assay buffer, 2 µl enzyme mix, 2 µl substrate mix). To measure the background, 50 µl of background mix (48 µl assay buffer, 2 µl substrate mix) was added to 50 µl PBS. Cells grown in glucose-free DMEM (Thermo Fisher Scientific) for 24 h were used as a negative control. The plate was incubated at room temperature for 30 min in the dark before measuring the OD at 450nm.

Further details regarding the methods are available as supplemental information.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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