The Role of Wee1 in the Hypoxia-Induced DNA Damage Response and its Potential as a Therapeutic Target

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Abstract

The majority of tumours have a poor vasculature which results in low oxygen supply. This, coupled with the high oxygen demand often leads to regions of hypoxia. The physiological relevance of this includes resistance to radiotherapy and chemotherapy, as well as activation of specific signalling cascades. Chk1, a component of the DNA damage response, is activated in acute hypoxia even in the absence of DNA damage. Loss or inhibition of Chk1 sensitises cells to hypoxia/reoxygenation. Wee1 is purportedly a downstream target of Chk1 and this study suggests this is the case in mammalian cells. Wee1 is involved in G_2-M progression; inhibitors of Wee1 force cells into premature mitosis causing damage as well as radiosensitising cells even under hypoxic conditions.

Wee1 also regulates genomic stability via inhibition of the endonuclease Mus81-Eme1 in S phase cells. Previous reports suggest damage induced by Wee1 inhibition is Mus81 dependent however no evidence for this was seen in this study in normoxia or hypoxia. However, a novel finding was the involvement of Mus81 in reoxygenation-induced restart suggesting this nuclease does play a significant role in cellular adaptation to hypoxia.

Overall Wee1 inhibitors have been shown to be effective in normoxic and hypoxic conditions and successfully radiosensitise hypoxic cells which are usually resistant to therapy. This suggests this combination treatment should be investigated further in clinical studies.

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List of Abbreviations

53 BP1 ~ p53 binding protein 1

ADM ~ Adrenomedullin

AMF ~ Autocrine motility factor

ATR ~ ATM-and Rad3-related

°C ~ Degrees centigrade

Cdc2 ~ Cell division control protein 2 homolog

Chk1 ~ Checkpoint protein 1

CO_2 ~ Carbon dioxide

Cs^{137} ~ Caesium 137, radioactive isotope

DDR ~ DNA damage response

DEPC water ~ Diethylpyrocarbonate treated water

DMEM ~ Dulbecco's modified eagle's medium

DMSO ~ Dimethyl sulfoxide

DNA ~ Deoxyribonucleic acid

DNA-PKcs ~ DNA dependent protein kinase catalytic subunit

DSB ~ Double stranded break

EDTA ~ Ethylenediaminetetraacetic acid

FBS ~ Foetal bovine serum

g~ G-force

GAPDH ~ Glyceraldehyde - 3 - phosphate dehydrogenase

GLUT1 ~ Glucose transporter 1

Gy ~ Gray

γH2AX ~ Phosphorylated histone 2A.X

H3 ~ Histone 3

HCl ~ Hydrochloride

HIF ~ Hypoxia-inducible factor

HR ~ Homologous recombination

HU ~ Hydroxyurea

γ-irradiation ~ Gamma-irradiation

kDa ~ Kilodalton

MET ~ Met proto-oncogene

MMP2 ~ Matrix metalloproteinase 2

MMR ~ Mismatch repair

Mus81 ~ Crossover junction endonuclease Mus81

Myt1 ~ Myelin transcription factor 1

NER ~ Nucleotide excision repair

NHEJ ~ Non-homologous end joining

p21 ~ Cyclin dependent kinase inhibitor 1

PBS ~ Phosphate buffered saline

PDGFB ~ Platelet-derived growth factor B

Phosphatidyl inositol 3-kinase-like kinases ~ PIKK

PI ~ Propidium iodide

PARP ~ Poly(ADP-ribose) polymerase

RNA ~ Ribonucleic acid

ROS ~ Reactive oxygen species

SD ~ Standard deviation

SDS ~ Sodium dodecyl sulphate

SE ~ Standard error

SER ~ Sensitivity enhancement ratio

Ser ~ Serine

siRNA ~ Silencing RNA

SNAI1 ~ Snail

SSB ~ Single-stranded break

ssDNA ~ Single-stranded DNA

TBS ~ Tris buffered saline

TBS-T ~ Tris buffered saline – tween

TERT ~ Telomerase

TGFα ~ Transforming growth factor α

Thr ~ Threonine

Tlk1 ~ Tousled-like kinase 1

Tyr ~ Tyrosine

UPR ~ Unfolded protein response

UTB ~ Urea-Tris-Bicine lysis buffer

VEGF ~ Vascular endothelial growth factor

VIM ~ Vimentin

Chapter 1. Introduction

1.1 The Hallmarks of Cancer

Cancer is a disease involving uncontrolled growth of abnormal cells that also have the ability to invade distant tissues. The progression of the disease is characterised by multiple genetic alterations which drive tumorigenesis; this includes both gain of function mutations in oncogenes and loss of function of tumour suppressor genes (Hanahan and Weinberg, 2000). Mathematical modelling was able to predict pathogenesis of a tumour requires 4 to 7 stochastic events in the majority of cancers since normal healthy cells have multiple defences to overcome tumorigenesis; this explains the relatively rare incidence of cancer (Renan, 1993). The idea of multistep progression in cancer has been built upon for many years; in 1976 Nowell outlined the clonal-evolution model which described how a tumour population is derived from a single normal cell (Nowell, 1976). The concept involves a normal cell acquiring a mutation providing it with a growth advantage thus allowing its proliferation over other cells and the beginning of a tumour cell population. Further along the cell lineage cells can acquire additional mutations due to genetic instability, often these will be detrimental and so cells harbouring them are deleted, but occasionally mutations may provide a survival advantage over other cells and these survive. The addition of further mutations and increased genetic and biological abnormalities gives tumour cells their pathogenic characteristics (Nowell, 1976). Since mutations are random events it is difficult to neatly class these changes based on cancer subtype and often they are patient-specific. However, work has been done to identify the main features of cancer known as the hallmarks of the disease (Hanahan and Weinberg, 2000).

The original hallmarks of cancer comprised of a list of six; ability to self-sustain growth signalling, ability to evade anti-growth signalling, resistance to apoptosis, limitless replicative potential, ability to induce angiogenesis, and activating tissue metastasis and invasion (Hanahan and Weinberg, 2000). More recently it has been suggested that two additional features should be added; reprogramming of metabolism and evasion of the immune system. In addition the role of the surrounding tissues and the tumour microenvironment must also be taken into account since these have an important effect on enabling tumour cell characteristics (Hanahan and

Weinberg, 2011). However, there is no set path to tumorigenesis and these hallmarks may occur in different order and involve different mutations. Furthermore, cancers may have mutations in redundant pathways causing issues for therapy; this can lead to selective pressure in the tumour cells if targeted therapy is used to inhibit only one pathway (Hanahan and Weinberg, 2011).

The hallmarks of cancer can all aid in adaptation to the tumour microenvironment. These surroundings are produced as a result of changes brought about by the tumour cells as well as their associated cells and tissues, for example fibroblasts, endothelial cells, pericytes, cancer stem cells and immune inflammatory cells (Hanahan and Weinberg, 2011). Overall the elements of the tumour microenvironment that effect cell survival are hypoxia, the consumption of glucose and nutrients, pH, lactate concentration, protein concentration and interstitial fluid pressure (Milosevic et al., 2004). One major alteration in tumour physiology affecting these factors is vasculature growth and development. In a tumour, established host vessels tend to disintegrate or are obstructed and are replaced with abnormal tumour vessels which have incorrect vessel wall development, irregular architecture, increased vascular permeability and form chaotic networks (Vaupel et al., 1989). The arrest in host vessel growth combined with tumour vessel abnormalities decreases the exchange of oxygen, nutrients, growth factors and waste products such as hydrogen ions, lactic acid and necrosis products which characterise the tumour microenvironment (Vaupel et al., 1989). Compounding these issues is the high interstitial fluid pressure found in tumours. Since capillaries are incompletely formed and hyperpermeable, plus there is a decreased function of lymphatic drainage in tumours, there is an increase in interstitial protein concentration changing the osmotic gradient in the capillary, and this leads to haemoconcentration and an increase in interstitial fluid pressure (Milosevic et al., 2004). A high interstitial fluid pressure has been associated with radioresistance and reduced response to chemotherapy (Milosevic et al., 2004).

As mentioned previously there is also a decrease in exchange of waste products in the tumour vasculature leading to tissue acidosis; the change in tumour metabolism contributes to this effect. In cancerous tissue there is an increase in glycolysis even if O_2 levels are sufficient for oxidative phosphorylation within the mitochondria. Warburg first described the increase in aerobic glycolysis in the 1950s, hence it is termed the Warburg effect; the increase can be from 10% in normal tissues to over 50% in tumours (Denko, 2008; Warburg, 1956). Glycolysis is the conversion of

glucose to pyruvate and ATP producing lactate as a waste product; although the process is less efficient for production of ATP it conserves oxygen for alternative uses. The transcription factor HIF-1α upregulates enzymes required for glycolysis and so aids in the tumours adaptation to low oxygen levels (Denko, 2008). The HIF family of transcription factors are activated in hypoxia, a characteristic of tumours due to poor vasculature, and this will be discussed in more detail. The net result of the increase in aerobic glycolysis is tissue acidosis and this has been shown to inhibit cell proliferation, reduce DNA synthesis, produce negative feedback on glycolysis, shift cells from G_1 to G_0 and enhance metastases (Vaupel et al., 1989). The pH in tumours is in the range of 5.85–7.68 compared to normal subcutis which is between 7.00-8.03 and this can further enhance hypoxia due to the stiffening of erythrocyte membranes decreasing oxygen exchange (Wike-Hooley et al., 1984). Low pH may also affect response to therapy since it can cause radiosensitivity in some cell lines although this is probably masked in some places by hypoxia causing radioresistance. Also hydrogen ion concentration can affect drug uptake with low pH increasing drug concentration in tissues and making chemotherapy more effective, however this effect is drug-dependent since pH may have different direct effects on certain agents (Wike-Hooley et al., 1984).

The component of the tumour microenvironment that will be focused on in this study is the induction of hypoxia. Hypoxia is the reduction in oxygen availability restricting the function of organs, tissues or cells (Hockel and Vaupel, 2001).

1.2 Hypoxia

Hypoxia occurs in the majority of tumours and is of significant clinical importance due to its association with poor prognosis, increased metastasis and resistance to therapy (Bristow and Hill, 2008; Hammond and Giaccia, 2004; Rofstad et al., 2000). These features identify hypoxia as a serious hurdle for treatment to overcome and provide a purpose for investigating hypoxic pathways in cancer. Data supporting this shows hypoxic tumours are more likely to develop recurrence locally and metastasise independent of initial treatment, even if this was surgery (Hockel et al., 1996). Hypoxia in tumours ranges from the normal physiological level for the organ they are located in to as low as 0.01% O_2; the severity, duration and final outcome of hypoxia

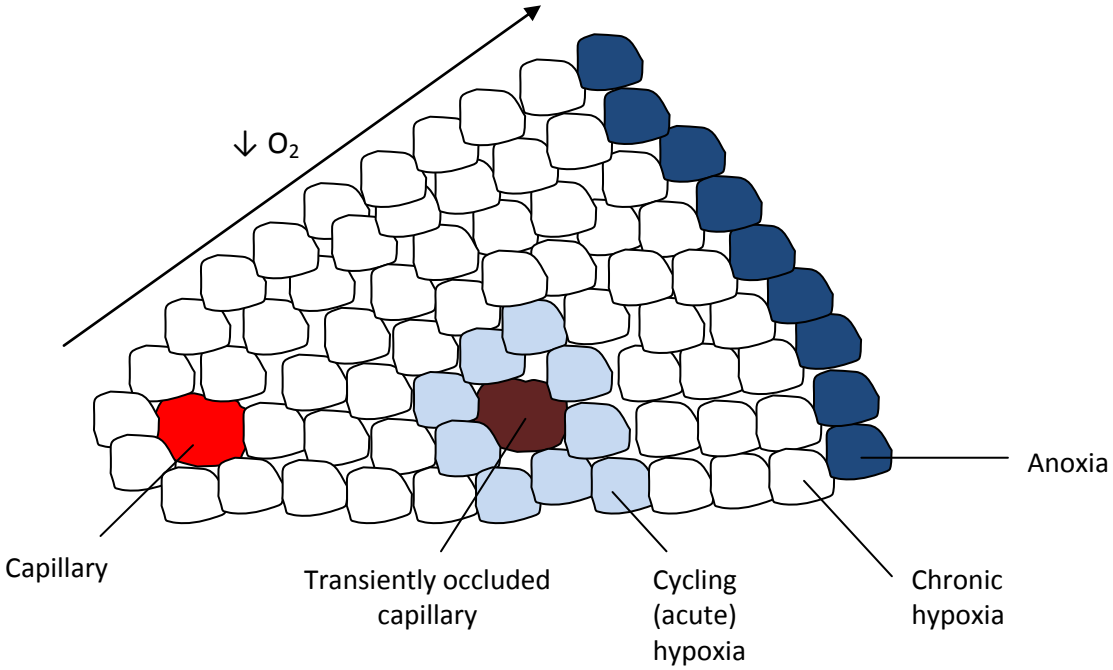


Figure 1.1. Modes of hypoxia. *A schematic representation of acute versus chronic hypoxia as discussed in the text.*

can all affect which pathways are activated and have differential effects on DNA repair and genetic stability (Bristow and Hill, 2008).

The two modes of hypoxia, acute and chronic, arise from different mechanisms and have different consequences (Figure 1.1). Chronic hypoxia was first to be identified and found to be due to cells being further than the critical distance for O_2 diffusion from the nearest blood vessel. The study showed tumours consisted of a necrotic, chronically hypoxic centre surrounded by a supportive layer of stroma, which never exceeds 180µl in width, this is consistent with the diffusion limit distance of O_2 (Cardenas-Navia et al., 2008). Nutrients diffuse to the centre from the stroma but O_2 was found to be the limiting factor which determines cell death (Thomlinson and Gray, 1955). However, this model was not thought a good representation of the tumour microenvironment *in vivo* since tumour oxygenation is highly unstable; one study showed that in 3 tumour types 30-50% of the image was fluctuating (Cardenas-Navia et al., 2008). Some studies have shown variation in oxygen tension within a time frame of 3 minutes, potentially due to spontaneous obstruction of blood flow, whereas others suggest longer cycling times of up to an hour, which are more likely to be a result of vascular remodelling (Bayer and Vaupel, 2012; Yasui et al., 2010). So an additional mode of hypoxia was suggested; acute, or intermittent, hypoxia arises from fluctuation in perfusion within the tumour vasculature and is also characterised by periods of reoxygenation (Brown, 1979; Cardenas-Navia et al., 2008). The size of

these fluctuations was found to be tumour type specific and larger with decreasing maturity of tumour vasculature or increasing tumour size. However, cycling and chronic hypoxia are not mutually exclusive since there is evidence of both within the same tumour samples, although cycling hypoxia appears only to be present in larger tumours (>500mg) (Bayer and Vaupel, 2012; Yasui et al., 2010).

Resistance to therapy is a major obstacle in cancer treatment and any information on how to prevent this is invaluable. Hypoxic cells are chemoresistant for a variety of reasons; mainly the poor tumour vasculature prevents efficient delivery and diffusion of drugs, but other factors include poor proliferation in hypoxic environments, inability of the drug to function in low oxygen and gene amplifications that may affect drug action (Shannon et al., 2003). Radioresistance is another issue; hypoxic tumour cells are 2-3 times more radioresistant than oxic cells, this is known as the oxygen enhancement ratio (OER) as illustrated in Figure 1.2 (Brown, 1979; Gray et al., 1953). Primarily this effect is due to the reliance of radiotherapy on the production of reactive oxygen species to produce DNA damage; radiation interacts with intracellular water producing these oxygen free radicals and so presence of O_2 increases DNA damage (Gray et al., 1953; Hockel and Vaupel, 2001).

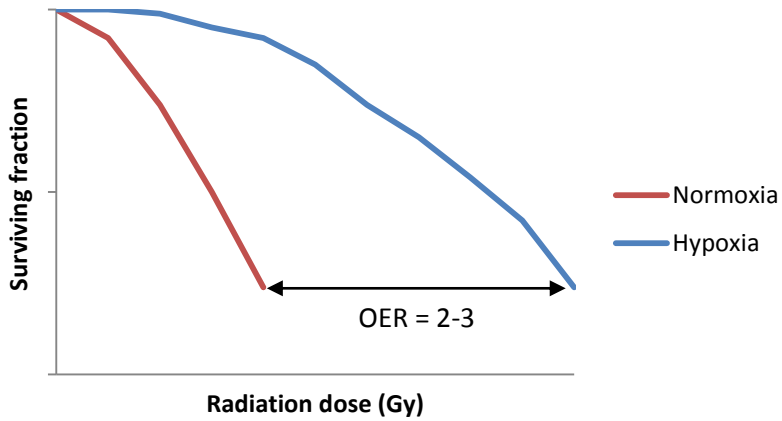


Figure 1.2. The oxygen enhancement ratio (OER) in severely hypoxic versus oxic cells. *Schematic illustration of OER. Normoxia represents 20% O_2. Hypoxia represents 0.1% O_2.*

Tumours adapt to the decrease in oxygen concentration within the tumour microenvironment either by increasing oxygenation, by increased angiogenesis, or decreasing oxygen and energy consumption. This is achieved by modulating their

expression of particular genes; predominantly through the activation of the HIF family of transcription factors. In hypoxia, HIF-1 acts to upregulate genes to aid in cancer cell survival including angiogenesis (e.g. VEGF, PDGFB), metabolic reprogramming (e.g. GLUT1, GAPDH), growth factor signalling (e.g. TGFα, ADM), invasion and metastasis (e.g. MMP2, AMF, MET), epithelial-mesenchymal transition (e.g. SNAI1, VIM) and stem cell maintenance (e.g. TERT, NANOG, OCT4) (Semenza, 2012). HIF-1 has also been found to be upregulated after radiotherapy, even though this tends to cause reoxygenation, suggesting it plays a role in radioresistance (Dewhirst et al., 2007). This upregulation is partly due to the formation of mRNA-protein complexes in hypoxia, which are disaggregated upon reoxygenation to release HIF-1 regulated proteins, but also partially due to HIF-1 stabilisation in the presence of free radical species produced by radiotherapy (Dewhirst et al., 2007). In chronic hypoxia it seems HIF-2α is active whilst HIF-1α is destabilised, this means cells are more likely to undergo apoptosis but changes in gene expression as described above can also occur (Bayer and Vaupel, 2012). Overall, alterations in protein expression are a key factor in driving a more aggressive phenotype and can contribute to chemo- and radioresistance.

In chronic hypoxia translation may be inhibited potentially as a means of conserving energy when the cells are under stress. This is either a rapid response via the unfolded protein response (UPR) or a delayed response in chronic hypoxia involving inhibition of transcript recruitment by eIF4F (Koritzinsky et al., 2006). The UPR aids hypoxic cell survival by inhibiting translation. A component of the UPR, PERK, causes an inhibitory phosphorylation of eIF2α (eukaryotic initiation factor 2α) which prevents the exchange of GDP for GTP on eIF2 thus inhibiting translation (Koumenis and Wouters, 2006). As well as decreasing ATP usage, the UPR can also increase genes for protein folding and redox homeostasis to further promote cell survival (Koumenis and Wouters, 2006). So regulation of translation in hypoxia promotes energy homeostasis for cell survival but can also increase hypoxia-induced proteins depending on the severity and duration of hypoxia (Koumenis and Wouters, 2006).

Another method for adaptation to hypoxia is activation of the DNA damage response (DDR) in hypoxic tumour cells. Potentially the reliance on the DDR could be a useful therapeutic target for successfully decreasing hypoxic tumour cells.

1.3 The DNA Damage Response in Hypoxia

The DDR is activated in order to maintain genomic integrity via activation of cell cycle checkpoints, DNA repair pathways and in severe cases apoptosis (Zhou and Elledge, 2000). In this way cells have time to repair DNA before re-entering the cell cycle or if the extent of damage is too great cells undergo apoptosis (Jalal et al., 2011). The hypoxia-induced DDR also aims to preserve genomic integrity by protecting stalled replication forks. However, the DDR in hypoxia is unique in a number of ways; there is no detectable DNA damage present and DNA repair is repressed (Hammond et al., 2004; Olcina et al., 2010).

The response to DNA breaks is mediated by three phosphatidyl inositol 3-kinase-like kinases (PIKKs); ATM, ATM-and Rad3-related kinase (ATR) and DNA dependent protein kinase catalytic subunit (DNA-PKcs) (Jackson and Bartek, 2009; Stiff et al., 2006; Toledo et al., 2011; Tse et al., 2007). ATM and DNA-PKcs typically respond to double stranded breaks (DSBs) whereas ATR is activated in the presence of single stranded breaks (SSBs) caused, for example, by stalled DNA replication. ATM and ATR are able to sense DSBs or SSBs respectively, but optimal activation occurs in the presence of other activators; Mre11-Nbs1-Rad50 (MRN complex) for ATM and the Rad17 clamp and Rad9-Rad1-Hus1 (9-1-1 complex) for ATR (Jalal et al., 2011; Tse et al., 2007). The signal from the PIKKs is then amplified by mediator proteins and effector molecules act to induce DNA repair, checkpoint activation or apoptosis (Olcina et al., 2010).

ATM is believed to be autophosphorylated at serine (ser) 1981 in response to chromatin remodelling initiated by ionising radiation, and presumably other agents producing DSBs; this is necessary for conversion from the inactive dimer form to the active monomer form of ATM (Bakkenist and Kastan, 2003). There are reports which contradict this being the mechanism of ATM activation; it was found that a mouse model with a mutation at the autophosphorylation site was still able to activate ATM (Pellegrini et al., 2006). So although this autophosphorylation may not be the initial signal in the ATM pathway or essential for ATM activity it may still play a role in activation at a later stage. ATM then acts to phosphorylate histone H2AX at ser139 (γH2AX) on chromatin flanking DSBs (Jackson and Bartek, 2009). γH2AX acts to maintain mediator proteins including 53BP1, MDC1 and BRCA1 that amplify the ATM signal at the site of damage to promote repair. Another ATM target is Chk2;

ATM-mediated Chk2 activation via phosphorylation at threonine (thr) 68 promotes p53 activation and inhibits Cdc25A and Cdc25C (Kumareswaran et al., 2012; Olcina et al., 2010; Tse et al., 2007). This acts to activate DNA damage checkpoints and arrest the cell cycle to allow time for DNA repair (Jackson, 2002).

ATR is recruited by an alternative mechanism. ATR-interacting protein (ATRIP) complexes with ATR and is attracted to single-stranded DNA (ssDNA) coated in recombinant protein A (RPA) (Stiff et al., 2006). γH2AX is also activated by ATR and, similar to the case of ATM, remains at the site of damage to augment the kinase signalling to downstream targets (Dai and Grant, 2010). A principle target of ATR is Chk1, which is phosphorylated at ser317 and ser345 for its activation, and the mediator proteins Claspin and TopBP1 strengthen this activation. (Hammond et al., 2003; Tse et al., 2007). Chk1 has overlapping functions with Chk2 but can also phosphorylate and consequently inhibit tousled-like kinase 1 (Tlk1) involved in chromatin remodelling. Curiously, one report has shown that ATR activity is not required for Tlk1 inhibition but it is ATM acting upstream of Chk1 in this case (Krause et al., 2003). This provides evidence for the notion that ATR and ATM have overlapping functions and may not be as separated in their pathways as previously thought. Further support for this comes from Jazayeri *et al.* (2006) who demonstrated ATM could act upstream of ATR by promoting nuclease activity of the MRN complex. This allows the processing of DSBs to initiate RPA coating ssDNA thus recruitment of ATR, although this is restricted to S and G_2 phases of the cell cycle (Jazayeri et al., 2006). Another effector molecule potentially targeted by Chk1 is Wee1; in *Xenopus* Wee1 is phosphorylated at ser549 by Chk1 although this is yet to be demonstrated in mammalian cells (Lee et al., 2001). These pathways are summarised in Figure 1.3 and 1.5.

The DDR in hypoxia is activated by replicative stress and involves ATR and ATM. This response is initiated by stalling of replication forks correlating with a depletion of nucleotides within the cell, it has been suggested that the O_2-dependency of ribonucleotide reductase results in the decline in nucleotides in hypoxia although this was not tested experimentally (Pires et al., 2010b; Probst et al., 1989). The block to replication affects both the initiation and elongation stages of replication and leads to stalling of replication forks and the failure of origin firing (Pires et al., 2010a). Upon reoxygenation the enzymes critical for DNA synthesis, such as ribonucleotide reductase, are hypothesised to be catalytically active therefore acutely hypoxic cells

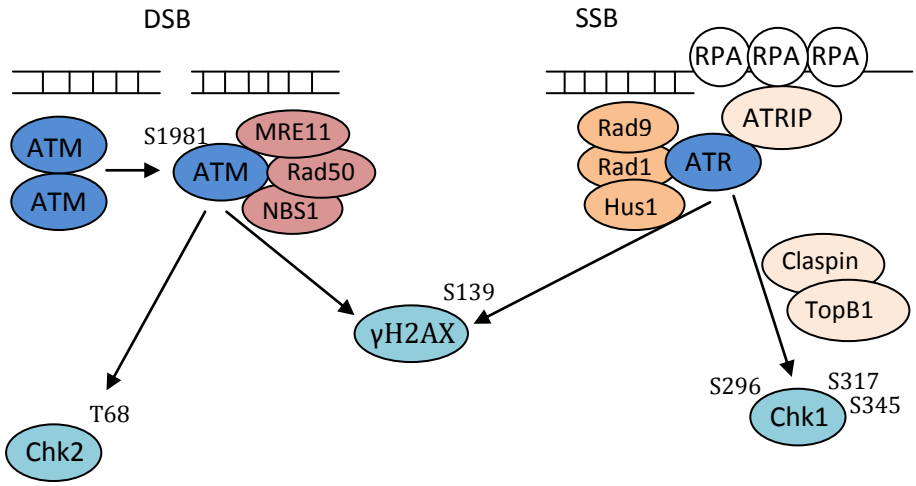


Figure 1.3. The DDR. *Schematic illustration of the signalling pathways involved in the DDR. Phosphorylation sites indicated adjacent to molecules.*

may undergo replication restart however, chronically hypoxic cells undergo disassembly of the replisome and are unable to utilise nucleotides even if they are able to be synthesised (Pires et al., 2010b; Probst et al., 1989). However, it has never been investigated whether nucleotide levels return to normal in reoxygenation after chronic hypoxia. Replisome disassembly is likely to be the main reason for replication restart being unable to occur in these conditions (Pires et al., 2010b). Stalled replication forks induced by hypoxia contain regions of ssDNA which may become coated with RPA to activate ATR and subsequently Chk1 (Hammond and Giaccia, 2004; Olcina et al., 2010; Toledo et al., 2011). In hypoxia fork collapse producing DSBs has only been described in conditions where ATR or Chk1 are limiting since these seem to play a role in protecting stalled replication fork integrity (Hammond and Giaccia, 2004). Persistent stalled forks may also collapse in normoxia; this is a controlled event regulated by the nuclease Mus81 so is not associated with pathogenicity, however the role of Mus81 in hypoxic conditions is yet to be elucidated (Hanada et al., 2007; Toledo et al., 2011). Following the production of these DSBs ATM and ATR are activated in the DDR as expected (Toledo et al., 2011).

ATM also plays a role in hypoxia and is phosphorylated in the absence of damage and independently of the MRN complex (Bencokova et al., 2009). There have been reports of ATR acting upstream of ATM phosphorylation at ser1981 in normoxic conditions, and potentially a similar mechanism may be occurring in hypoxia. This was demonstrated after UV exposure or replication fork stalling and was discovered to be 53-BP1-independent, γH2AX-independent and ATM-independent but ATR-

dependent, which is a surprising result since this is an autophosphorylation site on ATM (Stiff et al., 2006). However, another proposed mechanism of ATM activation in hypoxia is due to chromatin modifications and the activity of MDC1. Under hypoxic conditions MRN does not play a role and ATM forms diffuse nuclear foci unlike in the presence of damage where foci are distinct. It is proposed that ATR-phosphorylated γH2AX recruits MDC1 to stalled replication forks where it acts as a mediator for ATM signalling (Bencokova et al., 2009). ATM phosphorylates Chk2 in hypoxic conditions to cause a G_2 arrest after reoxygenation. This arrest occurs due to Chk2-mediated phosphorylation of Cdc25C to allow more time for DNA repair proteins to be expressed. ATM plays the same role in reoxygenation, although in these conditions DNA damage is detected due to ROS formation (Freiberg et al., 2006; Hammond et al., 2003).

1.4 Repair in Hypoxia

As mentioned previously hypoxia has the surprising characteristic of repressing DNA repair pathways and so can contribute to the major enabling hallmark for tumorigenesis; genomic instability (Bayer and Vaupel, 2012; Bristow and Hill, 2008; Kumareswaran et al., 2012; Rofstad et al., 2000). Hypoxia can have effects on gene expression to drive tumorigenesis and metastasis to increase genomic instability. Such effects on the genome are also illustrated by studies showing an increase in mutation rate under severe acute hypoxia; this increased further with cumulative number of exposures to transient hypoxia (Cairns and Hill, 2004; Reynolds et al., 1996). The mutation rate was increased 3.4 fold when cells were exposed to 1mmHg for 4 hours in the study from Reynolds and colleagues (Reynolds et al., 1996).

Another mechanism by which hypoxia increases genomic instability is the inhibition of certain DNA repair pathways (Bindra et al., 2005; Chan et al., 2009; Meng et al., 2005; Yuan et al., 2000). DSBs are highly lethal lesions repaired via homologous recombination (HR) or non-homologous end joining (NHEJ) (Hartlerode and Scully, 2009; Jackson, 2002). However, both genes involved in HR (RAD51, RAD52, RAD54, BRCA1 and BRCA2) and NHEJ (Ku70, DNA-PKcs, DNA ligase IV and Xrcc4) are downregulated in chronic hypoxia; this is independent of HIF or cell cycle distribution for HR genes (Kumareswaran et al., 2012; Meng et al., 2005). However, there is some disparity amongst reports; the reduction in gene expression

does not always correlate with decreased protein expression, for example Ku70 (Meng et al., 2005). Other studies found HR proteins downregulated but not at the transcript level, suggesting alterations in translation, with no change in NHEJ repair (Chan et al., 2008).

There is also inhibition of the mismatch repair (MMR) pathway via repression at the promoters for MLH1 and MSH2; these genes normally ensure correction of DNA base mismatches (Bindra and Glazer, 2007). There is a HIF-dependent mechanism whereby HIF displaces MYC from the gene promoter of MLH1 and MSH2; MYC plays a role in maintaining genetic stability so reduction in its binding and expression seen in hypoxia has further effects on decreasing genetic stability (Koshiji et al., 2005). Repression of nucleotide excision repair (NER) has also been associated with hypoxia; this pathway repairs lesions distorting the DNA double helix. Two proteins involved in the pathway, XPC and XPD, are transcriptionally regulated by HIF-1α whereas RAD23B is decreased by miR-373 activated by HIF-1α (Chan and Bristow, 2010).

Abnormal or reduced repair can lead to chromosomal aberrations and genomic instability if cells continue to divide, which may be the case when tumour cells have adapted to low oxygen concentrations (Bristow and Hill, 2008). One study showed cells irradiated and maintained under continual hypoxic conditions had an increase in chromosome and chromatid breaks, fragmented DNA, telomeric fusions and reciprocal translocations. If these aberrations occurred in oncogenes or tumour suppressor genes, cells could potentially gain a mutator phenotype leading to malignant disease (Kumareswaran et al., 2012). This fits with hypoxia driving aggressive forms of cancer. It also suggests a reliance on alternative pathways to maintain genomic integrity for hypoxic cell survival since certain forms of repair are inhibited.

The repression of DNA repair in hypoxia provides another possible therapeutic target for depleting hypoxic cells using context synthetic lethality. This term describes the lethal interaction between loss of pathway A through therapeutic intervention and loss of pathway B through cellular context, for example hypoxia, whereas loss of either pathway alone is compatible with cell viability (Chan and Bristow, 2010; Chan et al., 2010; Olcina et al., 2010). The main success in this area is the use of poly(ADP-ribose) polymerase (PARP) inhibitors which cells with defects in HR are particularly sensitive to (Chan et al., 2010; Hegan et al., 2010; Liu et al., 2008;

McCabe et al., 2006). PARP is involved in SSB repair however, if its activity is inhibited these breaks can cause replication fork collapse when the cell undergoes replication and HR is required for resolution of these forks (Bryant et al., 2005; Lord and Ashworth, 2008; McCabe et al., 2006). This explains their effective application in $BRCA^{-/-}$ breast cancers and since hypoxic cells have repressed BRCA and RAD51 signalling PARP inhibitors are also of use in this context (Chan et al., 2010). PARP inhibitors in combination with radiotherapy provide more encouraging results since the inhibitor ABT-888 was able to radiosensitise hypoxic cells (Liu et al., 2008). Another synthetic lethal interaction which could be exploited in hypoxia is inhibition of DNA polymerase β or DNA polymerase γ in cells defective in the MMR proteins MSH2 or MLH1 respectively (Martin et al., 2010). This interaction causes an increase in oxidized DNA base damage that if unrepaired can lead to lethal lesions such as DSBs however, no clinically useful inhibitors are yet available (Martin et al., 2010). The potential for using synthetic lethality to target hypoxic cells deficient in certain DNA repair pathways is shown in Figure 1.4.

Despite the encouraging studies mentioned here in targeting hypoxic cells, there is still a significant need to find drugs that can decrease hypoxic cell survival and hopefully improve patient prognosis. An intriguing new approach is inhibiting part of the DDR, such as ATR, Chk1 or targets further downstream to improve selectivity.

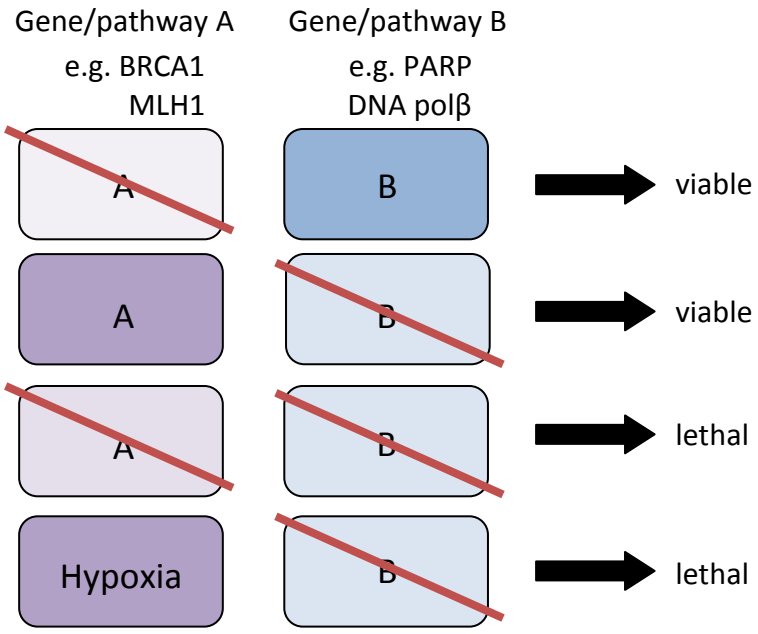


Figure 1.4. The concept of contextual synthetic lethality. *Diagram to illustrate synthetic lethality. Potential combinations of pathways which could be targeted by this approach are shown above.*

1.5 Targets of Chk1 in Hypoxia

Chk1 is an essential serine/threonine kinase activated by ATR by phosphorylation at ser317 and 345 (Tse et al., 2007). Chk1 is also able to undergo autophosphorylation at ser296 and this has been detected in normoxic (Clarke and Clarke, 2005) and hypoxic conditions (Hammond, unpublished data). Chk1 plays a role in cell cycle checkpoint activation and maintaining replication fork stability. Downstream targets of Chk1 are mainly involved in S phase and G_2/M phase arrest. These include Cdc25A and Cdc25C, which are inhibited thus causing an S or G_2 arrest respectively. Cdc25A is phosphorylated at ser76 and 124 leading to its ubiquitination and Cdc25C is phosphorylated at ser216 leading to proteasomal degradation and cytoplasmic sequestration by binding 14-3-3 proteins (Jackson, 2002). It has also been found that autophosphorylation of Chk1 at ser296 promotes binding to 14-3-3γ which facilitates the Chk1-mediated degradation of Cdc25A (Kasahara et al., 2010). Chk1-mediated phosphorylation of p53 at ser20 is also involved in G_1 and G_2 cell cycle arrest or apoptosis; p53 has reduced binding to Mdm2 and increased binding to its coactivators/corepressors and promoters of target genes (Jalal et al., 2011). Other targets of Chk1 are involved in DNA repair (Rad51 for homologous repair, FANCE for Fanconi Anaemia pathway), mitotic spindle checkpoint (Aurora B, Plk1), transcription (histone H3) and chromatin remodelling (Tlk1) (Jackson, 2002). As mentioned previously there have also been reports in *Xenopus* and yeast of Wee1 phosphorylation and activation by Chk1 to induce a G_2 arrest (Lee et al., 2001; O'Connell et al., 1997). These targets are illustrated in Figure 1.5.

However targets of Chk1 in hypoxic conditions have been shown to vary. For example downregulation of Cdc25A protein and mRNA is not Chk1-dependent in hypoxia but is mediated by p21 and miR-21 (de Oliveira et al., 2009). Also other Cdc25 family members are not phosphorylated by Chk1 in hypoxic conditions (de Oliveira et al., 2009). Chk1 targets that have been confirmed in hypoxia include Tlk1 and p53 (Hammond et al., 2002; Lee et al., 2012; Pires et al., 2010a). Chk1 phosphorylates Tlk1 at ser695 causing its deactivation. Tlk1 is normally involved in chromatin formation and is most active during S phase. It is proposed that Tlk1 inhibition may contribute to the S phase arrest seen in the DDR, but this is not seen experimentally since the effect of nucleotide depletion masks this result (Pires et al., 2010a). The activation of p53 in hypoxia has been found to be ATR-dependent

however the role of Chk1 was previously unclear (Hammond et al., 2002). It has now been elucidated that Chk1 activates p53 via phosphorylation and inhibition of MDMX at ser367, leading to its interaction with 14-3-3γ. This removes the inhibitory effect caused by MDMX on p53 and promotes p53 stability (Lee et al., 2012). The activation of Wee1 in mammalian cells under hypoxia has not been investigated previously although from existing data from yeast and *Xenopus* studies in normoxia it can be hypothesised that Wee1 is a downstream target of Chk1 (Lee et al., 2001; O'Connell et al., 1997).

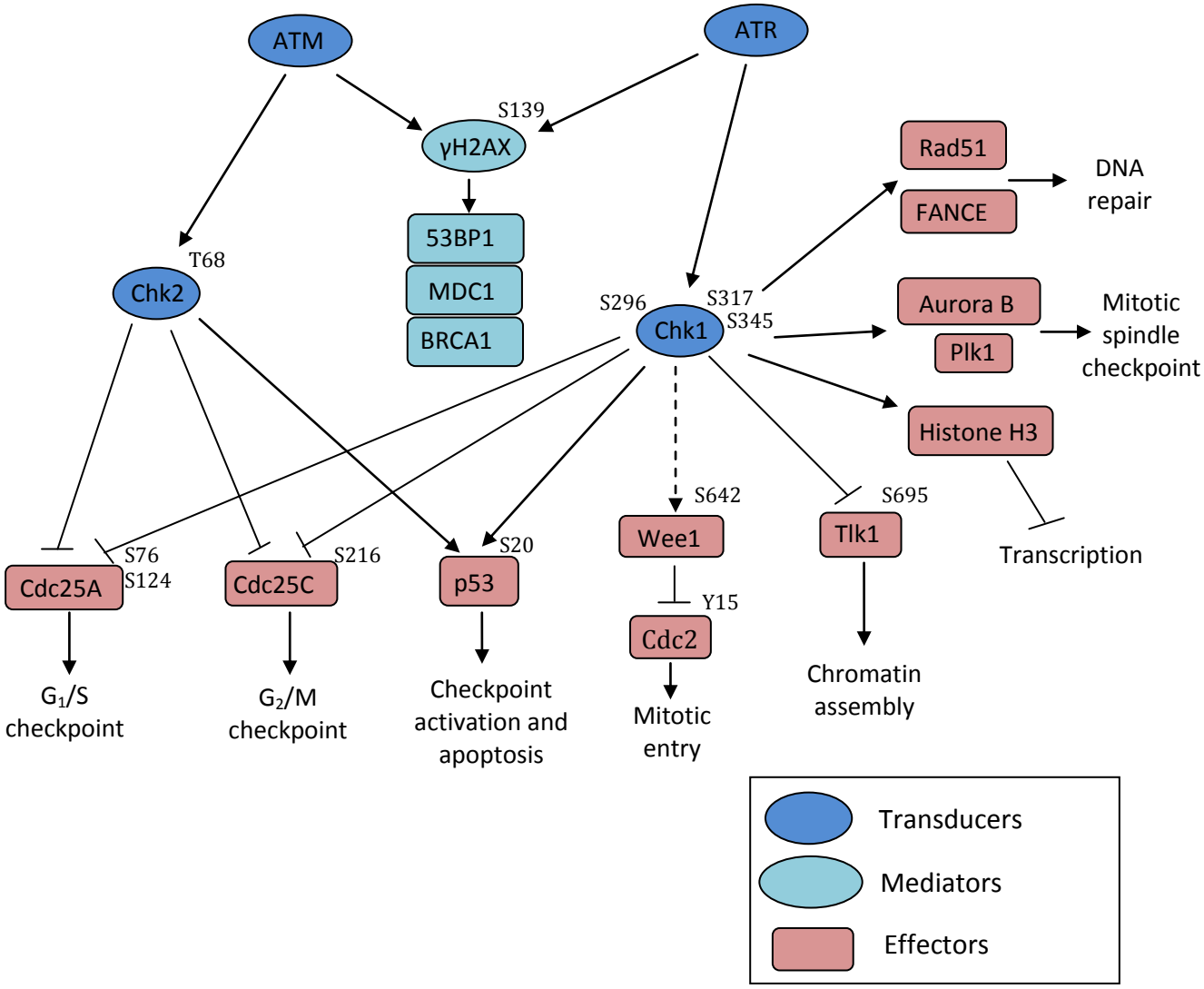


Figure 1.5. Targets of Chk1 in hypoxia. *Diagram of signalling pathways involving checkpoint proteins. Phosphorylation sites are indicated. For Cdc25A, Cdc25C and p53 sites are those phosphorylated by Chk1.Function of specific molecules shown in the key.*

1.6 Targeting the Hypoxia-Induced DDR Therapeutically

Since the DDR is activated in hypoxic cells it provides a potential therapeutic target. Studies investigating this have found hypoxic cells are indeed sensitive to loss or inhibition of DDR components such as ATR and Chk1 but since these are essential genes only partial depletion is desired (Hammond et al., 2004; Hammond et al., 2006; Olcina et al., 2010; Pires et al., 2012). Checkpoint inhibitors have been successful in cancer cells, particularly in combination with DNA damaging chemotherapeutics, since cells often have defects in checkpoint pathways thus rely heavily on any remaining pathways to overcome DNA damage (Garrett and Collins, 2011). For example ATR and Chk1 inhibitors are especially effective in p53 null cells since these cells suffer loss of the G_1 checkpoint and have increased reliance on the G_2 checkpoint, which is abrogated by these inhibitors (Garrett and Collins, 2011). Another theory to explain the efficacy of these agents in p53-deficient cells is that checkpoint inhibition induces S phase damage which, when combined with loss of the G_1 checkpoint permitting less restrictive S phase entry, leads to increased replicative stress and cell death (Toledo et al., 2011). These agents also appear to have significant effects in hypoxic cells that are usually resistant to therapy. The selective ATR inhibitor VE-821 was found to decrease hypoxic tumour cell viability due to decrease in ATR-mediated signalling and radiosensitised both normoxic and hypoxic cells (Pires et al., 2012). This is thought to be due to the loss of maintenance of replication fork integrity, which ATR usually provides, and the inability of hypoxic cells to repair replication-associated DNA damage caused by ATR inhibition, since DNA repair pathways are repressed in hypoxia (Pires et al., 2012). In accordance with this, the Chk1 inhibitor CEP-3891 was found to decrease hypoxic cell viability to a greater extent than normoxic cells, again due to loss of replication fork integrity and genomic stability with the loss of Chk1 (Hammond et al., 2006). Some caution is needed concerning timing of the use of these drugs; if Chk1 is inhibited during reoxygenation of severely hypoxic cells with their replisome still intact, they can restart replication and bypass the checkpoint for detecting damage and potentially, escape apoptosis thus contributing to genomic instability (Pires et al., 2010a). The ability to target hypoxic cells could potentially improve patient prognosis since this population are responsible for poor response to therapy and are highly metastatic; this is an essential area for further research. The success of hypoxic cell targeting will likely involve use of

agents to improve the tumour vasculature and delivery of drugs to the tissues where they are needed (Olcina et al., 2010).

1.7 Wee1 and its Role in the DDR

Cell cycle checkpoints are a major outcome of the DDR and allow adequate time for DNA repair. In conditions permissive for cell proliferation Cdc25C activates Cdc2 (also known as Cdk1) via dephosphorylation; this allows Cdc2 to complex with Cyclin B to promote mitotic entry. Upon DNA damage, in G_2 in mammalian cells a checkpoint response is activated which involves inhibitory phosphorylation of Cdc2 both by Wee1 (at tyrosine (tyr) 15) and Myt1 (at tyr15 and thr14) as illustrated in Figure 1.6 (Domingo-Sananes et al., 2011; O'Connell et al., 1997; Watanabe et al., 2005).

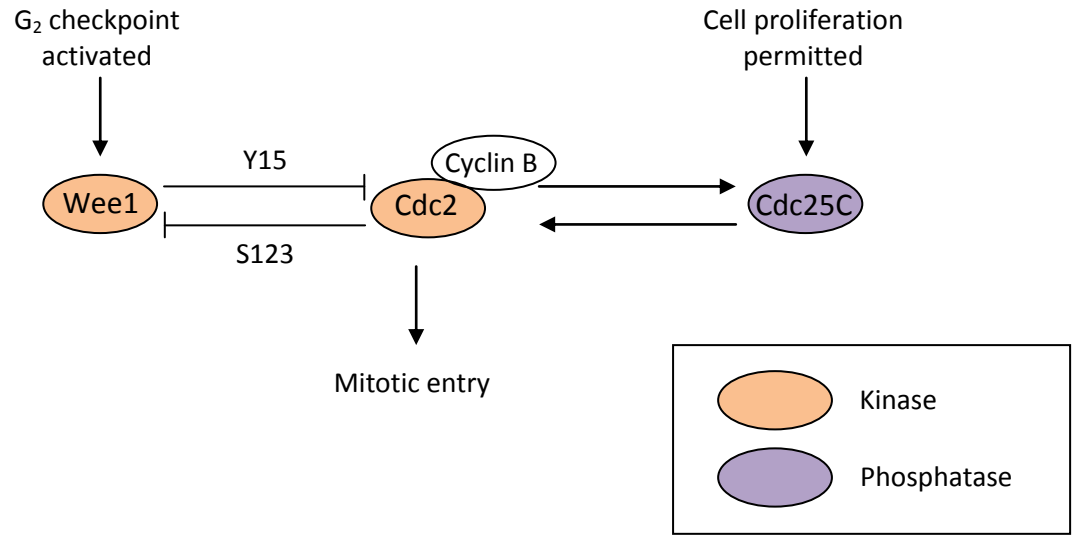


Figure 1.6. Activity of Wee1 at the G_2 checkpoint. *Diagram to illustrate the role of Wee1and Cdc25C in regulating Cdc2. Phosphorylation sites indicated. Function of specific molecules shown in the key.*

Phosphorylation of Cdc2 by Wee1 also occurs in interphase to regulate entry into mitosis (Domingo-Sananes et al., 2011). However, some Cdc2/Cyclin B complexes will remain unphosphorylated and as cyclin levels rise more of these become active. Eventually Cdc2 activity will reach a level where it is able to downregulate Wee1 via phosphorylation at ser123 and cause an activating phosphorylation on Cdc25C, so it inhibits its inhibitor and activates its activator, thus causing exit from G_2 and entry

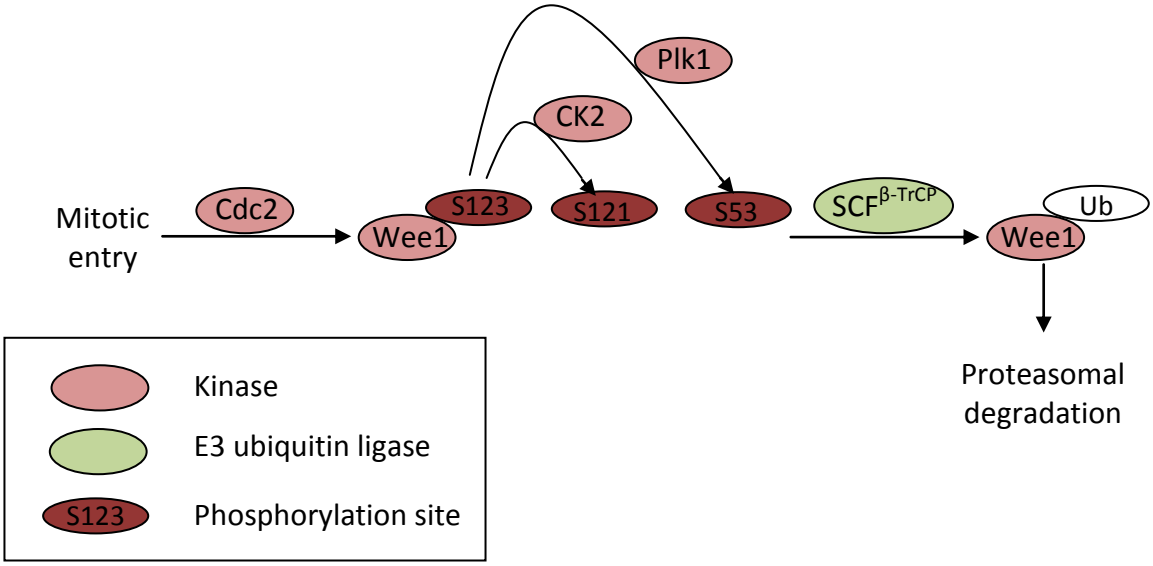


Figure 1.7. Wee1 degradation. *Diagram to illustrate sequential phosphorylation of Wee1 and its targeting for degradation. Function of molecules shown in key.*

into mitosis. Cyclin B then undergoes proteasomal degradation causing Cdc2 activity to decrease and the cycle starts again (Domingo-Sananes et al., 2011). Phosphorylation of Wee1 by Cdc2 initiates degradation by the proteasome after ubiquitination by E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$. The initial phosphorylation at ser123 of Wee1 primes phosphorylation by polo-like kinase 1 (Plk1) at ser53 and casein kinase 2 (CK2) at ser121 which creates phosphodegrons on Wee1 for β-TrCP recognition (Figure 1.7) (Watanabe et al., 2005). This regulation ensures Wee1 activity is highest in S and G_2 phases and inactivated in mitosis (Wang et al., 2000).

The upstream signals acting on Wee1 have so far only been elucidated in yeast and *Xenopus*. Wee1 was first identified in *Schizosaccharomyces pombe* when Paul Nurse showed mutant cells obtained a reduced cell size and advanced into mitosis (Nurse, 1975). Much later it was shown that Chk1 directly phosphorylates yeast Wee1 *in vitro* in order for its activation (O'Connell et al., 1997). In *Xenopus* egg extracts Chk1 was found to phosphorylate Wee1 at ser549 *in vitro* which allowed Wee1 to bind 14-3-3 proteins leading to increased Wee1 kinase activity against Cdc2 and intranuclear distribution (Figure 1.8) (Lee et al., 2001). A mutant of Wee1 with serine exchanged for alanine at position 549 was much less effective at delaying entry into mitosis further supporting this phosphorylation site in Wee1 activation. Also interaction with 14-3-3 is highest during interphase and is reduced during mitosis as expected considering Wee1's function in cell cycle regulation (Lee et al., 2001). Interaction

with 14-3-3 proteins has also been seen in human cells although the 14-3-3 subtype involved is disputed; Wang *et al.* identified 14-3-3β binding in increasing Wee1 stability and activity *in vitro* and *in vivo* whereas Rothblum-Oviatt *et al.* identified 14-3-3σ (Rothblum-Oviatt et al., 2001; Wang et al., 2000). In the study led by Wang they suggest Wee1 association with 14-3-3β prolongs Wee1 protein half-life thus has effects on protein levels and kinase activity, however findings from Rothlbum-Oviatt's study show 14-3-3 binding also causes an accumulation of a 14-3-3 binding mutant form of Wee1 suggesting this association has an indirect effect on Wee1 accumulation (Rothblum-Oviatt et al., 2001; Wang et al., 2000). Possibly Wee1 is able to bind to multiple isoforms of 14-3-3 to modulate its stability and function. Interestingly depletion of Chk1 from egg extracts did not abolish Wee1 binding to 14-3-3 suggesting Wee1 is also targeted by other kinases (Lee et al., 2001). It appears 14-3-3 has a significant role in regulating the G_2-M transition since it is able to promote degradation of Cdc25C and activation of Wee1; potentially both these activities are downstream of Chk1 (Jackson, 2002; Lee et al., 2001).

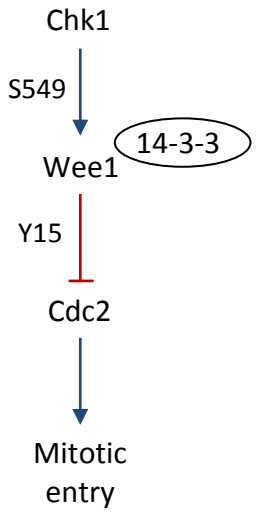


Figure 1.8. Wee1 activation in Xenopus. *Diagram to show activation of Wee1 by Chk1 promoting 14-3-3 binding. Phosphorylation sites indicated. Blue arrows represent activation, red lines represent inhibition.*

The kinase responsible for the activating phosphorylation on Wee1 in mammalian cells remains unclear. Evidence of Chk1-mediated activation in yeast and *Xenopus* has been extended to mammals however some studies suggest this may not be the case. UCN-01 is a protein kinase inhibitor that affects Chk1 activity; treatment of cells with UCN-01 was unsuccessful in affecting 14-3-3 binding to Wee1 suggesting

interactions between 14-3-3 and Wee1 are mediated by other kinases in addition to Chk1 or excluding Chk1 (Rothblum-Oviatt et al., 2001). However, it was also shown that binding to 14-3-3 proteins required prior phosphorylation of Wee1 at ser642 which is the analogous site in human Wee1 to ser549 in *Xenopus*; so this is potential evidence for Chk1 playing a role in human cells (Perry and Kornbluth, 2007). In addition another study illustrated UCN-01 inactivated murine Wee1, just prior to the activation of Cdc2 and mitotic entry, via upstream actions since purified Wee1 in vitro was resistant to UCN-01; this implies Chk1 is able to activate Wee1 (Yu et al., 1998). Phosphorylation at ser642 is also involved in negative regulation of Wee1. Akt was demonstrated to bind directly and phosphorylate Wee1 at ser642; this promoted binding of Wee1 to 14-3-3θ (but not 14-3-3β or 14-3-3σ), which in turn caused accumulation of Wee1 in the cytoplasm and encouraged cell cycle progression since Wee1 needs to be localised in the nucleus to cause cell cycle arrest (Katayama et al., 2005). Autophosphorylation of Wee1 has also been reported at tyr295 and tyr362 in humans and tyr90, tyr193 and tyr362 in *Xenopus*; in *Xenopus* this activation did not affect ability to phosphorylate Cdc2 but it did positively regulate the ability to inhibit mitotic entry (Katayama et al., 2005; Perry and Kornbluth, 2007). In light of these conflicting reports, one of the aims of this study is to investigate the affect of Chk1 signalling on Wee1 activation.

1.8 Therapeutic Potential of Targeting Wee1

As discussed above, inhibition of the hypoxia-induced DDR has already had some success with ATR and Chk1 inhibitors. However, targeting this signalling further downstream will hopefully provide a higher level of specificity therefore fewer side effects and this is part of the rationale for inhibiting Wee1 (Kawabe, 2004). Tumour cells frequently have a defective G_1 checkpoint (often due to p53 or Rb mutation) so rely heavily on the G_2 arrest to give time to repair any damaged DNA before mitosis. Wee1 inhibition abrogates this G_2 arrest and causes premature mitotic entry; when used in combination with chemotherapy or irradiation the damage the cells acquire remains unrepaired leading to mitotic catastrophe (De Witt Hamer et al., 2011). However, since non-transformed healthy cells have all their checkpoints fully functional in most cases they are able to repair any damage induced by these agents therefore do not suffer toxicity (De Witt Hamer et al., 2011). Wee1 also has potential

as a target in cancer therapy since there is evidence of its overexpression in hepatocellular carcinoma, breast cancer, osteosarcoma and glioblastoma suggesting its inhibition will have a greater effect on cancerous as opposed to healthy normal tissue (De Witt Hamer et al., 2011; Mir et al., 2010; Murrow et al., 2010; PosthumaDeBoer et al., 2011).

The current Wee1 inhibitors available are based on pyrimidine and pyrrolo-carbazole derivatives; three of these are used in this study so will be analysed in more detail but a fourth compound to mention is PD0407824, which has activity against both Wee1 and Chk1 (Palmer et al., 2006). PD0166285 is a non-selective Wee1 inhibitor which at higher concentrations also has effects on Src nonreceptor tyrosine kinase (c-Src), fibroblast growth factor receptor-1 (FGFR1), epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor beta subunit (PDGFRβ) (Panek et al., 1997). However, due to the relatively low specificity and toxicity associated with the compound it has not been investigated clinically (Bridges et al., 2011). Wee1 inhibitor II is another potent Wee1 inhibitor however the only inhibitor to have entered clinical trials is MK1775 which is the most selective inhibitor available (see Table 1.1) (De Witt Hamer et al., 2011). The most effective use of these agents has been in combination with either chemotherapy or radiotherapy so that cells not only enter mitosis prematurely, but also enter with DNA damage to cause apoptosis; effects have been seen both *in vitro* and *in vivo* as discussed below (De Witt Hamer et al., 2011).

1.8.1. Wee1 inhibitors combined with chemotherapy

The use of Wee1 inhibitors combined with chemotherapy *in vitro* has been studied in many cell lines all of which have shown promising results. Firstly, MK1775 used with Gemcitabine, Carboplatin or Cisplatin decreased the growth of human colorectal cells (WiDr), and the increase in cell death was shown to correlate with an increase in premature mitotic entry, whereas MK1775 alone had minimal antiproliferative effects as illustrated by colony survivals and caspase activation (Hirai et al., 2009). This study also found anti-tumour effects only in p53-deficient cell lines (including H1299 and MIA-PaCa-2) and this was explored further using an isogenic matched pair of TOV21G (ovarian adenocarcinoma) cells. MK1775 inhibited Cdc2 phosphorylation in both cell types however abrogation of the G_2 arrest in TOV21G cells expressing wildtype p53 was less efficient than in those expressing a short hairpin RNA for p53,

and only these cells were sensitised to chemotherapy (Hirai et al., 2009). The same group also found MK1775 potentiated the anti-tumour effects of 5-fluorouracil in p53-deficient cell lines causing mitotic catastrophe and apoptosis, as well as Pemetrexed, Doxorubicin, Camptothecin and Mitomycin C (Hirai et al., 2010). However, the effects of drugs targeting microtubules were not enhanced by MK1775, this was attributed to their inability to induce the G_2 checkpoint therefore Wee1 inhibition would not enhance their mode of action (Hirai et al., 2009). Wee1 inhibition by siRNA or PD0166285 has also been effective in combination with Temozolomide in glioblastoma cells with no adverse effects on the cell cycle or cell viability seen in primary human fibroblasts or astrocytes (Mir et al., 2010). Interestingly, there was no correlation between p53 status and sensitivity to Wee1 inhibitors found in this study. Although p53 wildtype cells were sensitised the least by PD0166285 these cells also did not overexpress Wee1 mRNA unlike other cell lines tested which may be the reason for this result (Mir et al., 2010). Another study found synergy between Gemcitabine and MK1775 only in p53-deficient breast cancer cell lines *in vitro* and suggested that the mechanism of cell death with Wee1 inhibition was to force mitotic entry of S phase arrested cells (Aarts et al., 2012). In this model chemotherapy is required to arrest cells in S phase until mitotic proteins are expressed at a sufficient level to allow mitotic entry, then Wee1 inhibition forces premature entry into mitosis before DNA has been repaired. Gemcitabine combined with MK1775 treatment caused abnormal mitosis characterised by unaligned and partially condensed chromosomes, and apoptosis was initiated upon exit from mitosis (Aarts et al., 2012). This study also showed Chk1 inhibition with AZD7762 in replacement of Gemcitabine was effective in inhibiting the intra-S phase checkpoint leading to premature mitotic entry in combination with MK1775 (Aarts et al., 2012). Wee1 inhibition with Wee1 inhibitor II was also found to enhance anti-tumour activity of TRAIL agonists and SRC kinase inhibitors *in vitro* (Cozzi et al., 2012; Garimella et al., 2011). Pretreatment with Wee1 inhibitor increased cell death seen with TRAIL in triple-negative breast cancer cells compared with either treatment alone (Garimella et al., 2011). This enhancement correlated with an increased surface expression of death receptors and caspase activation however the mechanism at work may be similar to the one seen with ATM inhibition; this promotes the downregulation of cFLIP, an antiapoptotic protein, and upregulation of TR2 receptors for TRAIL (Garimella et al., 2011). Since Wee1 is further downstream in the DDR it may be having similar

effects. Wee1 inhibitor II also increased cell death of Burkitt lymphoma cells treated with SRC kinase inhibitors; these inhibitors can prevent the AKT-mediated inhibition of Wee1 thus triggering a G_2/M arrest via Cdc2 inhibition by Wee1. So the use of Wee1 inhibitors in this case promotes apoptosis as opposed to cell cycle arrest in cancer cells (Cozzi et al., 2012).

The effects of Wee1 inhibitors are not only seen *in vitro* since *in vivo* studies have also shown positive results. Firstly MK1775 in combination with Gemcitabine was found to decrease tumour growth to a greater extent than either treatment alone in WiDr tumour xenografts with no increase in toxicity in animals (Aarts et al., 2012; Hirai et al., 2009). This correlated with a decrease in Cdc2 phosphorylation and an increase in the mitotic marker phospho-histone H3, showing effects were due to Wee1 inhibition (Hirai et al., 2009). Similar results were gained when WiDr human colon cancer xenografts were treated with 5-fluorouracil or its prodrug Capecitabine combined with MK1775, or MX-1 human breast cancer xenografts were treated with Capecitabine combined with MK1775 (Hirai et al., 2010). Another study focused on the effects of Wee1 inhibition with Gemcitabine in pancreatic tumour xenografts; again the combination enhances tumour growth inhibition and caused tumour shrinkage but only in p53-deficient tumours (Rajeshkumar et al., 2011). These effects were longer lasting than with Gemcitabine alone (Rajeshkumar et al., 2011). Combining checkpoint inhibitors with Gemcitabine is an attractive rationale for therapy since Gemcitabine may activate cell cycle checkpoints leading to resistance to therapy; in this case a G_2 checkpoint was activated hence the use of Wee1 inhibition (Rajeshkumar et al., 2011). Also Gemcitabine has a similar effect to hypoxia since it inhibits DNA synthesis; this implies inhibiting Wee1 in hypoxic conditions may also have anti-tumour effects (Plunkett et al., 1995). Overall the use of Wee1 inhibitors is efficacious in decreasing the viability of tumour cells whilst showing no short-term toxicity on non-transformed healthy cells.

1.8.2. Wee1 inhibitors combined with irradiation

The sensitisation to radiotherapy by Wee1 inhibition has also been investigated. Wang *et al.* discovered PD0166285 decreased the phosphorylation of Cdc2 induced by irradiation in colon, lung and cervical cancer cell lines (Wang et al., 2001). However, only p53-deficient cells, such as HT29 colon carcinoma cells, were radiosensitised by this agent; the G_2 arrest induced by irradiation was abrogated and

cell death increased with a sensitivity enhancement ratio (SER) of 1.23 (Wang et al., 2001). These results were further supported by data showing PA-1 ovarian cancer cell lines wildtype for p53 were not radiosensitised by PD0166285 whereas PA-1 cells with p53 degraded were (Wang et al., 2001). The dependence on p53 status for radiosensitisation was also seen in lung cancer cells (H1299) expressing temperature sensitive p53; cells without functional p53 had an SER of 1.35 whereas p53-expressing cells had an SER of 1.09 indicative of no enhancement (Li et al., 2002). These cells also showed weak inhibition of Cdc2 phosphorylation with Wee1 inhibitor compared to p53-deficient cells thus no disruption of the G_2 arrest following irradiation (Li et al., 2002). The proposed mechanism for these p53 effects is that p53 repressed cyclin B1 gene expression and this contributes to low Cdc2 activity therefore cells remain arrested in G_2; since Cdc2 is relatively inactive anyway the removal of the inhibitory phosphorylation from Wee1 makes little difference and entry into mitosis is not promoted. Thus with p53 active, cells are less likely to undergo mitotic catastrophe as well as being less reliant on the G_2 checkpoint (Li et al., 2002). Radiosensitisation by MK1775 was also seen in non-small cell lung, breast, and prostate cancer cell lines mutant for p53 whereas those which were p53 wildtype failed to have an SER above 1 (Bridges et al., 2011). This radiosensitisation was identified as being due to a lack of time to repair DNA rather than inhibition of DNA repair itself since cells treated with MK1775 entered mitosis prematurely with a high level of γH2AX foci and micronuclei present in the next cell cycle indicative of DNA damage (Bridges et al., 2011). An increase in unrepaired DSBs identified by γH2AX foci and fragmented nuclei was also seen in glioblastoma cell lines when irradiation was combined with PD0166285 treatment, this is indicative of mitotic catastrophe which is likely to have occurred since cells were unable to repair DNA damage induced by irradiation before premature mitotic entry (Mir et al., 2010). Results from this study and from Sarcar *et al.* show these effects were not dependent on p53 status in gliobastoma cell lines; the latter also showed a supra-additive increase in mitotic catastrophe when MK1775 was combined with irradiation (Mir et al., 2010; Sarcar et al., 2011). Wee1 inhibition by PD0166285 was also able to radiosensitise osteosarcoma cell lines regardless of p53 status (p53 wildtype, mutated and null cells used); this is particularly promising since this cell type is considered relatively radioresistant (PosthumaDeBoer et al., 2011). The ability of p53 wildtype cells to be radiosensitised may be explained by the cells harbouring other mutations affecting the

p53 pathway, but no mutation in p53 itself, so cells are still reliant on the G_2 arrest thus Wee1 inhibition is still effective (PosthumaDeBoer et al., 2011; Sarcar et al., 2011). Wee1 inhibition was also effective combined with the more clinically relevant treatment of fractionated radiotherapy, not just single dose irradiation. In this case continual administration of MK1775 in combination with 2Gy every 24h diminished the recovery rate of glioblastoma cells, which is calculated as the percentage survival after fractionated therapy divided by percentage survival after equivalent single dose irradiation (Sarcar et al., 2011).

The combined use of irradiation and Wee1 inhibition *in vivo* is also promising for enhancing cell kill in tumours regularly treated with radiotherapy. Xenografts consisting of p53-deficient non-small cell lung cancer cell line Calu-6 were radiosensitised *in vivo* by MK1775 using a variety of different sequences of drug and radiation, although the greatest tumour growth delay was seen when using irradiation and MK1775 twice daily on the same days for 5 days (Bridges et al., 2011). This shows the importance of administering the drug and radiotherapy close in time; others have noted that the transient phosphorylation of Cdc2 inducing a G_2 arrest occurs for 12h after irradiation and DNA damage can be repaired in 12-24h after irradiation, therefore cells are more susceptible to Wee1 inhibition in the first 12 hours after irradiation (PosthumaDeBoer et al., 2011). However, cells do not need to be arrested in G_2 for Wee1 inhibition to have effect, as long as the G_2 checkpoint is impaired cells will enter mitosis prematurely with these agents. This is shown by the sensitization of cells by Wee1 inhibitor even when administered directly after irradiation before a G_2 arrest has taken effect (PosthumaDeBoer et al., 2011). Wee1 inhibitors have also been effective radiosensitisers in *in vivo* glioblastoma models (Mir et al., 2010; Sarcar et al., 2011). In orthotopic glioblastoma models (using U251 or highly invasive E98 cells) where cells were injected subcutaneously, PD0166285 combined with single dose irradiation caused significant tumour regression, reduction in tumour burden, and prolonged survival compared to only moderate improvements with either therapy alone (Mir et al., 2010). U251 xenografts grown in the hind leg of mice also showed a significantly higher growth delay when MK1775 was combined with fractionated radiotherapy compared to either treatment alone (Sarcar et al., 2011). The *in vitro* mechanism was also found to occur *in vivo* since MK1775 was able to inhibit the reduction in mitotic ratio caused by irradiation (Sarcar et al., 2011). All these *in vivo* studies observed no animal toxicity (Bridges et al., 2011; Mir et al., 2010; Sarcar et

al., 2011). These studies show that radiosensitisation by Wee1 inhibitors is possible however *in vitro* studies have only been carried out in normoxic conditions; this study aims to address the effects of Wee1 inhibitors in hypoxic conditions which are resistant to radiotherapy to assess if their radiosensitising effects extend to this population.

1.8.3. Use as monotherapy

Previous reports suggest Wee1 inhibition induces premature mitotic entry, but a decrease in tumour cell survival is only seen when Wee1 inhibitors are used in combination with DNA damaging agents. Despite this there have been some reports of Wee1 inhibitors inducing tumour cell death when used as monotherapy (Kreahling et al., 2011; Murrow et al., 2010). Wee1 inhibitor II or siRNA against Wee1 was able to induce DNA damage, S phase arrest, decrease cell proliferation and increase caspase-mediated apoptosis in breast cancer cell lines but had no adverse effects on a non-transformed mammary epithelial cell line despite similar expression levels of Wee1 and its activity (Murrow et al., 2010). Since tumour cells are highly genetically unstable anyway and often contain defects in DNA repair, this may explain their sensitivity to Wee1 inhibition (Mir et al., 2010). On the other hand, non-transformed cells escape cell death induced by these inhibitors due to lack of inherent DNA damage and/or a functional G_1 checkpoint allowing time for DNA repair (Murrow et al., 2010). Another study showed single agent activity of MK1775 in sarcoma cell lines independent of their p53 status (Kreahling et al., 2011). The apoptosis that was detected after Wee1 inhibition demonstrated morphological features of mitotic catastrophe and patient-derived tumour explants *ex vivo* also showed increased cell death with MK1775 treatment (Kreahling et al., 2011). However, it should be noted that these effects were seen when using 500nM MK1775 whereas all other studies mentioned here use 100-300nM MK1775 so this elevated dose may be the reason for discrepancy in results when using Wee1 inhibitors as single agents (Bridges et al., 2011; Hirai et al., 2010; Hirai et al., 2009; Kreahling et al., 2011; Mizuarai et al., 2009; Sarcar et al., 2011).

In addition, Wee1 inhibition appears to have effects that block microtubule stability and affect mitotic spindle formation (Garcia et al., 2009; Hashimoto et al., 2006). PD0166285 in mouse melanoma cells inhibited microtubule polymerisation and caused imperfect adhesion within 4h, and this was associated with arrested cell

proliferation although no cell death was observed until 24h (Hashimoto et al., 2006). These effects may still be linked to cell cycle regulation by Wee1 since changes in cellular architecture is required for cell cycle progression. This includes complete recognition of the cytoskeleton by proteins that are targets of Cdc2-cyclin B1 phosphorylation; some of these are involved in proper assembly of the mitotic spindle and rearrangements of actin during mitosis (Hashimoto et al., 2006). Thus deregulation of the cytoskeleton by Wee1 inhibition may add to its effects on the cell cycle, however whether PD0166285 has a direct or indirect effect on microtubules is unknown (Hashimoto et al., 2006). Wee1 is also required for phosphorylation of kinesin-5 motor protein to mediate its movement along microtubules to allow correct positioning and assembly of the mitotic spindle (Garcia et al., 2009). So this provides further evidence that inhibition of Wee1 may also deregulate cell cycle progression via its effects on the cytoskeleton. These findings may also explain why Wee1 mutants in yeast divide at half the size of wildtype cells, which gave the protein its name (Nurse, 1975).

Another important aspect of therapy is the identification of biomarkers that indicate successful target engagement and biological efficacy. It has been indicated that a decrease in the phosphorylation of Cdc2 at tyr15 is a suitable read-out for effective Wee1 inhibition and the increase in phospho-histone H3 levels indicate mitotic entry therefore the abrogation in G_2 arrest (Hirai et al., 2009). This was demonstrated in various tumour types both *in vitro* and *in vivo* and these biomarkers showed a good correlation with anti-tumour efficacy (Hirai et al., 2009). However, the difficulty in obtaining tumour biopsies for biomarker analysis makes finding biomarker expression in skin samples desirable, and in particular the use of mRNA gene signatures is preferred since this requires a much smaller amount of biopsy tissue than when using immunohistochemical markers (Mizuarai et al., 2009). In order to determine this WiDr xenografts (p53-deficient) and matched rat skin samples (p53 wildtype) were treated with Gemcitabine and MK1775 then analysed for changes in gene expression. A common signature of genes modulated in both was determined as the Wee1 inhibition signature and included genes known to regulate S and G_2 cell cycle checkpoints and progression (Mizuarai et al., 2009). This is an encouraging step in showing surrogate tissues can be used to analyse the efficacy of Wee1 inhibition however, the role of the genes identified in the anti-tumour effect of Wee1 inhibitors still needs to be investigated and their use as biomarkers in human tissues is yet to be

determined (Mizuarai et al., 2009). Also, from data discussed above, biomarkers indicating p53 deficiency in tumours would be useful in predicting the efficacy of Wee1 inhibition (Hirai et al., 2009).

Overall evidence suggests Wee1 inhibition is a good therapeutic strategy in targeting cancerous cells whilst having no toxic effects on non-transformed cells. However, there may be potential limitations if some cancer cells are able to survive forced mitotic entry due to having only sublethal DNA damage; their daughter cells may become resistant to combination therapy due to natural selection. However, since DNA damage occurs randomly it is likely these cells would eventually acquire additional DNA damage leading to mitotic catastrophe (De Witt Hamer et al., 2011). Most data comes from preclinical studies and further clinical evaluation of these agents is definitely needed. Table 1.1 outlines the clinical studies that have been completed or that are currently underway. Results from the trial which commenced in February 2008 have so far found no dose limiting toxicity associated with monotherapy although adverse effects include nausea/vomiting, fatigue and haematological effects when MK1775 was combined with chemotherapy (Schellens et al., 2009). Overall, data suggests MK1775 targets Wee1 effectively and is well tolerated in humans (Schellens et al., 2009).

Table 1.1 Wee1 inhibitors in trials (Information obtained from clinicaltrials.gov)

Date	**Sponsor**	**Title**	**Phase**	**Status**
Feb 2008 – Apr 2013	Merck	Dose escalation study of MK1775 in combination with either Gemcitabine, Cisplatin, or Carboplatin in adults with advanced solid tumours	1	Recruiting
Jan 2010 – Jun 2011	Merck	Dose escalation study of MK1775 in combination with 5-FU or 5-FU/CDDP in patients with advanced solid tumour	1	Terminated
May 2010 – Jun 2011	Merck	MK1775 in combination with Topetecan/Cisplatin in patients with cervical cancer	1 + 2a	Terminated
Jul 2010 – Sep 2011	Netherlands Cancer Institute + Merck	MK1775 and Carboplatin to treat ovarian cancer	2	Unknown

Jun 2012 – Sep 2014	Merck	MK1775 in combination with Paclitaxel and Carboplatin versus Paclitaxel and Carboplatin alone for platinum sensitive ovarian tumours with p53 gene mutation	2	Recruiting

1.9 Project Aims

The main aim of the project is to identify whether Wee1 inhibition can be used to target hypoxic cells in tumours. Encouraging results come from the successful targeting of other DDR proteins potentially acting upstream of Wee1 in hypoxia, such as ATR and Chk1, implying Wee1 inhibition may also be successful (Hammond et al., 2006; Pires et al., 2012). Whether Chk1 is the activating kinase for Wee1 in mammalian cells will be examined. The effect of Wee1 inhibition in a range of oxygen concentrations will also be looked into. Since severe hypoxia can induce replication arrest this may decrease the efficacy of these agents if a functional cell cycle is required for them to take effect. Of particular interest is the ability of Wee1 inhibitors to radiosensitise tumour cells and whether this effect extends to hypoxic cells. Since these cells are radioresistant it is especially important to find more effective ways of decreasing their viability so this will be investigated. Wee1 has also been implicated in maintaining genomic stability and the signalling pathways involved will be explored in this study.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Laboratory and Tissue Culture Reagents

The reagents used in this study are listed in Table 2.1.

Table 2.1 Laboratory and tissue culture reagents

Name	**Supplier**
Acrylamide / Bis Solution, 30%, 29:1	BioRad
Agarose	Sigma-Aldrich UK
Ammonium persulfate	Fisher Scientific
β-mercaptoethanol	Fisher Scientific
Blocking Buffer LiCor	LiCor
Bovine serum albumin (BSA)	Fisher Scientific
Bromophenol blue	Fisher Scientific
Chloroform	Fisher Scientific
Crystal violet	Sigma-Aldrich UK
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich UK
Dharmafect 1	Fisher Scientific
Diethyl pyrocarbonate carbonate (DEPC)	Fisher Scientific
Dimethylsulphoxide (DMSO)	Fisher Scientific
Ethylenediaminetetraacetic acid dipotassium salt	Sigma-Aldrich UK
Ethanol	Fisher Scientific
Ethidium bromide	Sigma-Aldrich UK
Foetal bovine serum (FBS)	Autogen Bioscience
Glacial acetic acid	Fisher Scientific
Isopropanol	Fisher Scientific
McCoys 5A medium	Gibco
Methanol	Fisher Scientific
Paraformaldehyde	Fisher Scientific
Penicillin-streptomycin	Sigma-Aldrich UK
Pepsin	Sigma-Aldrich UK

Phosphate buffer saline	Fisher Scientific
Propidium iodide	Sigma-Aldrich UK
Ribonuclease A (RNase A)	Sigma-Aldrich UK
Sodium Dodecyl Sulphate (SDS)	Fisher Scientific
SYBR green	Applied Biosystems
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma-Aldrich UK
Trypsin 0.05% Ethylenediaminetetraacetic acid dipotassium salt (trypsin-EDTA)	Sigma-Aldrich UK
Tris base	Sigma-Aldrich UK
Tris-glycine	Fisher Scientific
Tris(hydroxymethyl)aminomethan hydrochloride (Tris-HCl)	Fisher Scientific
Trizol	Ambion
Tween 20	Fisher Scientific
Urea	Fisher Scientific

2.1.2 Antibodies

The antibodies used for western blotting in this study are listed in Table 2.2 and 2.3. Antibodies for FACS analysis are listed in Table 2.4.

Table 2.2 Primary antibodies for western blotting

Name	**Species**	**Dilution**	**Molecular weight (kDa)**	**Supplier**
β-Actin	Mouse monoclonal	1:10000	43	Santa Cruz Biotechnology/ Insight Biotechnology
Cdc2	Rabbit polyclonal	1:1000	34	Cell Signalling
Phospho-Cdc2 (Tyr15)	Rabbit polyclonal	1:1000	34	Cell Signalling
Chk1	Mouse monoclonal	1:500	56	Santa Cruz Biotechnology
Phospho-Chk1 (ser296)	Rabbit polyclonal	1:500	56	Cell Signalling

Phospho-Chk1 (ser317)	Rabbit polyclonal	1:500	56	Cell Signalling
Phospho-Chk1 (ser345)	Rabbit polyclonal	1:500	56	Cell Signalling
GAPDH	Mouse monoclonal	1:2000	36	Novus Biologicals
HIF-1α	Mouse monoclonal	1:500	120	BD Transduction Laboratories
H2AX	Rabbit monoclonal	1:5000	15-17	Calbiochem
γH2AX (ser139)	Mouse monoclonal	1:5000	15-17	Millipore
KAP1	Rabbit polyclonal	1:10000	110	Bethyl Laboratories
Mus81	Mouse monoclonal	1:1000	61	Abcam
p53 (DO-1)	Mouse monoclonal	1:1000	53	Santa Cruz Biotechnology
Wee1	Mouse monoclonal	1:500	98	Santa Cruz Biotechnology
Phospho-Wee1(ser642)	Rabbit monoclonal	1:500	95	Cell Signalling

Table 2.3 Secondary antibodies for western blotting

Name	**Species**	**Dilution**	**Supplier**
Alexa Fluor 680 anti-mouse IgG	Goat	1:10000	Invitrogen
Alexa Fluor 800 anti-mouse IgG	Goat	1:10000	Invitrogen
Alexa Fluor 680 anti-rabbit IgG	Goat	1:10000	Invitrogen
Alexa Fluor 800 anti-rabbit IgG	Goat	1:10000	Invitrogen

Table 2.4 Antibodies for FACs analysis

Name	Species	Dilution	Supplier
pH3ser10	Rabbit	1:100	Cell Signalling
Alexa Fluor 488 anti-rabbit	Goat	1:200	Invitrogen

2.2 Methods

2.2.1 Tissue Culture

H1299 (non-small cell lung carcinoma), RKO (colorectal carcinoma), RKO $HIF^{+/+}$, RKO $HIF^{-/-}$, U2OS (osteosarcoma), and WI38 (normal lung fibroblast) cells were cultured in DMEM supplemented with 10% FBS and Penicillin-Streptomycin. RKO $HIF^{+/+}$ and $HIF^{-/-}$ were a gift from D. Chan (UCSF). HCT116 (colorectal carcinoma), HCT116 $Mus81^{-/-}$, HCT116 $Mus8^{-/- + Mus81}$, HCT116 $p53^{-/-}$, and HCT116 $p53^{+/+}$ were cultured in McCoys with 10% FBS and Penicillin-Streptomycin. Mus81 knockout cell lines were obtained from K. Miyagawa (University of Tokyo) and matching p53 cell lines from B. Vogelstein (Johns Hopkins, Baltimore). Cells were grown in a humidified incubator at 37°C and 5% CO_2. Passaging cells involved removing media, washing with PBS and dissociating cells from the flask at 37°C for 1 minute with trypsin-EDTA. Cells were then suspended in fresh media and transferred to fresh flasks.

2.2.1.1 Mycoplasma testing

Mycoplasma testing was carried out every 3 months using the PlasmoTest kit (Invivogen). HEK-Blue cells were grown in DMEM supplemented with 10% FBS, Penicillin-Streptomycin and Normocin (1:500 dilution) (Invivogen) and passaged at least 48h before assay in selection media (DMEM with 10% FBS, Penicillin-Streptomycin, Normocin and HEK-Blue Selection ATB (1:250 dilution) (Invivogen)). Cells to be tested were seeded in antibiotic free media for at least 48h before testing at 100% confluency. Cells were then harvested and 500µl was heated for 15min at 100°C. After cooling to room temperature 50µl of sample was added to each well of a 96-well plate. Two samples for each cell line were tested and a positive and negative control was also plated. HEK-Blue cells were then rinsed in PBS and detached before obtaining a cell pellet. The pellet was resuspended in pre-warmed HEK-Blue detection medium (Invivogen) at a density of approximately $2.5x10^5$ cells/ml. 200µl

of these HEK-Blue cells were then added to each well and incubated at 37°C for 24h. A negative result was indicated by a pink/light purple colour and a positive result was indicated by a purple/blue colour.

2.2.1.2 Drug treatments

Three Wee1 inhibitors were used; PD0166285 (Pfizer), Wee1 Inhibitor II (Calbiochem) and MK1775 (Merck). In addition the Chk1 inhibitor Gö6976 (Calbiochem) was used. All drugs were made into stock solutions by dissolving in DMSO and aliquots were stored at -20°C. PD0166285 was used at 0.5µM, Wee1 Inhibitor II at 10µM and MK1775 at 0.2µM.

Adriamycin (Pharmacia Company) was used as a positive control for DNA damage and induction of the DNA damage response; stock solution was made by dissolving in water and the drug was used at 2µM. Further detail of drug treatments is provided in figure legends.

2.2.1.3 Hypoxia treatment

Hypoxic conditions were achieved using an 'in vivo$_2$ 400' Ruskinn chamber for oxygen concentrations of 0.5% to 2%. Anoxic conditions were achieved using a Bactron II chamber (Shell Labs); the oxygen concentration is maintained below 0.02% at all times. Cells were also cultured at 3% O_2 using a humidified incubator set to this concentration. Cells were incubated in the chambers for the time indicated in figure legends and harvested within the chamber unless experiments required reoxygenation.

2.2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Treated cells were harvested by aspirating media using an ILMVAC Biovac 106, washed with PBS then scraped into 1ml PBS. The sample was centrifuged at 13300*g* using a Heraeus Fresco 17 centrifuge for 4min at 4°C to obtain a cell pellet. The supernatant was then removed and the pellet resuspended in 50-80µl UTB (9M urea, 75mM Tris HCl (pH7.5), 0.15M β-mercaptoethanol) to lyse cells. After incubating at -20°C for at least 1h, samples were sonicated for 20s to shear DNA using a Model 300VT Ultrasonic Homogenizer (BioLogics Inc.) and centrifuged at 13300*g* for 15min at 4°C. The supernatant from this process was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). UTB was used as a blank when

calibrating the meter and absorbance was read at 280nm. An average of 2 readings of 2µl of sample was used as the final protein concentration.

To separate proteins by molecular weight SDS-PAGE was carried out using 25-60µg of protein. The appropriate volume of protein sample was added to sample buffer (3.3% SDS, 6M urea, 17mM Tris-HCl (pH7.5), 0.07M β-mercaptoethanol, 0.01% bromophenol blue) to make a total volume of 10µl to add to each well. Sample was boiled at 100°C for 5min then loaded into 7.5%, 10%, or gradient (4-20%) SDS-polyacrylamide precast gels (BioRad) or prepared SDS-polyacrylamide gels (recipe shown in Tables 2.5 and 2.6 (Sambrook and Russell, 2001)). Precast gels were run for 200V for 30min and prepared gels were run at 120V for approximately 1h 15min in Tris-glycine running buffer (25mM Tris base, 192mM glycine, 0.1%(w/v) SDS). A protein ladder was loaded into lane 1 of each gel; either Blue Wide Range Protein Ladder from Geneflow Ltd. or Precision Plus Protein Kaleidoscope Standards from BioRad was used.

2.5 Recipe for stacking gel

Reagent	**Volume**
H_20	6.1ml
0.5M Tris-HCl pH 6.8	2.5ml
Acrylamide	1.3ml
10% SDS	100µl
10% APS	100µl
TEMED	20µl

2.6 Recipe for separating gel

Reagent	**Volume dependent on gel percentage**		
	7.5%	10%	12%
H_20	7.4ml	6.07ml	5.2ml
1.5M Tris-HCl pH 8.8	3.75ml	3.75ml	3.75ml
Acrylamide	3.65ml	4.95ml	5.8ml
10% SDS	150µl	150µl	150µl
10% APS	75µl	75µl	75µl
TEMED	18µl	18µl	18µl

2.2.3 Western Blotting

SDS-polyacrylamide gels were transferred to a nitrocellulose membrane in transfer buffer (20%(v/v) methanol, 25mM Tris base, 150mM glycine) at 100V for 1h. Membranes were washed in TBS and subsequently incubated in blocking buffer (LiCor buffer diluted 1:2 in TBS) for 1h to prevent non-specific binding of primary antibody to the nitrocellulose. The membrane was incubated in primary antibody overnight at 4°C (1h for β-actin) using dilutions listed in Table 2.2; antibody was diluted using 1:2 LiCor buffer:TBS-T (0.1%). After washing three times in TBS-T for a total of 30min, membranes were incubated in secondary antibody (Table 2.3) for 1h diluted to 1:10000 in 1:2 LiCor buffer:TBS-T. Membranes were then washed twice in TBS-T for a total of 20min followed by TBS for 10min before being developed using a LiCor Odyssey Infrared Imager. All incubations and washes were done whilst shaking on a Stuart See-Saw Rocker SSL4 or Stovall Belly Dancer. LiCor Odyssey software was used to quantify protein bands relative to control blots.

2.2.4 Quantitative – Polymerase Chain Reaction (qPCR)

2.2.4.1 RNA preparation

H1299 and RKO $HIF^{+/+}$ or $HIF^{-/-}$ were cultured in 0.1% O_2 for 0, 8, 16 or 24h. After the allocated time media was removed from the dish and cells were lysed and harvested in 300µl trizol reagent per 6cm dish. Samples were then incubated for 5min at room temperature in eppendorfs before addition of 60µl chloroform. Each tube was shaken vigorously for 15s then incubated at room temperature for 2-3min. To separate the samples into phases, they were centrifuged using a Heraeus Fresco 17 centrifuge at 12000*g* for 15min at 4°C. The upper aqueous phase containing RNA was transferred to a fresh tube and 150µl isopropanol was added. Samples were incubated at room temperature for 10min then centrifuged at 12000*g* for 10min at 4°C to obtain the RNA pellet. The supernatant was then removed and the RNA was washed using 1ml of 75% ethanol (diluted in DEPC water). The sample was vortexed gently to lift the pellet and centrifuged at 7500*g* for 5min at 4°C. The wash was then removed and the RNA was air dried for approximately 5min to remove as much excess ethanol as possible. Care was taken to avoid drying the RNA completely since this can decrease the pellets solubility. The pellet was resuspended in 20-50µl DEPC water depending on the size of the pellet and incubated at 56°C for 10min. The RNA was then

quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) using DEPC water as a blank; the average of 2 readings of 2µl was taken as the final concentration. The volume of RNA needed for 1µg was calculated for cDNA preparation.

2.2.4.2 cDNA preparation

The preparation of cDNA from RNA was carried out using the Verso™ cDNA Kit (Thermo Scientific). PCR reactions consisted of 4µl 5x cDNA synthesis buffer, 2µl dNTP mix, 1µl RNA primer, 1µl RT enhancer, 1µl verso enzyme mix and 3µl H_2O. For each sample 1µg of RNA in 8µl DEPC water was added to the mix to make a total of 20µl. The run method for PCR was 60min at 42°C for cDNA synthesis followed by 2min at 95°C to inactivate the reaction mixture followed by a holding stage at 4°C. cDNA was then quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) using DEPC water as a blank to ensure a concentration of 100ng/µl had been achieved.

2.2.4.3 qPCR

qPCR is used to measure transcript levels whilst the PCR reaction proceeds. Primers are listed in Table 2.7. PCR reaction mixtures were set up containing 12.5µl SYBR green, 2.5µl forward primer (1µM for Mus81, 2.5µM for Eme1, 2.5µM for Wee1, 3µM for Glut1, 5µM for 18s), 2.5µl reverse primer (same concentrations as forward primer), and 2.5µl DEPC water. cDNA was used at 1:50 dilution and 5µl was added to each well containing the reaction mixture described above. Additional cDNA was prepared from extracts by K. Leszczynska. No template controls (NTC) contained 5µl DEPC water in replacement of cDNA. The reaction plate was then sealed and centrifuged at 1400*g* for 30s. The 7500 Real-Time PCR System from Applied Biosystems was used to run samples. The run method consisted of a first holding stage (50°C for 20s), second holding stage (95°C for 15min), cycling stage (95°C for 15s, 60°C for 30s, 72°c for 30s for 40 cycles), and melt curve stage (95°C for 15s, 60°C for 1min, 95°C for 30s, 60°C for 15s). Normoxic samples acted as a reference and 18s primers were used as an endogenous control. The fold change (FC) of the transcript of interest was then calculated against the reference sample using ΔC_T values using the calculations below:

$$\Delta C_T = C_T mean\ of\ gene\ of\ interest - C_T mean\ of\ reference\ gene$$

$$\Delta\Delta C_T = \Delta C_T - \Delta C_T\ of\ control$$

$$FC = 2^{-\Delta\Delta C_T}$$

(Livak and Schmittgen, 2001)

R (R Development Core Team) was used for statistical analysis of qPCR data. Statistical significance was measured by determining *P* values using a students paired t test to compare 2^{-C_T} values. Triplicate C_T values from three internal repeats were used for this. *P*<0.05 is indicated by an asterisk, *P*<0.01 indicated by a double asterisk and *P*<0.001 indicated by a triple asterisk in figures.

Table 2.7 Oligonucleotide primers for qPCR

Name	**Sequence (5'-3')**	**Supplier**
Eme1 Forward	CTGAACCGAGTCAGCCTGGAAA	Sigma-Aldrich UK
Eme1 Reverse	GCACCTGTATGTCTGCGAGCAA	Sigma-Aldrich UK
Glut1 Forward	ATACTCATGACCATCGCGCTAG	Sigma-Aldrich UK
Glut1 Reverse	AAAGAAGGCCACAAAGCCAAAG	Sigma-Aldrich UK
Mus81 Forward	TGACCTCTCCAAACCCTCTC	Sigma-Aldrich UK
Mus81 Reverse	AAACACTTCTCGCACCGACT	Sigma-Aldrich UK
Wee1 Forward	TCAGTATTGCTGTCCGCTTC	Sigma-Aldrich UK
Wee1 Reverse	TTGCCATCTGTGCTTTCTTG	Sigma-Aldrich UK
18s Forward	GCCCGAAGCGTTTACTTTGA	Sigma-Aldrich UK
18s Reverse	TCCATTATTCCTAGCTGCGGTATC	Sigma-Aldrich UK

2.2.5.4 Gel Electrophoresis

Agarose gel electrophoresis of the qPCR products was carried out on 2% agarose gels made in 50X TAE buffer (242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5mM EDTA pH8.0 in 1l dH_2O) containing 5µl ethidium bromide. Each well was loaded with 5µl loading buffer and 25µl PCR reaction mixture and run at 100V for 40min in 1X TAE buffer. 2-Log DNA ladder (New England Biolabs) was used to measure product size. This was to ensure the correct products were produced with the primers used.

2.2.5 siRNA Transfections

Chk1 siRNA transfections were carried out using the sequence indicated in Table 2.8 or scramble siRNA (proprietary sequence) from Qiagen in RKO cells grown in antibiotic free media. $0.2x10^6$ cells were seeded per well the day before transfection. For transfection two reaction mixtures were set up; mixture 1 contained 3.125µl of 20µM siRNA, 121.875µl DEPC water and 125µl of DMEM (serum-free) per well; mixture 2 contained 5µl Dharmafect and 245µl DMEM (serum-free) per well. Each mixture was incubated for 5min at room temperature then combined to incubate for a further 20min at room temperature. The final reaction mixture (500µl) for each well was then diluted by adding 2ml DMEM + FBS. The old media was aspirated from cells and 2ml of the transfection mixture added. Cells were harvested after incubation at 37°C for 48h. Any exposure to hypoxic conditions was included in this time. Optimal knockdown was seen at 48h using 25nM Chk1 siRNA.

Numerous methods were utilised in the optimisation of Wee1 siRNA. Initially U2OS cells were transfected with each individual siRNA at 50nM (listed in Table 2.8) for 24, 48 or 72h or a scramble sequence. $0.3x10^6$ cells were seeded per well the day before transfection in antibiotic free media. Similar to above, two reaction mixtures were set up; mixture 1 contained 5µl of 20µM siRNA, 20µl DEPC water and 25µl of DMEM (serum-free) per well; mixture 2 contained 4µl Dharmafect and 346µl DMEM (serum-free) per well. Each mixture was incubated for 5min at room temperature then combined to incubate for a further 20min at room temperature. The final reaction mixture (400µl) for each well was then diluted by adding 1.6ml DMEM + FBS. The old media in each well was then replaced with this transfection mix. Further optimisation was required and this method was also used to carry out the following transfections:

1. All 3 siRNA at 50nM simultaneously for 24, 48 or 72h in U2OS cells.
2. Double transfection in U2OS cells for 24, 48 and 72h.
3. 25nM or 50nM of each siRNA for 24 or 48h in U2OS cells.

Finally the transfection mix was set up as for Chk1 siRNA and RKO cells were transfected with either 25nM or 50nM of the 3 Wee1 siRNAs simultaneously. Protein was harvested after 24, 48, 72 or 96h. The results for all methods are shown in the appendix although optimal knockdown was not achieved.

Table 2.8 Oligonucleotides for siRNA transfection

Name	**Sequence (5'-3')**	**Supplier**
Chk1	GGCUUGGCAACAGUAUUUCGGUAUA	Invitrogen
Wee1-1	GGAAAAGGGAAUUUGAUG [dT][dT]	Sigma-Aldrich UK
Wee1-2	GGGAAUUUGAUGUGCGACA [dT][dT]	Sigma-Aldrich UK
Wee1-3	AAUAUGAAGUCCCGGUAUA [dT][dT]	Sigma-Aldrich UK

2.2.6 Fluorescence-activated Cell Sorting (FACS)

FACS was used to analyse any changes in the cell cycle due to treatment of the cells. Cells were harvested after treatment (indicated in figure legends) by aspirating media, washing in PBS then lysing by incubating in 1ml Trypsin-EDTA for 3min at 37°C. Cells were resuspended in 10ml PBS then centrifuged at 1800*g* for 5min using a MSE Mistral 1000. After aspirating the supernatant, the pellet was resuspended by adding ice cold 70% ethanol drop-wise to a total volume of 3ml whilst vortexing on a VWR VV3 vortex. Cells were then incubated at -20°C for at least 1h.

Samples were stained with pH3ser10 to determine the population of cells in mitosis. Samples were centrifuged at 1800*g* for 5min and the ethanol supernatant discarded. Blocking was carried out by resuspending the pellet in 5ml 2% FBS/PBS then centrifuging at 1800*g* for 5min. Once the supernatant was discarded cells were resuspended in 100µl pH3ser10 antibody (diluted 1:100 in 2% FBS/PBS) and incubated for 1h at room temperature in the dark. Samples were agitated after 30min. After incubation samples were resuspended and washed in 5ml 2% FBS/PBS and centrifuged at 1800*g* for 5min. The supernatant was then discarded and cells were incubated in secondary antibody for 1h at room temperature in the dark; Alexa Fluor 488 anti-rabbit was diluted 1:200 in 2% FBS/PBS. Samples were blocked once more by resuspension in 2% FBS/PBS. Details of the antibodies used are listed in Table 2.4.

The DNA within cells was then stained with the intercalating agent propidium iodide (PI). Samples were centrifuged at 1800*g* for 5min, the supernatant was then aspirated and the pellet resuspended in 1ml PI stain (10µg/ml PI, 100µg/ml RNase A in PBS). Samples were incubated for 30min at 37°C then ran on a Becton Dickinson FACSort. CellQuest Pro and ModFit LT were used for analysis.

2.2.7 Clonogenic Assay

Colony survival assays were carried out to determine the ability of cells to survive and form colonies after various treatments. Cells were first seeded into dishes so approximately 100 colonies form on the control dish. For this the number of cells in the starting cell suspension was counted using a haemocytometer (Marienfeld); 10µl was pipetted into the chamber to allow the number of cells per ml to be calculated thus total number of cells. The number of cells seeded per well in 6-well plates was 300 cells for H1299 and HCT116 cell lines. In 6cm dishes this was increased to 500 cells for H1299. For experiments involving irradiation, cells were seeded depending on the dose of γ-irradiation administered; 300 cells for 0Gy, 600 cells for 2Gy, 900 cells for 4Gy, 1200 cells for 6Gy and 1500 cells for 8Gy. Each treatment was plated in triplicate. Cells were allowed to adhere to the dishes for 3h before treatment was begun; either exposure to drug, hypoxic conditions or both. In the latter case cells were exposed to MK1775 for 2h then irradiated. Media was replaced 18h after irradiation or at the time point stated for drug treatments. After treatment dishes were left in a quiet incubator for 8 days for H1299 or 10 days for HCT116 cell lines.

Once colonies had formed media was aspirated from dishes and 2ml crystal violet solution (5mg/ml crystal violet, 50% methanol, 20% ethanol, 30% water) was added to each for at least 20min. Crystal violet solution was then removed and dishes destained in a basin of warm water before being left to air dry. Each colony was defined as containing 50 cells or more and these were counted using a Stuart Scientific colony counter. Statistical analysis was carried out using Microsoft Excel. The plating efficiency (number of cells counted/number of cells seeded) was then calculated and used to determine the surviving fraction (100 x (plating efficiency of treated/plating efficiency of untreated); this was plotted as a percentage on a logarithmic scale. Standard error (SE) was calculated as standard deviation (SD)/√number of samples (n). The sensitivity enhancement ratio (SER) was calculated as surviving fraction of cells treated with DMSO/surviving fraction of cells treated with drug.

2.2.7.1 γ-irradiation

Cells were exposed to the doses of irradiation stated in figure legends using a Gamma Service® GSR D1 irradiator containing a Cs^{137} source. Plates were placed on

Shelf 1 and the dose rates of the system were 1.938 Gy/min and 1.233 Gy/min depending on the distance from the source, as determined by the supplier.

Samples cultured in hypoxic conditions were maintained at the same oxygen concentration during irradiation by transporting dishes in sealed boxes. Dishes were removed from the boxes 10min after irradiation to allow any molecular pathways to take effect before reoxygenation.

2.2.8 DAPI Staining

Cells were visualised by DAPI staining to determine if apoptosis was occurring. 20,000 HCT116 wildtype or Mus81$^{-/-}$ cells were seeded onto coverslips in 6-well plates. Cells were then treated as indicated in figure legends and washed in PBS before being fixed in 4% fixation buffer (4% (w/v) paraformaldehyde (PFA), 2% (w/v) sucrose, 1X PBS) for 15min. To remove any traces of PFA cells were then washed in PBS. Ethanol-cleaned slides were mounted using Prolong-gold mounting medium with DAPI (Invitrogen) and left to dry in the dark for 24h. Slides were then sealed with nail varnish and analysed using a Nikon 90i microscope.

Chapter 3. Results: The activity of Wee1 in hypoxia and potential for its inhibition

3.1 Hypoxia induces a DDR

The potential activation of Wee1 was first investigated in mild hypoxia (1% O_2) in H1299 cells. At this oxygen concentration the DDR is not activated; in accordance with this no phosphorylation of Chk1 is observed, however there does appear to be a slight decrease in total Chk1 protein levels after chronic exposure (16h) to hypoxia (Figure 3.1A). Wee1 levels illustrate there is no change in total or phosphorylated protein. The total level of Cdc2, the downstream target of Wee1, is also unaffected in mild hypoxia although the phosphorylated protein at tyrosine 15 appears to decrease slightly after 16h exposure to 1% O_2.

In order to investigate the involvement of Wee1 in the DDR, H1299 cells were exposed to severe hypoxia (0.1% O_2) as indicated or reoxygenation (8h 0.1% O_2 followed by 24h normoxia) and western blotting for proteins involved in the DDR was carried out (Figure 3.1B). Chk1 total levels as well as its 3 phosphorylated forms (Chk1ser296, Chk1ser317 and Chk1ser345) increased in acute hypoxia but started to decrease by 8h. Upon reoxygenation total levels and Chk1ser296 appear to be upregulated compared to this 8h time point. Total Wee1 and its phosphorylated form (Wee1ser642) showed a similar pattern of transient upregulation since protein levels increase after acute hypoxic exposure but again a decrease is seen by 8h. The correlation between the two proteins suggests they may be linked. Levels of Wee1 do not appear to be affected by reoxygenation.

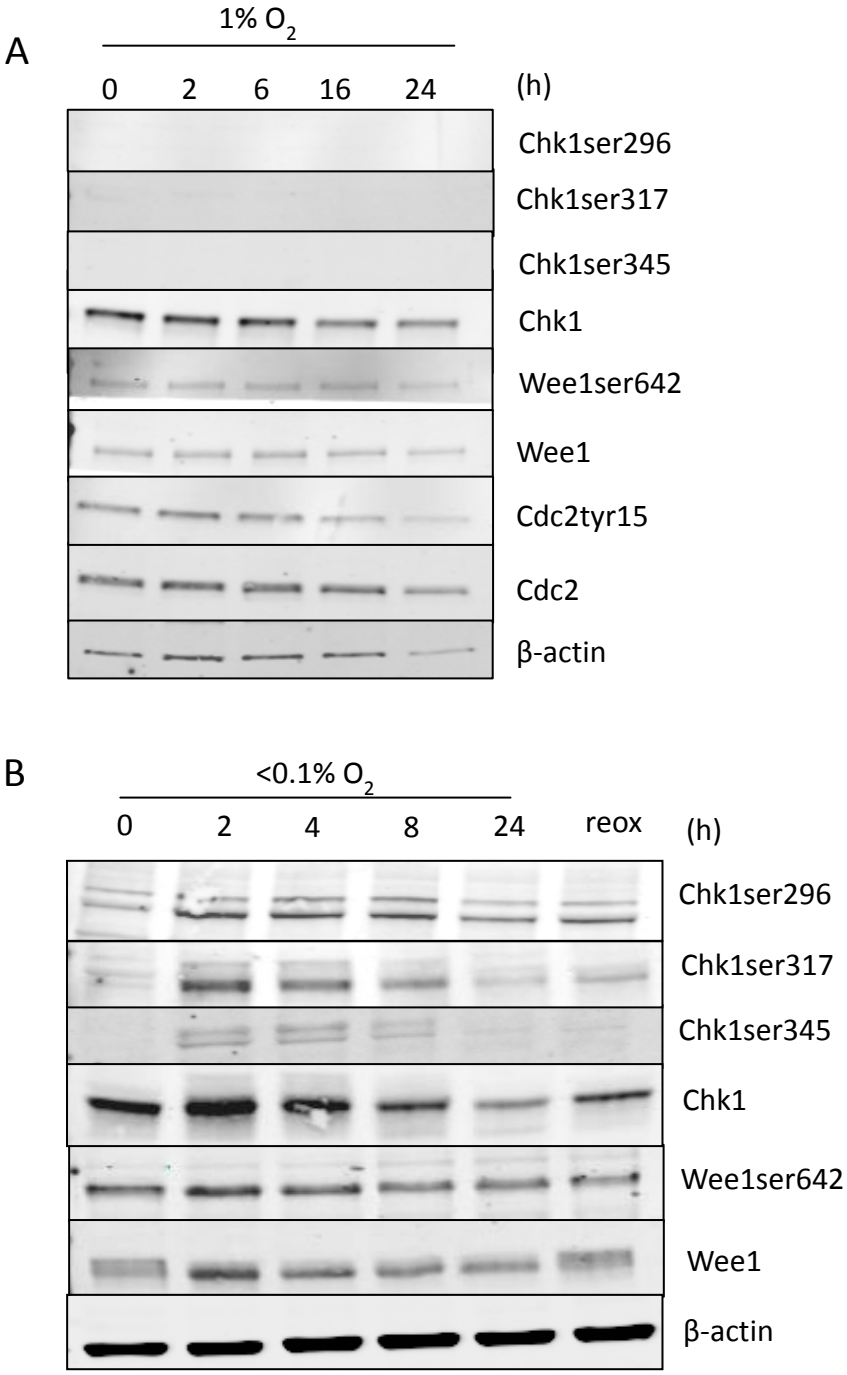


Figure 3.1. Severe hypoxia induces a DDR (A) H1299 cells were exposed to 0, 2, 6, 16 or 24h of hypoxia (1% O_2). Western blotting was carried out for Chk1, Wee1, Cdc2 and their phosphorylated forms. (B) Western blot to show effects of 0, 2, 4, 8 or 24h of severe hypoxia (<0.1% O_2), or reoxygenation (8h hypoxia followed by 24h reoxygenation), on H1299 cells. Blotting was carried out for Chk1 and Wee1 and their phosphorylated forms. β-actin was used as a loading control.

3.2 Wee1 decreases at the transcript level in hypoxia

Since Wee1 levels were shown to decrease in response to hypoxia (Figure 3.1B), we investigated if this also occurred at the transcript level; this was achieved using quantitative PCR (qPCR). In Figure 3.2A H1299 cells were exposed to 0.1% O_2 for the indicated durations and cDNA analysed using the normoxic sample as a reference from which the fold change of each gene could be calculated. 18S ribosomal RNA transcript levels were used as a control since 18S expression level should remain constant across the sample set. GLUT1 is a gene which is upregulated in response to hypoxia (Semenza, 2012); after 8h this is increased 5.67 fold. By 24h the gene has been downregulated but still shows an increase from the normoxic conditions of 2.90 fold. In contrast, Wee1 decreases significantly and by 8h the transcript is 0.37 fold that of starting levels in normoxia (P<0.001). This level of Wee1 remains fairly constant up to 24h exposure to hypoxia.

In hypoxic conditions the transcription factor HIF-1α regulates the transcription of many genes in order for the cells to adapt to the microenvironment (Semenza, 2012). This regulation can involve induction or repression; therefore it is possible that HIF-1α is playing a role in Wee1 downregulation (Eltzschig et al., 2005; Manalo et al., 2005; Narravula and Colgan, 2001). In order to test this RKO cells with loss of HIF-1α or able to express HIF-1α were exposed to 0.1% O_2 as indicated (Figure 3.2B). Transcript levels for GLUT1 and Wee1 were then determined again using 18S as a control and the normoxic sample for $HIF^{+/+}$ cells as a reference. GLUT1 is known to be regulated by HIF-1α so as expected GLUT1 levels increase in RKO $HIF^{+/+}$ cells 4.93 fold by 8h and follow the same pattern as in Figure 3.2A. However in RKO $HIF^{-/-}$ cells the level of GLUT1 in normoxic samples was already lower than RKO $HIF^{+/+}$ cells at 0.92 fold (Figure 3.2B). This decreased further to 0.46 fold by 24h in hypoxia. In contrast, there does not seem to be a dramatic effect on Wee1 transcript level due to HIF-1α knockout. There is a decline seen in $HIF^{+/+}$ cells at 24h to 0.41 fold with a similar decrease seen at the earlier time point of 16h in RKO $HIF^{-/-}$ where the fold change is 0.53. Overall Wee1 mRNA levels stay relatively constant in hypoxia although levels do decrease slightly; this correlates with a mild decrease in protein levels.

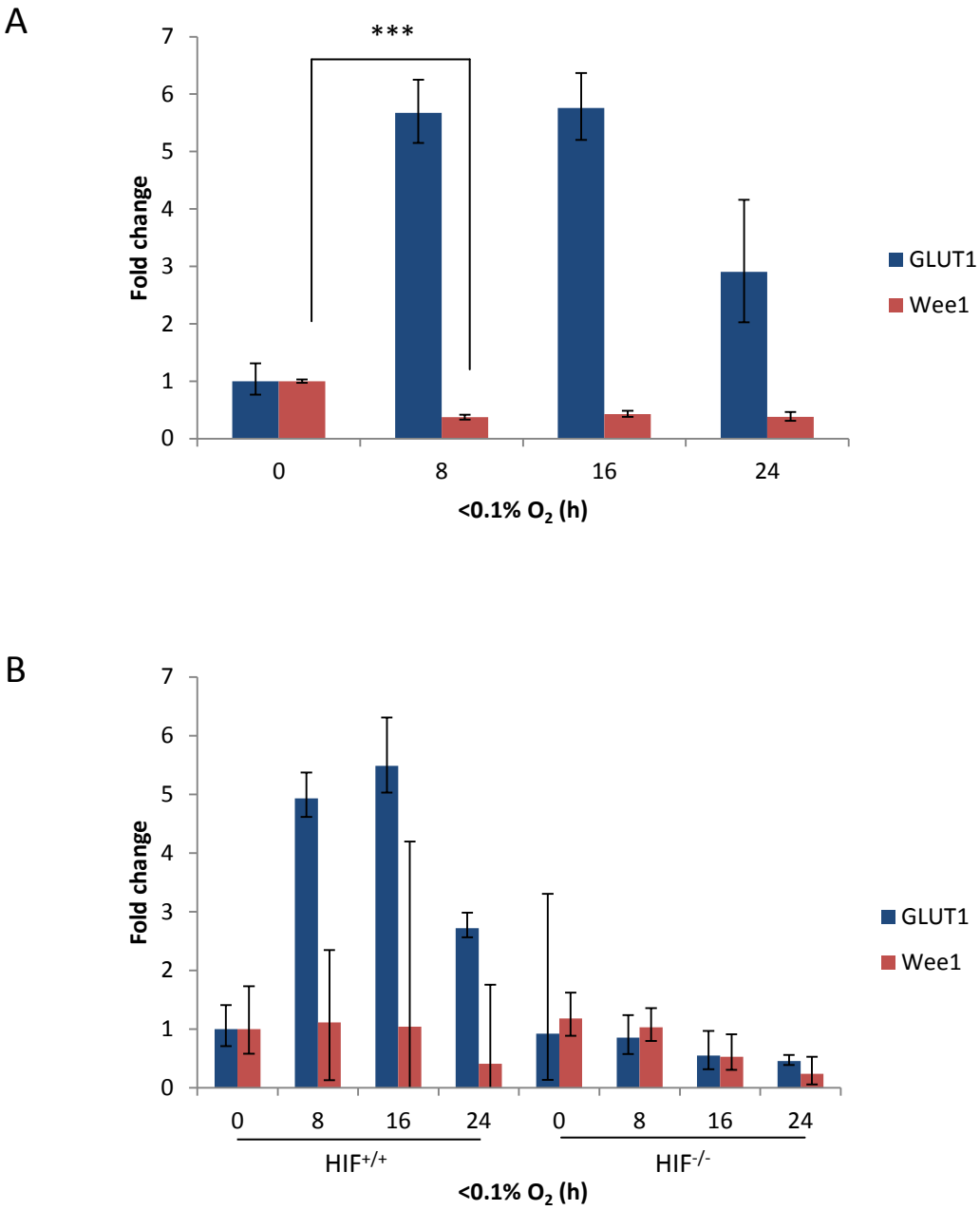


Figure 3.2. Wee1 is decreased in severe hypoxia independent of HIF activity. (A) H1299 cells were cultured in severe hypoxia (<0.1% O_2) for 0, 8, 16 or 24h. cDNA was analysed by qPCR for Glut1 and Wee1. Normoxic sample was used as an endogenous control and 18S primers were used as a reference. n=2 (B) RKO $HIF^{+/+}$ or $HIF^{-/-}$ cells were cultured and analysed by qPCR as in (A). Normoxic $HIF^{+/+}$ sample was used as an endogenous control and 18S primers were used as a reference. Significant difference between means using a paired t test is shown by a triple asterisk (*P*<0.001). Error bars represent three technical repeats. n=1

3.3 Chk1 potentially contributes to signalling to Wee1 in hypoxia

The upstream signalling to Wee1 in mammalian cells is still unknown although Chk1 has been proposed to be involved (Lee et al., 2001; O'Connell et al., 1997; Perry and Kornbluth, 2007; Yu et al., 1998). To examine this signalling in hypoxia a Chk1 inhibitor, Gö6976, as well as siRNA against Chk1 was used to inhibit its activity.

Figure 3.3A shows H1299 cells treated with Gö6976 (200nM) in hypoxia (0.1% O_2) as indicated with corresponding time points treated with DMSO as a control. Both total and phosphorylated forms of Wee1 are decreased when Gö6976 is added showing that inhibition of Chk1 decreased Wee1 levels and activation. The phosphorylation site on Wee1 (serine 642) corresponds to the site in *Xenopus* reportedly phosphorylated by Chk1 (ser549) (Lee et al., 2001; Perry and Kornbluth, 2007). Taken together these findings imply Chk1 may play a role in phosphorylating Wee1 in mammalian cells. In accordance with this Chk1 inhibitor also decreased the level of phosphorylated Cdc2 at tyr15; this is the site on Cdc2 phosphorylated by Wee1 (Domingo-Sananes et al., 2011). As a positive control for the induction of damage, cells were treated with Adriamycin (Adr) (2μM) for 6h which causes DSBs. This helps elucidate effects due to Chk1 inhibition rather than DNA damage in general. Total Wee1 and phosphorylated Wee1 were decreased compared to initial levels upon addition of Adriamycin, however exposure to hypoxia for 6h may be the actual reason for this depletion. The phosphorylation of Cdc2 is increased with Adriamycin supporting a role for this protein in the G_2 checkpoint response for repair of DNA damage (Domingo-Sananes et al., 2011).

However, results using Chk1 siRNA contradict results obtained using chemical inhibition of Chk1. RKO cells were either transfected with Chk1 siRNA (25nM) or mock transfected then exposed to hypoxia (0.1% O_2) for the indicated periods. A negative control was obtained using scramble siRNA (50nM) to show any changes in protein levels are due to Chk1 siRNA not the transfection process. As seen in Figure 3.3B, Wee1 and phosphorylated Wee1 did not change in expression across the samples, which is surprising considering results shown in Figure 3.3A and the previous data illustrating Wee1 is decreased in hypoxia (Figure 3.1B). However, siRNA transfected cells display a decrease in phospho-Cdc2(tyr15) compared to mock transfected controls, since this is the main target of Wee1 this potentially shows Chk1 depletion may have some effect on Wee1. However, it is unwise to extrapolate

these results since Chk1 may be signalling via another kinase to have effects on Cdc2 phosphorylation. Thus the upstream signalling to Wee1 may involve Chk1 but from these contradicting results a final conclusion cannot be drawn.

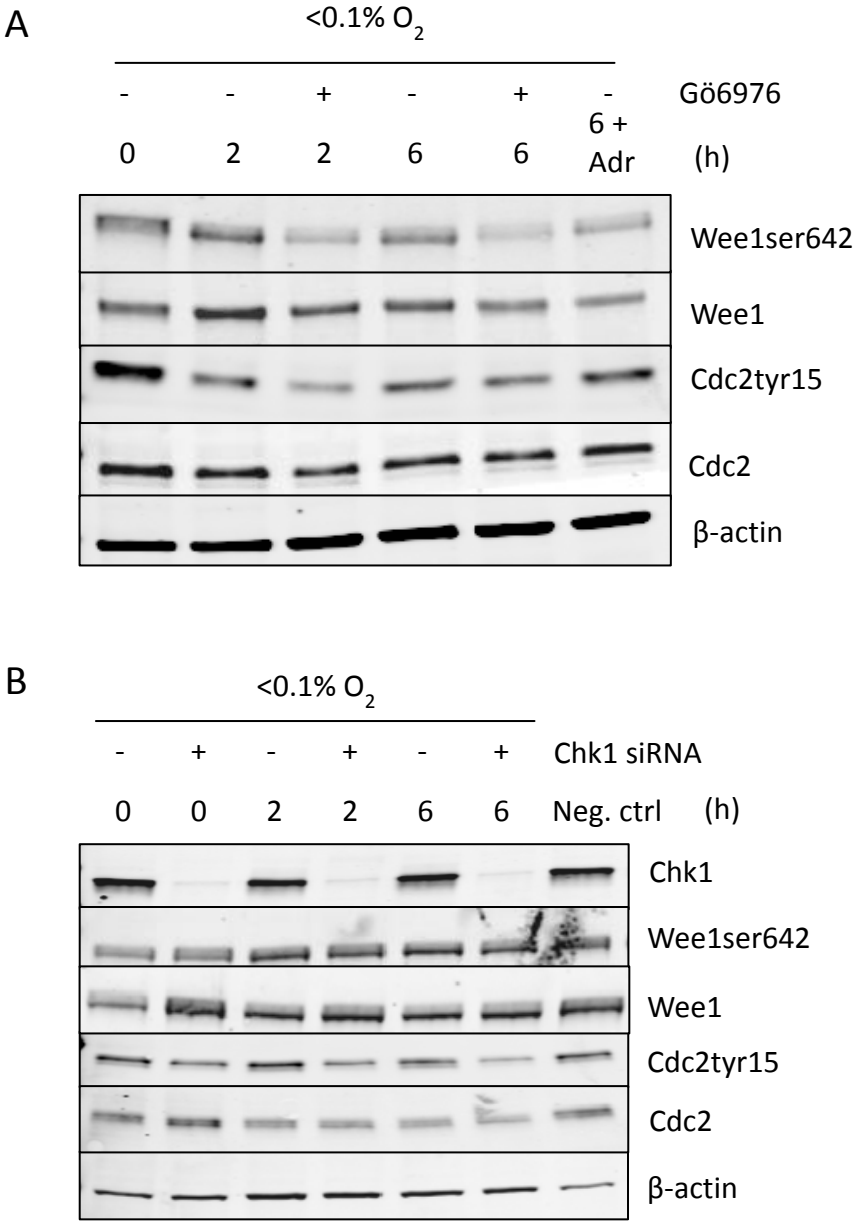


Figure 3.3. Wee1 is a potential Chk1 target in hypoxia. (A) H1299 cells were treated with the Chk1 inhibitor Gö6976 (200nM) (+) or DMSO (-) whilst in severe hypoxia for 0, 2 or 6h. Western blotting was carried out for Wee1 and its target Cdc2. Adriamycin (2μM) for 6h was used as a positive control for induction of the DDR. (B) RKO cells were transfected with siRNA for Chk1 (25nM) or mock transfected for 0, 2 or 6h. Scramble siRNA (50nM) for 6h was used as a negative control for Chk1 depletion. Western blotting for Chk1, Wee1 and Cdc2 proteins are shown. β-actin was used as a loading control.

3.4 Wee1 inhibitors induce damage in both transformed and non-transformed cells

There are currently 3 Wee1 inhibitors used in preclinical studies and these have been shown to decrease phosphorylation of Cdc2 at tyr15 to abrogate the G_2 arrest causing premature mitotic entry (De Witt Hamer et al., 2011). If cells are damaged, which is often the case in cancer which is highly genetically unstable, or treated with agents causing DNA damage premature mitotic entry can lead to mitotic catastrophe and cell death (De Witt Hamer et al., 2011). However, Wee1 inhibitors have also been shown to induce DNA damage as monotherapy (Kreahling et al., 2011; Murrow et al., 2010). The effects of these inhibitors on transformed (H1299) and non-transformed (WI-38) lung cells in normoxic conditions were investigated in Figure 3.4 to show their efficacy as well as potential issues with toxicity in healthy tissue.

H1299 cells were treated with either PD0166285 (0.5μM), Wee1 inhibitor II (10μM) or MK1775 (0.2μM) in normoxic conditions. All three inhibitors decreased phospho-Cdc2(tyr15) suggesting they successfully inhibited Wee1. This occurred as early as 6h for PD0166285 whereas MK1775 was the least potent taking 16h to deplete phospho-Cdc2(tyr15) to a similar level; however depletion was maintained up to 24h by all inhibitors (Figure 3.4A). These results may be due to PD0166285 being the least specific inhibitor and its inhibition of both Wee1 and Myt1, which phosphorylate Cdc2 at this site. Interestingly, total Cdc2 levels were decreased for all Wee1 inhibitors during the time course suggesting the proteins stability was affected. This could be due to Wee1 inhibition or an effect caused by a decrease in Cdc2 phosphorylation.

All 3 inhibitors showed an increase in the phosphorylation of histone H2AX known as γH2AX. This is a marker of DNA damage and levels were increased by just 2h treatment with PD0166285, again suggesting it has a high potency, whereas MK1775 took 6h to induce γH2AX and Wee1 inhibitor II appears to be the least toxic taking 16h (Figure 3.4A). The positive control using Adriamycin showed induction of γH2AX as expected. This data supports results obtained in other studies showing the mechanism of action of Wee1 inhibitors and that they are able to induce DNA damage.

Currently the only Wee1 inhibitor to have entered human trials is MK1775 so this inhibitor was chosen for further study in non-transformed human cell lines (Figure

3.4B) (De Witt Hamer et al., 2011). WI-38 cells were treated with MK1775 (0.2μM) with corresponding time points treated with DMSO as a control. Wee1 inhibition was successful in decreasing the phosphorylation of Cdc2(tyr15) after just 2h exposure to the drug and this effect was maintained until 24h. Similar to results shown in Figure 3.4A, the expression of total Cdc2 protein was also decreased by Wee1 inhibition. γH2AX was increased by 2h treatment with MK1775 and was dramatically increased by 6h. This indicates these agents have toxic effects on healthy cells which need to be considered for dosing and treatment schedules.

To ensure these effects were due to inhibition of Wee1 rather than off target effects, it was attempted to knockdown Wee1 expression using siRNA. Optimisation for the use of siRNA is described in the methods section and shown in Figure A1 however, sufficient knockdown of Wee1 was never achieved as illustrated with blots for total Wee1 protein and phospho-Cdc2(tyr15), the Wee1 target.

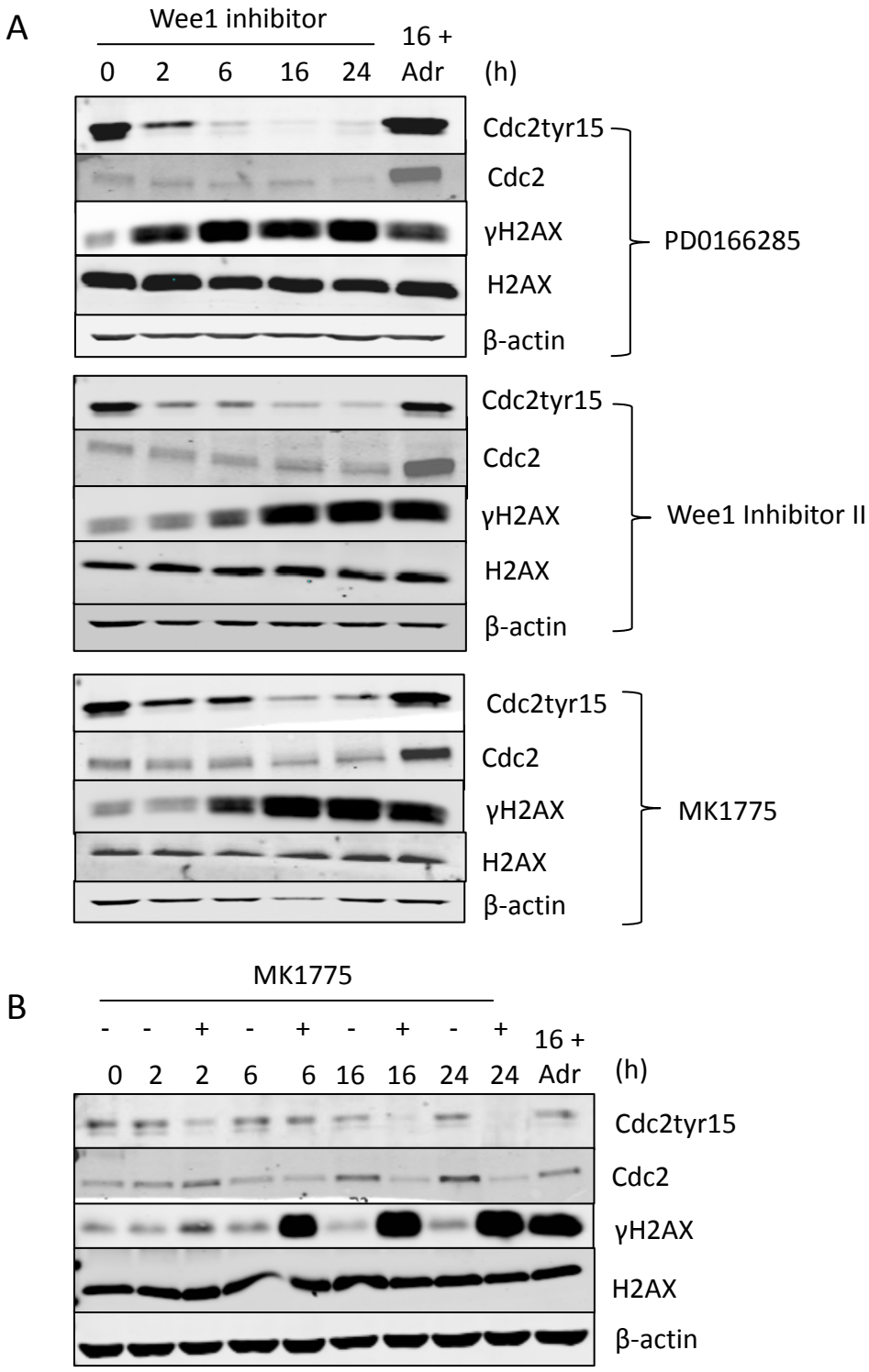


Figure 3.4. Wee1 inhibition leads to DNA damage in transformed and non-transformed cells. (A) H1299 cells were treated with Wee1 inhibitors (PD0166285 0.5μM, Wee1 Inhibitor II 10μM, MK1775 0.2μM) for 0, 2, 6, 16 or 24h. Western blotting was used to show levels of Cdc2 proteins and γH2AX. (B) Western blot to show phosphorylated Cdc2 and γH2AX in WI-38 cells treated with MK1775 (0.2μM) (+) or DMSO (-) for 0, 2, 6, 16 or 24h. Adriamycin (2μM) for 16h was used as a positive control for damage in all experiments. Experiments conducted in normoxia (20% O_2). β-actin was used as a loading control.

3.5 Wee1 inhibition is independent of p53 status

Preclinical data presents conflicting results on whether Wee1 inhibitor efficacy is dependent on p53 status (Aarts et al., 2012; Bridges et al., 2011; Hirai et al., 2009; Li et al., 2002; Mir et al., 2010; PosthumaDeBoer et al., 2011; Rajeshkumar et al., 2011; Sarcar et al., 2011; Wang et al., 2001). Previous reports suggest p53-deficient cells are unable to arrest in G_1 therefore rely on the G_2 checkpoint to repair DNA damage and are more sensitive to Wee1 inhibition (De Witt Hamer et al., 2011). However, p53 wildtype cells have both cell cycle checkpoints functional so even when Wee1 is inhibited the cell is still able to repair damage at the G_1 checkpoint before entering mitosis, therefore will evade mitotic catastrophe and Wee1 inhibitors lose their efficacy (De Witt Hamer et al., 2011). On the other hand some studies show Wee1 inhibition is able to decrease tumour cell viability and sensitise cells to both chemotherapy and radiotherapy independent of p53 status (Mir et al., 2010; PosthumaDeBoer et al., 2011; Sarcar et al., 2011).

All experiments in this study investigating Wee1 inhibitors up to this point were done in H1299 cells which are p53 null so comparison of different p53 backgrounds had not been addressed. To answer this question over p53 status, HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with MK1775 (0.2μM) for the indicated times under normoxic conditions. As shown in Figure 3.5, phosphorylation of Cdc2(tyr15) was decreased in both cell lines and at the early time point of 2h drug treatment. This suggests Wee1 inhibition was successful in preventing Wee1-mediated phosphorylation of Cdc2 independent of p53 status. This has been described before since p53 status does not affect the DDR signalling but rather the efficacy of Wee1 inhibition against cancer cells (Hirai et al., 2009). Data also shows an increase in γH2AX with increasing exposure to MK1775, indicating that Wee1 inhibition is able to cause DNA damage in both p53 backgrounds. Overall these results show both checkpoint and DNA damage signalling are affected by Wee1 inhibition in p53 wildtype cells suggesting they are efficacious in these cells. However, previous reports show these molecular changes did not correlate with the drugs ability to abrogate the G_2 arrest and promote mitotic entry in p53 wildtype cells; this needs to be elucidated in future work.

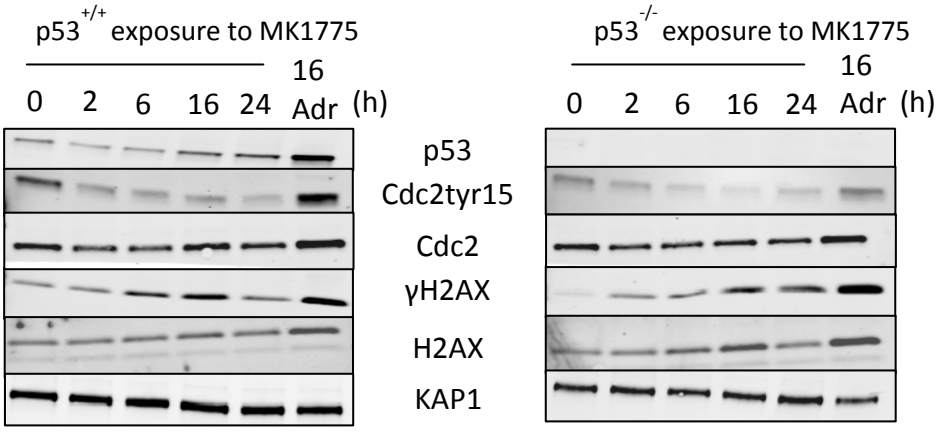


Figure 3.5. Wee1 inhibition induces DNA damage independent of p53 status. HCT116 cells with or without knockdown of p53 were treated with MK1775 (0.2μM) for 0, 2, 6, 16 or 24h. Western blotting indicates levels of p53, phosphorylated Cdc2, γH2AX and total protein levels. Adriamycin (2μM) for 16h was used as a positive control for damage. Experiments conducted in normoxia (20% O_2). KAP1 was used as a loading control.

3.6 Wee1 inhibitors damage tumour cells in both normoxic and hypoxic conditions

Although Wee1 inhibitors have been shown to be active in normoxic conditions no studies have examined their effects in hypoxic conditions. Due to their ability to sensitise cells to various DNA damaging therapies, and the fact that hypoxic cells are particularly resistant to both chemo- and radiotherapy, the potential for Wee1 inhibitors to enhance sensitivity to therapy in these cells is promising (Bristow and Hill, 2008; De Witt Hamer et al., 2011).

To look into effects of Wee1 inhibitors in hypoxia H1299 cells were treated with MK1775 (0.2μM) for the indicated durations with corresponding time points treated with DMSO as controls. These cells were exposed to either normoxia (20% O_2) or hypoxia (1% O_2) for the duration of drug treatment (Figure 3.6A). In both oxygen concentrations phospho-Cdc2(tyr15) was decreased upon treatment with Wee1 inhibitor illustrating the drug was active in these conditions. Phospho-Cdc2(tyr15) was also decreased by MK1775 when cells were treated in severe hypoxia (0.1% O_2) providing further support for the potential effective use of Wee1 inhibitors in a range of oxygen tensions (Figure 3.6B).

Furthermore damage is induced by MK1775 independently of oxygen tension since γH2AX signalling does not differ between 20% and 1% O_2 (Figure 3.6A). This shows Wee1 inhibitors may have efficacy in resistant areas of tumours which is promising for improving therapy of hypoxic cells. γH2AX signalling was also detected in severe hypoxia (0.1% O_2) however in this case γH2AX is induced anyway due to the hypoxia-induced DDR (Olcina et al., 2010). This means it is not able to determine whether Wee1 inhibition is able to induce damage in this level of hypoxia due to masking of the signal, a better readout may be 53BP1 which indicates damage but will not be affected by hypoxic signalling.

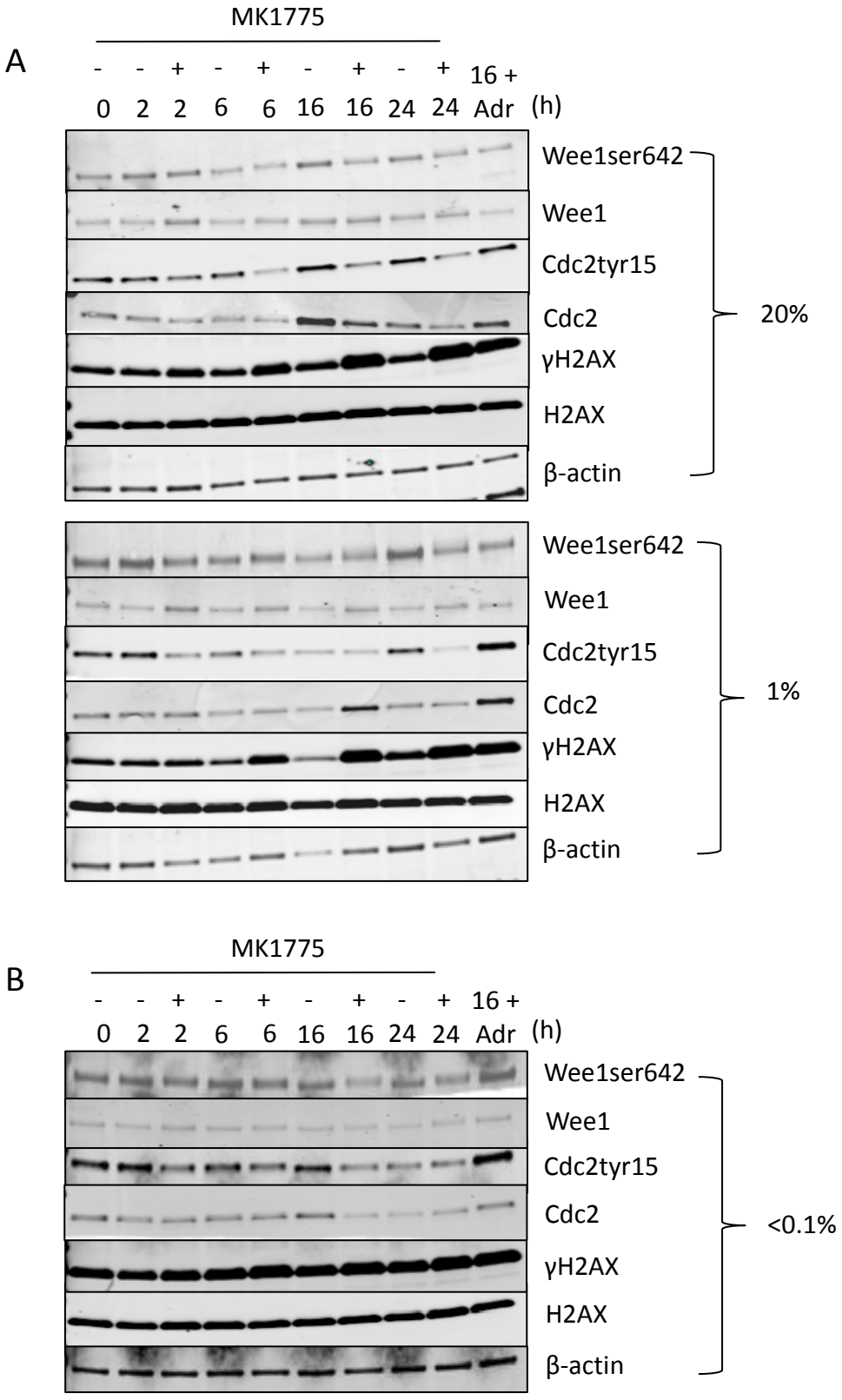


Figure 3.6. MK1775 is active in a range of oxygen concentrations. (A) H1299 cells were treated with MK1775 (0.2μM) (+) or DMSO (-) for 0, 2, 6, 16 or 24h under normoxic (20% O_2) or hypoxic (1% O_2) conditions. The same experiment was conducted in severe hypoxia (<0.1% O_2) in (B). Western blotting was carried out for phosphorylated Wee1, Cdc2 and γH2AX. Adriamycin (2μM) for 16h was used as a positive control for damage. β-actin was used as a loading control.

3.7 Wee1 inhibition affects the cell cycle profile in normoxia and hypoxia

To further investigate the effects of Wee1 inhibition in a range of oxygen concentrations, FACS was used to analyse changes in the cell cycle. Wee1 inhibition removes the inhibitory phosphorylation on Cdc2 allowing it to form a complex with cyclin B1 to promote mitotic entry (Domingo-Sananes et al., 2011). Since Figure 3.6 shows Wee1 inhibition correlated with a decrease in phospho-Cdc2(tyr15) in both normoxia and hypoxia, we investigated whether this molecular change translated into changes in the cell cycle.

Figure 3.7 shows data from H1299 cells exposed to normoxia (20% O_2), mild hypoxia (1% O_2) or severe hypoxia (0.1% O_2) and treated with PD0166285 (0.5μM) or DMSO control for the times indicated. In normoxia and mild hypoxia as early as 2h drug treatment the G_2 population was diminished and there was an increase in G_1 population, this effect was still seen after 24h. This matches effects of this drug seen in Figure 3.4 where phospho-Cdc2(tyr15) is decreased at 2h and this activation of Cdc2 explains the cell cycle effects seen. In severe hypoxia (0.1% O_2) the G_2 population was also decreased to 0% after just 2h PD0166285 and this was still seen at 24h. However, even in the control sample a decrease in the G_2 population is seen at 24h, potentially because this level of hypoxia induces a replication arrest so cells are unable to progress through the cell cycle and enter G_2 (Pires et al., 2010b).

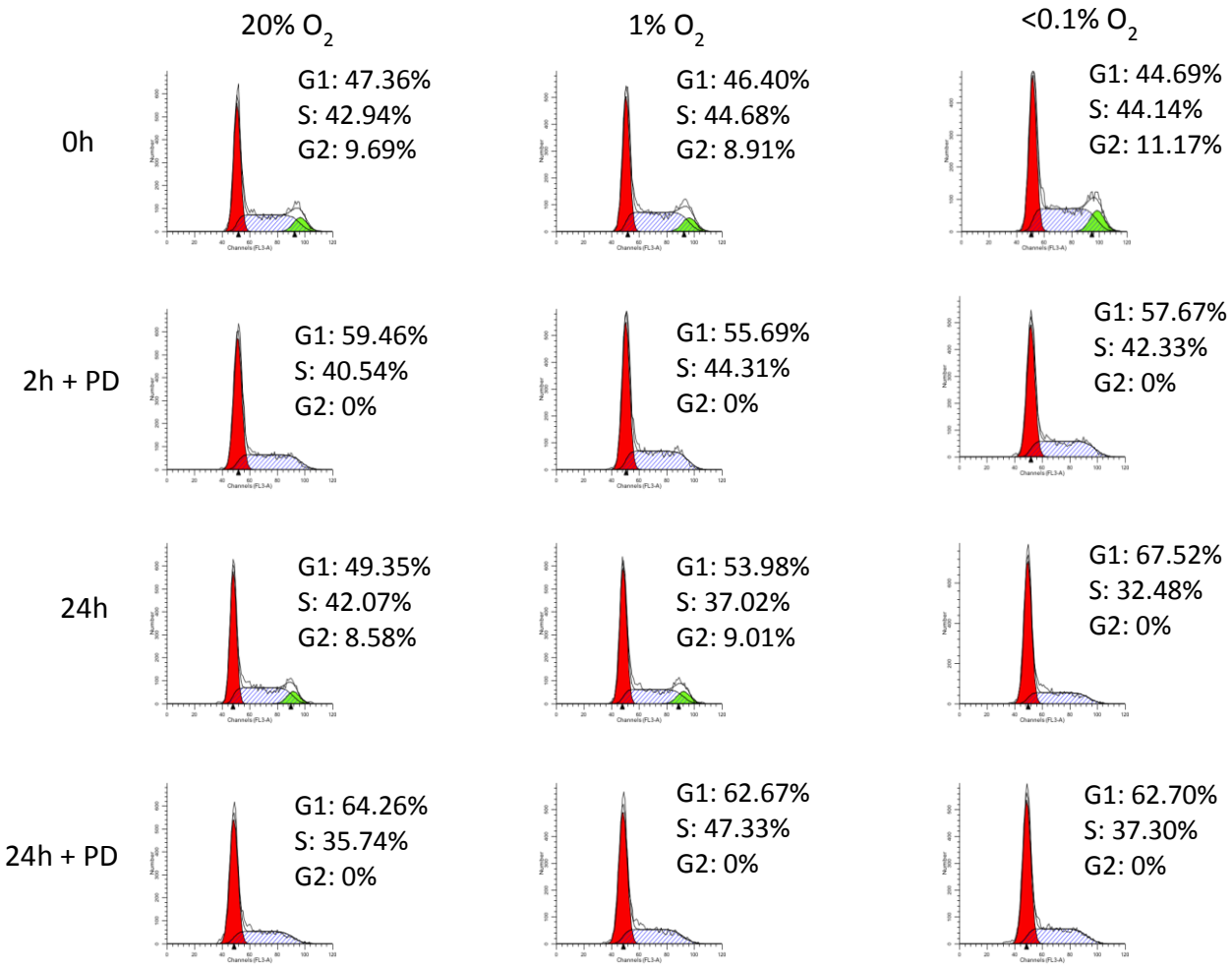


Figure 3.7. Wee1 inhibitors affect the cell cycle profiles of normoxic and hypoxic cells. H1299 cells were exposed to PD0166285 (0.5μM) or DMSO for 2 and 24h in normoxia (20% O_2), hypoxia (1% O_2) or severe hypoxia (<0.1% O_2). FACS analysis was used to calculate the percentage population in each cell cycle phase, this is indicated beside each profile.

3.8 Wee1 inhibition promotes premature mitotic entry in normoxia and hypoxia

In order to investigate the effects of the decline in G_2 population, the mitotic population was quantified for the same three oxygen tensions as in Figure 3.7. In Figure 3.8 MK1775 (0.2μM) was administered to H1299 cells for the indicated times and cells were cultured in normoxia (20% O_2), mild hypoxia (1% O_2) or severe hypoxia (0.1% O_2) for these same time points. All oxygen tensions show an increase in mitotic population after 2h MK1775 (2.15% increase from 0h for normoxia, 1.55% for 1% O_2, 2.01% for 0.1% O_2). This supports the mechanism of action of these agents in promoting premature mitotic entry via Cdc2 activation. In normoxia and mild hypoxia the mitotic population has increased further by 6h MK1775 although there is a slight decrease by 24h MK1775 in normoxia but this is not seen in mild hypoxia where the mitotic population continues to increase. Similar to Figure 3.7, any effects caused by Wee1 inhibition in severe hypoxia (0.1% O_2) may be masked by the replication arrest this lack of oxygen can produce. Although there is an increase in mitotic population after 2h MK1775 at this oxygen concentration, by 6h treatment the population has decreased back to initial levels and by 24h the population is less than the population at 0h. Again this can be attributed to the inability of cells to progress through the cell cycle further than S phase, thus they cannot be affected by the loss of G_2 arrest and enforcement of premature mitotic entry by Wee1 inhibition.

Overall this data suggests the mechanism of action of Wee1 inhibitors is functional in both normoxia and mild hypoxia. There is also evidence for its ability to work in severe hypoxia, however this is more difficult to determine due to effects of hypoxia on the cell cycle masking effects caused by Wee1 inhibition.

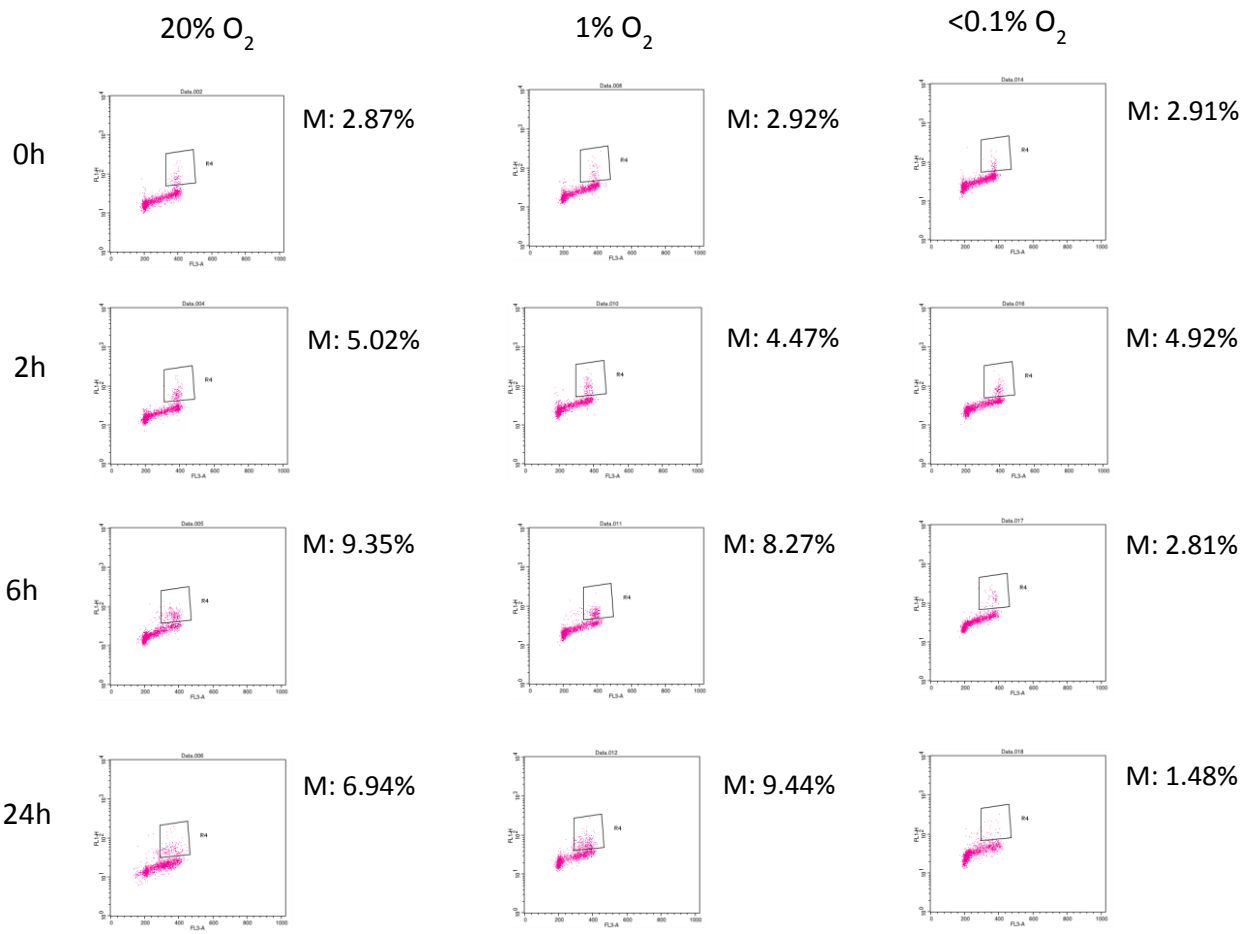


Figure 3.8. Wee1 inhibitors promote premature mitotic entry. FACS analysis with pH3ser10 staining was used to calculate the mitotic population of H1299 cells treated with MK1775 (0.2μM) for 0, 2, 6 or 24h in normoxia (20% O_2), hypoxia (1% O_2) or severe hypoxia (<0.1% O_2). Percentage of cells in mitosis indicated adjacent to each profile.

3.9 Wee1 inhibition decreases tumour cell survival in both normoxic and hypoxic conditions

Figure 3.6 illustrated damage signalling was induced by MK1775 in all oxygen tensions tested, but whether this translated into increased cell kill was still unanswered. To address this H1299 cells were grown for colony survivals in normoxia (20% O_2), hypoxia (1% O_2) or severe hypoxia (0.1% O_2) and left to form colonies for 8 days (Figure 3.9). For the same time points cells were treated with MK1775 (0.2μM) or DMSO so any intrinsic effects of different oxygen concentrations was taken into account. For all oxygen concentrations cell viability was decreased when MK1775 was administered. However the difference in cell survival when comparing DMSO treated controls with MK1775 treated cells is greatest in normoxic conditions with a change in surviving fraction of 94.33% (117.17% for 30h DMSO treated vs. 22.84% for 30h MK1775). Mild hypoxia illustrated the next biggest difference of 32.21% after 30h exposure (83.02% with DMSO to 50.81% with MK1775) whereas in severe hypoxia the decrease in survival fraction with MK1775 was 5.09% (40.56% with DMSO to 35.47% with MK1775) (Figure 3.9).

This is a promising result as it suggests Wee1 inhibitors are effective in physiologically relevant conditions and will have effects throughout a tumour. However, it should still be taken into consideration that efficacy does not appear as great in hypoxia as in normoxia. This is only the case for Wee1 inhibitors used as single agents, whether combination therapy of Wee1 inhibitors with chemotherapy or radiotherapy has similar efficacy in normoxic and hypoxic cells needs to be determined.

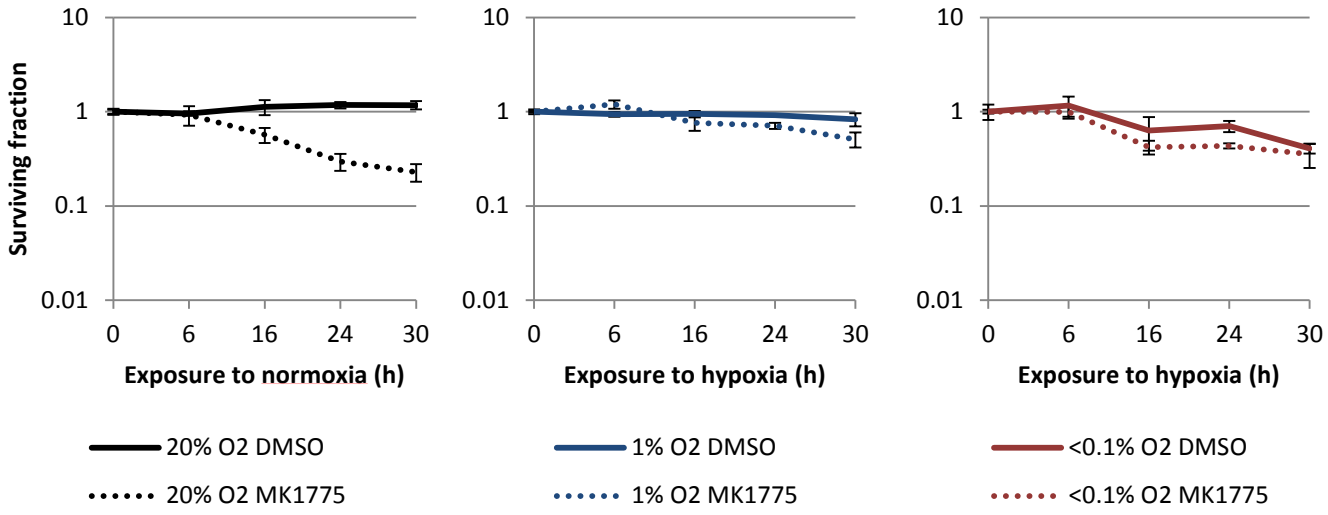


Figure 3.9. MK1775 decreases cell viability in a range of oxygen concentrations. Colony survival for H1299 cells treated with MK1775 (0.2μM) or DMSO for 0, 6, 16, 24 and 30h. Cells were exposed to normoxic (20% O_2), hypoxic (1% O_2) or severely hypoxic (<0.1% O_2) conditions. Error bars represent ± standard error calculated from the standard deviation of a single experiment run in triplicate.

3.10 MK1775 induces cell death in a range of oxygen concentrations

The reason for the decrease in survival illustrated in Figure 3.9 was looked into using morphological methods. Several reports have shown Wee1 inhibition induces mitotic catastrophe and apoptosis has also been detected after Wee1 depletion (Bridges et al., 2011; Kreahling et al., 2011; Sarcar et al., 2011; Wang et al., 2004). HCT116 cells were treated with DMSO as a control or MK1775 (0.2μM) for 6h either in normoxia or hypoxia (0.1% O_2). Cell death was identified by nuclear fragmentation, as shown by the images in Figure 3.10B and D. The percentage of dead cells was then quantified and it was found that upon treatment with Wee1 inhibitor in normoxia this population increased to 25.76% compared to 0% in controls (Figure 3.10A). So the induction of cell death supports the results seen in colony survivals for normoxic cells.

Interestingly, the same pattern was seen in severely hypoxic conditions (0.1% O_2) although the increase in cell death was 7.61% compared to no induction in controls (Figure 3.10C). This agrees with data shown in Figure 3.9 which shows severely hypoxic cells are sensitised less to MK1775 than normoxic cells. Potentially the cell cycle arrest caused by hypoxia prevents cells progressing through the cell cycle thus the mechanism of action of these inhibitors, which is to induce mitotic catastrophe, is unable to take effect. The cell death seen here needs to be characterised further since apoptosis and mitotic catastrophe share the main feature of multinucleation and fragmented nuclei (Caruso et al., 2011). To elucidate whether mitotic catastrophe is occurring other immunohistochemical markers and the incidence of cell death specifically in mitotic cells would need to be analysed.

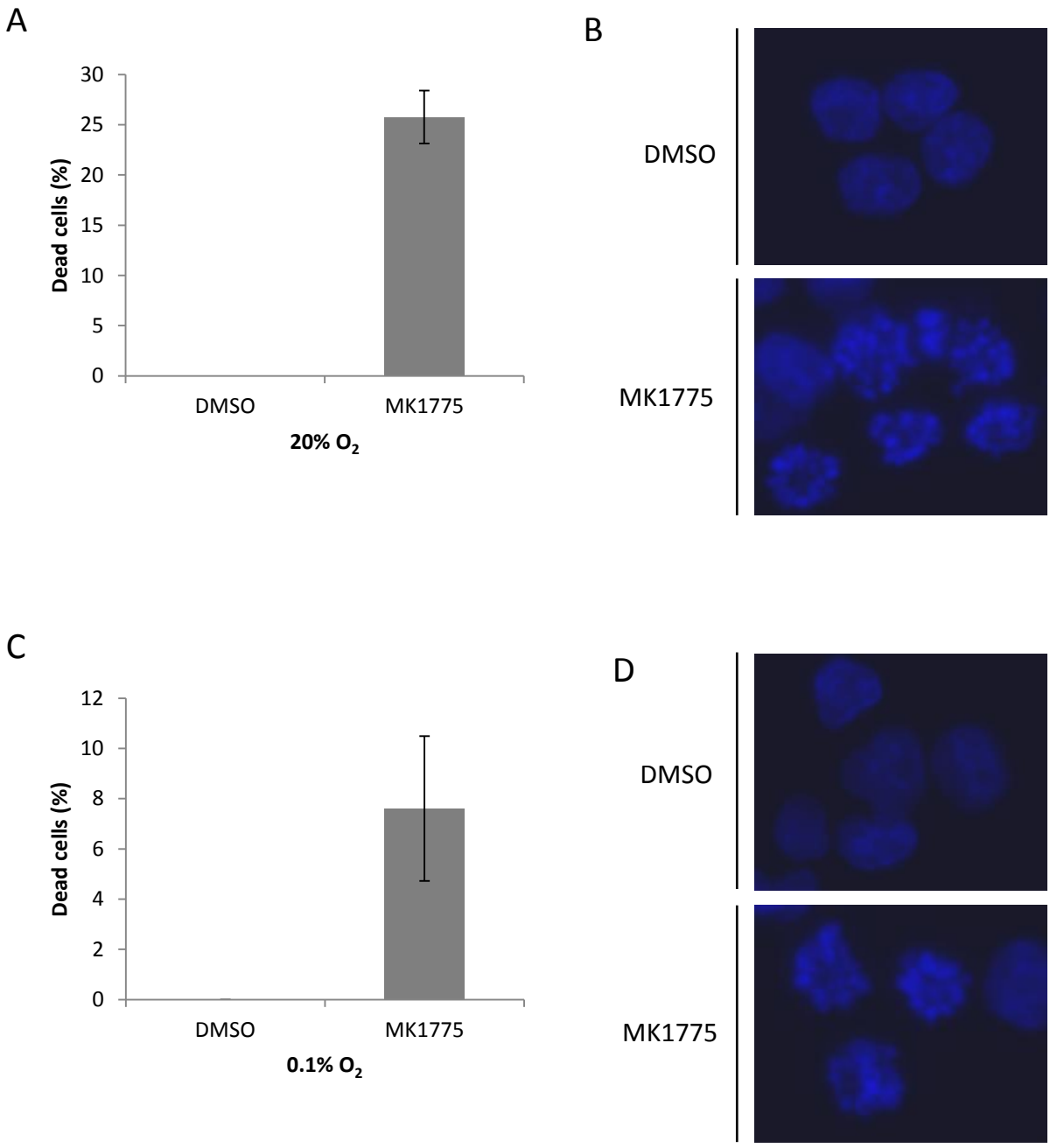


Figure 3.10. MK1775 induces cell death in a range of oxygen concentrations. (A) HCT116 cells treated with DMSO or MK1775 (0.2μM) for 6h in normoxic (20% O_2) conditions. Bar graph illustrates percentage of dead cells visualised by DAPI staining. Error bars represent ± standard error of results from approximately 200 cells and 10 fields of view. (B) Microscope images of HCT116 cells from (A) stained with DAPI. (C) Same experiment as (A) with cells exposed to hypoxic conditions (<0.1% O_2). (D) Microscope images of HCT116 cells from (C).

3.11 Wee1 inhibitors can radiosensitise normoxic and hypoxic cells

Preclinical data implies Wee1 inhibition is most successful when used as combination therapy either with chemotherapy or radiotherapy as it appears to enhance the efficacy of these treatments (De Witt Hamer et al., 2011). Since hypoxic cells are resistant to therapy, whether Wee1 inhibitors are able to sensitise this population is an interesting question, which may lead to improvement in treatment. Data displayed in Figure 3.9 suggests Wee1 inhibitors are slightly less effective in decreasing hypoxic cell viability as they are in affecting normoxic cells. To elucidate whether this is the case when Wee1 inhibitors are used as combination therapy colony survivals were set up using H1299 cells. The day after cells were seeded, cells were treated with MK1775 (0.2μM) or DMSO for controls for 2h, for this duration of time cells were grown either in normoxia (20% O_2), hypoxia (1% O_2) or severe hypoxia (0.1% O_2). Cells were then treated with γ-irradiation whilst being maintained at the oxygen tension they were originally cultured in; cells grown in hypoxic chambers were transported and irradiated in sealed perspex boxes. This methodology was validated previously (Pires et al., 2012). These boxes were not opened until ten minutes after irradiation had been administered in order to allow any signalling to take effect before reoxygenation. Plates were then left in normoxic conditions for 8 days.

Figure 3.11A shows increasing doses of irradiation decreased cell viability. The survival of cells was decreased by Wee1 inhibition compared to DMSO controls for all irradiation doses tested. This implies Wee1 inhibitors are able to radiosensitise cells in a range of oxygen tensions relevant to tumour cells. The sensitivity enhancement ratio (SER) for each condition was calculated for cells irradiated with 2Gy and is shown in Figure 3.11B, also illustrated is the percentage surviving fraction of MK1775 treated cells relative to DMSO controls. The SER was calculated by dividing the surviving fraction of cells treated with DMSO by the surviving fraction of cells treated with MK1775 for a particular dose of irradiation; values greater than 1 show sensitisation was achieved and less than 1 show no sensitisation. The bar graph shows a similar decrease in cell viability is seen in all oxygen tensions, however the SER at 2Gy is highest for cells grown in 1% O_2 (2.35) with normoxic cells having an SER of 1.83 and severely hypoxic cells (0.1% O_2) having a SER of 1.87. These results suggest both normoxic and hypoxic cells are radiosensitised by Wee1

inhibition and suggests this regimen of combination therapy should be considered in clinical trials.

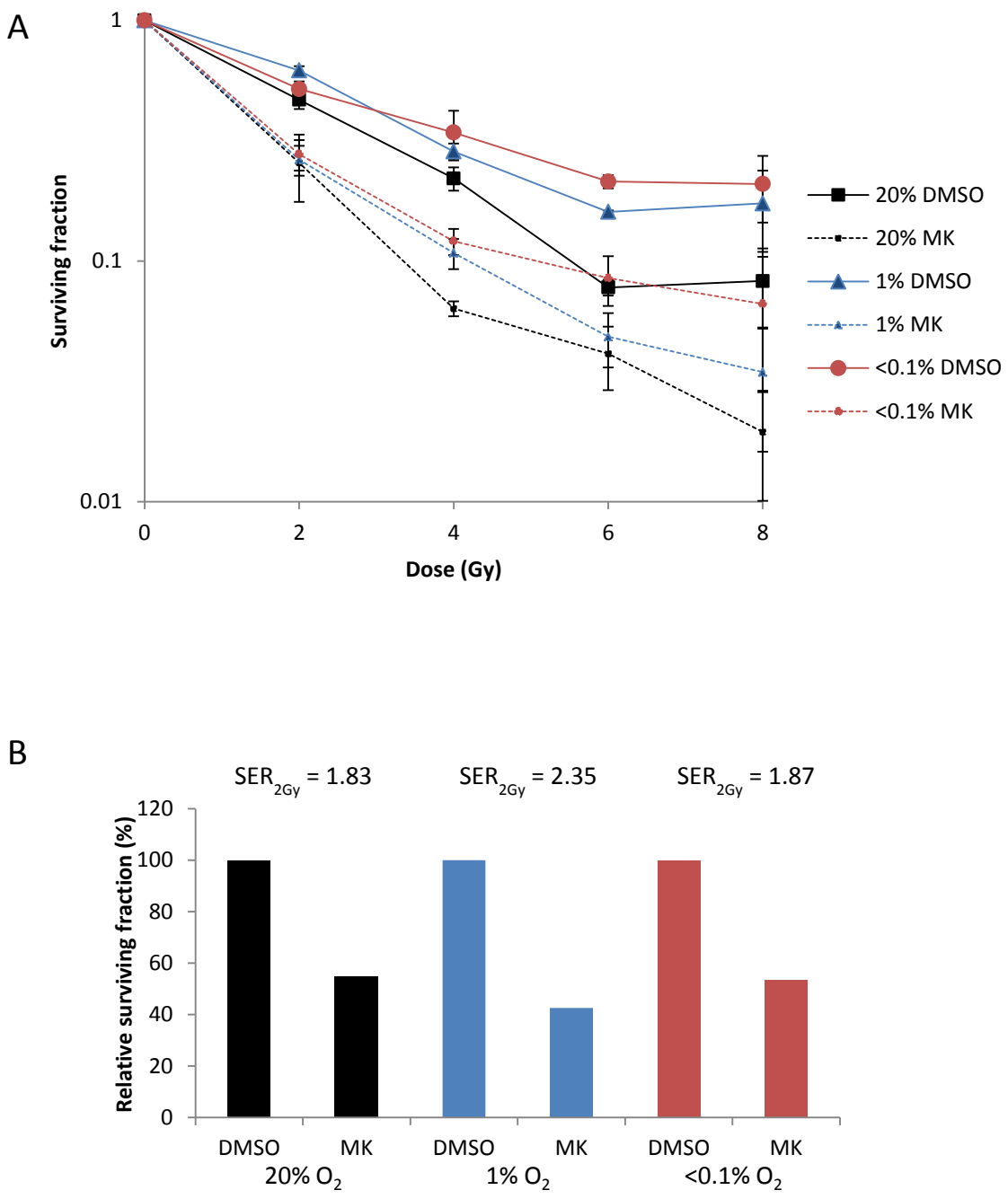


Figure 3.11. MK1775 can radiosensitise hypoxic cells. (A) Colony survival of H1299 cells treated with MK1775 (0.2μM) for 2h followed by irradiation with 0, 2, 4, 6 or 8Gy at the oxygen concentrations indicated. DMSO treated and unirradiated cells used as controls. Error bars represent ± standard error calculated from the standard deviation of a single experiment run in triplicate. (B) Percentage surviving fraction after treatment with 2Gy relative to untreated controls for each oxygen concentration investigated. SER at 2Gy for each oxygen tension tested is also shown.

Chapter 4. Results: Wee1-Mediated Regulation of Mus81

4.1 The Mus81-Eme1 endonuclease

Wee1 has been implicated in the maintenance of genomic stability and its depletion leads to induction of DNA damage (Beck et al., 2010; Dominguez-Kelly et al., 2011; Murrow et al., 2010). Protection of genomic integrity by Wee1 has been suggested to be via its negative regulation of Cdc2 (Cdk1) and Cdk2 in order to maintain cell cycle checkpoints, however more recent evidence proposes its involvement in regulating the structure specific endonuclease Mus81-Eme1 (Beck et al., 2010; Dominguez-Kelly et al., 2011). Eme1 is the binding partner for Mus81 and is essential for endonuclease activity of Mus81 although it is not catalytically active (Osman and Whitby, 2007). *In vitro* the Mus81-Eme1 complex cleaves nicked Holliday junctions, 3'-flap structures and aberrant replication fork structures although little work has been done *in vivo* (Hiyama et al., 2006; Osman and Whitby, 2007). Overall Mus81-Eme1 is involved in cleaving stalled replication forks to prevent the formation of aberrant structures during replication which could cause lethal damage to the cells; in this way the endonuclease preserves genomic integrity and allows replication to continue independent of new origin firing even though it does this by causing transient DSBs itself (Hanada et al., 2007). DSBs are highly toxic lesions so this pathway is likely to be a final resort for cells to overcome stalled replication forks and will be late-acting (Hanada et al., 2007). However, there is still some uncertainty over the exact role of Mus81 in the repair of stalled replication forks. There is evidence for its actions both upstream and downstream of HR machinery; Mus81 may cleave stalled replication forks to initiate HR, or it may cleave recombination intermediates produced by HR processing damaged replication forks (Osman and Whitby, 2007). There is evidence of Rad51, a component of the HR, being activated upstream of Mus81 due to replication stalling. This implies HR may be required to generate the substrate from stalled replication forks for Mus81 to act on, rather than Mus81 cleaving stalled replication forks directly (Hanada et al., 2007).

It has been proposed that Wee1 acts to negatively regulate Mus81 activity in S phase to prevent any unnecessary cleavage of forks and thus it plays a role in maintaining genomic integrity (Dominguez-Kelly et al., 2011). These findings imply that Wee1 inhibition may remove regulatory control from Mus81 thus the damage

associated with these inhibitors is due to inappropriate Mus81 activity; this has been demonstrated with siRNA (Dominguez-Kelly et al., 2011). Further support for Wee1 negatively controlling Mus81 comes from findings showing its potential activating kinase, Chk1, is involved in Mus81 regulation. Cds1, the yeast homolog of Chk1 has been shown to prevent the replisome dissociating from stalled replication forks so in the absence of Cds1 'collapsed' forks with accumulation of aberrant DNA structures results. Without the protection of the replisome these forks are physically accessible to Mus81 (Froget et al., 2008). This has implications for Mus81 control in severe hypoxia since these conditions can result in replisome disassembly from stalled replication forks therefore potentially inappropriate fork cleavage by Mus81 can occur. However, direct interaction between Chk1 and Mus81 has not been seen in mammalian cells *in vivo* suggesting this mechanism may differ from yeast although, mammalian Chk1 does appear to be involved in Mus81 regulation since DNA damage seen with Chk1 inhibitors is due to Mus81-Eme1 cleaving replication forks to produce DSBs (Forment et al., 2011; Froget et al., 2008). Taken together these findings suggest Chk1 and Wee1 are involved in Mus81 regulation, since Chk1 and Wee1 have been implicated in the same signalling pathway this shared activity is not surprising (Figure 4.1). Figure 4.2A illustrates the proposed model for regulation of Mus81 by Wee1; Wee1 either inhibits Mus81-Eme1 directly or via inhibition of Cdk2, this should allow normal replication fork progression and prevent inappropriate Mus81 activity at stalled replication forks which may activate the DDR (Dominguez-Kelly et al., 2011). The regulation of Mus81 by Wee1 was explored in this study.

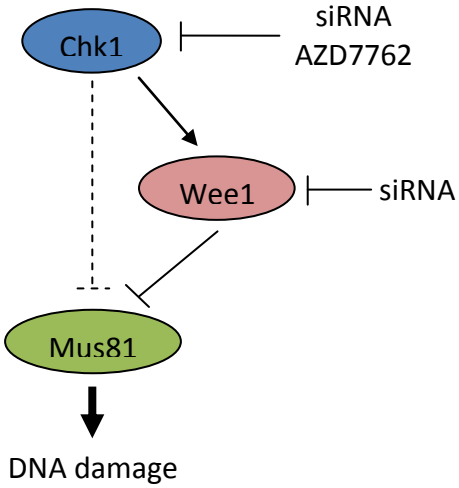


Figure 4.1. Regulation of Mus81. *Diagram to show regulation of Mus81 by Chk1 and Wee1 in mammalian cells. Methods of decreasing Chk1 and Wee1 activity which have been reported to affect Mus81 activity are indicated. Details in text.*

Mus81 has been identified as being necessary for normal growth and viability of cells and its depletion is associated with tumorigenesis (McPherson et al., 2004; Osman and Whitby, 2007; Wu et al., 2011). Mus81 may be important for survival, even in the absence of exogenous damage-inducing agents, due to the frequent aberrations in replication in mammalian cells, although its deletion in germline cells indicates this gene is nonessential for DSB processing in development (Blais et al., 2004; McPherson et al., 2004). Several studies have looked into the role of Mu81 in sensitivity to DNA damage. Mus81-deficient cells were found to be sensitive to DNA crosslinking agents such as Mitomycin C, Cisplatin and bifunctional Nitrogen Mustard (NH2 can form interstrand crosslinks as well as N-alkylpurine adducts) (Dendouga et al., 2005; Hiyama et al., 2006; McPherson et al., 2004). However, only a slight increase or no increase in sensitivity was seen in $Mus81^{-/-}$ cells treated with ionising radiation, UV light or hydroxyurea (Dendouga et al., 2005; Hiyama et al., 2006; McPherson et al., 2004). This shows there is no DSB processing defect in cells lacking Mus81 but they are sensitive to agents causing collapse of replication forks and interstrand crosslinks (Hanada et al., 2007). This would suggest $Mus81^{-/-}$ cells would not be sensitised to hypoxia, however there is evidence of $Eme1^{-/-}$ cells showing decreased viability in hypoxic conditions (Hammond, unpublished data). Whether results are similar for Mus81 knockout cells was investigated in this study.

4.2 Mus81$^{-/-}$ cells are viable and respond to hypoxia

In order to investigate the role of Mus81 in inducing the DNA damage seen when Wee1 is inhibited, the use of knockout cell lines was employed; these included HCT116 Mus81$^{-/-}$ and with Mus81 reintroduced (denoted Mus81$^{-/- + Mus81}$) (K. Miyagawa, University of Tokyo). Figure 4.2B illustrates Mus81 expression is successfully depleted in Mus81$^{-/-}$ cells and when Mus81 is reintroduced it is expressed to a similar extent as in wildtype HCT116 cells. There is a slight induction of γH2AX in both knockout cell lines that may indicate damage induced by their manipulation.

The response to hypoxia in these cells was characterised by exposing HCT116 Mus81$^{-/-}$ and HCT116 Mus81$^{-/- + Mus81}$ to hypoxia (0.1% O_2) for the periods indicated (Figure 4.2C). HIF-1α was induced after 2h hypoxia and was still present after 24h although by this time expression had decreased. There was also an increase in γH2AX levels after 2h hypoxia as expected. These results imply these cells have a normal response to hypoxia and are an appropriate model to use in this study. Total Wee1 protein and phospho-Wee1(ser642) were decreased after 16h in hypoxia in both Mus81$^{-/-}$ and Mus81$^{-/- + Mus81}$ cells, although this decrease was more prominent in knockout cells. This also fits with the hypoxia response seen in other cancer cell lines suggesting the genetic manipulation of the HCT116 cell line has not affected their hypoxic signalling pathways to a great extent.

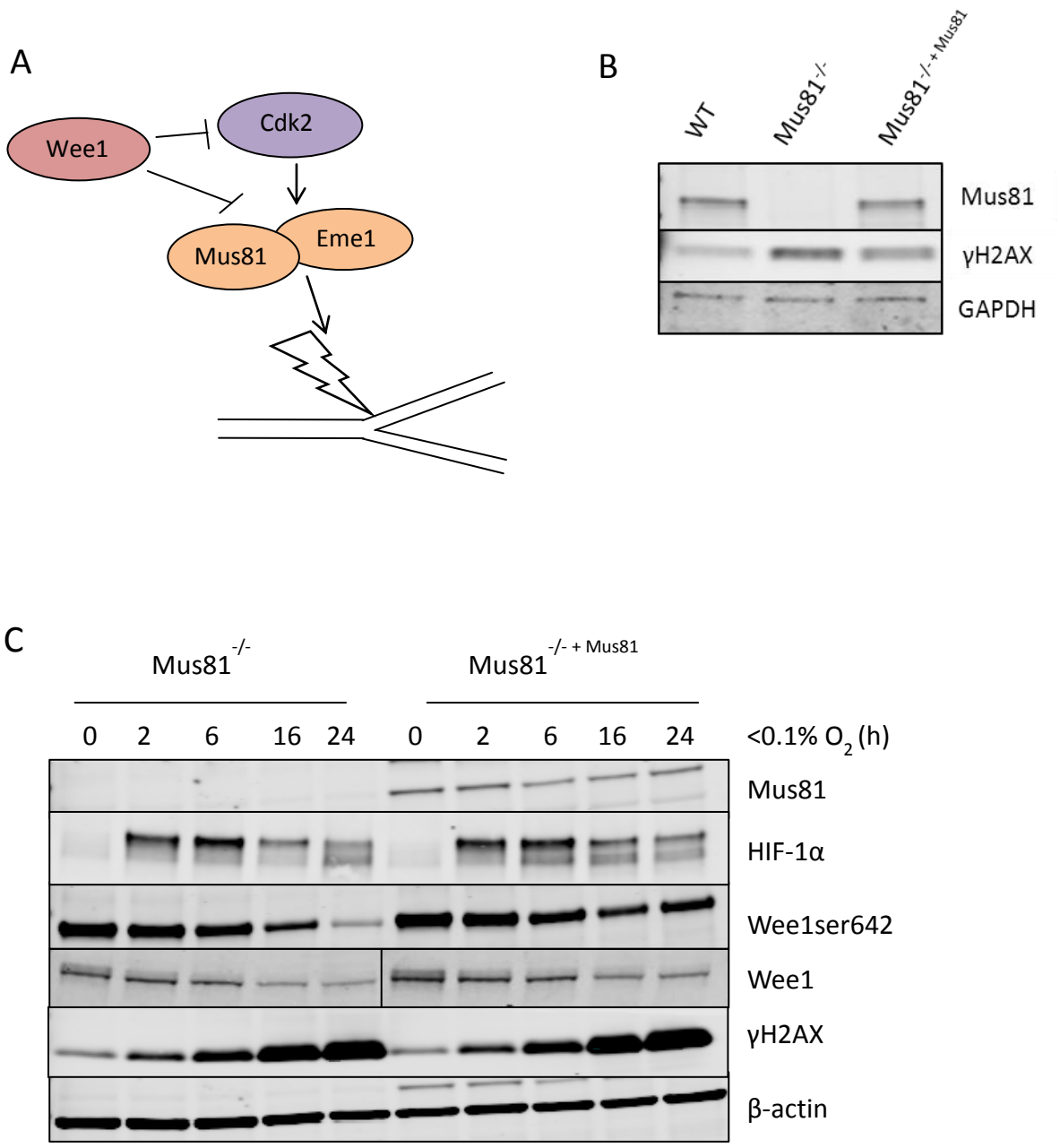


Figure 4.2. HCT116 cell lines are viable with Mus81 knockout. (A) Proposed model of Wee1 regulation of Mus81/Eme1. (B) Western blotting to characterise HCT116 knockout cell lines. (C) Western blot to show the response of HCT116 cell lines to severe hypoxia (<0.1% O_2) exposure for 0, 2, 6, 16 or 24h. β-actin or GAPDH were used as loading controls.

4.3 The role of Mus81 in genomic integrity

Evidence suggests that Wee1 maintains genomic stability via regulation of Mus81-Eme1 and so it can be hypothesised that Mus81 may be involved in producing the DNA damage seen when cells are treated with Wee1 inhibitors (Dominguez-Kelly et al., 2011; Forment et al., 2011). To test this hypothesis, HCT116 Mus81$^{-/-}$ and HCT116 Mus81$^{-/- + Mus81}$ cells were treated with MK1775 (0.2μM) for the indicated times in normoxic (20% O_2) or hypoxic (0.1% O_2) conditions (Figure 4.3). γH2AX is induced mildly in both cell lines by MK1775 in normoxia, this is surprising considering the large induction of γH2AX in H1299 cells treated with this compound (Figure 3.4A, 3.6A). The induction of γH2AX is greater in hypoxic conditions and increases from 2h but this is likely an effect of the severe level of hypoxia rather than the drug. There does not appear to be a difference in damage levels when comparing the two cell lines, this disagrees with our hypothesis which implies cells lacking Mus81 will have less damage than those expressing Mus81 when treated with Wee1 inhibitor. Phospho-Wee1(ser642) levels remain unchanged in cells exposed to normoxia but cells grown in hypoxic conditions show a slight decrease in this protein after 16h which correlates with previous data from this study.

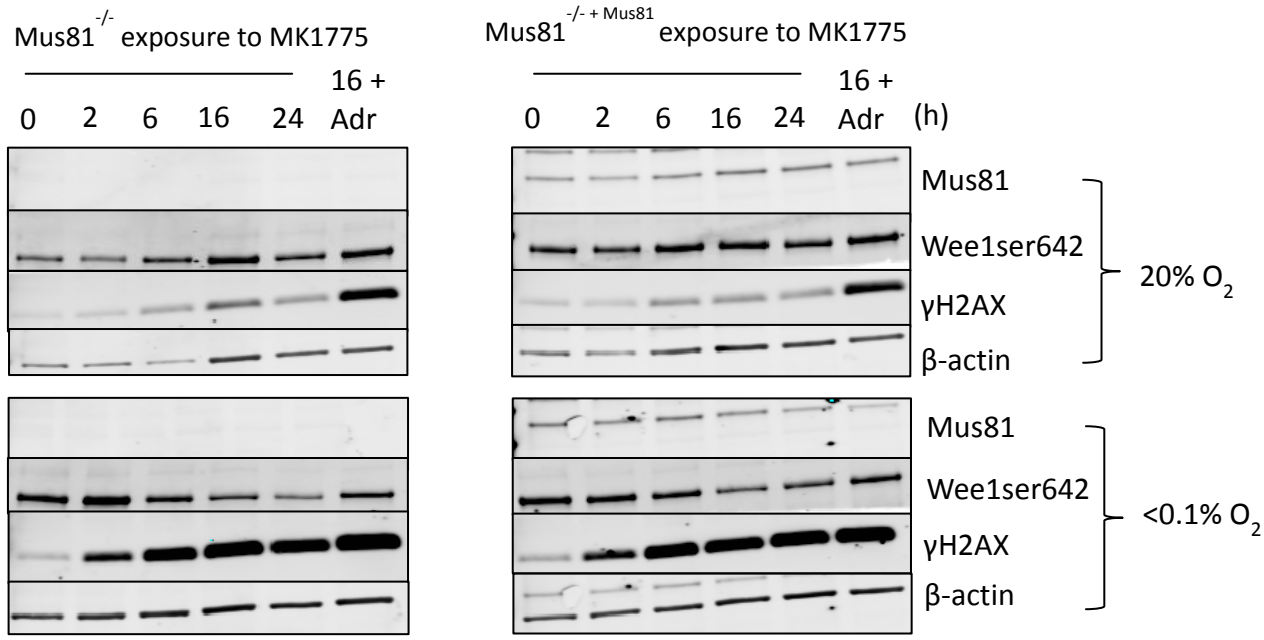


Figure 4.3. The role of Mus81 in genomic integrity. HCT116 Mus81 knockout cells ($Mus81^{-/-}$) or with Mus81 reintroduced ($Mus81^{-/-+Mus81}$) were treated with MK1775 (0.2μM) for 0, 2, 6, 16 or 24h. Western blotting was used to show Mus81, phosphorylated Wee1 and γH2AX levels. Adriamycin (2μM) for 16h was used as a positive control for damage. β-actin used as a loading control.

4.4 Loss of Mus81 does not increase sensitivity to hypoxia or reoxygenation

It was decided to investigate the role of Mus81 in tumour cell survival further by examining the effects of hypoxia and γ-irradiation on Mus81 knockout cells. Firstly a colony survival assay was carried out using HCT116 and HCT116 Mus81$^{-/-}$ cells grown in hypoxia (0.1% O_2) for the indicated times then left to form colonies for 10 days (Figure 4.4A). Results from this experiment show loss of Mus81 did not increase sensitivity to hypoxia since the surviving fraction for both cell lines are very similar. The same cell lines were also treated with the stated doses of γ-irradiation and allowed to form colonies for 10 days; again neither cell line showed increased cell viability over the other suggesting loss of Mus81 does not sensitise these cells to γ-irradiation (Figure 4.4B). This fits with previous findings in yeast and mammals showing Mus81 depletion sensitises cells to agents that collapse replication forks but not those that induce DSBs, such as γ-irradiation (McPherson et al., 2004). Similarly, hypoxia leads to replication fork stalling but collapse of forks is not seen unless components of the DDR such as ATR and Chk1 are limiting therefore Mus81 is unlikely to have a prominent role in adaptation to hypoxia (Hammond and Giaccia, 2004).

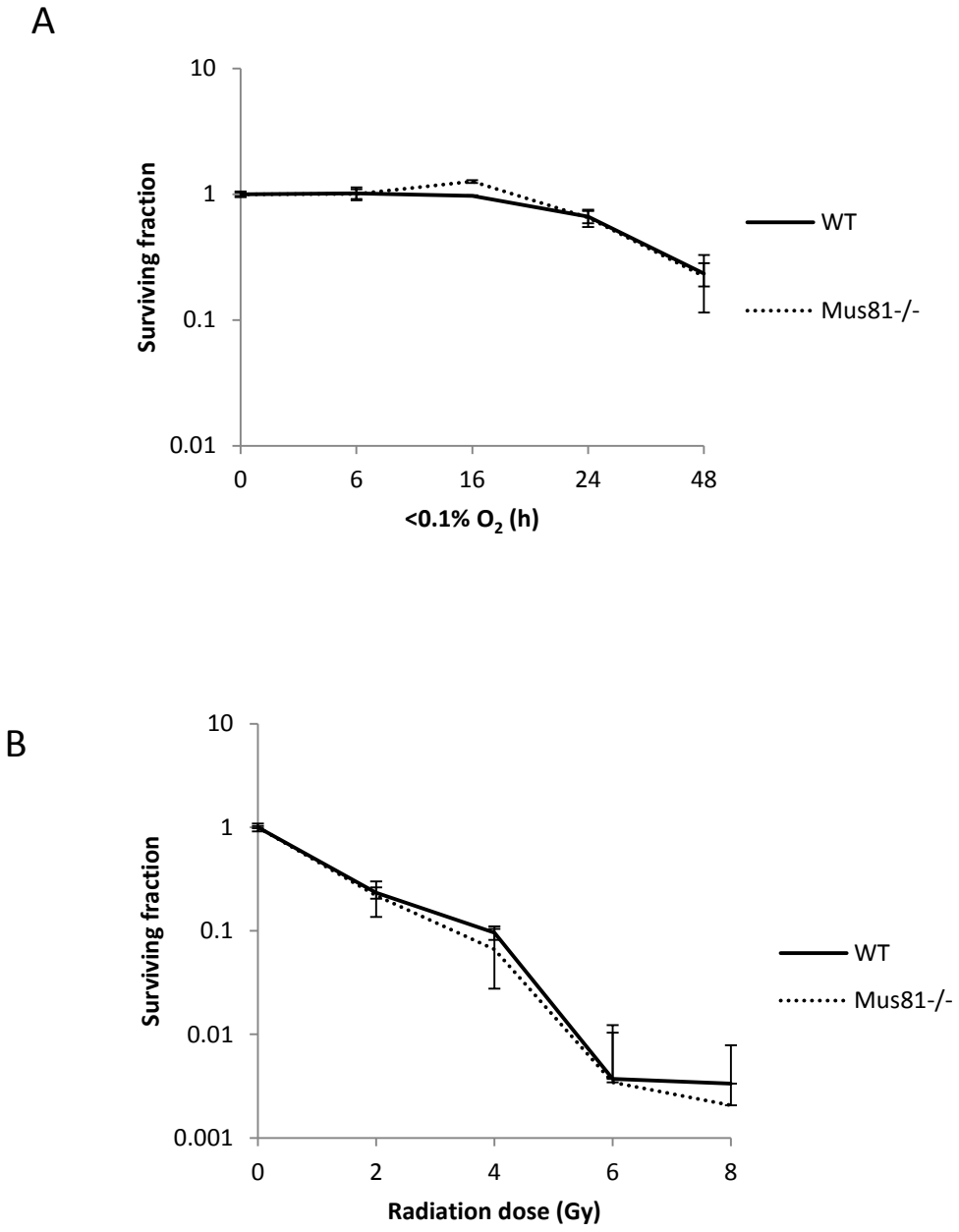


Figure 4.4. Loss of Mus81 does not increase sensitivity to hypoxia or irradiation. (A) Colony survival of HCT116 cell lines exposed to severe hypoxia (<0.1% O_2) for 0, 6, 16, 24 and 48h. (B) Colony survival of HCT116 cell lines treated with 0, 2, 4, 6 or 8Gy. Error bars represent ± standard error calculated from the standard deviation of a single experiment run in triplicate.

4.5 Mus81 protein level decreases in hypoxia

To further assess the effect of hypoxia on Mus81, several cell lines were exposed to severe hypoxia. Figure 4.5A shows H1299 cells exposed to hypoxia (0.1% O_2) as indicated or reoxygenation (8h 0.1% O_2 followed by 24h normoxia). Mus81 protein level is greatly reduced after 8h hypoxia and declines further after 24h, however expression returns to initial levels after reoxygenation. To elucidate whether this was a cell type specific effect WI-38 (non-transformed lung), U2OS (osteosarcoma) and RKO (colorectal carcinoma) cells were employed to see the effects of hypoxia (0.1% O_2) on Mus81 expression shown in Figure 4.5B. A decrease in Mus81 expression was seen in all cell lines after 6h hypoxia (14.73% decrease for WI-38, 14.75% for U2OS and 30.32% for RKO). By 24h levels in WI-38 and U2OS were still decreased (10.9% and 19.22% respectively) however RKO cells showed Mus81 expression increased by 14.71% after 18h hypoxia. Still the overall pattern from all four cell lines looked at here is that Mus81 protein is decreased in hypoxic conditions. In agreement with data shown earlier Wee1 expression was decreased in hypoxia for all cells in Figure 4.5B and γH2AX was induced.

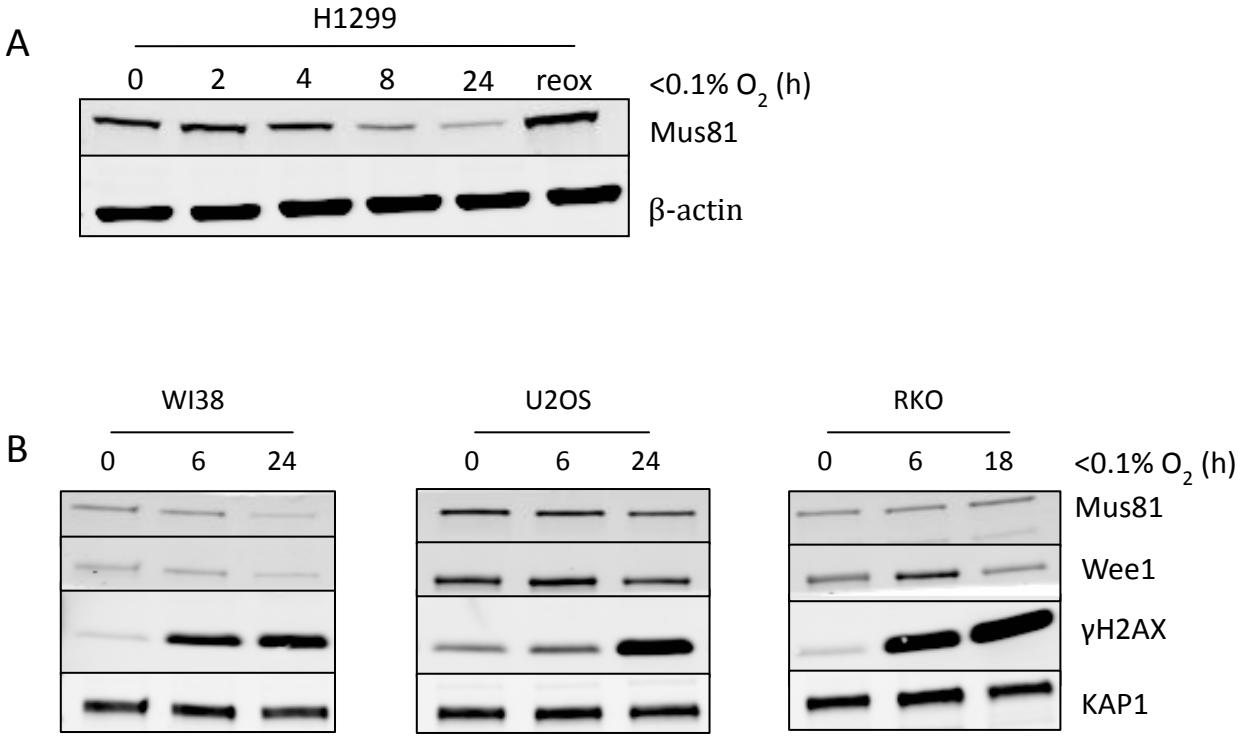


Figure 4.5. Mus81 protein level decreases in severe hypoxia in a variety of cell lines. (A) Western blot to show Mus81 levels in H1299 cells exposed to 0, 2, 4, 8 or 24h severe hypoxia (<0.1% O_2) or reoxygenation (8h hypoxia followed by 24h reoxygenation). (B) Western blot to show Mus81 levels in WI-38 and U2OS exposed to 0, 6 and 24h hypoxia (<0.1% O_2) and RKO cells exposed to 0, 6 and 18h hypoxia (<0.1% O_2) . β-actin or KAP1 were used as a loading control. Quantification shown in figure A2.

4.6 The decrease in Mus81 protein expression is dependent on oxygen tension

Figure 4.5 demonstrates Mus81 is decreased in severe hypoxia (0.1% O_2) so the next query was what range of oxygen tensions does this occur at. In Figure 4.6A H1299 cells were exposed to severe hypoxia (0.1% O_2) as indicated. HIF-1α was induced by this treatment. Mus81 levels decreased by 57.54% after 8h and was depleted almost entirely by 48h (5.62% Mus81 expression) as shown by the bar graph.

Milder hypoxic conditions were used in Figure 4.6B. H1299 cells were exposed to 1% O_2 as indicated. In these conditions Mus81 expression was not decreased until 48h hypoxia (31.37% decrease) illustrated by the bar graph. Again HIF-1α was induced at all time points.

Figure 4.6C shows Mus81 was not decreased in H1299 cells cultured in 3% O_2. The western blot suggests Mus81 protein expression remains constant and the bar graph shows only small fluctuations occur between time points (the greatest being a 10.42% increase at 8h and a 9.56% decrease at 48h). HIF-1α was induced to a lesser extent in Figure 4.6C compared to 4.6A or B. Overall these results suggest the decrease in Mus81 in hypoxia is dependent on the level of oxygen, since a decline is seen below 3% O_2, and the duration of exposure, since longer exposure times in milder hypoxia are required to achieve a decline in Mus81.

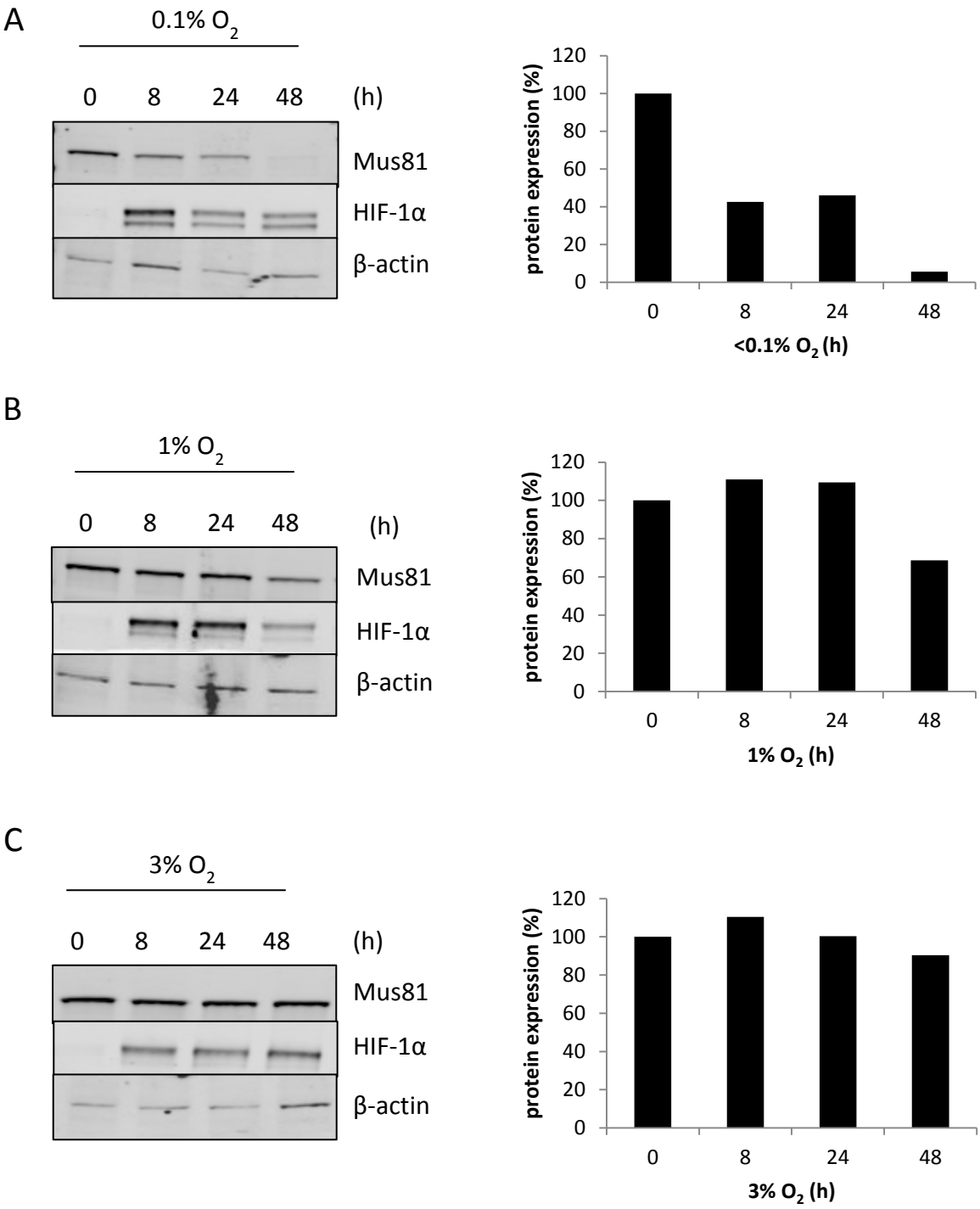


Figure 4.6. The decrease in Mus81 is oxygen dependent. (A) H1299 cells were exposed to severe hypoxia (<0.1% O_2) for 0, 8, 24 or 48h. Western blotting was carried out for Mus81 and HIF-1α. Bar graph represents Mus81 protein expression relative to control. (B) The same experiment was carried out as (A) but at 1% O_2. (C) The same experiment as (A) was carried out at 3% O_2. β-actin was used as a control.

4.7 Mus81 is not regulated by p53 but may be regulated by HIF

To investigate if Mus81 regulation was occurring at the transcriptional level its regulation by p53 and HIF-1α was considered; knockout cell lines were used for this purpose.

Firstly HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were exposed to hypoxia (0.1% O_2) as indicated (Figure 4.7A). The response of these cells to hypoxia was not different as shown by similar blots for HIF-1α expression, also p53 blots provide evidence that p53 is absent in the correct cell line. Mus81 levels appear to be unaffected by both hypoxia and p53 status in this experiment (note the apparent decrease after 24h in $p53^{-/-}$ cells is due to different loading). The bar graph below shows the quantification of Mus81 levels; interestingly there is a slight increase in Mus81 in both cell lines although this increase occurs earlier in $p53^{+/+}$ cells after 16h hypoxia (to 111.21%) whereas this is not seen until 24h hypoxia in $p53^{-/-}$ cells (to 137.05%).

Secondly the affect HIF-1α had on Mus81 regulation was investigated. RKO $HIF^{+/+}$ and $HIF^{-/-}$ cells were cultured in hypoxia (0.1% O_2) as indicated (Figure 4.7B). Blots for HIF-1α show the absence of this protein in the correct cell line and p53 response is similar in both cell lines. Data from this experiment shows Mus81 protein level increases in $HIF^{-/-}$ cells but decreases in $HIF^{+/+}$ cells upon exposure to hypoxic conditions. As shown in the bar graph, Mus81 levels are 53.02% of initial levels at 16h in $HIF^{+/+}$ cells but have increased to 175.29% in $HIF^{-/-}$ cells. This suggests HIF is involved in the regulation of Mus81 and its presence may be necessary for the downregulation of Mus81 in hypoxia.

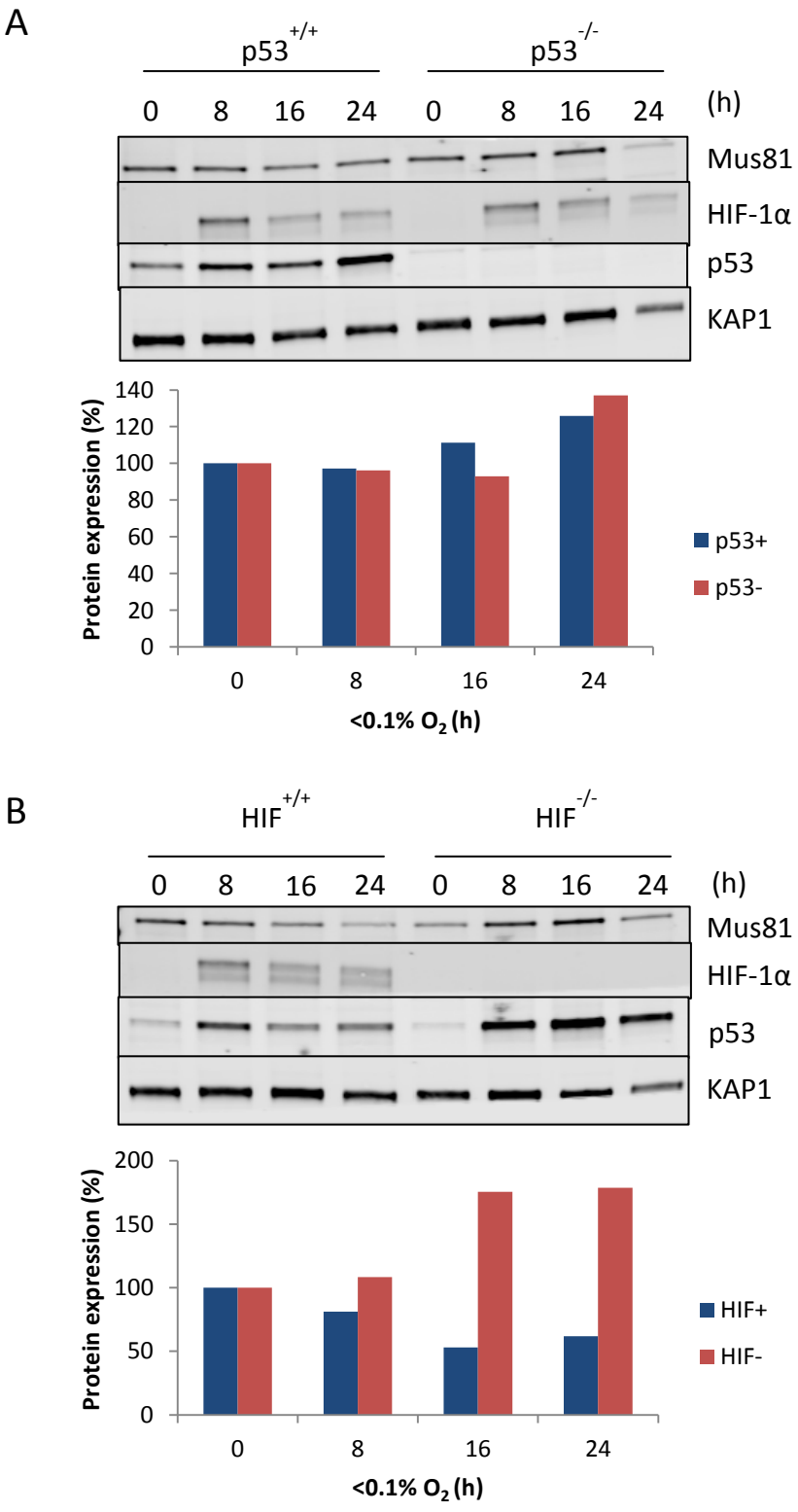


Figure 4.7. Mus81 levels are not affected by p53 status but may be regulated by HIF. (A) HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were exposed to severe hypoxia (<0.1% O_2) for 0, 8, 16 or 24h. Western blotting was carried out to determine Mus81, p53 and HIF-1α levels. (B) The same experiment as (A) was carried out for RKO $HIF^{+/+}$ or $HIF^{-/-}$ cells. KAP1 was used as a loading control. Bar graphs represents Mus81 protein expression relative to control.

4.8 Mus81 does not decrease at the transcript level in hypoxia

From the results shown in Figure 4.7 it appears that HIF plays a role in regulating Mus81 at the protein level, since HIF is a transcription factor it was decided to see if this regulation was actually occurring at the transcript level. RKO $HIF^{+/+}$ and $HIF^{-/-}$ cells were cultured in hypoxic conditions (0.1% O_2) as indicated. cDNA was then run for qPCR using 18S as a control and the normoxic sample for $HIF^{+/+}$ cells as a reference from which fold change of genes could be calculated. As seen in previous experiments GLUT1 was dramatically increased 4.93 fold after 8h hypoxia in $HIF^{+/+}$ cells, however since this gene is regulated by HIF-1α no increase is detected in $HIF^{-/-}$ cells and there is actually a steady decrease in GLUT1 levels in these cells to 0.46 fold by 24h (Figure 4.8A). Results for changes in Mus81 gene do not show a great difference for either cell line. In $HIF^{+/+}$ cells Mus81 transcript does decrease slightly but only to 0.45 fold by 16h hypoxia ($P<0.01$) and 0.46 fold by 24h hypoxia ($P<0.01$). In $HIF^{-/-}$ cells levels fluctuate but do not vary significantly, also initial levels are significantly lower in these cells than $HIF^{+/+}$ ($P<0.01$). Overall Mus81 transcript level does not appear to be regulated by HIF. Eme1 levels also decrease in $HIF^{+/+}$ cells with hypoxia exposure, after 8h levels are 0.40 fold ($P<0.05$) that of levels in normoxia and this has diminished to 0.07 fold after 24h ($P<0.01$). Since Mus81 and Eme1 are binding partners it is possible that their regulation is tightly linked. It would be interesting to determine Eme1 protein levels in hypoxia in future studies. Eme1 initially decreases in $HIF^{-/-}$ cells dropping from 0.68 to 0.15 fold after 8h hypoxia, but after this time levels increase to 0.80 fold; neither change is significant. Although this pattern may suggest HIF plays some role in Eme1 regulation, there does not appear to be a great difference in levels between the cell lines. Overall there is little evidence for HIF having transcriptional regulation over Mus81.

To ensure the effects on Mus81 at the transcript level were not due to cell type effects, the experiment was repeated in H1299 cells exposed to hypoxia (0.1% O_2). cDNA was obtained from the cells and a qPCR was run using 18S as a control and the normoxic sample as a reference from which fold change of GLUT1, Mus81 and Eme1 could be calculated. Figure 4.8B shows GLUT1 expression increases dramatically by 5.67 fold after 8h hypoxia and remains at this level until 24h, however by 48h GLUT1 is only 2.9 fold higher than initial levels. In contrast Mus81 expression remains

relatively constant (1.23 fold at 8h (P<0.05), 1.52 fold at 16h (P<0.01), 1.19 fold at 24h (P<0.01)). The decrease in Eme1 in hypoxia is significant to 0.62 fold after 8h hypoxia (P<0.05) and this is decreased further by 24h (0.46 fold, P<0.01). So although Mus81 appears to be unaffected at the transcript level in hypoxia, potentially the decrease in Eme1 is affecting the proteins stability.

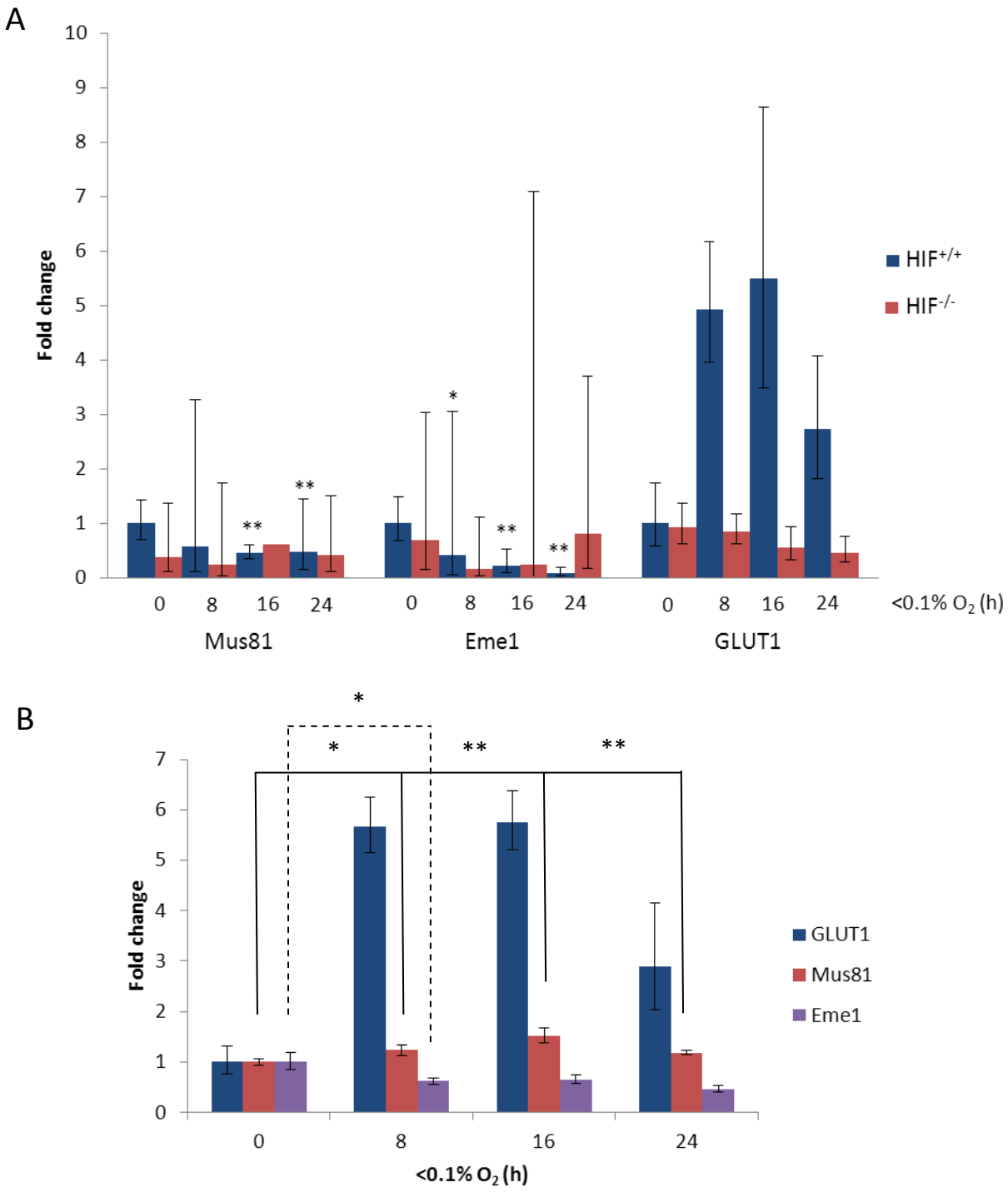


Figure 4.8. Mus81 is not regulated at the transcriptional level in hypoxia. (A) RKO HIF$^{+/+}$ or HIF$^{-/-}$ cells were exposed to 0, 8, 16 or 24h severe hypoxia (<0.1% O_2). qPCR was then used to analyse the transcript levels of GLUT1, Mus81 and Eme1. Normoxic HIF$^{+/+}$ sample was used as an endogenous control and 18S primers were used as a reference. (B) The same experiment as (A) was carried out in H1299 cells. Normoxic sample was used as an endogenous control and 18S primers were used as a reference. Significant difference between means using a paired t test is shown by an asterisk (P<0.05) or a double asterisk (P<0.01). Error bars represent three technical repeats. n=2

4.9 Mus81 has a potential role in reoxygenation-induced replication restart

The activity of Mus81 at stalled replication forks suggest stalled forks produced in severe hypoxia may also be substrates of Mus81, so this endonuclease may play a role in allowing replication restart upon reoxygenation after acute hypoxia.

This theory was tested by exposing H1299 cells to either acute (4h) or chronic (16h) hypoxia (0.1% O_2) followed by increasing periods of reoxygenation. Cells are capable of reoxygenation-induced replication restart after 4h but not after 16h (Pires et al., 2010b). Western blotting shows Mus81 protein levels decrease slightly after 4h hypoxia (62.94%) but upon reoxygenation quickly increase to higher than original levels (113.65% after 4h reoxygenation) (Figure 4.9A); this is quantitated in the bar graph (Figure 4.9B). In contrast cells exposed to chronic hypoxia show a decrease in Mus81 protein (to 38.66%) but this does not recover and is still at 57.26% after 6h reoxygenation. Replication restart after reoxygenation has been shown in acute but not chronic hypoxia, since disassembly of the replisome occurs after longer hypoxia exposure, so these results indicate Mus81 could be involved in this restart process (Pires et al., 2010b). A model for the role of Mus81 in reoxygenation-induced replication restart is illustrated in Figure 4.9C.

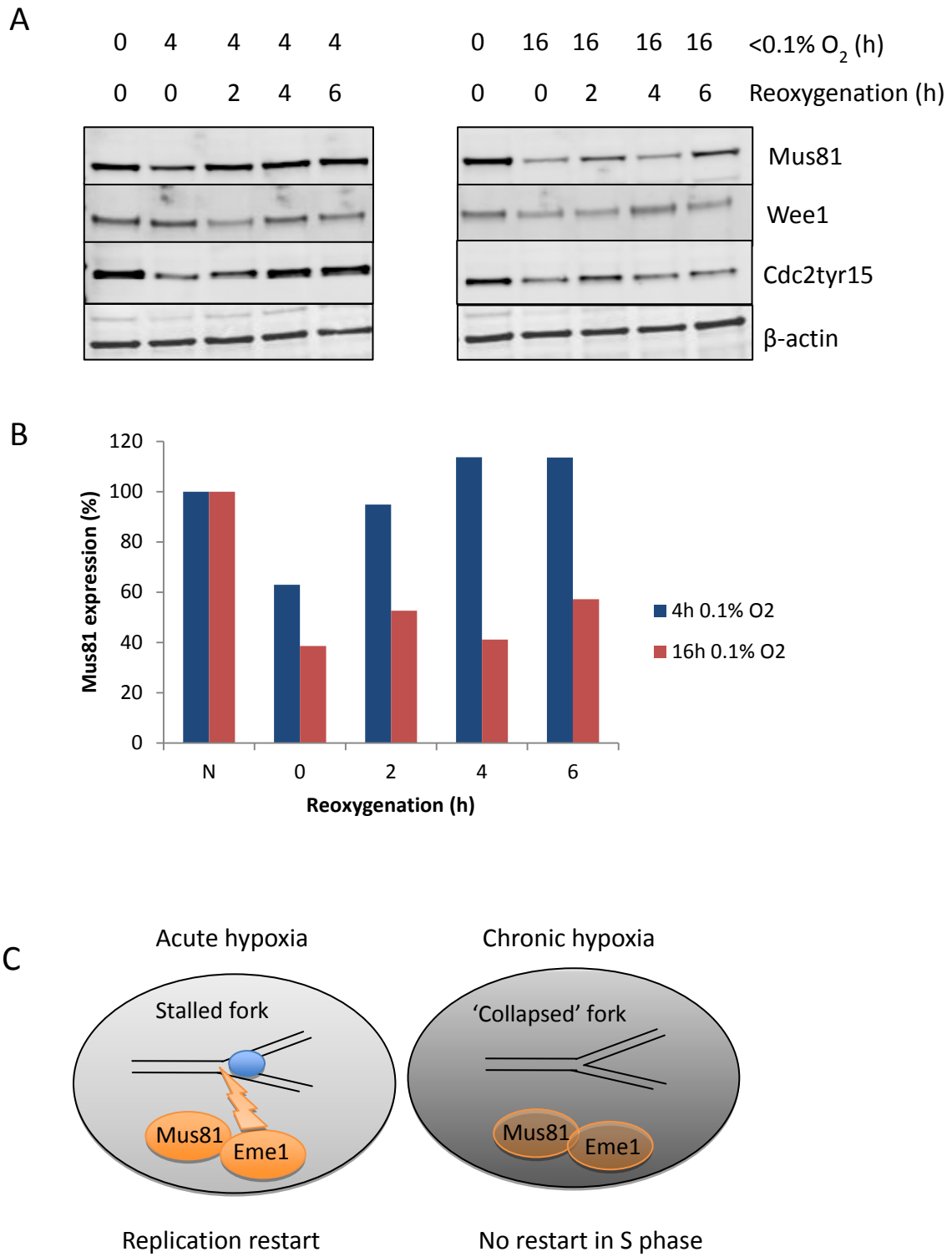


Figure 4.9. Mus81 has a potential role in reoxygenation-induced replication restart. (A) Western blot to show Mus81, Wee1 and phosphorylated Cdc2 levels in H1299 cells after acute hypoxia (4h) or chronic hypoxia (16h) followed by different periods of reoxygenation (0, 2, 4 or 6h). β-actin was used as a loading control. (B) Bar graph represents Mus81 protein expression from (A) relative to control. N = normoxia (20% O_2). (C) Proposed model of reoxygenation-induced replication restart involving Mus81-Eme1.

Chapter 5. Discussion

5.1 The use of Wee1 inhibitors in Hypoxia

Wee1 is a kinase involved in the G_2 cell cycle checkpoint as well as maintaining genomic stability in S phase (Dominguez-Kelly et al., 2011; Sorensen and Syljuasen, 2011). We found a decrease of this protein in hypoxia (0.1% O_2); this may be due to cell cycle effects since the replication arrest, which occurs in severe hypoxia creates an increase in G_1 population but Wee1 activity and protein level is highest in S and G_2 phases (Watanabe et al., 1995). Previous studies characterising this protein have mainly been carried out in yeast and *Xenopus*. These studies suggest Chk1 phosphorylates and activates Wee1 (Lee et al., 2001; O'Connell et al., 1997). This study provides additional evidence of this. Chemical inhibition of Chk1 suggested Chk1 phosphorylates Wee1. However Chk1 siRNA did not decrease Wee1 phosphorylation but was able to decrease phosphorylation of Cdc2 at tyr15, a characterised target of Wee1 (Domingo-Sananes et al., 2011; Lee et al., 2001). Nevertheless, it cannot be ruled out that Chk1 is affecting another kinase involved in phosphorylation of Cdc2 at this site or that the Chk1 inhibitor was having effects on another kinase to decrease Wee1 phosphorylation. Another study shows results opposing the idea that Chk1 acts upstream of Wee1; findings show dual inhibition of Chk1 and Wee1 has additive effects on decreasing DNA replication speed and cell viability suggesting they function in different pathways (Davies et al., 2011; Dominguez-Kelly et al., 2011). So potentially Chk1 does not activate Wee1 in mammals but the majority of evidence suggests otherwise. Overall this study provides some indication that Chk1 is the kinase activating Wee1 in hypoxia, which has never been shown before in mammalian cells or these oxygen tensions, however a solid conclusion cannot be drawn from the results shown here.

Wee1 is an attractive target for cancer therapy for several reasons and numerous studies have already investigated their anti-tumorigenic efficacy. Firstly, Wee1 is overexpressed in several tumour types including glioblastoma, osteosarcoma, breast, and hepatocellular carcinoma (De Witt Hamer et al., 2011; Mir et al., 2010; Murrow et al., 2010; PosthumaDeBoer et al., 2011). Since it may play a role in maintaining genomic stability, expression of Wee1 may protect cancerous cells from obtaining lethal amounts of DNA damage making inhibition of Wee1 advantageous

(Magnussen et al., 2012). Wee1 has also been associated with increased tumour progression and decreased relapse-free survival in melanomas (Magnussen et al., 2012). Secondly, the mechanism of action of Wee1 inhibition is particularly effective in cancer cells with p53 mutations which therefore lack a G_1 checkpoint and rely heavily on the G_2 checkpoint for DNA damage repair (Kawabe, 2004). Thirdly, Wee1 depletion has been correlated with increased apoptosis (Wang et al., 2004). Finally, preclinical trials with these agents have shown promising results and no toxicity in non-transformed healthy tissue or animals, this may be in part due to expression of wildtype p53 in non-tumorigenic tissue (Aarts et al., 2012; Bridges et al., 2011; Hirai et al., 2009; Mir et al., 2010; Murrow et al., 2010; Sarcar et al., 2011).

The potential of using Wee1 inhibitors in hypoxic conditions is intriguing for several reasons. These include: (1) inhibitors of the DDR pathway have already been shown to be efficacious in hypoxia therefore Wee1 may also be a good target (Hammond et al., 2006; Olcina et al., 2010; Pires et al., 2012), (2) Wee1 acts further downstream in the DDR pathway so targeting it therapeutically will hopefully have fewer side effects than targeting ATR and Chk1 kinases which phosphorylate many downstream targets (Kawabe, 2004), (3) Wee1 inhibitors have been shown to sensitise cells to chemotherapy and radiotherapy in normoxia, since hypoxic cells are particularly resistant to therapy these drugs may have valuable actions in these conditions (Bristow and Hill, 2008; De Witt Hamer et al., 2011).

We found Wee1 inhibitors are able to reduce the phosphorylation of Cdc2 at tyr15 and cause premature mitotic entry in hypoxic (1% and 0.1% O_2) cells suggesting they are efficacious in a range of oxygen tensions. These agents were also able to induce DNA damage signalling in hypoxia (1% O_2); this effect may also occur in severe hypoxia but the induction of the DDR by these conditions masks the result. Damage was also seen in non-transformed cells after similar exposure times as in tumour cell lines; this suggests there may be issues with the toxicity profile of these drugs. So far only short term effects have been elucidated whereas the damage found here suggests long term effects may occur, so although Wee1 inhibitors do not appear to be toxic they may cause malignant progression in the long term. The studies mentioned previously which found no toxicity in non-transformed cells were using different read-outs which may be the reason for discrepancy from results shown here (Bridges et al., 2011; Mir et al., 2010; Murrow et al., 2010; Sarcar et al., 2011). γH2AX protein expression was identified in one study, although in this case Wee1 siRNA did not

induce γH2AX in non-transformed breast cells but did in cancerous cell lines (Murrow et al., 2010). This may suggest the induction of γH2AX is an effect of the drug (MK1775) rather than loss of Wee1 signalling in general. Unfortunately our study did not include the use of Wee1 siRNA since sufficient knockdown of the protein was not achieved even after several optimisations using previously published oligonucleotide sequences (Dominguez-Kelly et al., 2011; Murrow et al., 2010). Further work needs to be done to address this.

This study is the first to identify Wee1 inhibitors as effective radiosensitisers in hypoxic conditions. Although it does not appear that Wee1 inhibition radiosensitises hypoxic cells to a greater extent than normoxic cells, it still suggests these agents could be successfully used in targeting the hypoxic cell population; there is a great need for therapeutics in this area due to these cells' aggressive characteristics and resistance to therapy. Radiosensitisation may be explained due to cells accumulating in phases which are more sensitive to radiotherapy. Upon Wee1 inhibition the population of G_2 cells supposedly decreases with a corresponding increase in mitotic population, however we have also identified a slight increase in the G_1 population. Potentially Wee1 inhibitors may be successful in radiosensitising hypoxic cells since they aid in their progression from the relatively radioresistant G_1/S phase to the radiosensitive mitotic phase (Pawlik and Keyomarsi, 2004). However, the radiosensitising effects can also be attributed to the general cytotoxicity of these drugs, not just their cell cycle effects, and are likely to be due to multiple factors. Another factor which may be involved in Wee1 inhibitor-mediated sensitisation of cells to therapy is the impairment of HR (Krajewska et al., 2012). Wee1 inhibition impedes HR potentially via the inappropriate activation of Cdc2, which has been shown previously to cause phosphorylation of BRCA2 leading to inhibition of DNA repair (Krajewska et al., 2012). This suggests that Wee1 inhibition would reduce the repair of damage induced by chemotherapy or radiotherapy thus enhancing their effects and effectively sensitising tumour cells to these therapies. Whether this would have additive effects when combined with the inhibition of HR seen in hypoxia is yet to be elucidated but would imply sensitisation by Wee1 inhibition would be successful in these conditions.

Overall the use of Wee1 inhibitors as radiosensitisers in hypoxic conditions is an exciting outcome of this study and suggests that this use should be considered in future clinical trials. Also results show Wee1 inhibitors were not as effective as

monotherapy in hypoxic cells indicating caution should be taken with current studies which may appear efficacious but are not having the same effect in physiological tumour conditions where the oxygen tension is considerably lower than experimental conditions.

5.2 The regulation of Mus81 by Wee1

The rationale for the inhibition of Wee1 concerns its negative phosphorylation of Cdc2 (Cdk1) in G_2, however it is the negative regulation of Cdk2 in S phase which is involved in controlling DNA replication (Figure 5.1) (Dominguez-Kelly et al., 2011). Potentially Cdk2 is able to phosphorylate and activate Mus81, thus the inhibition of Cdk2 by Wee1 prevents inappropriate endonuclease activity which may induce DNA damage. It is also possible that Wee1 directly interacts with Mus81 as evidence for this is shown by co-immunoprecipitation studies (Dominguez-Kelly et al., 2011).

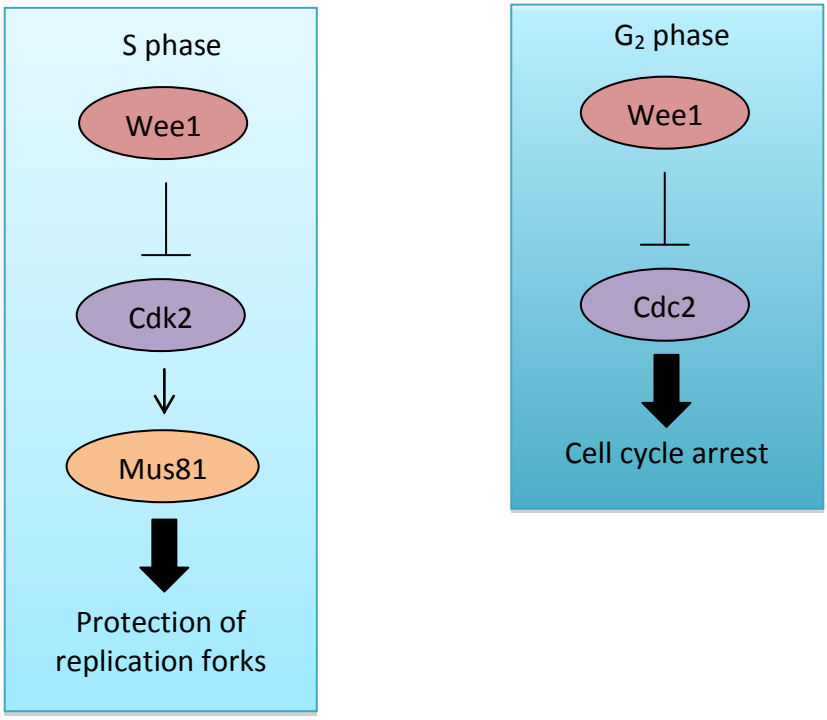


Figure 5.1. The regulatory effects of Wee1 in the cell cycle. *Diagram to illustrate the roles of Wee1 in the S and G_2 phases of the cell cycle via inhibition of Cdk2 and Cdc2 respectively.*

The model for Wee1 regulation of Mus81 implies that inhibition of Wee1 would remove this regulatory mechanism causing overactivation of Mus81 and DNA

damage. Indeed damage is seen after Wee1 inhibition in this study but no evidence was found for dependence on Mus81. In both normoxia and hypoxia γH2AX was induced by Wee1 inhibition even in the absence of Mus81. Potentially this result may show DDR signalling is still active rather than damage, in order to determine whether this is the case another marker of DNA damage needs to be investigated. One example of this could be examining the production of 53BP1 foci in Mus81-deficient or Mus81 expressing cells treated with Wee1 inhibitor; fewer foci would be expected in Mus81-deficient cells. DNA damage could also be detected by COMET assay with the hypothesis being that the comet tail would be shorter in $Mus81^{-/-}$ cells after Wee1 inhibition. These techniques may provide a clearer answer. Another explanation for discrepancy from previous studies may be due to the difference in systems used; our study used cells with permanent Mus81 knockdown which is likely to have different effects on signalling than the transient knockdown achieved by siRNA transfection used in the current literature (Dominguez-Kelly et al., 2011).

The effect of Mus81 knockout on cell survival was investigated in this study and results support those seen previously since $Mus81^{-/-}$ cells were not sensitised to γ-irradiation over wildtype cells, although at the highest dose (8Gy) there does appear to be some sensitisation. The effect of hypoxia on $Mus81^{-/-}$ cell survival was also examined; these cells were not sensitised to hypoxia potentially because these conditions do not cause collapse of replication forks or interstrand crosslinks. Considering the suggested role of Mus81 in cleaving stalled replication forks Mus81 may be considered important for allowing replication restart upon reoxygenation. However, the majority of these cells were exposed to chronic hypoxia so would be unable to restart due to disassembly of the replisome thus removal of Mus81 would not have significant effects (Pires et al., 2010b). Another explanation may be that the removal of the replisome in chronic hypoxia may allow Mus81 access to the fork which would contribute to potentially lethal DNA damage seen in reoxygenation, however this does not occur in $Mus81^{-/-}$ cells so no difference in survival is seen when compared to wildtype cells (Froget et al., 2008). However, there may be little difference in phenotype between wildtype and $Mus81^{-/-}$ cells since our data shows Mus81 expression remains depleted after chronic hypoxia even when reoxygenation occurs, so in these conditions these two cell types would be expected to react similarly.

The hypothesis that Mus81 is important for reoxygenation induced replication restart was investigated further in this study. Severe hypoxia can induce stalling of replication forks so it is possible that Mus81 plays a role in processing these forks upon reoxygenation to allow replication restart. An initial observation was the decrease in Mus81 protein expression in hypoxia in a variety of cancerous cell lines and non-transformed cells, this occurred after just 6h exposure to 0.1% O_2 in some cases. Although little change in Mus81 transcript level was detected, results do show a slight decrease in Eme1 transcript and this may be playing a role in decreasing Mus81 protein levels. The XPF family of endonucleases which Mus81 belongs to function as heterodimers with their binding partner being catalytically inactive but vital for stability and nuclease function of the functional monomer, in this case Eme1 has been shown to be vital for Mus81 activity but potentially it is also of importance for protein stability, as our results would suggest (Osman and Whitby, 2007). Another reason for the downregulation of Mus81 in hypoxia may be tumour cells adapting to the microenvironment by decreasing production of any unnecessary proteins to conserve energy during this time of low oxygen and nutrients. Cell cycle effects may also play a role; Mus81 expression is highest in S and G_2 phases, however the replication arrest induced in hypoxia causes a decrease in these populations thus Mus81 is depleted. Regulation of Mus81 could also involve a feedback mechanism; Wee1 levels decrease in response to hypoxia and this would remove the inhibitory control on Mus81 making it more active, perhaps in this case the cell would decrease Mus81 levels to compensate for its overactivation. Another theory is that Mus81 is decreased in hypoxia so that upon reoxygenation, when DNA damage is induced, cells are unable to restart and incorporate damage which will lead to genetic instability. This would depend on the role Mus81 plays in reoxygenation-induced restart.

Findings suggest Mus81 is required for replication restart upon reoxygenation since levels decreased in cells unable to restart whereas expression remained constant in cells able to restart suggesting this protein was needed. In addition, $Mus81^{-/-}$ cells formed fewer colonies in hypoxic conditions at time points where restart can occur suggesting Mus81 is critical for this process. So in these conditions Mus81 contributes to genomic instability, since cells often obtain damage upon reoxygenation and if these cells are allowed to restart they will incorporate these aberrations. Mus81 is usually considered anti-tumorigenic so this could be a reason

why the cell would decrease its expression in hypoxia. Overall this study is the first to elucidate the role of Mus81 in hypoxia and reoxygenation.

In conclusion this study has shown the role of Wee1 in the hypoxia-induced DDR and illustrates the potential efficacy of Wee1 inhibition in combination with radiotherapy for targeting hypoxic cells. The search for novel therapeutics targeting hypoxic cells is urgently needed since this population represents physiologically relevant conditions, which are often overlooked in the drug discovery process. Hopefully Wee1 inhibitors will gain some success in this area.

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Appendix

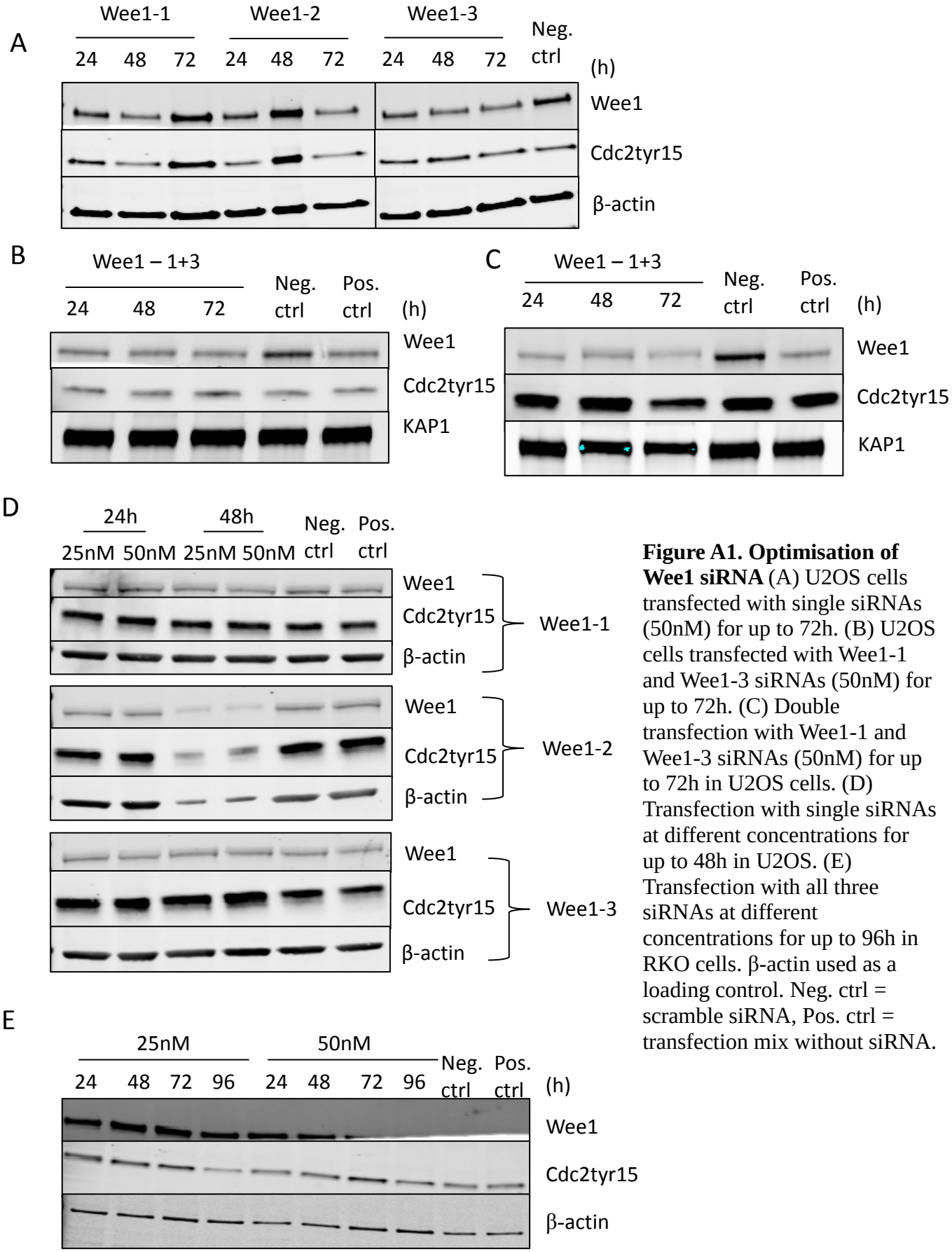


Figure A1. Optimisation of Wee1 siRNA (A) U2OS cells transfected with single siRNAs (50nM) for up to 72h. (B) U2OS cells transfected with Wee1-1 and Wee1-3 siRNAs (50nM) for up to 72h. (C) Double transfection with Wee1-1 and Wee1-3 siRNAs (50nM) for up to 72h in U2OS cells. (D) Transfection with single siRNAs at different concentrations for up to 48h in U2OS. (E) Transfection with all three siRNAs at different concentrations for up to 96h in RKO cells. β-actin used as a loading control. Neg. ctrl = scramble siRNA, Pos. ctrl = transfection mix without siRNA.

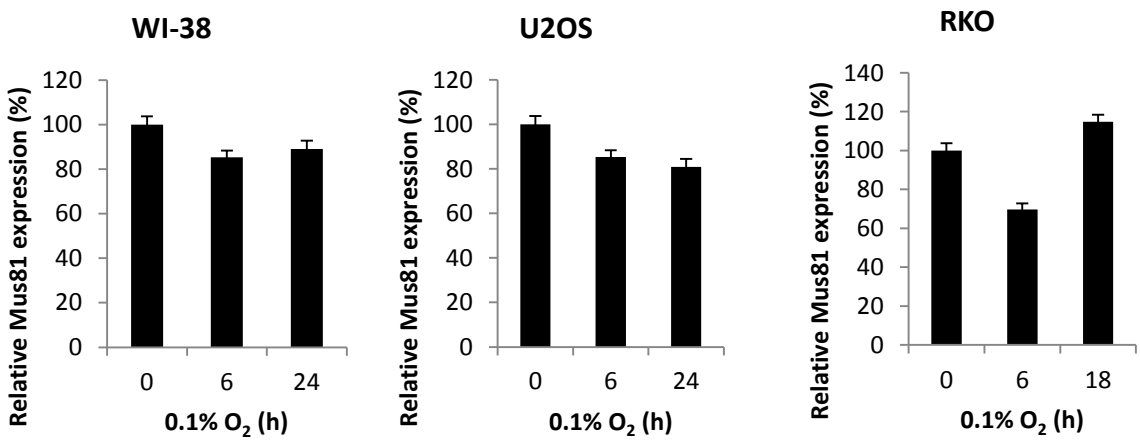


Figure A2. Mus81 protein level decreases in severe hypoxia in a variety of cell lines. Quantification of Mus81 levels in figure 4.5B (WI-38 and U2OS exposed to 0, 6 and 24h hypoxia (<0.1% O_2) and RKO cells exposed to 0, 6 and 18h hypoxia (<0.1% O_2)). Plotted relative to normoxic sample. Error bars represent 3 measurements.