Regulation of p53-dependent cell death responses in normoxia and hypoxia

Afshan Ahmed

University College London

Thesis submitted for the degree of

Doctor of Philosophy

2011

Declaration

I, Afshan Ahmed confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgments

Firstly, and most of all for Margaret
My supervisor, my mentor, my mummy and my friend,
Thank you for always seeing the ‘sunshine’ in me.

For Nick, Tom B, Deepa and Tom C
You are all ‘amazing’,
Thank you for looking after me.

For Akill Uncle
You made me realise this was all about my happiness,
Thank you for keeping me strong,

and finally for Sabby
You inspire me in more ways than you know,
Thank you ☺

Abstract

Hypoxia, defined as low oxygen tension, is a common characteristic of growing tumours. Tumour cells adapt to their hypoxic microenvironment by inducing angiogenesis and escaping cell death. In doing so, tumour cells become resistant to radiotherapy and many forms of chemotherapy. Hypoxia signalling and angiogenesis is mediated by the hypoxia-inducible factor (HIF) transcriptional complex. HIF can cross-talk to the p53 tumour suppressor protein, a critical regulator of cell cycle and cell death responses to stress.

This study aims to understand how cell death responses are regulated in tumour cells by HIF and p53, in normoxia and in hypoxia. Recently, activation of p53 by the small molecule RITA has been investigated. Flow cytometry, comet assays and western blot analysis have been used to reveal a novel p53-dependent DNA damage response that activates cell cycle checkpoints, and induces significant cell death of hypoxic tumour cells. Activation of p53 also achieves anti-angiogenic effects, both *in vitro* and *in vivo* by inhibition of HIF-1α protein synthesis and HIF target genes, including VEGF.

The MEK-ERK MAPK pathway has also been investigated as a critical modulator of p53-dependent cell death in normoxia and hypoxia. Inhibition of MEK1/2 by the MAPK signalling inhibitor PD98059 significantly inhibits p53 induction and cell death responses by RITA. The anti-tumour effect of p53 activation in response to RITA is therefore dependent on MEK-ERK signalling. By understanding p53 interactions with HIF signalling, and the role of p53 in responding to DNA damage and apoptotic signals, this study has potential to improve the therapeutic targeting of resistant tumours with deregulated angiogenic and cell death pathways.

Table of Contents

List of Tables and Figures

Chapter 1

Introduction

1.1 Overview of cancer

The earliest records of cancer date back to almost 1600 BC when Egyptians documented the first methods of treatment for tumours. Later, the ancient Greek physician Hippocrates (ca.460 BC-370 BC) described the appearance of solid tumour sections as ‘carcinos’ (meaning crab or crayfish in Greek), from which the name cancer was derived. Today, one in three people are likely to develop cancer during their lifetime in the UK and in 2008, up to 27% of all deaths in the UK were caused by cancer. Globally, cancer accounts for 13% of all deaths, making cancer the leading cause of mortality in the developed world (Jemal et al., 2011). With greater understanding of the basic principles of tumour development, and significant progress in screening programmes, earlier detection, and new cancer treatments, the survival rate for patients diagnosed with cancer has doubled over the last forty years. Despite this, patients with complex and diverse tumours still have poor survival outcomes and there continues to be a clear need for further improving treatment regimes and patient care.

There are over 200 types of cancers originating from distinct cells within the body. Therefore, understanding cancer as a disease is challenging, as each tumour type will have unique characteristics defined by where and when it originated. Clearly, tumour evolution is a multistep process, and over time, every tumour will undergo distinct changes that contribute towards the formation of a complex tumour microenvironment. Such changes have been categorised into defined processes and are commonly referred to as the hallmarks of cancer. The interactions that various hallmarks of cancer have between each other and the impact this makes on the tumour phenotype will be discussed in detail later. To begin studying molecular pathways that are involved in cancer, and how these can be targeted for therapy, it is first important to address the basic principles of how normal cells become transformed into tumour cells.

1.1.1 The building blocks of life

Cells are the basic building blocks of all forms of life and are organised into distinct tissues and organs within the human body. Every cell must grow, divide and die efficiently, according to the demands and function of the tissues in which it resides. The instructions for these processes lie within the genetic material of every cell. Normal cells that suffer from genetic defects undergo either repair, or cell death so that they can be replaced with healthy cells. However, some cells which have damaged DNA

bypass these mechanisms and continue to survive and replicate in an uncontrolled way, posing a significant threat to the surrounding tissue.

1.1.2 Deregulated cell control in cancer

Cancer is defined as a disease in which normal cells lose control of their cell cycle. There are multiple cell types in the body, and tumours can arise from any of these cells. Tumours which form as a result of uncontrolled cell division are either benign or malignant. Benign tumours are not cancerous, they can be removed from the organ with surgery and do not reoccur, whilst malignant tumours are those formed by cancer cells. Malignant tumours are the most threatening tumours due to the ability of cells to invade surrounding tissues and form tumours at distant organs in the body. This process is known as metastasis and is one of the hallmarks of cancer. It is by metastasis that tumour cells ensure nutrient and oxygen supplies are not limiting factors.

1.2 The causes of cancer

Genes are comprised of nucleotide base pair sequences that are inherited by every cell to provide the coding instructions for functional proteins. Cancer is a common genetic disease, and is formed by changes in the DNA sequence of specific genes. Such changes, or mutations, provide significant growth advantages to cells and lead to the accumulation of further changes that promote the cancer phenotype. The majority of genetic mutations in cancer are somatic mutations, i.e. those that are acquired during life (Anand et al., 2008; Futreal et al., 2004). Environmental and lifestyle factors such as smoking, alcohol, pollution, and diet play an important role in determining the likelihood of acquiring somatic mutations. The remainder are inherited mutations which significantly increase the susceptibility of developing cancer. For example, women that inherit mutations in the *BRCA1/BRCA2* genes have a 75% risk of developing either breast cancer, or ovarian cancer during their lifetime (Roukos, 2009).

The first draft of the human genome was published in 2001, describing a vast collaborative effort to dissect the human genetic code (Lander et al., 2001; Venter et al., 2001). Such information has been invaluable for mapping genetic data to identify cellular signatures and understand evolutionary conservation, gene transcription programmes, and associations or alterations in genes that underlie numerous disease types (Lander, 2011). The information provided by the human genome, along with technical advances in DNA sequencing, high throughput mutation detection, and

bioinformatics has significantly advanced the identification of several gene variants, or cancer genes that are critical for the development of cancer. Before the human genome project was published, approximately 80 cancer genes involved in the formation of solid tumours had been described. In 2004, the number of cancer genes identified had increased to almost 300, providing important clues to the molecular mechanisms by which cancers arise, and greatly improving opportunities for treatment (Futreal et al., 2004). Various projects are currently underway to catalogue the genes that have an increased susceptibility to mutations in various cancer types. These include the Cancer Genome Project (being conducted by the Wellcome Trust Sanger Institute), the Cancer Genome Atlas (National Institute of Health), and the International Cancer Genome Consortium (ICGC).

Gene sequencing has also been instrumental in designing clinical trials for targeted agents. Initial sequencing of human cancer genes involved those that were known to be deregulated during tumour progression. Somatic point mutations in the *BRAF* gene, for example, were identified in over 50% of melanomas. However targeted inhibitors of the *BRAF* signalling pathway showed poor response rates until patients genes could be sequenced and treated according to their particular *BRAF* mutation status (Davies et al., 2002). In other clinical studies, treatment of patients with non small cell lung cancer using Gefitinib (Iressa) showed poor efficacy (Giaccone et al., 2004a; Giaccone et al., 2004b; Herbst et al., 2004). The discovery of *EGFR* (epidermal growth factor) mutations in nearly 10-15% of lung cancers allowed better predictions for sensitivity to Gefitinib and significantly improved response rates (Costanzo et al.; Lynch et al., 2004; Paez et al., 2004).

1.2.1 The genetic footprints of cancer

Cancer associated genes are often referred to as either oncogenes, or tumour suppressor genes (Kinzler and Vogelstein, 1997). Oncogenes have a gain of function effect and initiate tumour formation by enhancing cell proliferation. Point mutations in the *RAS* oncogene for example are common in lung, colorectal, and pancreatic cancers (Downward, 2003b). Another example of a common oncogene is the *c-MYC* oncogene, encoding a nuclear protein that regulates transcription of various genes involved in cellular proliferation, differentiation and apoptosis. Amplification or overexpression of c-MYC by mutation is found in 15-25% of breast tumours (Nass and Dickson, 1997).

Unlike oncogenes, tumour suppressor genes inhibit tumour formation by negatively regulating cell division. Genetic mutations in tumour suppressor genes that lead to their loss of function promote the malignant phenotype. The first tumour suppressor protein to be identified was the retinoblastoma protein, encoded by the human retinoblastoma gene, *Rb*. Since then, numerous other tumour suppressors have been discovered, including the p53 tumour suppressor protein, now the most studied tumour suppressor protein whose deregulation is involved in cancer progression. Originally studied as a tumour suppressor gene involved in the pathogenesis of lung cancer (Takahashi et al., 1989), mutations in the *p53* gene are now shown to occur in up to 50% of all human cancers (Hollstein et al., 1991).

Alleles are copies of genes, often found on each chromosome that is inherited through the germline. Sequence variations in alleles are either inherited, or arise spontaneously by somatic mutation (Futreal et al., 2004). Mutational activation of an oncogene is often coupled with loss of several tumour suppressor genes throughout different stages of tumour development (Vogelstein et al., 1988). In fact, it is now accepted that multiple independent mutational events are required for transition of a benign tumour to malignancy (Kinzler and Vogelstein, 1996a). This hypothesis was proposed as early as 1953 by the simple observation that cancer death rates increase with age, therefore, cancer depends on a series of mutational events in genes that accumulate throughout life (Nordling, 1953). Later, statistical analysis of the death rate for women with gastric cancer suggested that between three to seven mutational events were required for the development of cancer (Ashley, 1969). Today the most accepted model for tumour formation by genetic mutation is that proposed by Knudson in 1971. Using statistical analysis, Knudson hypothesised that retinoblastoma is initiated by as little as two independent mutational events in the retinoblastoma gene, *Rb* (Knudson, 1971). That is, individuals who have a greater risk of developing cancer often inherit one mutated allele, while the other is mutated somatically later in life. Such individuals are therefore heterozygous for cancer causing genes, and although they have a greater risk of developing cancer compared to those that are homozygous, it is not until the second wildtype allele is mutated that the malignant phenotype of tumours is unmasked.

Knudson's so called 'two hit' hypothesis is proven in the development of colorectal cancer, the second leading cause of cancer related deaths in the United States (Parker et al., 1996). Colorectal cancers develop over many years. Mutational events in the adenomatous polyposis coli (*APC*) gene significantly increase the risk of developing colorectal cancers. Individuals that inherit germline mutations of APC have a greater

predisposition to both familial adenomatous polyposis (FAP), and hereditary non-polyposis colorectal cancer (HNPCC), compared to the general population. However, such cancers are only initiated following an additional somatic mutation that inactivates the remaining wildtype *APC* allele inherited from the unaffected parent (Ichii et al., 1992). The probability by which a second mutational hit is achieved in the *APC* gene is further increased by defects in important DNA repair pathways, thereby increasing genomic instability and the number of mutations that accumulate over time (Huang et al., 1996a).

Studies suggest that not only the nature, but the order of genetic changes are important for determining tumour progression (Jen et al., 1994a). Again, the colorectal cancer model illustrates this well. Although *p53* is mutated in over 80% of colorectal cancers, mutations in *p53* are not necessary for the initiation of colorectal cancer (Baker et al., 1990; Garber et al., 1991). Mutations in the *p53* gene are therefore considered to be late events in colorectal tumourigenesis (Baker et al., 1990). Similarly, mutations in *KRAS* are common in colon cancer, but are not necessary for tumour initiation (Jen et al., 1994b). Only biallelic inactivation of the *APC* tumour suppressor gene is considered as an important determinant of tumour onset, and it is important that mutations in *APC* alleles precede those in *KRAS* and *p53* alleles during tumour development.

Oncogenes and tumour suppressor genes are further categorised as either ‘gatekeepers’, or ‘caretakers’. Gatekeeper genes function to maintain normal cell proliferation. The *APC* gene is an example of a gatekeeper gene, as inactivation of a single *APC* allele enhances colonic epithelial cell proliferation. Although this change in proliferation rate is not sufficient to induce malignancy, it contributes towards tumour formation by increasing the likelihood of cells acquiring further genetic changes as they divide (Kinzler and Vogelstein, 1996b). Other examples of gatekeeper genes include the retinoblastoma gene (*Rb*) which, when mutated, is involved in initiating transformation of retinal epithelial cells, and the *VHL* gene (von Hippel-Lindau) involved in the transformation of kidney cells (Knudson, 1993). Caretaker genes are responsible for maintaining genomic integrity and genetic instability following loss of caretaker alleles accelerates the mutation frequency in other alleles, including those defined as gatekeepers, to promote tumour formation. Common examples of caretaker genes include *BRCA1* and *BRCA2*, involved in DNA repair which are often mutated in breast cancers, and the *MSH2* gene, a mismatch repair gene that is often mutated in hereditary nonpolyposis colorectal cancer.

Li-Fraumeni syndrome is a rare familial disorder involving increased predisposition to leukaemia, breast and brain cancers. Li-Fraumeni syndrome results from a germline mutation of *p53*, and the risk of developing cancer increases with age following somatic mutation of the remaining *p53* allele (Malkin et al., 1990). Similarly, germline inactivation of the *VHL* tumour suppressor gene gives rise to *VHL* hereditary cancer syndrome, involving increased predisposition to neuronal and retinal hemangioblastomas, clear cell renal carcinomas, and pheochromocytomas (Kim and Kaelin, 2004). Individuals with *VHL* disease are therefore *VHL* heterozygotes, they harbour one wildtype *VHL* allele, and another that is genetically inactivated. Renal cell carcinoma and hemangioblastomas are only formed following somatic inactivation of the remaining wildtype *VHL* allele. Like the *APC* gene in colorectal cancer, *p53* and *VHL* also serve as early gatekeeper genes, initiating the process of deregulated cell growth and promoting accumulation of further genetic changes that are required for cancer initiation and progression.

Clearly, cancer is a complex disease, and the involvement of numerous cancer-associated genes is critical in determining how efficiently cells progress from a normal to a cancerous phenotype. Although the number of cancer causing genes is vast, the cell cycle and cell death pathways that they regulate can be arranged into distinct categories. These categories have helped researchers to simplify the properties that are commonly shared by each cancer, and are often referred to as the hallmarks of cancer. Studying the hallmarks of cancer enables an understanding of the cell signalling pathways that are deregulated by specific genes and how these pathways are shared amongst distinct malignancies.

1.2.2 The hallmarks of cancer

All cells regulate proliferation, differentiation and death by complex pathways that interact with one another. Many cancers share common traits in their molecular, biochemical and cellular characteristics. Hanahan and Weinberg first defined six hallmarks that were shared by all cancer cells (Hanahan and Weinberg, 2000). These included: 1. Sustained proliferative signalling, often by growth factors, 2. Evasion of growth suppressor pathways, those involving tumour suppressor genes, or ‘gatekeeper’ genes, 3. Resistance to cell death by mechanisms such as apoptosis, autophagy and necrosis, 4. Angiogenesis, 5. Invasion and metastasis, and, 6. Genomic instability and mutation, by loss of ‘caretaker’ genes that maintain genomic integrity (Hanahan and Weinberg, 2000). Since then, two further hallmarks of cancer have emerged (Hanahan and Weinberg). These are evasion of the immune system to inhibit

targeting of cancer cells by immune cells for destruction, and reprogramming of energy metabolism to fuel cell growth and division, an idea that was proposed by Warburg as early as 1930 (Warburg et al., 1927).

The hallmarks of cancer that we are interested in exploring are angiogenesis and evasion of cell death by apoptosis. Tumour cells which evolve to induce angiogenesis in a growing tumour mass while escaping cell death in a stressful microenvironment have a significant growth advantage (Graeber et al., 1996). Our laboratory aims to understand the relationship between angiogenesis and cell death as a basis for how these processes may be deregulated in tumour development. These hallmarks will be briefly introduced, and then the key regulators of the pathways involved will be discussed in greater detail later.

1.2.3 Control of cell survival and cell death

Cell survival is maintained by intracellular sensors that monitor stress signals such as hypoxia, DNA damage, loss of survival factors, nutrient deprivation and various signalling imbalances (Evan and Littlewood, 1998). Cellular stress can lead to either cell cycle arrest, or cell death, often by an organised process referred to as apoptosis. The p53 tumour suppressor protein maintains genetic stability by inhibiting cell cycle progression in response to stress (Lane, 1992; Tlsty, 1997). In this way, the cell controls for further damage and protects surrounding cells and tissues.

1.2.3.1 Deregulated apoptosis is a hallmark of cancer

Loss of cell cycle control by p53 is recognised as an important determinant of tumour development and deregulated p53 activity is linked to over 50% of human cancers (Harris, 1996; Vousden and Lane, 2007). The overall importance of p53 in regulating cell fate is shown by its association with the pathology of various neurodegenerative diseases such as Huntington's disease (Bae et al., 2005), Parkinson's disease (Bretaud et al., 2007), and Alzheimer's disease (Culmsee and Landshamer, 2006). Expression of p53 has also been shown to regulate survival, self renewal and differentiation of neural stem cells, as well as those found in the mammary epithelium (Cicalese et al., 2009; Meletis et al., 2006). Studies in which p53 has been associated with ageing have also been described (Arum and Johnson, 2007; Feng et al., 2007).

1.2.4 Regulation of angiogenesis

Cells need a continuous supply of oxygen and nutrients to survive. When the blood supply becomes limited, tumour cells induce the formation of new blood vessels by a highly regulated process described as angiogenesis (Carmeliet and Jain, 2000). Angiogenesis (also referred to as neoangiogenesis) is the process by which new capillaries branch from pre-existing vessels (Carmeliet, 2000; Risau, 1995). During embryogenesis, blood vessels may also develop by vasculogenesis, whereby endothelial cells are born from progenitor cell types, however angiogenesis only occurs in adult tissues at times of wound healing and during tumour growth.

1.2.4.1 Sustained angiogenesis is a hallmark of cancer

Angiogenesis is a common hallmark of cancer and contributes significantly to the invasive and metastatic potential of tumour cells into surrounding tissues and organs. In normal tissues, vessel structure and density are maintained by a dynamic and controlled angiogenic process, whereby oxygen supply meets demand. In the tumour microenvironment however, cell density and the demand for survival factors and oxygen exceeds the rate at which these can be supplied through the vascular network. Tumour cells therefore use distinct strategies to induce angiogenesis and receive the oxygen and nutrients they require. The resulting vascular networks formed within tumours are disorganised with irregular and ineffective blood flow, and the surrounding tumour tissues often suffer from acute and chronic regions of low oxygen tension (or hypoxia).

Tumours that contain large regions of hypoxia are associated with poor prognosis and poor patient survival. Such tumours promote an aggressive phenotype and are often associated with resistance to many standard lines of therapy. The transient opening and collapse of blood vessels through hypoxic tumours leads to increased genomic instability in hypoxic cells that are re-exposed to oxygen. Genomic instability increases mutation rates that deregulate key signalling pathways and promote tumourigenesis (Yuan and Glazer, 1998). Hypoxic tumours are also more invasive (Pennacchietti et al., 2003) and metastatic (Rofstad and Halsor, 2000).

The hypoxia inducible factor (HIF) is a transcription factor that maintains oxygen homeostasis and regulates angiogenesis (Bardos and Ashcroft, 2005). The HIF transcription factor is formed by dimerisation of various subunits that are part of the HIF family, and when activated, regulates expression of numerous genes involved in the

adaptive response to low oxygen concentration. HIF activation is deregulated across a broad range of tumours including colon, gastric, skin, pancreatic and renal carcinomas (Zhong et al., 1999) and is also known to play an important role in embryonic development (Ryan et al., 1998), stroke, and cardiovascular disease (Semenza and Wang, 1992).

As discussed, deregulation of both angiogenic and cell death responses are important hallmarks of cancer. The angiogenic response is a complex, controlled process that regulates vascular structure in both normal tissues and malignant tumours. Activation of HIF in response to hypoxia is central to angiogenesis. The mechanisms by which HIF transcription is activated in hypoxia will be discussed in greater detail below.

1.3 The HIF family

Hypoxia inducible factors are members of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors that bind specific sequences on DNA called hypoxia response elements (HREs). The HIF family is divided into the HIF-α subunits, of which there are HIF-1α, HIF-2α, and HIF-3α, and the HIF-β subunits, comprising HIF-1β, and HIF-2β. The active HIF transcription factor is a heterodimer consisting of an oxygen sensitive HIF-α subunit, complexed with a constitutively expressed HIF-1β subunit.

HIF-1 (comprising HIF-1α and HIF-1β) was discovered following identification of a nuclear factor binding sequence in the 3' enhancer region of the hypoxia induced erythropoietin gene (*EPO*, encoding a renal hormone that promotes haemoglobin synthesis), (Semenza et al., 1991). Subsequent studies showed that the HIF transcriptional complex is necessary for binding to this enhancer region in hypoxia, and inducing the transcription of erythropoietin (Wang and Semenza, 1995). Since then, both HIF-1α and HIF-2α subunits of the HIF-1 and HIF-2 transcription factors respectively, have been well studied. The physiological significance of HIF-1α in regulating angiogenesis is shown by *in vivo* mouse knockout models whereby deletion of *Hif-1α* leads to early embryonic lethality due to abnormal vascular development (Iyer et al., 1998; Ryan et al., 1998).

HIF-2α is also known as EPAS1 (endothelial PAS domain protein 1) and shares 48% amino acid sequence identity to HIF-1α (Tian et al., 1997). Compared to HIF-1α which is expressed at a fairly similar level in many cell types, HIF-2α expression is more variable and partially evident in endothelial cells, type II pneumocytes, cardiomyocytes

and hepatocytes (Wiesener et al., 2003). Deletion of *Hif-2α* however has distinct phenotypes *in vivo* depending on the mouse strain used. For this reason, elucidating the distinct functions of HIF-2α from HIF-1α has been challenging. In some models, *Hif-2α* knockout mice survive until adulthood (Scortegagna et al., 2005). In other studies, *Hif-2α* knockout mice are embryonic lethal, and this is shown to occur by numerous phenotypes including heart failure (Tian et al., 1998), respiratory distress syndrome (Compernolle et al., 2002), and severe vascular defects (Peng et al., 2000).

Regardless of these discrepancies, adult mice in which *Hif-2α* is deleted suffer from severe anaemia suggesting a critical for role for HIF-2α in regulating erythropoiesis in both physiological and stress induced conditions (Gruber et al., 2007). In humans, mutations in the *HIF-2α* gene are associated with erythrocytosis, a condition whereby erythropoietin synthesis is deregulated (Percy et al., 2008). Patients that suffer from erythrocytosis have increased erythropoietin levels, elevated haemoglobin counts, and blood volumes. Genome wide linkage analysis has shown that a single activating mutation in the *HIF-2α* gene is responsible for causing erythrocytosis in certain families (Gale et al., 2008). Furthermore, genetic selection of *HIF-2α* variants in Tibetan highlanders has also been shown to alter haemoglobin concentrations and protect against high altitude (Beall et al., 2010). Finally, in *VHL* defective hemangioblastomas, pheochromocytomas and renal cell carcinomas, HIF-2α overexpression upregulates the expression of genes that are important in promoting tumour progression (Kondo et al., 2003; Mandriota et al., 2002).

Although HIF-3α shares structural similarities with HIF-1α and HIF-2α, less is known about its regulation. HIF-3α protein is found in the human kidney, thymus, lung, brain, heart and liver (Hara et al., 2001; Makino et al., 2001). As with HIF-1α and HIF-2α, HIF-3α forms a heterodimer with its corresponding HIF-β subunit in response to low oxygen conditions (Gu et al., 1998). However, function of the activated HIF-3α transcription factor in mediating gene expression *in vivo* is not well understood (Gu et al., 1998). HIF-3α has a number of splice variants. The truncated HIF-3α variant that lacks a transactivation domain in its C terminus forms an IPAS protein (inhibitory domain PAS protein) that binds to HIF-1β and can inhibit HIF-1α mediated gene expression to repress tumour growth *in vivo* (Hara et al., 2001; Makino et al., 2001).

The HIF-β subunits of the HIF family are also known as aryl receptor nuclear transporter (ARNT) proteins, and three isoforms have been described: HIF-1β/ARNT1, HIF-2β/ARNT2, and HIF-3β/ARNT3. Like the HIF-α subunits, HIF-β subunits contain

bHLH, PAS and transactivation domains, however, they lack an oxygen-dependent degradation domain (ODD) allowing constitutive and ubiquitous expression in all tissues (Huang et al., 1996b). HIF-1β/ARNT1 was identified due to its requirement for dioxin (aryl hydrocarbon) receptor function in hepatocytes (Reyes et al., 1992). Later studies showed that disruption of the *Hif-1β/Arnt1* gene in mice led to inhibition of transcriptional responses by low glucose and oxygen, and early embryonic lethality due to severe vascular defects (Maltepe et al., 1997). HIF-2β/ARNT2 has 57% sequence similarity to HIF-1β/ARNT1, however it is only expressed in the brain and kidney, and has unique functions in embryonic development compared to HIF-1β/ARNT1 (Hirose et al., 1996). Less is known about HIF-3β/ARNT3 (also known as Mop3/BMAL1), although interactions with HIF-1α, and HIF-2α have been described (Hogenesch et al., 1998). A role for HIF-3β/ARNT3 in regulating circadian rhythm has been suggested due to its dimerisation with CLOCK (circadian locomotor output cycles kaput) (Bunger et al., 2000).

1.3.1 Oxygen dependent regulation of HIF-1α

While HIF-β (defined here as including HIF-1β and HIF-2β) is constitutively expressed, the HIF-α subunit (defined here as both HIF-1α and HIF-2α, unless otherwise specified) is stabilised transiently in response to low cellular oxygen. The oxygen dependent degradation domain (ODD) of HIF-α regulates protein stability by forming a complex with the pVHL ligase complex (Maxwell et al., 1999). With similarities to the SCF complex in yeast (Stebbins et al., 1999), the VHL protein (pVHL) binds to elongin B, elongin C (Kibel et al., 1995), cullin 2 (Cul2), (Pause et al., 1997), and Ring-box 1 (Rbx1) (Kamura et al., 1999) to form an E3 ubiquitin ligase complex. This complex is involved in binding and polyubiquitinating HIF-α for destruction by the 26S proteasome. Interactions between HIF-α and the pVHL ligase complex are regulated by prolyl hydroxylase enzymes (PHD, prolyl hydroxylase domain-containing proteins), also referred to as the egg-laying-defective nine (EGLN) enzymes. Members of the PHD hydroxylase family hydroxylate HIF-α at one of two conserved prolyl residues within the ODD domain to form a binding site for the pVHL ubiquitin ligase complex (Jaakkola et al., 2001). In HIF-1α PHD hydroxylation sites correspond to proline residues 402 and 564 within the ODD domain, and in HIF-2α, these correspond to proline residues 405 and 531 (Jaakkola et al., 2001; Masson et al., 2001; Patel and Simon, 2008).

In hypoxia, the Siah E3 ligase targets prolyl hydroxylases for proteasomal degradation, consequently inhibiting hydroxylation and degradation of HIF-α (Nakayama et al., 2004). The nuclear localization sequences (NLS) at the N-terminal and C-terminal

regions of HIF-α are important for localising stabilised HIF-α protein to the nucleus. Once HIF-α is translocated into the nucleus, the PAS domain of HIF-α interacts with HIF-β to form a heterodimer. The transactivation domain (TAD) of HIF-α binds to key transcriptional co-activators such as the CAMP-response element binding protein (CREB)-binding protein (CBP)/p300 to promote gene expression. HIF-1α transcriptional activity is also regulated by the iron dependent hydroxylase, factor inhibiting HIF (FIH), which hydroxylates HIF-α at specific asparagine residues. Hydroxylation by FIH inhibits recruitment of p300/CBP to the transactivation domain, thereby inhibiting HIF transcriptional activity (Mahon et al., 2001).

As well as binding of co-activators, many post-translational modifications also occur at the transactivation domain of HIF-α including phosphorylation and acetylation which further mediate activation of HIF transcription (Schmid et al., 2004). The HIF transcriptional complex binds to a specific consensus sequence in the promoter of key target genes known as the hypoxia response element (HRE). Binding of HIF to DNA has been shown to occur from cellular oxygen concentrations less than 6%, and is maximised at 0.5% (Jiang et al., 1996). When HIF transcription is activated, expression of numerous target genes are induced to promote cellular adaptation to low oxygen (Semenza, 1999). The structural domains of the HIF-α subunits, HIF-1α and HIF-2α, and of HIF-1β/ARNT are summarised in Figure 1.3.1. Regulation of the HIF-α subunits is summarised in Figure 1.3.2.

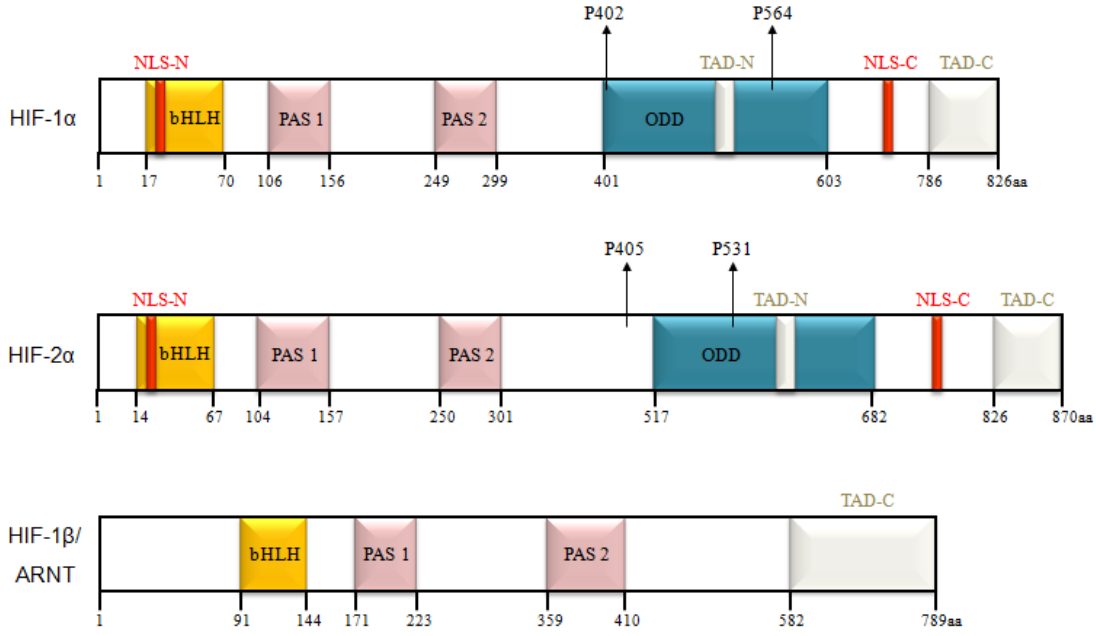


Figure 1.3.1: Schematic representation of HIF-1α, HIF-2α and HIF-1β/ARNT proteins

The HIF transcription factor belongs to the bHLH-PAS family of eukaryotic transcription factors. Both HIF-α and HIF-1β/ARNT subunits contain the highly conserved bHLH-PAS domains. While HIF-β is constitutively expressed in the nucleus, HIF-α activity and protein abundance is only increased by oxidative stress (hypoxia). The oxygen dependent degradation domain (ODD) is important in maintaining HIF-α protein stability. The ODD contains two prolyl hydroxylation sites at proline residues 402 and 564 (P402, P564) in HIF-1α, and at proline residues 405 and 531 (P405, P531) in HIF-2α. In normoxia, these sites are hydroxylated by prolyl hydroxylase enzymes that target the protein for proteosomal degradation by the pVHL E3 ligase complex. In hypoxia, prolyl hydroxylase enzymes are inhibited and HIF-α protein is stabilised. The N-terminus and C-terminus nuclear localisation signal (NLS) enable translocation of HIF-α to the nucleus, and binding to HIF-β to form an active HIF transcriptional factor complex. The PAS domain present in both HIF-α and HIF-1β/ARNT is responsible for dimerising the two partners. Transcriptional activity of HIF is regulated by the transactivation domains (TAD).

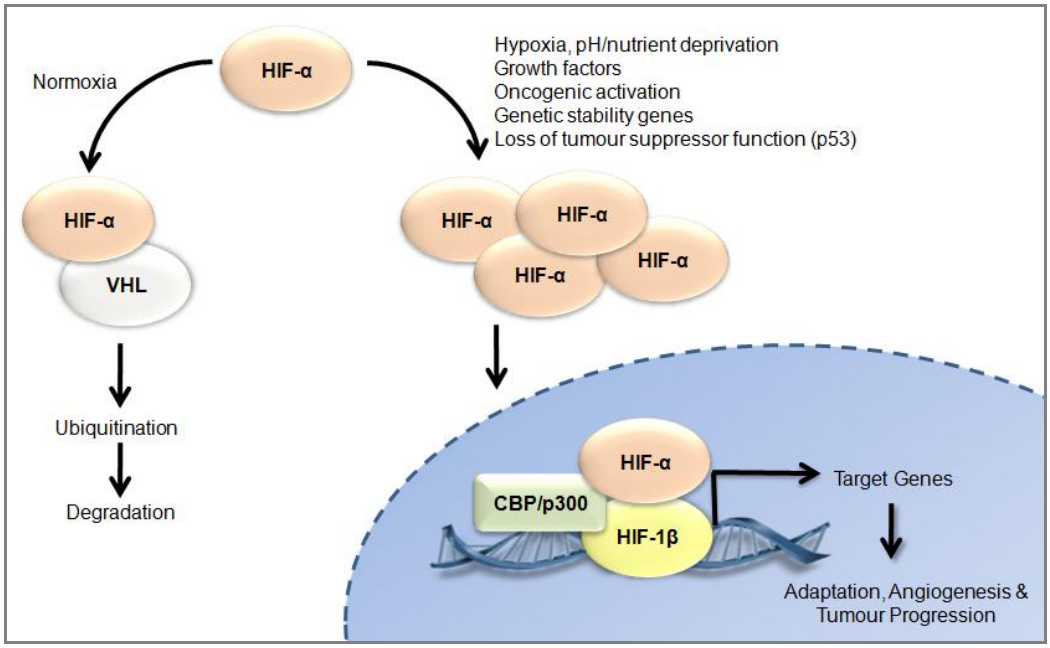


Figure 1.3.2: HIF-α interacts with HIF-1β/ARNT in the nucleus

HIF-α (HIF-1α or HIF-2α) is an important regulatory component of the HIF transcription factor. In normoxia, pVHL targets HIF-α for ubiquitination and proteosome mediated degradation. In response to a number of stressful stimuli including oxidative stress, oncogenic activation and loss of tumour suppressor function, HIF-α is stabilised, translocates to the nucleus and forms a complex with the constitutively expressed HIF-1β/ARNT subunit. The HIF-α and HIF-1β/ARNT complex binds to HRE in the promoters of target genes. Recruitment of the CBP/p300 co-activator forms an active HIF transcription factor, inducing transcription of target genes that are involved in cellular adaptation, angiogenesis, and tumour progression.

1.3.2 The role of HIF in hypoxic gene regulation

HIF (HIF-1 or HIF-2) is involved in regulating an array of target genes that promote adaptation to hypoxic stress. These include genes that are involved in metabolism, angiogenesis, oxygen delivery, cell growth and proliferation, metastasis and apoptosis (Semenza, 2001; Wenger et al., 1996). A selection of these genes have been shown in Figure 1.3.3. Many HIF target genes are involved in regulating angiogenesis such as transforming growth factor (*TGF*), and vascular endothelial growth factor (*VEGF*). HIF targeted genes that regulate metastasis include cadherin 1 (*CDH1*) which encodes the E-cadherin (epithelial cadherin) protein. E-cadherin is involved in maintaining tissue architecture and also regulates cell proliferation and cell death by its interactions with the catenin family of proteins (Conacci-Sorrell et al., 2002). Attenuation of *CDH1*

expression by HIF inhibits cell adhesion and promotes tumour invasion (Esteban et al., 2006; Imai et al., 2003). Lysyl oxidase (*LOX*) is also a HIF target gene, and is essential for hypoxia induced metastasis (Erler et al., 2006).

Metabolic adaptation to hypoxia is an emerging hallmark of cancer cells and numerous metabolic genes have been identified that are induced by HIF activity. To preserve energy during oxidative stress, cancer cells can efficiently switch from using mitochondrial respiration for ATP production, to using anaerobic glycolysis. This switch in metabolic signalling was originally discovered in the 1800's by Louis Pasteur, a microbiologist who observed changes in yeast growth under aerobic and anaerobic conditions. In anaerobic conditions, yeast growth was inhibited by the conversion of glucose to lactate, in a process described as fermentation. These changes are now referred to as the Pasteur Effect and describe switches in energy consuming pathways when cells have limited supplies of oxygen. Warburg later studied metabolic signals in cancer cells and described the switch to anaerobic glycolysis from mitochondrial respiration as an important response for cancer cell survival. Changes in cancer cell metabolism have since been referred to as the Warburg Effect (Hanahan and Weinberg, 2011; Warburg, 1956).

Many HIF target genes have been identified in hypoxic conditions that are involved in both inhibiting mitochondrial respiration and promoting anaerobic glycolysis. In normal cells, glucose is converted to pyruvate by glycolytic enzymes. Pyruvate is transported into mitochondria for conversion to acetyl coenzyme A (acetyl-CoA). The tricarboxylic acid (TCA) cycle begins when acetyl-CoA is catabolised and involves a series of chemical reactions leading to ATP production. In hypoxia, HIF induced expression of pyruvate dehydrogenase kinase 1 (*PDK1*) blocks conversion of pyruvate to acetyl-CoA by inhibiting the pyruvate dehydrogenase (PDH) enzyme complex (Kim et al., 2006; Papandreou et al., 2006). By enhancing *PDK1* activity, HIF inhibits mitochondrial respiration and promotes glucose metabolism by anaerobic glycolysis. When oxygen supply is limited in hypoxic cells, anaerobic glycolysis is an important pathway by which cell growth is slowed, and energy levels are maintained. Instead of converting pyruvate to acetyl-CoA, anaerobic glycolysis involves a series of enzymatic reactions whereby pyruvate is converted to lactate (Koukourakis et al., 2005). HIF has been shown to activate glucose transporter-1, and -3 (*GLUT-1* and *GLUT-3)* and increase glucose transport into the cell for glycolysis (Iyer et al., 1998). To further increase the efficiency of ATP production by anaerobic glycolysis, HIF also induces expression of numerous

genes that encode glycolytic enzymes involved in glucose conversion to lactate such as aldolase A (*ALDA*) and lactate dehydrogenase A (*LDHA*) (Semenza et al., 1996).

Structural data which show differences in transactivation domain sequence suggest that HIF-1α and HIF-2α may have independent target genes (Tian et al., 1997). Consequently, previous studies have attempted to unveil the differential pathways that HIF-1α and HIF-2α may control during tumourigenesis (Hu et al., 2003). Conditional knockout studies of *hif-1α* and *hif-2α* in hypoxic epithelial cells of the intestine, for example have shown that HIF-2α, and not HIF-1α is involved in regulating iron absorption by inducing expression of the divalent metal transporter 1 (*DMT1*) gene (Mastrogiannaki et al., 2009). In *VHL* defective renal cell carcinoma, it is thought that HIF-1α and HIF-2α have contrasting functions. HIF-1α inhibits tumour growth by activating pro-apoptotic target genes such as *BNIP3* (Bcl-2 nineteen kD interacting protein 3), while HIF-2α expression promotes tumour growth by inducing expression of the pro-tumourigenic target genes *VEGF* and *TGFα* (Kondo et al., 2002; Raval et al., 2005). These studies not only confirm the critical role of pVHL in regulating HIF-α protein stability during renal cell carcinogenesis, but also highlight the unique profile HIF-α subunits may have in inducing tumour specific gene expression.

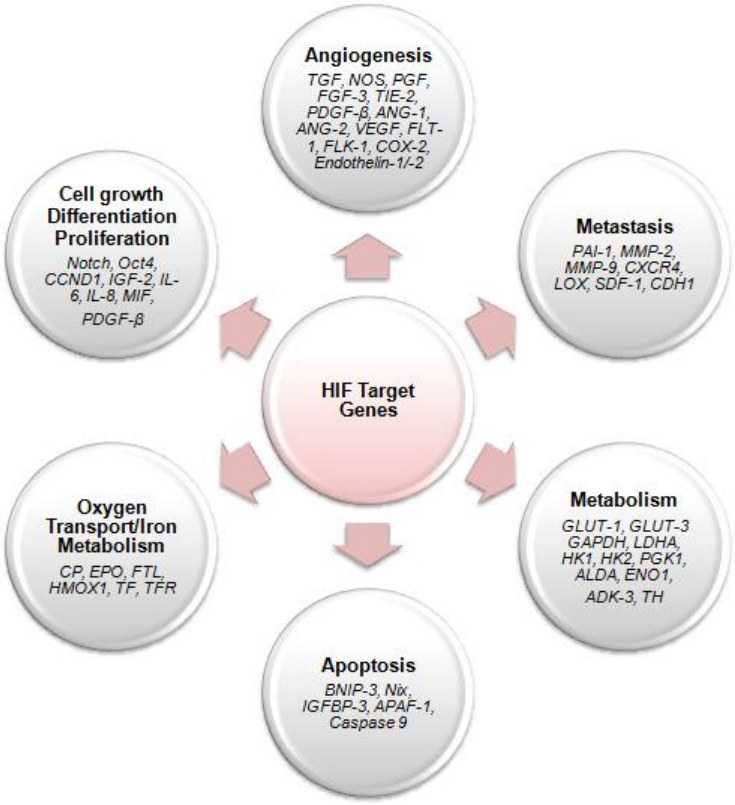


Figure 1.3.3: HIF target genes are involved in numerous cell pathways that promote tumourigenesis

HIF target genes are involved in controlling an array of cellular processes that promote tumourigenesis including angiogenesis, cell metabolism and metastasis. Over 100 HIF target genes have been described and some key genes involved are highlighted and are abbreviated in alphabetical order as follows: *ADK-3* (adenylate kinase-3), *ALDA* (aldolase A), *ANG-1/-2* (angiopoetin-1/-2), *APAF-1* (apoptotic peptidase activating factor-1), *BNIP3* (Bcl-2/adenovirus E1B 19kDa interacting protein 3), *CCND1* (cyclin D1), *COX-2* (cyclo-oxygenase-2), *CP* (ceruloplasmin), *CXCR4* (CXC chemokine receptor type 4), *ENO1* (Enolase 1), *EPO* (erythropoietin), *FGF-3* (fibroblast growth factor-3), *FLT-1* (VEGF receptor-1), *FLK-1* (VEGF receptor-2), *FTL* (ferritin light chain), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *GLUT-1/-3* (glucose transporter-1/-3), *HK1/-2* (hexokinase 1/-2), *HMOX1* (heme oxygenase 1), *IGF-2* (insulin like growth factor-2), IGFBP-3 (insulin-like growth factor binding protein-3), *IL-6/-8* (interleukin-6/-8), *LDHA* (lactate dehydrogenase A), *LOX* (lysyl oxidase), *MIF* (macrophage inhibitory factor), *MMP-2/-9* (matrix metalloprotenases-2/-9), *NOS* (nitric oxide synthase), *Oct4* (octamer binding transcription factor 4), *PAI-1* (plasminogen activator inhibitor-1), *PDGF-β* (platelet derived growth factor-β), *PGF* (placental growth factor), *PGK1* (phoshoglycerate kinase 1), *SDF-1* (stromal-derived factor-1), *TF* (transferrin), *TFR* (transferrin receptor), *TGF* (transforming growth factor), *TH* (tyrosine hydroxylase), *TIE-2* (UPAR, urokinase plasminogen activator receptor), *VEGF* (vascular endothelial growth factor).

As discussed, HIF is involved in promoting tumour development by inducing many target genes that regulate cellular adaptation to hypoxia. We are particularly interested in understating the role that HIF plays in regulating cell apoptosis. Hypoxic cells are often resistant to radiotherapy and chemotherapy induced cell death due to their confined location within tumours, as well as the molecular mechanisms that are controlled by HIF. Common HIF target genes that are involved in regulating apoptosis will be discussed further.

1.3.3 The role of HIF in tumour cell apoptosis

As well as promoting cancer cell survival through cellular adaptation, in some settings HIF also has an established role in promoting tumour cell apoptosis. In *VHL*-deficient tumours for example, HIF-1 (the active HIF transcription factor comprising HIF-1α and HIF-1β) inhibits tumour growth by inhibiting proliferation and inducing apoptosis (Acker et al., 2005). HIF-1 induced expression of pro-apoptotic BNip3 and the Nip3-like protein X (Nix) induces mitochondrial pore permbealisation, promoting mitochondrial dysfunction and apoptosis (Bruick, 2000). Furthermore, HIF has been shown to induce expression of insulin like growth factor binding protein-3 (IGFBP-3), (Tazuke et al., 1998). Hypoxic cells are sensitised to radiation induced apoptosis by IGFBP-3 mediated inhibition of insulin like growth factor-1 (IGF-1), resulting in higher pro-apoptotic Bcl-2 protein levels (Butt et al., 2000). HIF-1α has also been shown to promote p53-dependent apoptosis by stimulating the expression of apoptotic protease activating factor 1 (APAF-1) and its co-factor caspase 9 (Soengas et al., 1999).

Although HIF induces expression of target genes involved in apoptosis, the interplay between HIF and p53, the central node by which cell death is achieved, remains unclear. Some studies suggest that HIF and p53 have a negative relationship, in that high levels of HIF-1α in hypoxic tumour cells inhibit p53 transcriptional activity, and p53-induced apoptosis (Bertout et al., 2009). To understand the relationship between HIF and p53, the functional roles of p53 will be discussed in greater detail first, followed by how p53 activity can be modified in hypoxic cells to promote survival and tumour progression.

1.4 Regulation of cellular responses to exogenous stress by p53

The p53 protein is stabilised by stress stimuli including DNA damage, telomere erosion, aberrant proliferative signals, loss of key adhesion and survival signals, and non genotoxic forms of stress such as exposure to hypoxia and heat shock (Graeber et al.,

1996). Stabilisation of p53 is also induced by SV40 and adenovirus transformed cells (Lane and Crawford, 1979; Lowe and Ruley, 1993), as well as the expression of cellular oncogenes such as *Rb*, *MYC* and *RAS* (Prives, 1998). When activated, p53 regulates multiple signalling pathways including cell growth, differentiation, apoptosis, and senescence (Harris and Levine, 2005). Cellular responses mediated by p53 are summarised in Figure 1.4.1.

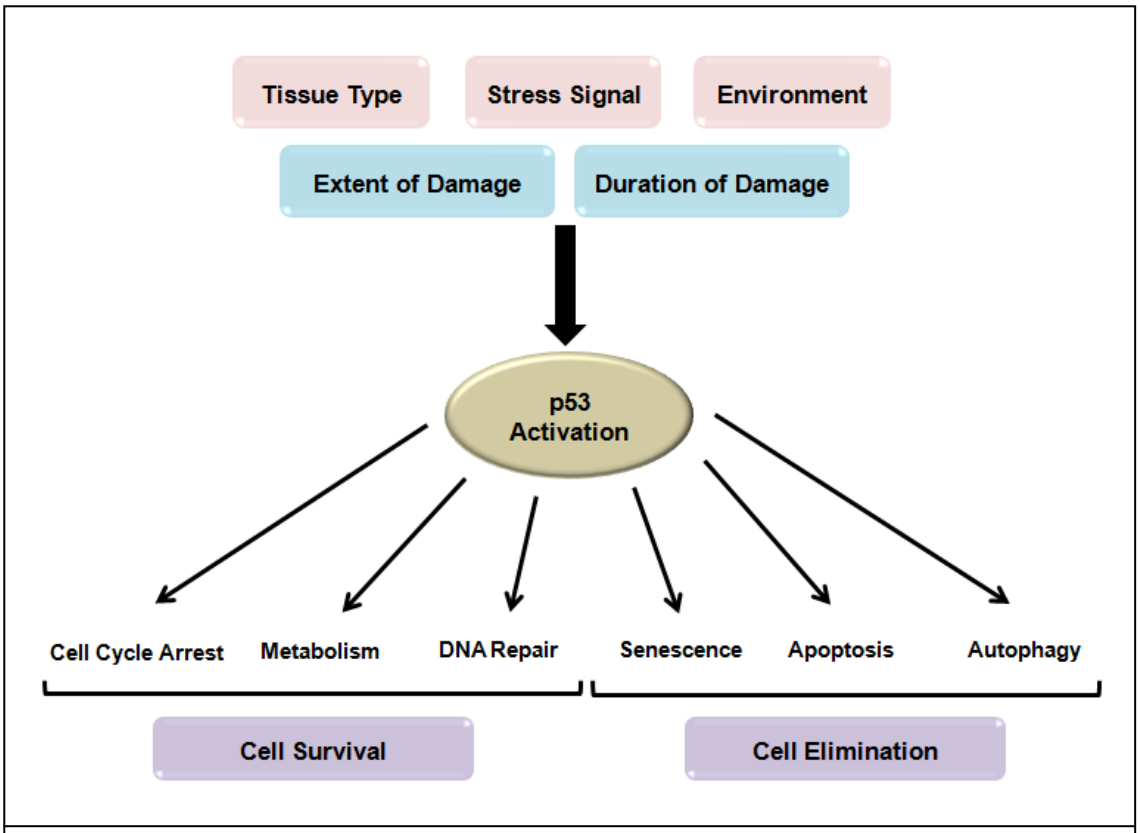


Figure 1.4.1: p53 regulates a variety of responses to cellular stress
Pathways regulated by p53 are activated according to the extent and duration of damage that cells are exposed to. Cell survival and cell death responses by p53 will vary according to tissue and tumour type, the specific stress signal induced, and impact of the cell's microenvironment. Numerous p53 mediated pathways are transduced upon p53 activation which are involved in either rescuing cells through stress and promoting cell survival, or inducing apoptosis in defective cells.

1.4.1 Control of p53 stability

Cellular levels of p53 protein are determined by rate of degradation rather than rate of synthesis (Vogelstein et al., 2000). In normal cells, p53 stability is regulated by ubiquitin mediated protein degradation via the proteosome. The HDM2 protein (originally identified in mice as murine double minute 2 protein, also referred to as MDM2) is an E3 ubiquitin ligase for p53 (Honda et al., 1997; Momand et al., 2000). In normal cells, basal levels of p53 are maintained by expression of HDM2 in an autoregulatory feedback loop (Haupt et al., 1997; Kubbutat et al., 1997).

When a stress signal is elicited, the nuclear localisation signal in the C-terminus of p53 mediates transport to the nucleus by interactions with the dynein motor protein and microtubule networks (Giannakakou et al., 2000). Once inside the nucleus, p53 binds to DNA and undergoes distinct conformational changes which promote p53 transcriptional activity (Halazonetis et al., 1993). Active p53 induces transcription of its downstream target gene *HDM2*. Induced HDM2 protein inhibits p53 activity by forming a complex with the N-terminal transactivation domain (residues 19-26) of p53 and targeting p53 for proteosomal degradation (Chen et al., 1993; Momand et al., 1992). Loss of p53 activity by HDM2 mediated export and degradation allows basal levels of p53 to be restored once the stress signal is removed (Haupt et al., 1997; Kubbutat et al., 1997). Interestingly, HDM2 can also directly repress p53 transcriptional activity by associating with and promoting histone modification on the chromatin of p53 target genes (Minsky and Oren, 2004). The importance of the p53-HDM2 feedback loop is demonstrated by *in vivo* studies whereby loss of *Mdm2* leads to embryonic lethality in a p53-dependent manner. Genetic loss of *Mdmx*, an HDM2 related gene also induces embryonic lethality suggesting that MDMX also regulates p53 (Parant et al., 2001).

Because HDM2 is a critical regulator of p53 function, regulation of HDM2 is also tightly controlled. HDM2 is phosphorylated at multiple sites *in vivo* (Ashcroft et al., 2002; Hay and Meek, 2000) and by a number of protein kinases in response to specific forms of stress (Khosravi et al., 1999; Mayo et al., 1997). Under normal conditions, in response to growth factors, the phosphatidyl inositol-3-kinase (PI3K) signalling pathway is activated and its downstream substrate AKT/protein kinase B (PKB) phosphorylates HDM2 at serine 166 and serine 186, leading to localisation of HDM2 to the nucleus and inhibition of p53 activity (Ashcroft et al., 2002; Mayo and Donner, 2001). ARF (alternate reading frame product of the *CDKN2A* locus), referred to as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice, stabilises p53 through its negative interaction with HDM2 (Sherr, 1998).

ARF counteracts hyperproliferation by inhibiting p53 directed ubiquitin ligase activity of HDM2 and also alters the subcellular localization of p53 by targeting p53 to the nucleosome for activation (Honda and Yasuda, 1999; Midgley et al., 2000).

1.4.2 Regulation of p53 activity by post-translational modifications

Once stabilised, p53 is post-translationally modified to promote its transcriptional activity in the nucleus (Fritsche et al., 1993). These modifications include phosphorylation (Meek, 1994), dephosphorylation, acetylation, ribosylation and SUMO-1 modification (Ashcroft et al., 2000; Gostissa et al., 1999; Ljungman, 2000; Muller et al., 2000). Post-translational modifications at lysine residues within the caroboxyl terminal of p53 are important in regulating p53 activity. While acetylation at lysine residues activate p53, methylation retains p53 in a transcriptionally repressed state (Huang and Berger, 2008).

Recently, Loewer et al. showed that post-translational modifications of p53 are critical in determining the basal dynamics of p53. In a normal cell cycle, p53 levels oscillate, corresponding with harmless DNA strand breaks (Loewer et al., 2010). Although p53 is stabilised during a normal p53 pulse, carboxyl terminal lysine residues remain methylated, rendering p53 transcriptionally inactive. However, p53 that is stabilised by more severe forms of extrinsic stress is acetylated at the carboxyl terminus to promote transcriptional activity and stress induced cell cycle arrest (Loewer et al., 2010). Investigating the basal dynamics of p53 regulation and the numerous post-translational modifications that determine p53 transcriptional responses allow greater understanding of the mechanism by which p53 is able to filter stress inputs and always remain on standby as a critical and immediate response to more severe forms of stress.

1.4.2.1 Regulation of p53 by phosphorylation

Post-translational phosphorylation of p53 in response to many forms of genotoxic and microenvironmental stress is also well studied (Ashcroft et al., 2000). Phosphorylation of p53 in response to DNA damage is mediated by a series of highly conserved checkpoint kinases (Albrechtsen et al., 1999). Both the DYRK2 (Taira et al., 2007) and HIPK2 kinases (Calzado et al., 2007) have recently been identified as important initiators of apoptosis by phosphorylating p53 under severe stress. Although numerous kinases regulate p53 activity by phosphorylation (Meek, 1994) it has also been demonstrated using a series of N-terminal and C-terminal p53 mutants that phosphorylation of p53 is not critical for DNA damage induced stabilisation of p53

(Ashcroft et al., 1999). Loss of post-translational modification by phosphorylation can reduce the efficiency of p53 activity, without causing complete loss of function, suggesting that a wide range of co-dependent modifications of p53 are required for regulating p53 activation (Ashcroft et al., 1999; Crook et al., 1994). Common p53 post-translational modification sites are shown in Figure 1.4.2.

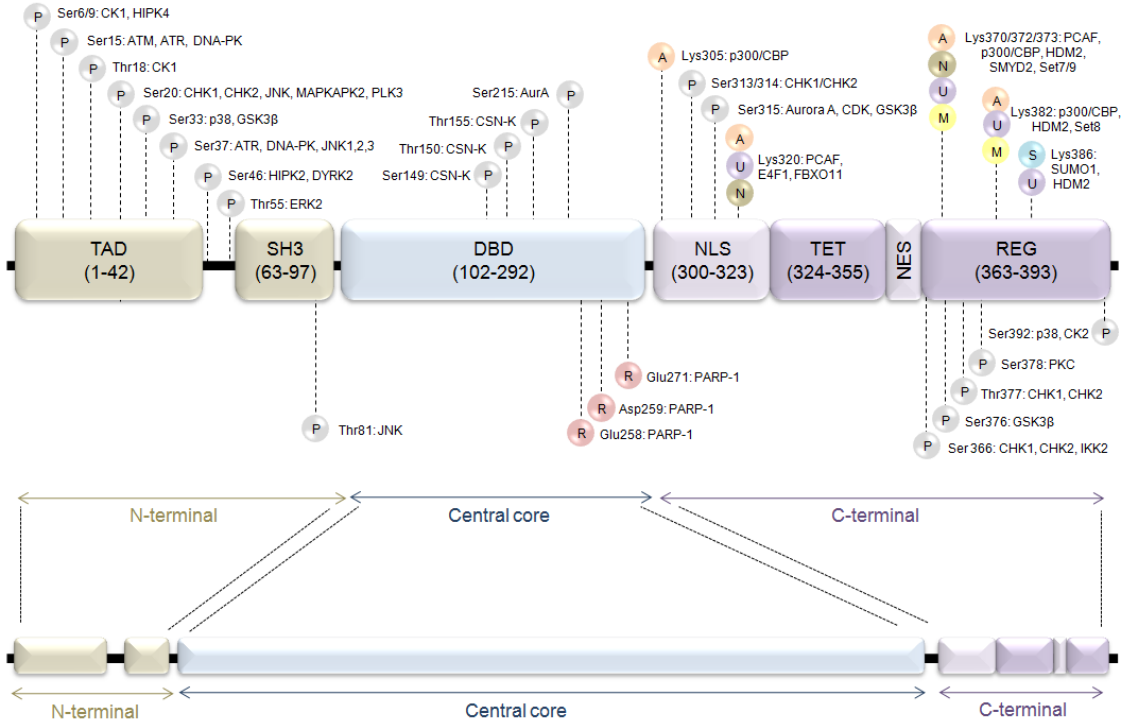


Figure 1.4.2: Post-translational modification of p53

The p53 protein has three functional regions important for stabilisation, localisation and transcriptional activity: the N-terminal, the central core, and the C-terminal region. The N-terminal region consists of the transactivation domain (TAD) for transcriptional activation of p53, and the Src homology 3-like domain (SH3), required for various protein interactions. The central core of the protein consists of the sequence specific DNA binding domain (DBD). The C-terminal region contains the nuclear localisation (NLS), tetramerisation (TET), nuclear export signal (NES), and regulatory (REG) regions. The domains illustrated are not to scale but amino acid length have been shown in brackets. An accurate representation of size is redrawn below. When stabilised, the p53 protein undergoes a number of post-translational modifications which influence p53 stabilisation and activity, including phosphorylation (P), acetylation (A), ubiquitination (U), neddylation (N), ribosylation (R), methylation (M), and sumoylation (S). Many target sites for post-translational modification of p53 by these mechanisms have been identified, and a few common sites are indicated along with the modifying proteins involved. Amino acid abbreviations: Ser (serine), Thr (threonine), Lys (lysine), Glu (glutamic acid), Asp (aspartic acid). Protein abbreviations (in alphabetical

order): AurA (Aurora A), ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), CDK (cyclin dependent kinase), CHK1/-2 (checkpoint kinase 1/-2), CK1/-2 (casein kinase 1/-2), CSN-K (casein kinase), DNA-PK (DNA dependent protein kinase), DYRK2 (dual specificity tyrosine phosphorylation regulated kinase 2), ERK2 (extracellular signal regulated kinase 2), E4F1 (E4F transcription factor 1), FBXO11 (F-box only protein 11), GSK3β (glycogen synthase kinase 3 beta), HDM2 (human double minute 2), HIPK2/-4 (homeodomain interacting protein kinase 2/-4), IKK2 (inhibitor of nuclear factor kappa-b kinase), JNK1/-2/-3 (c-Jun N-terminal kinase 1/-2/-3), MAPKAPK2 (mitogen activated protein kinase activated protein kinase 2), PARP-1 (poly ADP ribose polymerase), PCAF (p300/CBP associated factor), PKC (protein kinase C), PLK3 (polo-like kinase 3), p300/CBP (E1A binding protein p300/CREB binding protein), Set7/-8/-9 (SET domain containing lysine methyltransferase-7/-8/-9), SMYD2 (SET and MYND domain containing 4), SUMO1 (small ubiquitin related modifier 1).

1.4.3 Important co-activators for p53 transcriptional activity

Transcriptional co-activators are also important for activating p53 target genes. The apoptosis stimulating protein of p53 (ASPP) family of proteins, specifically ASPP1 and ASPP2 interact with p53, increasing p53 binding to DNA and promoting p53 target gene expression *in vivo* (Samuels-Lev et al., 2001). The p53BP1 and p53BP2 proteins identified by a yeast two-hybrid system also bind to the central DNA binding domain of wildtype p53 to increase p53 transcriptional activity (Iwabuchi et al., 1994). JMY (junction mediating and regulatory protein, p53 cofactor), (Shikama et al., 1999) and p300/CBP (CREB binding protein) are also important in regulating p53 transcriptional activity (Avantaggiati et al., 1997). Both JMY and p300 co-factors form a complex with activated p53 to potentiate its activity and drive the expression of p53 mediated cell death genes in response to stress (Shikama et al., 1999).

1.4.4 Novel regulators of p53 target gene expression

Chromatin immunoprecipitation studies have shown that although p53 remains associated to target gene promoters in the absence of stress, p53 activity is inhibited by specific transcriptional repressors (Jang et al., 2009; Kaeser and Iggo, 2002). Binding of p53 to target gene promoters allows for prompt p53 activation and target gene expression when cells are exposed to stress. Recently, a p53-Cabin1 (calcineurin binding protein) complex was described that regulated histone modification on the chromatin of p53 target genes such as *GADD45A* and *CDKN1A* (p21), maintaining p53 gene expression at basal levels (Jang et al., 2009). When cells were exposed to genotoxic stress, Cabin1 was degraded allowing p53 to bind target sequences,

associate with transcriptional co-factors and induce transcription of key genes required for a prompt stress response. Such studies provide important insight into basal p53 interactions and transcriptional responses that determine p53-dependent cellular responses to stress stimuli.

Moumen et al. show that the heterogeneous nuclear ribonucleoprotein K (hnRNP K) cofactor complexes with p53 in the absence of stress, suggesting that like Cabin1, hnRNP K may also facilitate p53 promoter complex formation at basal levels and stabilise p53 on chromatin to activate rapid transcriptional responses to stress (Moumen et al., 2005). It is also possible that hnRNP K may act as an anchor to recruit other co-factors which modify and optimise p53 activity. A recent study analysed p53-dependent transcription of large intergenic non-coding RNA's (lincRNA's), with particular focus on lincRNA-p21 transcription (Huarte et al., 2010). When activated, lincRNA-p21 was found to be recruited to p53 target gene promoters along with hnRNP K to form a transcriptional complex which represses a large subset of p53 target genes involved in stress responses. Interestingly however, although association between lincRNA-p21 and hnRNP K led to transcriptional repression of many p53 target genes, it also activated several pro-apoptotic genes necessary for p53-dependent cell death by DNA damage (Huarte et al., 2010). Specific complexes at p53 promoter sequences and specific p53 binding partners may therefore be critical in determining whether p53 transcription is activated or repressed under physiological conditions and in response to various stress stimuli.

1.4.5 Cell cycle responses controlled by p53

Although p53 has an established function in activating apoptosis in response to cellular stress, several p53 target genes also function to promote cell survival by inhibiting apoptosis. Cell cycle pathways that promote survival give cells an important opportunity to resolve DNA damage induced by genotoxic stress. Cell death responses are only activated if DNA damage is severe and irreparable. To ensure only healthy cells grow and proliferate, checkpoints are employed during each phase of the cell cycle. These checkpoints are complex, involving numerous factors that relay vital genomic signals through the cellf.

1.4.5.1 The cell cycle and checkpoint function

The eukaryotic cell cycle is comprised of distinct phases that ensure cells grow and divide with efficiency and accuracy. The G1 phase (growth phase) involves RNA

translation and protein synthesis of key molecules that are required for DNA duplication. The following DNA synthesis or replication phase is referred to as S-phase. The G2 phase occurs after S-phase and involves biosynthesis of key proteins required for mitosis, such as those that comprise the microtubules to which chromosomes attach prior to cell division. Finally, the M-phase (mitosis) is the process by which duplicated chromosomes are divided into two genetically identical daughter cells. Transition through the cell cycle is controlled by distinct heterodimeric complexes comprising cyclins and cyclin dependent kinases (CDKs). The cyclin is the regulatory subunit, while the CDK component forms the catalytic subunit through which the complex is activated. Many cyclin/CDK complexes have been identified to date which are involved in regulating numerous transcription factors that promote cell cycle phase transition (Harper and Adams, 2001). The different cell cycle phases have been illustrated in Figure 1.4.3.

Transition through each phase of the cell cycle involves a checkpoint response. Cell cycle checkpoints act as gatekeeper pathways that ensure accurate cell division by checking each phase is completed successfully before cells can enter the next phase. The G1-S checkpoint is induced to resolve damaged DNA before DNA undergoes duplication, the S-phase checkpoint monitors DNA damage during replication, and the G2-M checkpoint is activated to ensure that damaged DNA which has bypassed previous controls is resolved before mitosis (Hartwell and Weinert, 1989). Cells respond to checkpoints by undergoing transient growth arrest allowing activation of specific repair pathways, or induction of apoptosis to eliminate cells in which DNA damage cannot be repaired. Checkpoints are important mechanisms by which cells respond to both intrinsic and extrinsic forms of DNA damage.

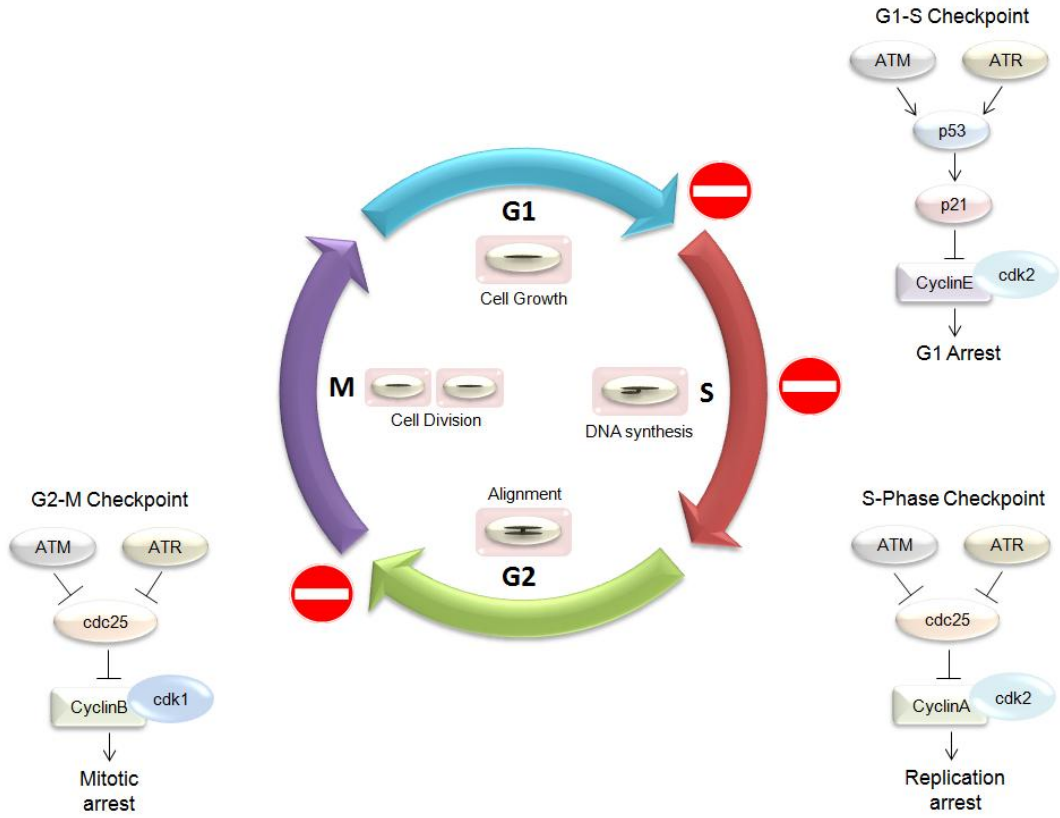


Figure 1.4.3: Cell cycle and checkpoint control

The eukaryotic cell cycle is comprised of distinct phases that involve cell biosynthesis and growth (G1 phase), DNA synthesis (S-phase), chromosome alignment (G2 phase), and cell division or mitosis (M-phase). Progression through each phase is controlled by cell cycle checkpoints. Three distinct checkpoints have been described, the G1-S checkpoint, the S-phase checkpoint, and the G2-M checkpoint. These checkpoints involve transduction of various signalling pathways that inhibit cyclin/CDK activity, and hence promote cell cycle arrest to ensure defects in cellular DNA are resolved before the cycle can continue.

1.4.5.2 Cell cycle checkpoints are activated by DNA damage

Checkpoints are comprised of DNA damage sensors, signal transducers, and effectors which together co-ordinate responses to delay the cell cycle in response to genetic stress, and initiate repair (Hartwell and Kastan, 1994; Zhou and Elledge, 2000). Often, deregulated activity of various sensors, transducers, and effectors promote tumour development by allowing cells with genetic defects to continue growth and replication (Hartwell and Kastan, 1994). Diseases such as Bloom syndrome (BS), Fanconi anaemia (FA), and ataxia telangiectasia (AT) are characterized by the inability of cells to repair DNA damage due to mutations in specific DNA repair genes, and these diseases are often associated with greater predispositions to cancer (Pierce and Jasin, 2001).

The DNA damage sensors, ataxia-telangiectasia mutated protein (ATM), and ataxia telangiectasia and Rad3 related protein kinase (ATR) are members of the PI3K kinase family. ATM exists as a homodimer and undergoes DNA damage induced autophosphorylation at serine 1981, allowing dissociation of the homodimer complex and phosphorylation of p53, as well as repair proteins like NBS1 (Nijmegen breakage syndrome 1) and BRCA1 (breast cancer 1), (Bakkenist and Kastan, 2003). ATR does not exist as a homodimer like ATM, but instead forms a complex with ATRIP (ATR-interacting protein), (Cortez et al., 2001). ATR is activated by replication stress induced under hypoxic conditions (Hammond et al., 2002), and in response to DNA damaging agents that alkylate and crosslink DNA base pairs (Zou and Elledge, 2003). Replication protein A (RPA) binds single stranded DNA breaks and stimulates recruitment of the ATR-ATRIP complex to sites of replication fork arrest. Activated ATR then induces phosphorylation of checkpoint kinase 1 (CHK1) to initiate checkpoint signalling (Zou and Elledge, 2003).

The importance of ATR function is demonstrated by *in vivo* studies whereby *atr* null mice are non-viable (Shechter et al., 2004; Zou et al., 2002). Interestingly, although ATM and ATR share common substrates, loss of *atm* does not lead to early embryonic lethality suggesting that ATR has a significant role in controlling replication checkpoints during the normal cell cycle, while ATM is important for regulating responses to external stress stimuli (Shiloh, 2003). Such studies also demonstrate a level of redundancy between the ATM and ATR sensor kinases.

Activation of ATM and ATR is involved in initiating signalling through all checkpoints, and these have been summarised in Figure 1.4.3. The G1-S phase checkpoint is activated by double strand DNA breaks induced during G1-phase. ATM phosphorylates its downstream checkpoint kinase 2 (CHK2), and ATR leads to active CHK1 function (Kastan and Lim, 2000). Both CHK2 and CHK1 phosphorylate p53 at specific residues (some of which are shown in Figure 1.4.2). Phosphorylation of p53 by CHK2 and CHK1 inhibits p53 binding with HDM2 (Chehab et al., 2000). As well as promoting p53 phosphorylation through CHK1, and CHK2, ATM and ATR also phosphorylate HDM2 directly, retaining MDM2 in the cytoplasm and preventing targeting of p53 to the proteasome (Maya et al., 2001). Activation of p53 increases expression of p53 target proteins such as p21 (also referred to as wild type p53-activated fragment 1, WAF1). The cyclin E/CDK2 complex is inhibited by p21 to induce G1 arrest while DNA damage is resolved (Kastan and Lim, 2000). The CDC25A phosphatase regulates CDK2 activity, and both ATM and ATR kinases also target CDC25A for proteasomal degradation directly, rendering CDK2 inactive and hence delaying cell cycle progression.

The S-phase checkpoint is activated by DNA damage induced during DNA replication. S-phase arrest inhibits replication fork progression when DNA is damaged, protecting against replication fork collapse and thereby inhibiting DNA strand breaks. As in the G1-S checkpoint, p53 is phosphorylated by CHK kinases to induce target genes involved in cell cycle arrest (Tibbetts et al., 1999). However, p53 is also recruited directly by the RecQ helicase BLM (Bloom's syndrome helicase) to sites of stalled replication forks where p53 promotes homologous recombination and resolves damaged DNA (Sengupta et al., 2003). ATM and ATR activation during the S-phase checkpoint can also target the CDC25A phosphatase to inhibit cyclinA/CDK2 activity and the downstream substrate CDC45 which is required for recruitment of DNA polymerase to initiate new origin firing (Bartek and Lukas, 2003).

The G2-M checkpoint prevents mitosis of cells that have unrepaired DNA damage (Xu et al., 2002). As with G1-S, and S-phase checkpoints, this involves activation of ATM and ATR to initiate effector responses involving p53 and its downstream targets $p21^{WAF/CIP}$, GADD45-α (growth arrest and DNA damage inducible 45-α), and 14-3-3δ (Taylor and Stark, 2001). Activation of the cyclin B/CDK1 complex is important in promoting mitosis, and inhibition of CDC25A by ATM and ATR inhibits cyclin B/CDK1 activity to induce G2 arrest while DNA breaks are repaired (Nyberg et al., 2002). In addition to p53 and CDC25A, ATM also activates the DNA repair protein BRCA1 in

response to DNA strand breaks induced by ionising radiation (Xu et al., 2001). Although cell cycle arrest is mediated through activation of p53 and induction of p53 target genes, p53 null cell types can also arrest in G2 by DNA damage (Kastan et al., 1991). CHK1 and CHK2 have additionally been shown to phosphorylate and target CDC25A in a p53-independent manner in response to UV light or irradiation (Falck et al., 2001). Selected pathways involved in the G1-S, S-phase, and G2-M checkpoints are shown in Figure 1.4.3.

In addition to ATM and ATR, H2AX (a member of the histone H2A family) also acts as an important DNA damage sensor. Histones are important for organising and packaging DNA into nucleosomes. The H2A nucleosomal histone family is one of five major families of histone proteins, and includes the highly conserved variant H2AX (Redon et al., 2002). H2AX represents between 2-25% of total cellular H2A (Redon et al., 2002) and undergoes several post-translational modifications in response to DNA strand breaks such as acetylation, phosphorylation, and ubiquitination which promote chromatin remodelling, and lead to the recruitment of various repair factors that resolve DNA strand breaks. Phosphorylation of H2AX by activated ATM, ATR and DNA-dependent protein kinase (DNA-PK) (Wang et al., 2005) at serine 139 is an early response to DNA double strand breaks (Rogakou et al., 1998). As well as phosphorylating H2AX, ATM also phosphorylates and activates BRCA1, 53BP1, and mediator of checkpoint signalling 1 (MDC1). These DNA repair factors are recruited to sites of phosphorylated H2AX on chromatin to form specific repair complexes (Stucki et al., 2005). H2AX deficient mice are hypersensitive to radiation, have DNA damage repair defects, and increased genomic instability (Celeste et al., 2002). The key sensors, transducers and effectors that are involved in the DNA damage response have been summarised in Figure 1.4.4.

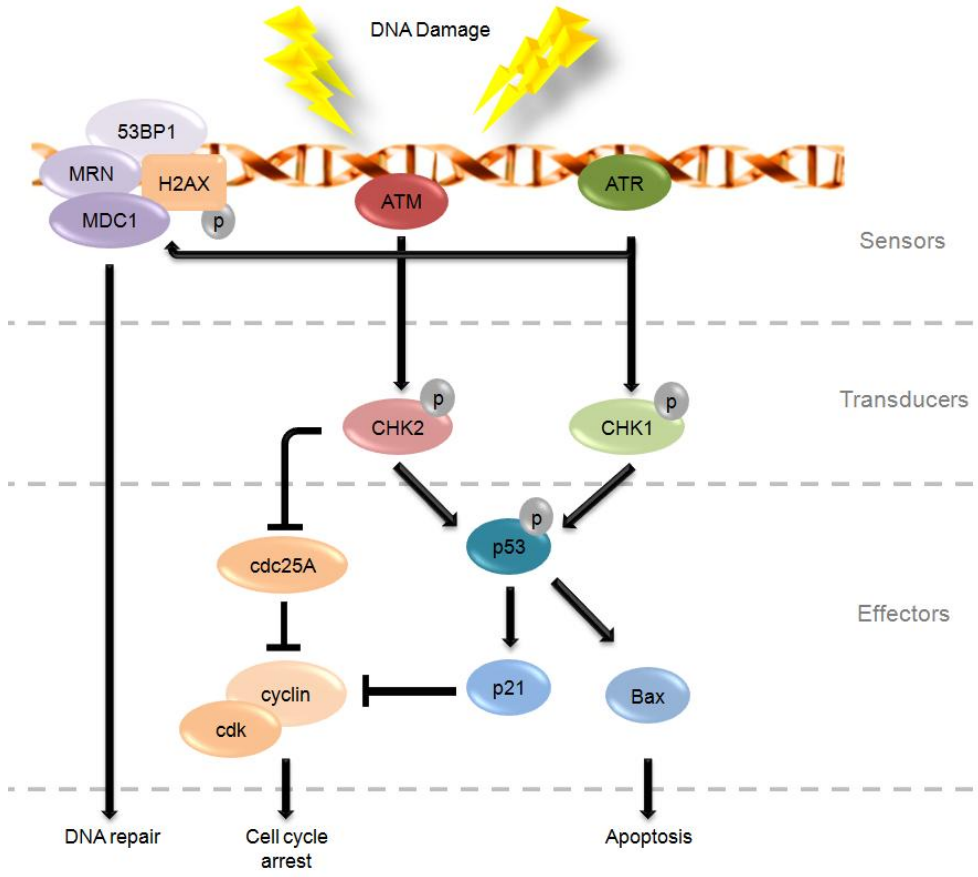


Figure 1.4.4: The DNA damage response

DNA damage is detected by the DNA damage sensors ATM and ATR that are involved in phosphorylating their substrates CHK2 and CHK1 respectively. CHK2 and CHK1 kinases phosphorylate p53 leading to transcriptional activation of p53 and induction of numerous p53 target genes involved in mediating cell cycle arrest, DNA repair, or apoptosis. Induction of p21 by p53 elicits cell cycle arrest by inhibiting CDK activity. ATM/CHK2 also inhibits CDC25A phosphatase by targeting CDC25A for proteasomal degradation, rendering CDK inactive, and hence delaying cell cycle progression. In addition, the ATM sensor kinase phosphorylates the histone protein H2AX, leading to chromatin remodelling and the recruitment of a number of DNA damage repair proteins. Many p53 target genes activate the apoptotic cell death response if DNA damage is beyond repair. These include Bax, PUMA and NOXA (only Bax is shown for simplicity).

1.4.5.3 The role of p53 in DNA repair and genomic integrity

As discussed, p53 is a crucial transcription factor that regulates an array of cellular pathways to determine cellular fate. Once stabilised in response to stress stimuli, p53 undergoes complex post translational modifications required for its sequence specific binding to target gene promoters (Vogelstein et al., 2000). Cells are continuously subjected to intrinsic and extrinsic genomic insults and p53 has a critical role in maintaining genomic integrity by both transactivation dependent and transactivation independent mechanisms.

In the transactivation dependent role of p53, p53 is an effector protein, and is phosphorylated at specific phosphorylation sites by the upstream kinases ATM-CHK2, and ATR-CHK1. Transcription of genes involved in eliciting DNA repair, apoptosis, or senescence are induced by binding of activated p53 to target DNA. However, p53 also has transactivation independent functions, and has been shown to bind DNA in a non-sequence specific manner. The C-terminal domain of p53 can bind with high affinity to DNA lesions caused by insertion and deletion mismatches, and is proposed to act as an important recruitment factor for DNA repair proteins (Lee et al., 1995; Liu and Kulesz-Martin, 2001). p53 is also localised to single and double stranded DNA breaks induced by ionising radiation (Bakalkin et al., 1995; Reed et al., 1995).

The importance of p53 in regulating DNA repair and maintaining genomic integrity is shown by p53's involvement in a number of DNA repair pathways (Fukasawa et al., 1996). Repair pathways are specific to the types of DNA damage that occur in cells which suffer from both intrinsic and extrinsic forms of genomic stress. The nucleotide excision repair pathway is an example of a DNA repair pathway that is activated in response to DNA damage induced by ultraviolet lesions, various carcinogens, and mutagens. Nucleotide excision repair involves an array of complex mechanisms that identify, remove, and replace damaged fragments of DNA. p53 has been shown to regulate the transcription of various genes that encode DNA repair proteins involved in nucleotide excision repair. These include $p48^{DDB2}$ (p48 xeroderma pigmentosum gene) which binds to DNA damaged sites in a p53 dependent manner (Hwang et al., 1999). In irradiated cells, $p48^{DDB2}$ also transports XPC (xeroderma pigmentosum complementation group C) to sites of DNA damage in irradiated cells leading to the recruitment of other repair proteins (Fitch et al., 2003). When induced by ultraviolet radiation, nucleotide excision repair efficiency is reduced in the absence of p53 (Smith et al., 1995). The base excision repair (BER) pathway is important in removing small

damaged lesions of DNA that arise as part of the normal cell division cycle. Co-operation between p53 signalling and activation of the base excision repair pathway has also been described and is controlled by levels of genotoxic stress (Offer et al., 1999).

During non-homologous end joining (NHEJ), damaged DNA strands are repaired without a template strand (Hoeijmakers, 2001a). Non-homologous end joining is regulated by a number of sensor proteins, including DNA-PK and XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells-4). In response to double strand DNA breaks, DNA-PK phosphorylates p53 to promote p53 transactivation, while inhibiting the negative regulation of p53 by HDM2 (Shieh et al., 1997). This pathway induces significant p53-dependent apoptosis. The importance of XRCC4 in regulating non-homologous end joining in response to double strand DNA breaks is shown by *in vivo* studies whereby *Xrcc4* knockout mice undergo significant neuronal apoptosis and have early embryonic lethality (Gao et al., 2000). Interestingly, this phenotype is rescued by loss of *p53*, suggesting that p53-dependent apoptosis is essential in determining the outcome of cells with severe genomic defects caused by loss of critical DNA repair proteins. Transactivation independent roles for p53 in non-homologous end joining have also been described (Yang et al., 1997).

In homologous recombination, DNA strand breaks are resolved using the adjacent DNA template. Wildtype p53 can interact with several repair proteins involved in homologous recombination including RAD51 and RAD54 (Linke et al., 2003). As p53 can recognise mismatched DNA bases as well as activate repair proteins, loss of p53 function results in severe DNA defects that promote spontaneous homologous recombination, leading to increased repair errors and genomic instability (Dudenhoffer et al., 1998). Conversely, by maintaining DNA integrity, wildtype p53 represses the homologous recombination pathway and also inhibits tumourigenesis (Akyuz et al., 2002). Of additional interest, *Atm* and *Rad52* deficient mice have reduced onset of T-cell lymphomas and increased survival rates compared to *Atm* deficient mice alone suggesting that excessive recombination events in the absence of ATM are an important determinant of tumour development (Treuner et al., 2004).

The RecQ family of helicases unwind paired DNA in the 3'-5' direction allowing localisation of repair complexes to resolve mismatched base pairs. RecQ helicases therefore have a critical role in maintaining genomic integrity, and deregulation of these helicases are involved in disorders such as Bloom syndrome (BS) and Werner

syndrome (WS) that are associated with an increased predisposition to cancer. Bloom syndrome helicase (BLM) and Werners syndrome helicase (WRN) are RecQ helicases that are early sensors of replication stress and have been shown to interact directly with p53. Complex formation between BLM and p53 is required for co-localisation of RAD51 to sites of stalled replication forks (Sengupta et al., 2003). Loss of BLM and p53 enhances the frequency of homologous recombination indicating that BLM and p53 proteins have a complimentary relationship in maintaining genomic integrity during replication (Sengupta et al., 2003). Furthermore, mice that are null for both *wrn* and *p53* have an increased incidence of tumour formation compared to mice that are *p53* null alone (Lebel et al., 2001).

Overall, p53 maintains DNA integrity by regulating a number of complex DNA repair pathways, and defects in or loss of wildtype p53 function has detrimental effects on cell viability by these mechanisms (Fukasawa et al., 1996). p53 is not only an effector protein activated by DNA damage sensors to modulate expression of genes involved in cell cycle arrest, apoptosis, and senescence, but also has complex functions in regulating DNA repair and recombination processes via transactivation independent pathways.

In previous sections, p53 stabilisation, post-translational modification and activation have been reviewed followed by p53 function in cellular maintenance. However, when cells are exposed to lethal forms of damage that cannot be repaired, they must undergo cell death to keep surrounding cells and tissues from damage. The involvement of p53 in cell death responses will be discussed below.

1.4.5.4 The role of p53 in cell death responses

Apoptosis is an evolutionarily conserved process that is essential for embryonic development, and for cellular homeostasis in adult tissues (Kerr et al., 1972). Cell death by apoptosis involves distinct molecular pathways and morphological changes in cells, ultimately leading to the removal of damaged cells from tissues. Apoptosis is divided into two distinct pathways, the intrinsic pathway which is activated by stress stimuli, and the extrinsic pathway which is executed by death receptors. Both intrinsic and extrinsic forms of apoptosis involve a relay of enzymatic events involving a family of cysteine aspartyl proteases called caspases (Chipuk and Green, 2006; Hill et al., 2004). Caspases are activated when cleaved, and promote further activation of downstream caspases by the same process.

The intrinsic cell death pathway is induced by internal stress stimuli such as DNA damage and hypoxia. Transcriptional activation of p53 in response to such stress is important for inducing numerous p53 target genes that regulate Bcl-2 proteins involved in the intrinsic cell death process. Interestingly, Bergamaschi et al. have shown that the apoptosis-stimulating protein for p53 (ASPP) proteins, ASPP1 and ASPP2, can preferentially co-operate with p53 to induce pro-apoptotic target gene expression, as opposed to those involved in cell cycle arrest in response to specific stress stimuli, and thereby promote apoptosis (Bergamaschi et al., 2004). The first pro-apoptotic Bcl-2 protein shown to be upregulated by p53 was Bcl-2 associated X protein (Bax), (Miyashita and Reed, 1995), and since then, several p53 pro-apoptotic target genes have been identified that are involved in regulating apoptosis such as NOXA, and PUMA (p53-upregulated modulator of apoptosis) (Oda et al., 2000a).

Pro-apoptotic Bcl-2 proteins induced by p53 localise to the mitochondrial membrane to interact with anti-apoptotic Bcl-2 protein complexes on the mitochondrial surface, and induce mitochondrial outer membrane permbealisation (MOMP), (Spierings et al., 2005). Activation of MOMP leads to the release of cytochrome C from the mitochondrial intermembrane space, and formation of the apoptotic protease activating factor 1 (APAF1) complex. Binding of APAF1 to caspase 9 forms an apoptosome complex that leads to activation of the downstream caspase cascade. The final caspase to be activated in this cascade is caspase 3 which completes the apoptosis pathway. Cells undergoing apoptosis have distinct morphological features including chromatin condensation, DNA fragmentation and cellular blebbing caused by the formation of apoptotic bodies that contain discarded cell fragments (Edinger and Thompson, 2004).

Accumulation of p53 in the cytoplasm is regulated by HDM2 (Dumont et al., 2003). As well as inducing pro-apoptotic Bcl-2 proteins by transactivation dependent mechanisms, p53 also has extranuclear functions and has been shown to rapidly accumulate in the cytoplasm following radiation (Erster et al., 2004; Erster and Moll, 2004). Cytoplasmic p53 has been shown to bind directly to Bcl-2 complexes on the mitochondrial membrane to induce MOMP and initiate apoptosis (Schuler and Green, 2005). Key cell death pathways that are mediated by p53 have been summarised in Figure 1.4.5.

The extrinsic pathway of apoptosis is activated when death receptors are ligated by the binding of death ligands. The tumour necrosis factor receptor (TNFR) family are death

receptors that bind to a number of ligands including CD95, TNF ligands, and the TNF-related apoptosis-inducing ligand (TRAIL), (Muppidi et al., 2004). Upon binding to death ligands, the TNFR death receptor recruits specific adaptor molecules such as the FAS associated death domain protein (FADD). Recruitment of FADD acts as a platform for binding and activating caspase 8, followed by activation of the BH3-interacting domain death agonist (Bid). Translocation of Bid to the mitochondrial membrane induces MOMP and the consequent series of events involved in intrinsic apoptosis.

Recently, autophagy has been described as an important mechanism induced by stress that can promote both cell survival and apoptosis. As with intrinsic apoptosis, p53 has been shown to regulate autophagy mediated cell death (Crighton et al., 2006). Autophagy is an evolutionarily conserved pathway that involves lysosomal mediated degradation of damaged cytoplasmic organelles (Debnath et al., 2005; Levine and Kroemer, 2008). Organelles such as mitochondria and the endoplasmic reticulum that are damaged by nutrient starvation and genotoxic stress are engulfed into double membrane vesicles called autophagosomes (Boya et al., 2005). Cellular contents within autophagosomes are transferred to lysosomes upon fusion, forming a degradation vesicle called the autolysosome. Lysosomal hydrolases complete autophagy by degrading the contents within the autolysosome (Baehrecke, 2005).

AMPK (5'-AMP-activated protein kinase) is a major regulator of energy homeostasis (Hardie, 2007) both in normoxic and hypoxic tumour microenvironments (Laderoute et al., 2006), and has a well established role in mediating autophagy by acting as a metabolic sensor (Meley et al., 2006). AMPK is an upstream regulator of the serine threonine kinase mTOR (mammalian target of rapamycin), which in turn regulates important signalling pathways involved in translation and cell division (Bohensky et al., 2010). One of the earliest studies investigating the role of p53 in autophagy showed that p53 could activate AMPK and induce autophagy by inhibiting mTOR in response to glucose starvation (Feng et al., 2005). Other studies explored the expression of p53 target genes and showed that damage regulated autophagy modulator (DRAM) was a p53 target gene that encoded a lysosomal protein involved in activating autophagy and inducing apoptosis (Crighton et al., 2006).

Intrinsic, extrinsic and autophagic mechanisms that lead to the execution of apoptosis involve various pro-apoptotic target genes that are induced by p53 transactivation in response to metabolic stress and genotoxic damage that is beyond repair. The importance of p53 as a tumour suppressor protein is shown by the range of pathways

that are regulated by p53. The role of p53 in mediating cell cycle checkpoints, DNA repair, and cell death responses has been reviewed in detail above. It is not surprising then that given the central function that p53 has in regulating cell growth, proliferation, and death, that p53 activity is lost in almost all cancers. Our laboratory is particularly interested in how p53 function is affected in hypoxic tumours that overexpress HIF protein levels, as these tumours are often the most difficult tumours to target with radiotherapy and chemotherapy. Regulation between HIF and p53 and what this means for specific tumour models will now be discussed further.

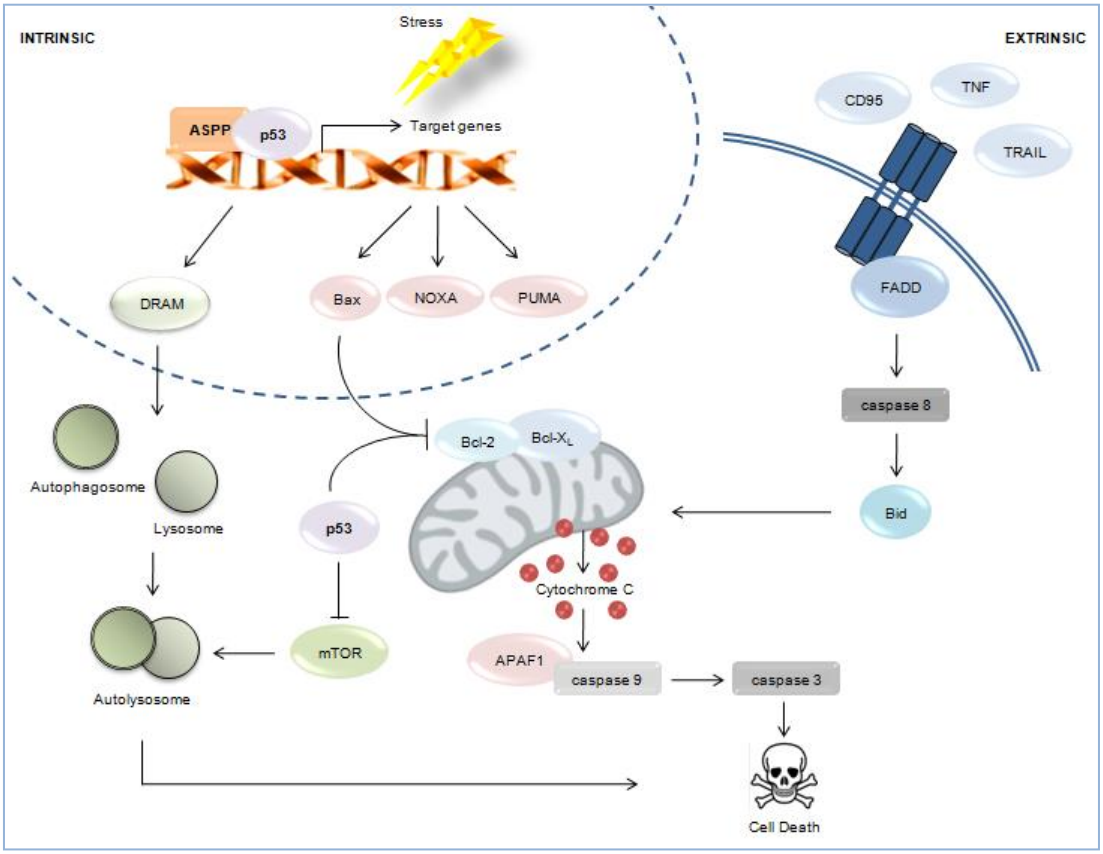


Figure 1.4.5: Regulation of apoptosis by p53

Apoptosis is regulated by the intrinsic and extrinsic pathways. Intrinsic apoptosis is induced by internal stress stimuli such as DNA damage. Activation of p53 in response to DNA damage leads to expression of pro-apoptotic target genes such as *Bax, NOXA and PUMA*. The ASPP protein family act as co-factors for p53 to induce pro-apoptotic target gene expression. Pro-apoptotic Bcl-2 proteins induced by p53 translocate to the mitochondria and disrupt anti-apoptotic Bcl-2 complexes, such as those formed between Bcl-2 and Bcl-xL, thereby inducing mitochondrial pore permbealisation, and consequent cytochrome C release. The APAF1 complex is activated by cytochrome C and initiates the caspase cascade leading to activation of caspase 3, and cell death by apoptosis. The extrinsic cell death pathway involves binding of the death ligands CD95, TRAIL, and TNF to the TNF death receptor. The receptor is activated by ligation and recruits adaptor proteins such as FADD which activate caspase 8, and pro-apoptotic Bid. The intrinsic response is executed by Bid following localisation of Bid to the mitochondrial membrane. Additionally, p53 may activate apoptosis through induction of autophagy, by either mTOR inhibition, or expression of the p53 target gene *DRAM*. Formation of autolysosomes during autophagy leads to degradation of cellular organelles, and activation of apoptosis.

1.5 Crosstalk between HIF-1α and p53

HIF-1α can induce p53 activation and p53 induced apoptosis in response to gamma irradiation (Moeller et al., 2005) and can interact directly with p53 to promote its stability (An et al., 1998; Chen et al., 2003). However, activated p53 can also have significant inhibitory effects on the HIF pathway and on tumour angiogenesis (Ravi et al., 2000; Yang et al., 2009b). Supporting studies indicate that activation of p53 by the small molecule RITA (reactivation of p53 and induction of tumour cell apoptosis) can inhibit HIF-1α protein and VEGF induction, and induce cell death and antiangiogenic effects in hypoxia (Yang et al., 2009b). Additionally, specific knockdown of HIF-1α in hypoxic cells containing wildtype p53 can reverse resistance to cisplatin by upregulating pro-apoptotic Bid, therefore activating cell death responses (Hao et al., 2008). The negative relationship between HIF and p53 activity suggests that in hypoxic tumours, p53 is transcriptionally repressed. Although some studies have addressed this relationship, and will be discussed in the next section, the mechanisms by which HIF and p53 crosstalk remain unclear.

1.5.1 p53 activity is repressed by HIF

Koumenis et al. previously suggested that although p53 accumulates in the cell under hypoxic stress, the protein is transcriptionally inactive and does not induce p53-dependent cell cycle arrest (Koumenis et al., 2001). Instead, additional signals such as DNA damage were required for p53 activity in hypoxia (Kaluzova et al., 2004). Furthermore, it has been known for some time that although p53 is stabilised by stress, multiple post-translational modifications are required for p53 activity, and these require a range of stress signals (Ashcroft et al., 2000).

Using mutant *C. elegans* with loss of *vhl*, Sendoel and colleagues have overexpressed *hif-1* specifically in sensory neurons of *C. elegans*, leading to transcriptional up regulation of the tyrosinase tyr-2. By a mechanism dependent on endocytosis, tyr-2 can target the *C.elegans* homologue of p53, cep-1, specifically in the gonads and inhibit cep-1 mediated apoptosis in response to stress. Sendoel et al. reveal novel complexities for HIF signalling pathways whereby HIF target genes can repress p53 activity in cells at both close proximity, and at distant regions within the tumour microenvironment (Ahmed and Ashcroft, 2010; Sendoel et al., 2010).

1.5.2 p53 function is deregulated in angiogenic tumours

VHL disease is a hereditary cancer syndrome whereby loss of *VHL* results in a greater predisposition to the development of highly vascular tumours (known as hemangioblastomas) of the central nervous system, and of the retina. VHL disease is also associated with the development of renal carcinomas and pheochromocytomas (Haase et al., 2001). Almost 80% of clear cell renal cell carcinomas involve the biallelic inactivation of *VHL* (Kaelin, 2002). As expected, loss of pVHL function in renal carcinomas also leads to the formation of tumours that have high HIF gene expression, and are therefore highly vascular (Gnarra et al., 1996; Takahashi et al., 1994). Renal cell carcinomas are a good model to assess the interplay between deregulated HIF and p53 because although mutations in p53 are rarely detected in renal cell carcinomas, they remain highly resistant to radiotherapy and chemotherapy. The mechanisms by which p53 function is deregulated in renal cell carcinomas are unclear, although some evidence suggests a level of crosstalk between p53 activity, and overexpression of HIF signalling due to loss of pVHL function (Gurova et al., 2004; Lowe, 1995).

Reintroduction of wildtype *VHL* into renal carcinoma cells can promote p53 accumulation, suggesting both p53 and pVHL tumour suppressor functions may co-operate in such tumour models (Galban et al., 2003). However, transactivation of stabilised p53 protein in renal cell carcinoma has also been shown to be suppressed (Gurova et al., 2004). Although the mechanism by which the transactivation function of p53 is inhibited in renal cell carcinoma is undefined, and even disputed by other studies (Warburton et al., 2005) it is an important basis for studies that use either p53 activating agents (Roberts et al., 2009; Yang et al., 2009b) or HIF inhibition (Bertout et al., 2009) to effectively sensitise renal carcinoma cells to chemotherapy.

Interestingly, HIF-independent functions for pVHL have also been described involving cell cycle and apoptotic pathways (Pause et al., 1998; Roe et al., 2006). As well as binding to HIF-1α (Maxwell et al., 1999) pVHL is proposed to bind directly with p53 and regulate p53 transcription in a HIF-independent manner (Roe et al., 2006). By binding to p53, pVHL inhibits p53 interactions with HDM2 leading to p53 stabilisation in response to genotoxic stress (Roe et al., 2006). Other studies cannot reproduce the association between pVHL and p53 that has been proposed by Roe et al. (Roberts et al., 2009), and such ambiguities make it challenging to understand the HIF-independent mechanisms by which p53 is regulated in renal carcinomas.

The association between pVHL loss of function and HIF-α overexpression in renal cell carcinomas has led to a number of studies in which the differential effects of HIF-1α and HIF-2α expression have been assessed (Carroll and Ashcroft, 2006; Raval et al., 2005). It is now considered that HIF-2α overexpression is a primary contributor to the progression of clear cell renal cell carcinoma (Kondo et al., 2002). Supporting studies have shown that HIF target gene expression in renal cell carcinoma cells is dependent on HIF-2α overexpression (Carroll and Ashcroft, 2006). Importantly, the mechanisms by which HIF-2α overexpression leads to chemoresistance remain unclear. The impact that loss of p53 activity has in renal cell carcinomas that overexpress HIF-2α will be discussed further.

1.5.2.1 Effects of HIF-2α on p53 activity in renal cell carcinoma

Xenograft mouse models show HIF-2α is important for progression of clear cell renal carcinoma (Kondo et al., 2003). The oncogenic functions of HIF-2α in renal cell carcinomas are strengthened by studies in which HIF-2α has been shown to co-operate with the proto-oncogene *c-myc* to enhance cell proliferation, and regulate genomic stability (Gordan et al., 2008). Furthermore, gradual increases in the distribution and expression of HIF-2α protein during renal cell carcinoma progression have been shown, which inversely correlate with HIF-1α expression (Mandriota et al., 2002).

The inhibitory effects of HIF-2α on p53 activity contribute significantly to the radioresistance of clear cell renal cell carcinomas (Bertout et al., 2009). Renal carcinoma cells expressing HIF-2α alone have decreased p53 transcriptional activity and inhibition of HIF-2α protein expression sensitises cells to radiation by activating p53-dependent cell death responses (Bertout et al., 2009). These studies provide an important rationale for the development of HIF-2α inhibitors, and for the use of p53 activating agents, either alone or in combination, to improve the therapeutic targeting of renal cell carcinomas.

Both HIF signalling and p53-mediated pathways have been discussed in detail as independent regulators of the cellular response to stress, and as important pathways that co-operate in specific tumours to promote tumour development. Clearly, targeting tumours in which these pathways are deregulated has been challenging, although progress has been made in the development of small molecule targeted agents and combination strategies designed to maximise tumour cell death. The most common strategies that are used to treat cancer will now be reviewed along with the progress

that targeted agents have made in the clinic. Current challenges in targeting angiogenic tumours with deregulated HIF-α, and those with deregulated p53 signalling will also be discussed.

1.6 Treating cancer

The most common methods by which cancer is treated are surgery, radiotherapy, and chemotherapy. Treatments are often used in combination to improve therapeutic outcome and responses are dependent on both tumour stage (an indication of how widespread the tumour is in the body), and tumour grade (defined according to the differentiated morphology of cancer cells within a tumour). There are several grading systems that are commonly used, and these depend on the tumour type assessed. At presentation, patients are often assessed for tumour size and location, to determine tumour stage, and a biopsy is taken to define tumour grade. These factors are then used to build an individual treatment plan that best predicts patient prognosis. If a tumour is detected during the early developmental stages, surgery is used to remove the tumour.

However, for tumours that are malignant, radiotherapy is often used in combination with chemotherapy or 'drug therapy' to induce lethal DNA damage in rapidly proliferating cancer cells, and cell death is achieved through apoptosis to inhibit tumour growth (Albain et al., 2009). Radiotherapy involves the use of ionizing radiation. Although radiation is targeted specifically to the tumour tissue, some healthy, surrounding tissues are also affected. Therefore, unwanted side effects are a common drawback of radiation therapy. Chemotherapy schedules are prescribed according to tumour type, age, and genetic makeup. Most chemotherapy agents are divided into categories according to the specific cell cycle pathway which they inhibit. Alkylating agents like cisplatin, for example modify cellular DNA by forming DNA cross links that damage DNA during replication (Alderden et al., 2006; Rosenberg et al., 1969). Normal cells can also be damaged by chemotherapy agents however normal cells are more efficient at activating cell cycle arrest and DNA repair compared to cancer cells, and can resume growth once treatment is over.

Targeted therapies interfere with specific cell signalling pathways that are deregulated in cancer cells. These can be either small molecules, or antibodies directed against specific molecules within the pathway of interest. Common targeted therapies clinically used include those that inhibit the epidermal growth factor receptor (EGFR), a receptor often overexpressed in many cancer cells which promotes tumour growth (Zhang et al.,

2007). Gefitinib (Iressa®) for example is a small molecule targeting EGFR and is used to treat non small cell lung cancer (Lynch et al., 2004; Paez et al., 2004). The monoclonal antibody Cetuximab (Erbitox®) also functions by inhibiting EGFR signalling and is used to treat patients with squamous cell carcinoma of the head and neck (Vermorken et al., 2008), and patients with colon cancer (Van Cutsem et al., 2009).

Other common targeted agents include Bevacizumab (Avastin®), a monoclonal antibody that inhibits blood vessel growth in tumours by targeting VEGF (Ferrara et al., 2004), and Sorafenib (Nexavar®), a small molecule used in renal cell (Escudier, 2007) and hepatocellular carcinomas (Keating and Santoro, 2009) that also targets blood vessel growth by inhibiting tyrosine kinase pathways (Wilhelm et al., 2008). Because targeted therapies are designed to inhibit signalling pathways that are deregulated in cancer cells, they reduce unwanted side effects and improve treatment responses.

Hormone therapy has been developed for tumours that require hormones for growth such as breast and prostate cancers. Selective estrogen receptor modulators (SERMS) such as tamoxifen inhibit binding of estrogen by blocking the estrogen receptor (Jordan, 2006). Aromatase inhibitors have also been developed which inhibit estrogen production (Mokbel, 2002). Other forms of cancer treatment include immunotherapy based approaches and vaccine development (Borghaei et al., 2009). Gene therapy is also being used to improve targeting of cancer cells (Gutierrez et al., 1992; Morgan et al., 2006).

Overexpression of HIF-α (HIF-1α and HIF-2α) is found in numerous tumours, and is associated with poor patient survival. For this reason, HIF is an important therapeutic target, and has been pursued for the development of agents that inhibit HIF activity and HIF target gene expression in several tumour models. However, targeting HIF is challenging because HIF-1α and HIF-2α have specific functions to either promote, or suppress tumour progression, and these functions largely depend on the tumour type in which they are expressed. The various strategies that have been designed to target the HIF pathways and the challenges which these agents face on the road to the clinic will now be discussed.

1.6.1 Strategies for targeting the HIF pathway

Polypeptides derived from the carboxyl-terminal transactivation domain of HIF-1α can be used to directly inhibit interactions between HIF-1α and its transcriptional co-activator p300 (Kung et al., 2000). HIF-1α protein may be targeted by use of HIF-1α

antisense plasmids (Sun et al., 2001) and inhibitory cDNA from which mRNA splicing produces truncated, transcriptionally inactive HIF-1α protein (Chun et al., 2001). Tirapazamine, a hypoxia-activated pro-drug forms highly reactive radicals in hypoxic cells that sensitise tumours to conventional therapy (Brown and Wilson, 2004). Tirapazamine was shown to synergise with cisplatin in Phase III clinical trials for non small cell lung cancer and improve survival rates (von Pawel et al., 2000). Hypoxia activated gene therapy has been explored as a means to target hypoxic cells specifically (Greco et al., 2006). However, as with most forms of gene therapy, the need for better systemic delivery is required and such challenges hinder further progress.

Heat shock protein 90 (HSP90) is a molecular chaperone involved in the folding and stabilisation of HIF-1α protein (Minet et al., 1999). HSP90 inhibitors such as 17AAG (17-allyl-aminogel-danamycin) and galdanamycin destabilise HIF-1α and promote its degradation, even in renal carcinoma cells that lack functional pVHL (Isaacs et al., 2002; Mabjeesh et al., 2002). Other agents that lead to inhibition of HIF-1α include activators of p53 such as RITA (Yang et al., 2009b) and nutlin-3 (LaRusch et al., 2007). Farnesyl transferase inhibitors that block oncogenic Ras signalling have also been shown to inhibit the HIF pathway (Chen et al., 2001).

In breast carcinoma cells, and renal cell carcinoma models, a reciprocal relationship was observed between HIF-1α and HIF-2α subunits (Carroll and Ashcroft, 2006). Loss of HIF-2α in MCF7 cells increases HIF-1α protein expression, suggesting that in this breast carcinoma model, efficient targeting of both subunits will be most beneficial for therapy (Carroll and Ashcroft, 2006). In renal carcinoma cells lacking pVHL function, HIF-2α has important functions in mediating HIF target gene expression and use of small molecule inhibitors against both HIF-1α and HIF-2α subunits achieves the most significant decrease in angiogenesis and tumour growth (Carroll and Ashcroft, 2006).

1.6.1.1 Hypoxic cells are resistant to treatment

Hypoxic tumours are amongst the most resistant tumours. Niches in which hypoxic cells form are located further away from blood vessels and therefore have less exposure to oxygen and nutrients. Delivery of cytotoxic agents that are used to kill tumour cells is also less efficient (Tannock, 1998). Most chemotherapies are designed to target rapidly proliferating tumour cells. However, cellular proliferation also decreases with distance from blood vessels, further guarding hypoxic cells from cytotoxic agents (Tannock, 1998). Other mechanisms that hypoxic cells employ to

acquire resistance include deregulated p53 activity allowing aggressive cells that are genetically unstable to colonise tumours, and regulation of the multidrug resistant (*MDR1*) gene (Comerford et al., 2002).

Many studies also demonstrate adaptive resistance strategies of tumour cells towards angiogenic therapy (Bergers and Hanahan, 2008). In the treatment of glioblastomas for example, anti-VEGF antibody treatment results in decreased tumour vessel density and increased apoptosis to prolong survival. However, anti-VEGF antibody treatment also leads to increased infiltration of surrounding vasculature, bypassing the need of tumours to initiate angiogenesis for survival (Rubenstein et al., 2000). Furthermore, anti-angiogenic agents show significant anti-tumour activity in mouse models of pancreatic neuroendocrine carcinoma and glioblastoma, however tumour adaptation is observed whereby cancer cells respond to prolonged treatment by promoting migration and invasion mechanisms that form more aggressive tumours (Paez-Ribes et al., 2009).

In some clinical schedules, use of combination therapy has the most significant anti-tumour results (Kerbel, 2001). Administration of cytotoxic chemotherapies alone such as the taxanes and platins has been associated with severe toxicity issues in multiple tissues and organs. Toxicity leads to serious and often lethal side effects including anaemia, cardiovascular failure, renal, neurological, and gastrointestinal disorders (Lokich and Anderson, 1998). Schedules involving co-administration of anti-angiogenics in combination with cytotoxics have shown increased survival benefits and sustained tumour regression, without toxicity and threatening signs of resistance (Klement et al., 2000). Combined administration of the monoclonal VEGF antibody bevacizumab with 5-fluorouracil, for example shows prolonged survival of colorectal cancer patients when compared to either agent alone (Fernando and Hurwitz, 2004).

Administration of anti-angiogenics can induce vascular normalisation, improving blood vessel structure and function to allow more efficient delivery of cytotoxic agents to tumour tissue (Jain, 2005). Recently, in murine models it was shown that dosing the receptor tyrosine kinase inhibitor, sunitinib prior to dosing with the DNA alkylating agent carboplatin had more significant effects on survival than dosing both agents simultaneously (Zhang et al., 2011). Such studies emphasise the importance of designing combination strategies carefully according to the mechanism and specific dosing requirements of each agent.

The tumour suppressor functions of p53 have been discussed in detail. As well as targeting HIF, exploring p53 agents that induce p53 mediated cell death is also an important means to inhibit tumour growth. Strategies that have been used to activate p53 function in tumours will be discussed next, including the clinical progress of some targeted agents.

1.6.2 Targeting p53 for cancer therapy

Solid tumours that express wildtype p53 are often resistant to cell death due to deregulated pathways that control p53 function. The most common pathway by which wildtype p53 activity is inhibited involves HDM2. *In vivo* studies show increased apoptosis in radiosensitive tissues and increased fatality when wildtype *p53* mice are crossed with *Mdm2* knockout mice (Ringshausen et al., 2006). Therefore, targeting HDM2 by small molecules is an effective strategy with which to activate wildtype p53 and induce p53-dependent cell death (Chene, 2003).

Therapeutic strategies commonly used to target p53 activity have been summarised in Figure 1.6.1. Small molecules that interfere with the p53-HDM2 interaction are well studied (Vassilev, 2007). Antisense oligonucleotides targeted against HDM2 show increased radiation responses (Zhang et al., 2004), and small molecules that specifically target the E3 ligase activity of HDM2 have also been described (Yang et al., 2005).

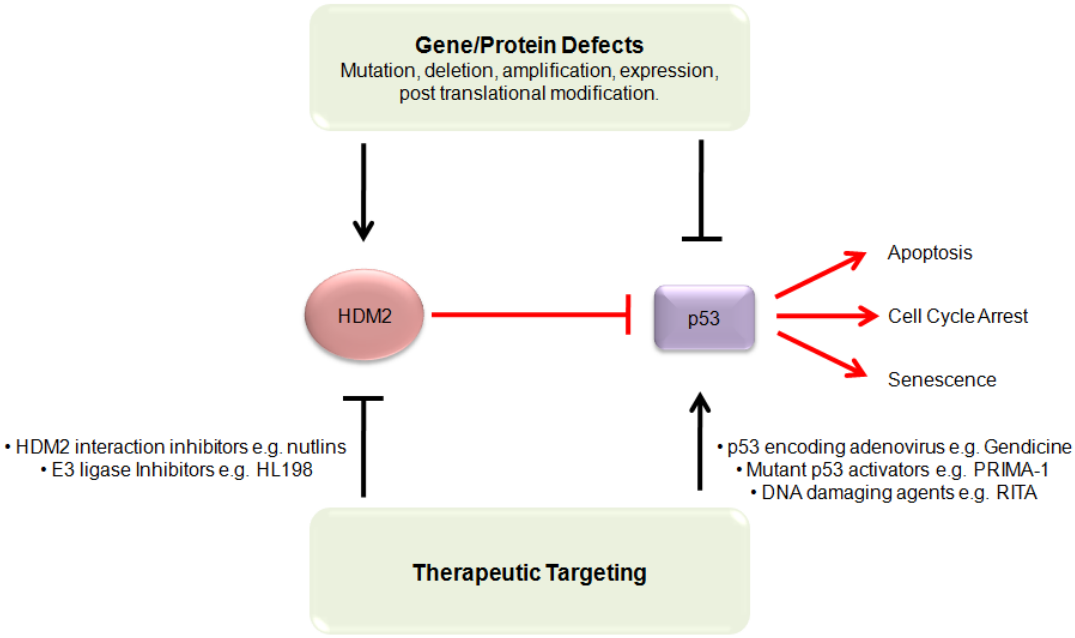


Figure 1.6.1: Therapeutic targeting of HDM2 and p53

HDM2 negatively regulates p53 function in normal cells and this relationship has important consequences for the cellular response to intrinsic and exogenous stress stimuli. The HDM2-p53 interaction is defective in cancer cells due to a number of genetic changes such as mutations and deletions affecting protein expression and function. Often these changes lead to cell phenotypes whereby HDM2 activity is hyper-activated, while p53 function is inhibited. Small molecule inhibitors have been designed to target HDM2 and its interaction with p53. These include the nutlins which inhibit HDM2 binding to p53, and HL198, an inhibitor that blocks the E3 ligase activity HDM2. Other small molecules target p53 directly to activate p53 function and these include PRIMA and RITA. Such strategies are important for altering the cellular response to stress so that sufficient p53 activation and tumour cell apoptosis is activated.

1.6.2.1 Clinical progress of HDM2 inhibitors

Small molecule HDM2 inhibitors are designed to target the inhibitory interaction between HDM2 and p53, thereby enabling p53 activity. Several HDM2 inhibitors are progressing through clinical trials including JNJ-26854165, a p53-activating tryptamine derivative (Johnson and Johnson, New Brunswick, NJ, USA). JNJ-26854165 is currently in Phase I for advanced solid tumours, and treatment of acute leukaemias (Kojima et al., 2010). The nutlins are the most well studied inhibitors of HDM2, and RG7112 (F.Hoffman, La Roche, Basel, Switzerland) is a small molecule taken from the nutlin series that is currently in Phase I trials for hematologic neoplasms and advanced

solid tumours (Cheok et al., 2010). Another class of HDM2 inhibitors known as the spiro-oxiadoles have been generated based on the crystal structure of the p53-HDM2 complex, and MI-219 is in preclinical development as an oral agent for the treatment of acute myeloid leukaemia (Ding et al., 2006).

Several groups have investigated the relationship between HDM2 and the HIF pathway, and effects of HDM2 inhibitors on HIF have also been described. Nutlin-3 stabilises p53 by targeting the p53 binding pocket on the surface of HDM2 and shows potent *in vivo* anti-tumour activity in xenografts (Supiot et al., 2008; Vassilev, 2007; Vassilev et al., 2004). Because HDM2 positively regulates HIF expression (Bardos et al., 2004), HDM2 inhibitors also target angiogenesis by p53-dependent (Secchiero et al., 2007), and p53-independent mechanisms (LaRusch et al., 2007).

Recently, HDM2 was shown to compete with FIH (factor inhibiting hypoxia inducible factor) for the C-terminal transactivation domain of HIF-1α (Lee et al., 2009). By competing with FIH, HDM2 inhibits HIF hydroxylation, and facilitates recruitment of p300 to the C-terminal transactivation domain of HIF-1α to induce HIF transcription. By binding to HDM2, nutlin-3 interferes with the HDM2-HIF interaction so FIH can bind to HIF-1α, increase hydroxylation and inhibit HIF mediated responses (Lee et al., 2009). Interestingly, Bortezomib is a proteasome inhibitor that can also inhibit HIF activity by increasing FIH interactions with HIF-1α in hypoxia, and achieves significant anti-angiogenic effects in solid tumours (Shin et al., 2008). Both studies with nutlin and bortezomib show the ability of targeted agents to inhibit a specific interaction between HDM2 and HIF-1α that is critical in the regulation of HIF activity.

Previous studies have explored the idea that a number of critical cell survival pathways can be targeted simultaneously to elicit maximum cell death responses in tumours without compromising tumour cell specificity (Grinkevich et al., 2009). PRIMA-1^{MET} for example, a small molecule that reactivates mutant p53 can synergise with cisplatin to induce tumour cell apoptosis and inhibit growth of tumour xenografts *in vivo* (Bykov et al., 2005). Many cytotoxic combinations with anti-angiogenics have also progressed into the clinic (Fernando and Hurwitz, 2004). More recently, ‘cyclotherapy’ has been described as a novel concept to minimise unwanted side effects by specifically targeting proliferating cells within tumours. Low dose nutlin-3 or actinomycin D (both at non-genotoxic concentrations) activate p53 to place normal cells into reversible cell cycle arrest, treatment is then followed with a mitotic inhibitor such as a taxane that selectively targets remaining proliferating cells within the tumour for cell death (Sur et

al., 2009). Cyclotherapy is promising because it utilises knowledge of tumour cell kinetics and mechanisms behind targeted small molecules to build specific treatment regimes. Combining low dose activators of p53 with specific inhibitors of the PI3K/Akt pathway has also been suggested (Bardos et al., 2004; Grinkevich et al., 2009).

Knowing that cancer is a disease that has a makeup unique to a particular individual and cancer type is critical in making accurate predictions of treatment responses and disease outcome. Careful consideration is taken when assessing the cell and tumour type that could be targeted by specific agents which affect the p53-HIF pathways, especially in renal cell carcinoma where inhibition of HIF-2α specifically has recently been shown to enhance tumour cell survival by promoting the angiogenic functions of HIF-1α (Carroll and Ashcroft, 2008). It is also important to consider the possibility that restoring activity of p53 to increase apoptosis could also select for cells that are defective in p53 function, resulting in tumour adaptation towards a more aggressive and metastatic phenotype (Kastan, 2007).

1.6.3 RITA: a small molecule activator of p53

In a cell-based screen of HCT116 cells that differentially express p53, compounds from the National Cancer Institute library were assessed for their ability to inhibit cell growth (Issaeva et al., 2004). The small molecule RITA (2,5-bis (5-hydroxymethyl-2-thienyl) furan, NSC652287) was isolated from this screen on the basis that HCT116 p53+/+ cells treated with a dose range of RITA showed significant inhibition of cell growth compared to HCT116 p53-/- cells (Figure 1.6.2). As well as cell proliferation assays using WST-1, long term growth was also assessed using the colony formation assay. At a 500nM dose (the dose whereby cell proliferation is inhibited by approximately 50%) RITA was also shown to inhibit the number of colonies formed in HCT116 p53+/+ cells to a greater degree compared with HCT116 p53-/- cells (Issaeva et al., 2004). Further studies proposed that RITA suppressed tumour growth *in vitro* and *in vivo* by binding with high affinity to the NH2-terminal domain of p53 resulting in a conformational change that inhibited the p53-HDM2 interaction and therefore stabilised p53 (Issaeva et al., 2004).

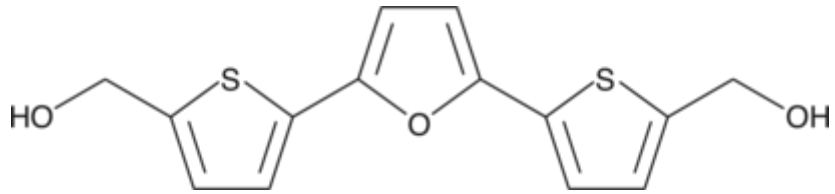


Figure 1.6.2: The structure of RITA

Chemical structure for NSC652287 (2,5-bis (5-hydroxymethyl-2-thienyl) furan). NSC652287 is a naturally occurring tricyclic bis-thiophene derivative. This compound was isolated from the National Cancer Institute library of compounds due to its role in activating p53 and inducing tumour cell apoptosis. NSC652287 was therefore named RITA (reactivation of p53 and *i*nduction of *t*umour cell apoptosis).

1.6.3.1 RITA elicits cell death responses

Since the original identification of RITA as an activator of p53 in 2004, subsequent studies have explored RITA's mechanism of action in greater detail. Activation of p53 by nutlin-3 induces cell cycle arrest in a number of tumour cell lines while RITA is primarily involved in promoting apoptosis (Tovar et al., 2006). By binding to HDM2, nutlin-3 disrupts the p53-HDM2 interaction, p53 is stabilised, and activated to induce transcription of genes involved in cell cycle control and growth arrest. RITA, however, is proposed to bind directly to p53 and inhibit its interaction with HDM2 (Issaeva et al., 2004). Genome wide oligonucleotide micro-array analysis was recently used to investigate the mechanisms which govern p53 dependent cell cycle and cell death responses to stress. Data from isogenic p53 positive and p53 negative HCT116 colon carcinoma cells was used to study differences in gene expression when p53 was activated by either nutlin-3 or RITA (Enge et al., 2009). RITA's unique p53-dependent activity was confirmed by observation of very few changes in global gene expression when HCT116 p53-/- cells were treated with RITA compared to HCT116 p53+/+ cells or MCF7 cells expressing wildtype p53 (Enge et al., 2009). RITA was shown to increase the expression profiles of a number of genes involved not only in the DNA damage pathway, but also in PI3K/Akt signal transduction, cytoskeleton regulation, immune responses and oestrogen/androgen signalling (Enge et al., 2009). As expected, expression of apoptosis genes was higher in HCT116 p53+/+ cells treated with RITA compared to those treated with nutlin-3. Mitotic arrest induced by nutlin-3 involved activation of p21 mediated cell cycle arrest (Enge et al., 2009).

Expression of the hnRNP K cofactor was identified as an important mechanism in determining the outcome of cell cycle responses in cells treated with either RITA or nutlin-3 (Enge et al., 2009). When cells are exposed to DNA damage, the DNA damage signalling kinases ATM and ATR activate hnRNP K (Moumen et al., 2005). Both p53 and hnRNP K are recruited to promoters of cell cycle arrest genes such as *GADD45* and *CDKN1A* (encoding p21) to activate transcription in response to stress (Moumen et al., 2005). The hnRNP K cofactor is a target for HDM2 and when RITA binds to p53, free HDM2 degrades hnRNP K so it is unable to mediate p53-dependent transcription of p21 and induce cell cycle arrest (Enge et al., 2009). Instead, p53 activates expression of genes involved in apoptosis. In comparison, nutlin-3, by binding to HDM2, inhibits HDM2's ability to target hnRNP K for degradation allowing recruitment of hnRNP K to p53 target gene promoters and cell cycle gene expression (Enge et al., 2009).

HDM2 mediated regulation of homeodomain interacting protein kinase 2 (HIPK2) is also involved in regulating cell cycle and cell death in response to p53 activation by nutlin-3 and RITA (Rinaldo et al., 2009). HIPK2 is a pro-apoptotic kinase activated in response to severe stress and induces p53 activity and DNA damage responses by phosphorylating p53 at serine 46 (Oda et al., 2000b). When bound to HDM2, nutlin-3 mediates HDM2-dependent proteasomal degradation of the HIPK2 kinase to inhibit apoptosis while activating cell cycle arrest. Transiently inhibited HDM2 protein levels by higher and extended doses of RITA lead to HIPK2 stabilisation and p53-dependent apoptosis (Rinaldo et al., 2009).

Also of interest is a recent study whereby RITA was shown to non-covalently bind thioredoxin reductase 1 (TrxR1) a protein involved in regulating redox dependent cellular pathways (Hedstrom et al., 2009). Binding of TrxR1 by RITA increases levels of reactive oxygen species to induce p53-dependant apoptosis suggesting a mechanism whereby RITA can target regions of tumours that suffer from oxidative stress (Hedstrom et al., 2009). Other studies from our lab have shown that RITA can elicit p53-dependent cell death in normoxia and hypoxia as well as induce p53-dependent DNA damage responses (Yang et al., 2009a; Yang et al., 2009b). Furthermore, DNA damage pathways that are induced by RITA also elicit cell cycle checkpoints involved in maintaining genomic integrity in response to stress (Ahmed et al., 2011).

1.6.3.2 Synergistic effects of p53 activation by RITA

Interestingly, 'oncogene addiction' has been described whereby tumour cells become dependent on oncogene activity for survival. However, oncogene addiction also results in tumours gaining an Achilles heel against which many highly targeted drug combinations can be used (Weinstein, 2002). It was recently shown that RITA can also inhibit specific oncogenes at higher doses (1μM and beyond) which contributes significantly to p53 mediated apoptosis. Grinkevich and colleagues used microarray data to identify several critical oncogenes selectively inhibited by RITA in HCT116 p53+/+ cells (Grinkevich et al., 2009). The AKT/PKB signalling pathway was inhibited, as was its downstream effectors mTOR and GSK3β. Further analysis of the mTOR pathway revealed significant inhibition of the mTOR substrate eIF4E which is involved in regulating the translation of several important oncoproteins, including c-myc (Averous et al., 2008; Averous and Proud, 2006). Inhibition of c-myc in response to RITA was found to occur at the level of transcription, translation, and protein stability (Grinkevich et al., 2009). RITA may therefore induce its p53-dependent cell death effects by not only activating pro-apoptotic genes and pathways (Enge et al., 2009), but also mediating downregulation of critical oncogenes (Grinkevich et al., 2009). Using RITA in combination with specific oncogene inhibitors was proposed as a strategy to maximise apoptotic responses.

1.6.3.3 Using RITA to activate p53 in hypoxia

Many agents activate p53 but are not effective in mediating tumour cell death in hypoxia. This is due to transcriptional repression of wildtype p53 in hypoxia, by unknown mechanisms (Ashcroft et al., 2000; Pan et al., 2004). RITA was used as a pharmacological tool in our laboratory to activate p53 and to understand the relationship between p53 and HIF. RITA was found to induce significant p53-dependent tumour cell apoptosis, not only in normoxia as was previously published (Issaeva et al., 2004), but also in hypoxia (Yang et al., 2009b).

Initial experiments by Yang et al. using flow cytometry showed a significant increase in the apoptotic population of hypoxic HCT116 p53+/+ cells treated with RITA and this was not observed in cells that were deficient or defective in p53 activity (Yang et al., 2009b). Analysis of the mechanisms for this response revealed the induction of a unique p53-dependent DNA damage response signal. Additionally, RITA was found to inhibit key signalling pathways such as the HIF pathway by affecting the translational machinery (Yang et al., 2009b). Further studies revealed a critical role for the MAPK

pathway in mediating p53-dependent DNA damage and cell death responses (Yang, unpublished). The cell pathways activated by RITA are summarised in Figure 1.6.2. The studies conducted so far in our laboratory provide a basis for understanding the crosstalk between p53 and HIF in cancers with deregulated apoptotic and angiogenic pathways.

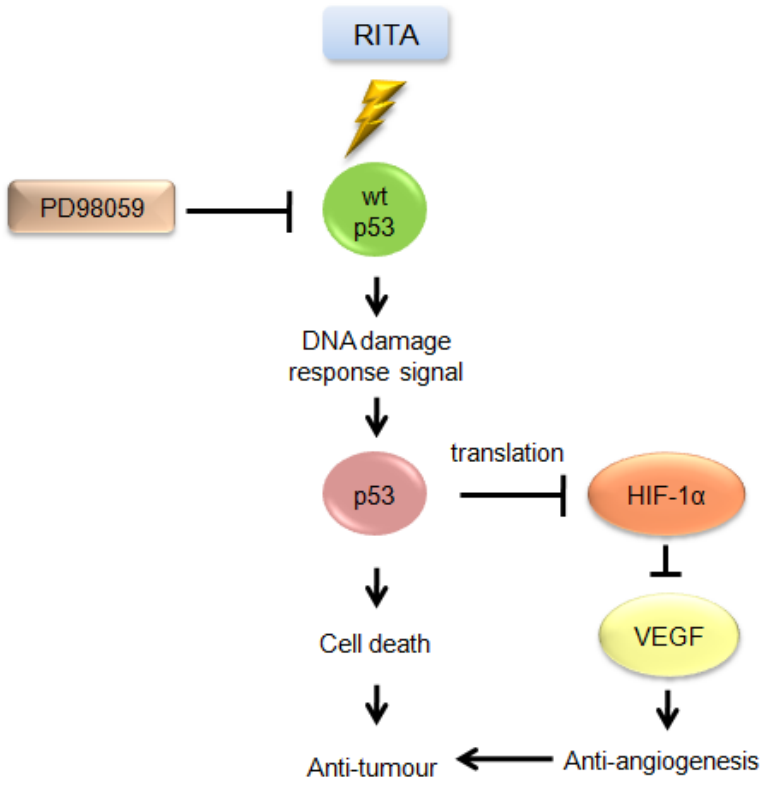


Figure 1.6.3: The RITA response
RITA elicits a DNA damage signal in wildtype p53 cells to activate p53 and induce significant cell apoptosis and anti-tumour effects, in both normoxia and hypoxia (Yang et al., 2009b). The HIF pathway is also targeted by inhibition of the translational machinery leading to anti-angiogenic effects. These responses are dependent on the presence of wildtype p53. RITA mediated effects are reversed by the MEK1/2 inhibitor PD98059, therefore the MAPK pathway is also important for RITA induced cell death and anti-angiogenic responses.

1.7 Project hypothesis

Small molecule activation of p53 can inhibit HIF mediated responses in normoxia and hypoxia to induce tumour cell death

1.8 Project aims

- Chapter 3: Understand how cell death responses are regulated in tumour cells by HIF and p53, in normoxia and hypoxia.

- Chapter 4: Investigate DNA damage responses induced by RITA and their impact on the cell cycle.

- Chapter 5: The MAPK pathway regulates p53-dependent cell cycle and cell death responses. Investigate members of the MAPK family which activate p53-dependent mechanisms to induce tumour cell apoptosis by RITA.

- Chapter 6: Investigate effects of RITA on HIF-α status and explore the translational mechanisms by which the HIF pathway is suppressed by RITA mediated induction of p53.

Chapter 2

Materials and Methods

2.1 Cell culture

The matched colorectal cell line HCT116 p53+/+ with wildtype p53, and its isogenic derivative in which p53 is lost by homologous recombination, HCT116 p53-/- have been described previously (Bunz et al., 1998). Tetracycline inducible HCT116-pCDNA5 (control) and HCT116-PERKΔC (PERK dominant negative) cell lines were a kind gift from Kasper Rouschop (University of Maastricht) and Brad Wouters (Ontorio Cancer Institute). The osteosarcoma Saos-2, breast adenocarcinoma MCF7, and renal carcinoma cell lines RCC4 VHL and RCC4 were purchased from American Type Culture Collection (ATCC). The 786-O EV and 786-O VHL renal cell carcinoma cell lines were a kind gift from Bill Kaelin (Harvard Hughes Medical Centre). All Cell lines were cultured in Dulbecco Modified Eagle Medium (GIBCO/Life Technologies) supplemented with 10% fetal calf serum (FCS, Harlan), 100IU/ml Penicillin, 100µg/ml streptomycin and 2mM glutamine (all from GIBCO/Life Technologies).

2.2 siRNA oligo duplexes and transient transfection

Custom made siRNA oligo duplexes to p53, HIF-1α and CHK1 were ordered from Dharmacon. Non-silencing control siRNA oligo duplex was obtained from Qiagen. ATM, ATR and p38α siRNAs were purchased from Dharmacon. Table 1 summarises the sequences and final transfection concentrations for specific custom made siRNA oligo duplexes. Transient transfections with siRNA oligo duplexes were performed using HiPerfect^{TM} transfection reagent (Qiagen) according to manufacturer's instructions.

Target Gene	Target Sequence	Transfection Concentration
p53	5'-GCATCTTATCCGAGTGGAA-3'	50nM
HIF-1α	5'-TACGTTGTGAGTGGTATTATT-3'	10nM
HIF-2α	5'-CCCGGATAGACTTATTGCCAA-3'	10nM
Non-silencing control	5'-AATTCTCCGAACGTGTCACGT-3'	As specified
CHK1 Target	5'-GGTGCCTATGGAGAAGTT-3'	25nM
CHK1 Reverse	5'-CTTGAAGAGGTATCCGUGG-3'	25nM

Table 1: Custom made siRNA oligo duplex sequences

2.3 Antibodies and staining reagents

Table 2 summarises primary and secondary antibodies used along with their conjugated labels, source, and specific dilutions according to application. Staining reagents have also been listed.

Antibody	Molecular Weight (kDa)	Source	Company	Application	Dilution
Phospho-AKT (Ser473)	60	Rabbit polyclonal	Cell Signalling #9271	Western blotting	1:1000
HIF-1α	120	Mouse monoclonal	BD Biosciences	Western blotting	1:1000
HIF-2α	100	Rabbit polyclonal	Abcam ab199	Western blotting	1:1000
Phospho-CHK1 (Ser345)	56	Rabbit polyclonal	Cell Signalling #2348	Western blotting	1:1000
CHK1	56	Rabbit polyclonal	Cell Signalling #2345	Western blotting	1:1000
Phospho-CHK2 (Thr68)	62	Rabbit polyclonal	Cell Signalling #2662	Western blotting	1:1000
CHK2	62	Rabbit polyclonal	Cell Signalling #2662	Western blotting	1:1000

Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	42,44	Rabbit polyclonal	Cell Signalling #9101	Western blotting	1:1000
p44/42 MAPK (ERK1/2)	42,44	Rabbit polyclonal	Cell Signalling #9102	Western blotting	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	46,54	Rabbit polyclonal	Cell Signalling #9251	Western blotting	1:1000
Phospho-MKK3/MKK6 (Ser189/207) (22A8)	40	Rabbit polyclonal	Cell Signalling #9236	Western blotting	1:1000
MKK3	40	Rabbit polyclonal	Cell Signalling #9232	Western blotting	1:1000
Phospho-p38 MAPK (Thr180/Tyr182)	43	Rabbit polyclonal	Cell Signalling #9211	Western blotting	1:1000
p38 MAPK	43	Rabbit polyclonal	Cell Signalling #9212	Western blotting	1:1000
AMPKα	62	Rabbit polyclonal	Cell Signalling #2532	Western blotting	1:1000
Phospho-AMPKα (Thr172)	62	Rabbit polyclonal	Cell Signalling #2531	Western blotting	1:1000
eIF-2α	38	Rabbit polyclonal	Cell Signalling #9722	Western blotting	1:1000
Phospho-eIF-2α (Ser51)	38	Rabbit polyclonal	Cell Signalling #9721S	Western blotting	1:1000
PARP	116 uncleaved, 89 cleaved	Rabbit polyclonal	Cell Signalling #9542	Western blotting	1:1000
mTOR	289	Rabbit polyclonal	Cell Signalling #2972	Western blotting	1:1000
Phospho-4E-BP1 (Thr37/46)	15-20	Rabbit polyclonal	Cell Signalling #9459	Western blotting	1:1000
α-tubulin	50	Mouse monoclonal	Sigma T6199	Western blotting	1:20,000
β-actin	42	Mouse monoclonal	AbCam Ab6276	Western blotting	1:10,000
Phospho-Histone H2A.X (Ser139)	15	Mouse monoclonal	Upstate #16.193 clone JBW301	Fluorescence	1:250

		Rabbit monoclonal	Cell Signalling #2577	Western blotting	1:1000
				Flow cytometry	1:100
Phospho-p53 (Ser46)	53	Rabbit polyclonal	Cell Signalling #2521	Western blotting	1:1000
Phospho-p53, 16G8 (Ser15)	53	Mouse monoclonal	Cell Signalling #9286	Western blotting	1:1000
p53	53	Mouse monoclonal	Calbiochem Ab-6 #OP43	Western blotting	1:1000
				Flow cytometry	1:80
		Rabbit monoclonal	Cell Signalling 7F5 #2527	Flourescence	1:200
Secondary anti-rabbit HRP conjugate		Rabbit	Amersham	Western blotting	1:5000
Secondary anti-mouse HRP conjugate		Mouse	Amersham	Western blotting	1:5000
Alexa Fluor 568 goat anti-mouse IgG		Mouse	Invitrogen A-11031	Flourescence	1:500
Alexa Fluor 488 goat anti-rabbit IgG		Rabbit	Invitrogen A-11034	Flourescence	1:500
Allophycocyanin (APC) crosslinked goat anti-rabbit IgG		Rabbit	Invitrogen, A-10931	Flow cytometry	1:200
Polyclonal goat anti-mouse IgG/FITC (fluorescein isothiocyanate) goat		Mouse	Dako F0479	Flow cytometry	1:10
DAPI (4',6'-diamidino-2-phenylindole)			Invitrogen D1306	Flourescence	1:50,000
Propidium Iodide			Sigma	Flow cytometry	10µg/ml

Table 2: Primary, secondary antibodies and staining reagents

2.4 Inductions and drug treatments

Hypoxia was achieved by incubating cells in 1% O_2, 5% CO_2 and 94% nitrogen in an LEEC dual gas incubator (GA-156) for 16 hours. RITA, 2,5-bis (5-hydroxymethyl-2-thienyl) furan was obtained from the National Cancer Centre Drug Therapeutic Program (NSC-652287). The compound was dissolved in 100% DMSO, stored at -20°C as 10mM aliquots and dosed between 0-1µM. Nutlin-3 (Calbiochem) was dissolved in 100% DMSO and dosed at a final concentration of 4µM. The MEK1/2 inhibitors PD98059 (Cell Signalling Technologies) and PD184352 (Calbiochem) were dissolved in 100% DMSO and stored at -20°C. PD98059 was dosed at final concentrations of 10µM or 50µM and PD184352 was dosed at 2µM. Etoposide (dosed at 25µM), Doxorubicin (dosed at 1µM), and Hydroxyurea (dosed at 1mM) were purchased from Sigma. The caspase 3 inhibitor Z-DEVD-FMK (Tocris Bioscience) was dosed at a final concentration of 50µM. All reagents were prepared according to suppliers instructions. Length of dosing was specific to each experiment.

2.5 Western blot analysis

Following treatment, cells were washed in ice-cold phosphate buffered saline (PBS) and lysed in 2x sample buffer (125mM Tris pH 6.8, 4% sodium dodecyl sulphate (SDS), 0.01% bromophenol blue, 10% β-mercaptoethanol, 10% glycerol). Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and assessed by western blot using standard procedures. All antibody dilutions were prepared in 5% Milk with 0.1% Tween in TBS buffer (140mM sodium chloride, 250mM Tris). See table 2 for specific antibody dilutions.

2.6 Fluorescence activated cell sorting (FACS)

Flow cytometry cell cycle profiles were acquired using CyAn^{TM} ADP technology (Dako). Total populations of cells, including those floating and adherent were collected and fixed in ice cold 100% methanol. Cells were blocked in 1% FCS/PBS and labelled with specific primary and fluorescent conjugated secondary antibodies (see table 2) diluted in blocking buffer. Prior to acquisition, cells were stained with 10µg/ml propidium iodide (Sigma) and ribonuclease was added at 100µg/ml (Sigma). Data was gated and quantified using CyAn^{TM} Summit v4.3 software.

2.7 Immunoflourescence

HCT116 p53+/+ and HCT116 p53-/- cells were seeded onto 96 well plates at 10,000 cells/well. Following treatment, cells were fixed for 1 hour in 4% paraformaldehyde in 1xPBS (phosphate buffered saline) at 4°C. Cells were then permeabilized with 0.5% Triton x-100 (Sigma) in PBS for 10 mins and washed using PBS. This was followed by a blocking step in IFF buffer (1% BSA (bovine serum albumin), 2% FCS in PBS) for 1 hour. A rabbit monoclonal antibody for p53 (Cell Signalling) was used followed by an Alexa Fluor 488 conjugated secondary antibody (Invitrogen). Cells were also labelled with a mouse monoclonal antibody against γH2AX at serine 139 followed by an Alexa Fluor 568 conjugated secondary antibody (Invitrogen), see Table 2 for details of all antibodies. Antibodies were diluted in IFF buffer and incubated on cells for 40 mins. Nuclei were visualised by DAPI staining (Invitrogen). Plates were analysed and quantified using the INCell 1000 Analyser (GE Healthcare).

2.8 Comet assay

The comet assay (Trevigen) was performed according to manufacturer's instructions. All cells (both floating and adherent) were harvested, mixed with low melting agarose at $2x10^5$ cells/ml (approx 1000 cells/slide), and spread onto pre-prepared comet slides. Cells were incubated in alkaline lysis buffer to select for single strand DNA breaks. Slides were then electrophoresed using alkaline buffer at 4°C, 18v, and 300mA for 40 mins. Following electrophoresis, slides were fixed, dried and stained using SYBR Green. Comets were viewed under a Zeiss fluorescent microscope at 20x magnification and images were captured over 20 fields of view for each slide using ImagePro™ software. The relative length and intensity of individual SYBR Green stained nuclei (comets) was proportional to DNA damage and was quantified using an algorithm for Olive tail moment on TriTek CometScore™ software. Over 100 comets were analysed for each treatment. A 20 min dose of 100µM hydrogen peroxide at 4°C was used as a positive control for DNA damage.

2.9 Real-time quantitative PCR (polymerase chain reaction)

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and 1µg was used for first-strand cDNA synthesis. First strand synthesis was performed using qScript cDNA SuperMix (Quanta Biosciences) according to manufacturer's instructions. Real-time quantitative PCR was performed using DyNAmo SYBR Green Quantitative PCR kit (Finnzymes Genetic Research Institute (GRI) Ltd) with a DNA Engine OPTICON 2 Continuous Fluorescence

Detection System (GRI). Primers for HDM2, p21 and β-actin were purchased from Invitrogen. Sequences are shown in Table 3. For each PCR reaction primers were used at a final concentration of 70nM. DNA was incubated for 16 mins at 95°C, denatured for 30 secs at 94°C and annealed at 60°C for 1 min followed by an extension step at 72°C for 1 min. Each cycle was quantified and repeated 40 times. A melting curve was performed from 65°C to 94°C and read every 0.4°C, holding for 1 sec. Expression of target gene mRNA relative to reference gene mRNA (β-actin) was calculated using the Relative Expression Software Tool (REST), (http://www.gene-quantification.de/download.html)

Target Gene	Forward primer	Reverse primer
β-actin	5'- CCCAGAGCAAGAGAGG-3'	5'- GTCCAGACGCAGGATG-3'
HDM2	5'-CTTCGTGAGAATTGGCTTCC-3'	5'-CAAAGCCCTCTTCAGCTTGT-3'
p21	5'-GTTCCTTGTGGAGCCGGAGC-3'	5'-GGTACAAGACAGTGACAGGTC-3'

Table 3: Primer sequences for real-time quantitative PCR analysis

Chapter 3

RITA activates p53-dependent DNA damage and cell death responses in normoxia and hypoxia

3.1 Introduction

In hypoxic tumours, oxygen concentrations can fluctuate widely and reach almost anoxic levels of 0.5% and below (Vaupel et al., 1991). In areas of severe hypoxia (<0.2% oxygen), cells stop proliferating and DNA synthesis is delayed. DNA strand breaks are formed during replication arrest leading to activation of a DNA damage response that involves induction of γH2AX and activation of ATR and CHK1 signalling. CHK1 mediates phosphorylation of p53 to induce replication arrest and thereby stabilise the replication fork machinery (Hammond et al., 2002; Hammond et al., 2004). By activating an ATR-dependent replication arrest that is mediated by p53, cells conduct DNA repair pathways that protect them against DNA strand breaks during hypoxia (Hammond et al., 2004). The ATM kinase is also phosphorylated at 0.02% oxygen to activate CHK2 and elicit similar cell cycle checkpoints (Bencokova et al., 2009).

DNA damage responses involving γH2AX, ATM-CHK2, ATR-CHK1 and p53 are important barriers against tumour development because they initiate repair mechanisms that maintain genomic integrity (Bartkova et al., 2005; Di Micco et al., 2008). However, pathways employed by cells to maintain viability have also been adapted by cancer cells to promote their development. Cell cycle arrest activated in severe hypoxia allows for resistance to many chemotherapies that target proliferating cells (Bencokova et al., 2009). Furthermore, hypoxia induced DNA damage responses involving γH2AX have been shown to enhance endothelial cell proliferation and neovascularisation to promote tumour growth *in vivo* (Economopoulou et al., 2009). In hypoxic environments of 1% oxygen, p53 activity is impaired (Ashcroft et al., 2000) and p53 requires additional signals such as genotoxic stress which is not present in hypoxic conditions alone for transactivation (Koumenis et al., 2001; Yang et al., 2009b). Therefore, it is of therapeutic interest to determine whether small molecules such as RITA can be used to activate p53-dependent cell death responses in normoxia and in hypoxia.

3.2 Hypothesis

RITA induces p53 and elicits significant cell death in p53 positive cells, both in normoxia and in response to hypoxia.

3.3 Aims

- Investigate p53 responses in cells that vary in p53 status using the small molecule RITA.
- Assess cell death responses and the mechanisms by which p53 is induced by RITA in normoxia and in hypoxia.
- Investigate whether RITA affects the HIF pathway in hypoxia.

3.4 Cell death by RITA is distinct compared to other p53-activating agents

RITA has been proposed to bind directly to p53, and stabilise p53 by inhibiting its association with HDM2. By stabilising wildtype p53, RITA can elicit p53-dependent cell death *in vitro*, and *in vivo* (Issaeva et al., 2004). Previous studies from our laboratory have shown that RITA induces p53-dependent cell death in normoxia and in hypoxia (Yang et al., 2009b). To investigate these findings in greater detail, RITA induced tumour cell death was compared to other DNA damaging agents like doxorubicin and hydroxyurea which also activate p53-mediated cell death responses.

DNA damaging agents used in the clinic achieve anti-tumour efficacy by eliciting p53-mediated apoptosis through distinct mechanisms. Etoposide is a topoisomerase inhibitor that targets cells during the S-G2 phases of the cell cycle, induces DNA strand breaks, and inhibits DNA synthesis (van Maanen et al., 1988). Doxorubicin, an anthracycline antibiotic also inhibits DNA synthesis, but does so by intercalating into DNA and inhibiting the activity of topoisomerase enzymes (Fornari et al., 1994). Hydroxyurea induces DNA damage by inhibiting ribonucleotide reductase and dNTP (deoxyribonucleoside triphosphate) production required for the G1-S phase transition of the cell cycle (Hakansson et al., 2006; Koc et al., 2004). Doxorubicin, etoposide and hydroxyurea have distinct cell cycle affects and elicit significant DNA damage responses that induce p53 and mediate tumour cell apoptosis.

To compare cell death responses induced by RITA with other p53-activating agents, flow cytometry was used to quantify sub-G1 cell populations as an indication of cell death. Doses were used at which etoposide, doxorubicin and hydroxyurea induce p53 to a similar degree as RITA, both in normoxia and in hypoxia. As shown in Figure 3.4.1, RITA induced significant cell death in HCT116 p53+/+ cells, both in normoxia, and in response to hypoxia. Cell death observed with RITA treatment was not observed to the same degree in cells treated with etoposide, doxorubicin, or hydroxyurea. Closer analysis of DNA flow cytometry profiles showed that as expected, etoposide elicited a

G2 arrest, doxorubicin an S-phase arrest, and hydroxyurea, a significant G1 arrest (Figure 3.4.1). In comparison, RITA did not elicit cell cycle arrest in HCT116 p53+/+ cells, instead the sub-G1 population of RITA treated cells was significantly induced suggesting that cell death is a primary response of HCT116 p53+/+ cells treated with this agent.

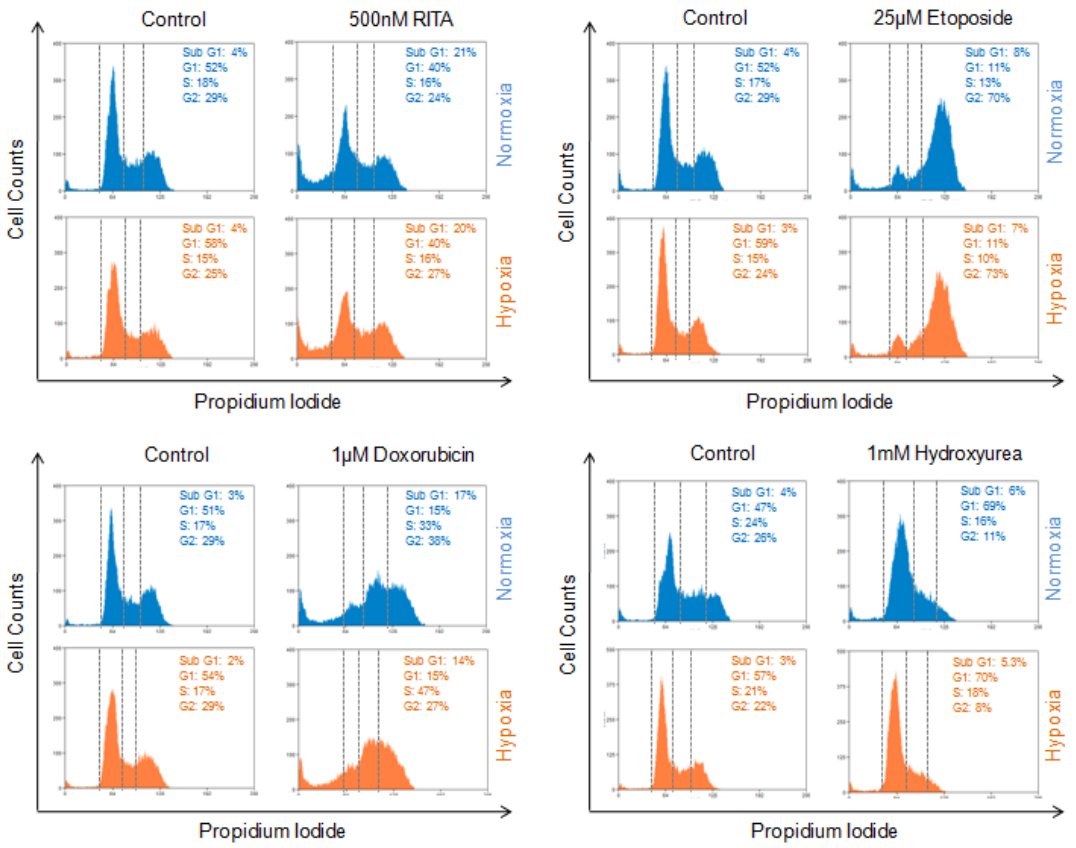


Figure 3.4.1: RITA mediated cell death is distinct compared to other p53 activating agents

Flow cytometry cell cycle profiles show changes in cell cycle phases when HCT116 p53+/+ cells are dosed with either 500nM RITA, 25μM etoposide, 1μM doxorubicin or 1mM hydroxyurea for 16 hours in normoxia (20% O_2, shown by blue profiles) or in hypoxia (1% O_2, shown by orange profiles). Gated regions are indicated by dashed lines to highlight different phases of the cell cycle, from left to right; sub-G1, G1, S, and G2 phase. The percentage of cells in each cell cycle phase is quantified and shown alongside each profile. Each flow cytometry profile shows propidium iodide fluorescent intensity on the x-axis, and cell counts on the y-axis. Data shown is representative of 3 independent experiments.

The sub-G1 population of cells from each flow cytometry profile was quantified as an indication of cell death. RITA treated cells had the most significant increase in sub-G1 compared to cells treated with etoposide, doxorubicin and hydroxyurea (Figure 3.4.2).

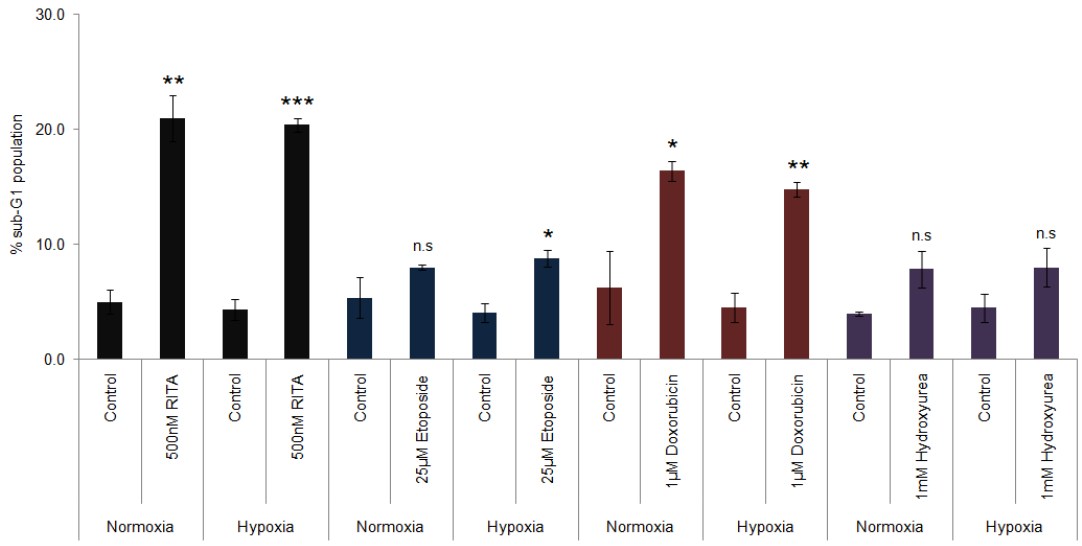


Figure 3.4.2: RITA activates cell death in normoxia and in hypoxia

Graph shows the percentage of HCT116 p53+/+ cells in sub-G1 that have been treated for 16 hours in normoxia (20% O_2), or in hypoxia (1% O_2), with either 500nM RITA, 25μM etoposide, 1μM doxorubicin or 1mM hydroxyurea. Cells were harvested for flow cytometry analysis and stained using propidium iodide to assess cell cycle profiles. The sub-G1 population for each treatment was gated and quantified. Data is averaged from 3 independent repeat experiments. An unpaired t-test was used to compare significance between control and treated cells and a two-tailed p-value <0.05 was considered significant (***p<0.0005, **p<0.005, *p<0.05). Standard error bars are shown in every graph unless otherwise indicated.

To confirm that RITA was mediating cell death by activating apoptosis, HCT116 p53+/+ cells were treated with RITA alone or in combination with the caspase 3 inhibitor Z-DEVD-FMK (Figure 3.4.3). Cells dosed with 500nM RITA in the presence of caspase inhibitor showed a significant reduction in sub-G1 content, suggesting that sub-G1 cells are those that are undergoing apoptotic cell death (Figure 3.4.3).

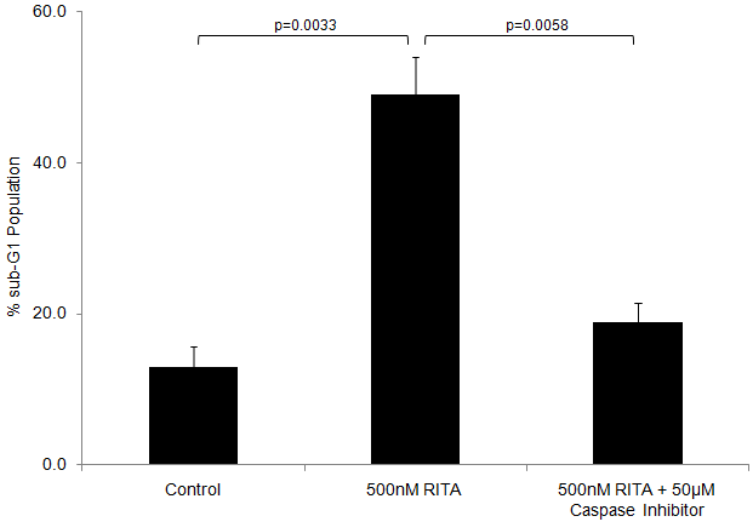


Figure 3.4.3: RITA mediates cell death by activating apoptosis

Graph shows the percentage of HCT116 p53+/+ cells in sub-G1 that have been treated with 500nM RITA alone, or in combination with the caspase inhibitor Z-DEVD-FMK for 24 hours in normoxia. Following treatment, cells were harvested for flow cytometry and stained using propidium iodide. Cell cycle DNA profiles for each treatment were gated and quantified for sub-G1. Data shown has been averaged from 3 independent experiments. An unpaired t-test was used to assess significance and a two tailed p-value of <0.05 was considered statistically significant.

As cell death was induced by RITA in hypoxia, western analysis was used to assess whether p53 stabilisation in response to RITA affected HIF-1α protein expression. As indicated by cleaved PARP, another marker for apoptotic cell death, 500nM RITA induced cell death both in normoxia and in hypoxia. This was in agreement with flow cytometry data shown in Figure 3.4.2 and published previously by our laboratory (Yang et al., 2009b). Interestingly, RITA could also inhibit HIF-1α protein expression in hypoxia. Effects on HIF-1α protein expression were not observed to the same extent in cells that had been treated with etoposide, doxorubicin, or hydroxyurea (Figure 3.4.4).

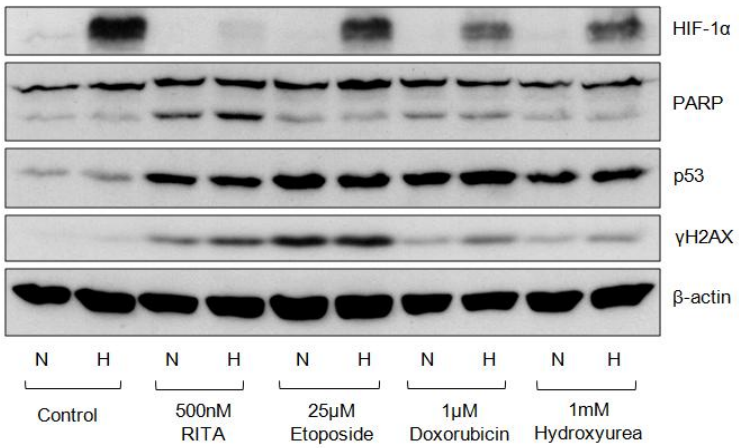


Figure 3.4.4: RITA inhibits HIF-1α protein expression

Western blot shows HIF-1α, cleaved poly ADP ribose polymerase (PARP), p53, and phosphorylated γH2AX protein levels in HCT116 p53+/+ cells treated with either DMSO, 500nM RITA, 25μM etoposide, 1μM doxorubicin or 1mM hydroxyurea for 16 hours in normoxia (N), or in hypoxia (H). β-actin was used as a loading control. Data shown is representative of 3 independent experiments.

Unlike other DNA damaging agents that promote cell cycle effects when p53 is activated, RITA primarily activates apoptosis, both in normoxia, and in hypoxia. As shown in Figure 3.4.4, RITA also inhibits HIF-1α protein expression in hypoxia (Yang et al., 2009b).

3.5 RITA elicits p53-dependent DNA damage responses

So far, studies from our laboratory by Yang et al. have shown that RITA can induce p53 and inhibit HIF-1α protein expression in hypoxia (Yang et al., 2009b). Further analysis has shown that these effects are distinct compared to other DNA damaging agents which activate p53 (Figure 3.4.4). Next, I aimed to investigate the mechanism by which RITA activates p53 in the cell. RITA's proposed mechanism of action was described to involve direct binding of RITA to the N-terminal domain of p53 leading to disruption of the p53-HDM2 interaction and stabilisation of p53 (Issaeva et al., 2004). In previous studies however, when p53 and HDM2 were both stabilised in the cell by the proteosomal inhibitor MG132, RITA was unable to disrupt p53-HDM2 complexes (Yang et al., 2009b). In contrast and as expected, the small molecule inhibitor of HDM2, nutlin-3 impaired p53-HDM2 binding leading to p53 activation (Yang et al., 2009b). Detailed studies using nuclear magnetic resonance (NMR) to assess conformational

shifts in p53 and HDM2 complexes upon RITA treatment also indicated that RITA does not interfere with the p53-HDM2 interaction *in vitro* and suggested that RITA may stabilise p53 by other mechanisms (Krajewski et al., 2005).

RITA can induce protein-DNA and DNA-DNA intrastrand cross-links *in vitro* suggesting that RITA may elicit DNA damage (Nieves-Neira et al., 1999; Rivera et al., 1999). To explore the possibility of whether RITA could induce a DNA damage response, phosphorylation of p53 was assessed at sites commonly activated by genotoxic stress within the N-terminus (Ashcroft et al., 2000; Jimenez et al., 1999). RITA was found to induce phosphorylation of p53 at serine 15 and serine 46 (Figure 3.5.1, Ahmed et al., 2011).

The DNA damage protein H2AX was also phosphorylated in response to RITA treatment (Figure 3.4.4, 3.5.1). H2AX is a member of the H2A family which are involved in organising DNA into chromatin, and also form part of the replication machinery. When phosphorylated at serine 139, H2AX referred to as γH2AX (gamma-H2AX) localises to points of DNA strand breaks both during DNA synthesis, and in response to genotoxic stress, forming a platform on which repair and checkpoint proteins can mediate a response (Bonner et al., 2008). Phosphorylation of p53 and induction of γH2AX in response to RITA was observed only in cells with wildtype p53 (Figure 3.5.1).

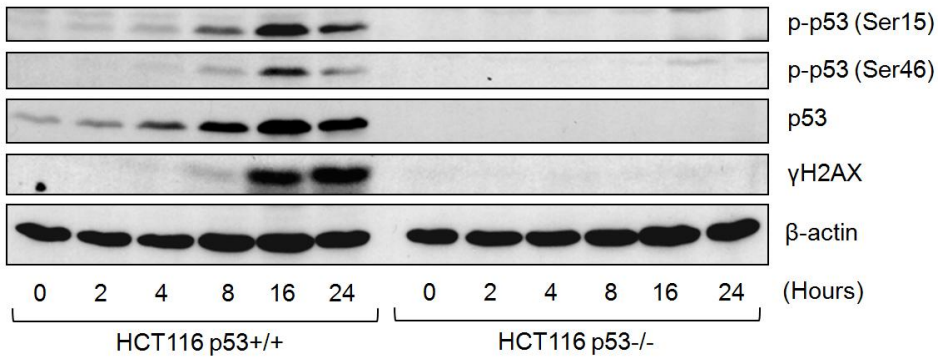


Figure 3.5.1: RITA induces phosphorylation of p53 and γH2AX
Western blot shows phosphorylated p53 at serine 15 and serine 46, indicated as p-p53 (Ser15) and p-p53 (Ser46), total p53, and γH2AX (phosphorylation site S139) protein levels in HCT116 p53+/+ and HCT116 p53-/- cells treated with 1μM RITA over a 24 hour timecourse in normoxia. β-actin was used as a loading control. A representative blot from 3 independent experiments is shown.

Fluorescent based INCell imaging was used to confirm induction of p53 and γH2AX protein in cells treated with RITA. HCT116 p53+/+ cells were treated with RITA, nutlin, or etoposide and assessed for changes in cell number, p53 nuclear intensity and γH2AX nuclear intensity. As expected, p53 protein was stabilised in cell nuclei treated with RITA, nutlin, and etoposide. However, γH2AX nuclear intensity was only induced in cells treated with either RITA or etoposide (Figure 3.5.2).

Basal levels of p53 protein are maintained in normal cells by the negative feedback loop between p53 and HDM2. HDM2 is both a transcriptional target and an E3 ligase for p53 (Haupt et al., 1997; Kubbutat et al., 1997). Recently, p53 protein expression was shown to oscillate in normal cells, and these pulses corresponded to transient, harmless breaks in DNA as cells progressed through the normal cell cycle (Loewer et al., 2010). The repeated firing of feedback loops induced by DNA damage sensors such as ATM are important mechanisms with which to re-evaluate DNA damage, and determine the length of each p53 response (Batchelor et al., 2008). However, despite p53 protein being stabilised in normal cells, p53 was not transcriptionally active (Loewer et al., 2010). Instead, the post-translational modifications on p53 following specific forms of genotoxic stress were thought to play an important role in determining p53 transcriptional activity (Loewer et al., 2010).

Changes in basal p53 protein expression were not observed when analysing p53 protein over whole cell populations (Loewer et al., 2010). In immunoflourescent experiments, I also noted that 2-5% of cells in the control population had elevated nuclear p53 protein expression that was similar in intensity to cells treated with RITA, nutlin and etoposide (Figure 3.5.2). The mismatch repair (MMR) pathway involves a number of proteins that recognise and repair mismatched DNA base pairs, and this pathway is often defective in hereditary non-polyposis colorectal cancer (Toft and Arends, 1998). The HCT116 colon carcinoma cell line is also defective in mismatch repair and therefore has greater sensitivity to genomic instability compared to other tumour cell lines (Robinson et al., 2003). It is therefore likely, that basal p53 expression may also be elevated in this cell line. Nevertheless, our observations are interesting because they highlight significant differences in p53 dynamics in individual cells, both at the basal level and in response to genotoxic stress.

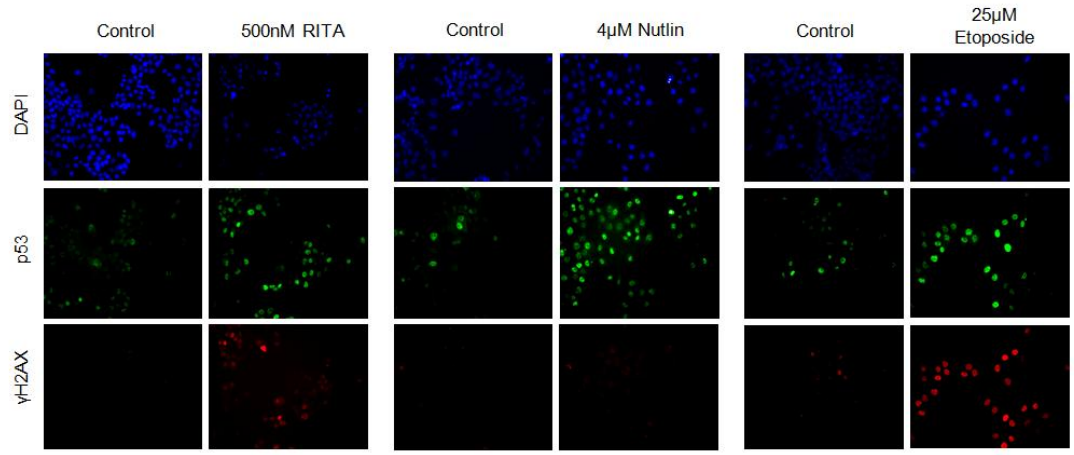


Figure 3.5.2: Immunoflourescence analysis of p53 stabilisation and γH2AX induction in response to RITA, nutlin and etoposide

Immunoflourescent images show HCT116 p53+/+ cells that have been treated with DMSO controls and either 500nM RITA, 4μM nutlin, or 25μM etoposide for 24 hours in normoxia. Cells were fixed following treatment and stained using DAPI (4'6'-diamidino-2-phenylindole, indicated by blue staining), p53 (with an Alexa Fluor 488 conjugated secondary antibody, indicated by green staining), and γH2AX (with an Alexa Fluor 568 conjugated secondary antibody, indicated by red staining). Images were acquired using INCell1000 software at 20x magnification. 10 fields of view were captured for each fluorescent wavelength, and each treatment. Images shown are representative of 3 repeat experiments.

Fluorescent images of HCT116 p53+/+ and HCT116 p53-/- cells treated with RITA and stained for cell nuclei, nuclear p53 protein, and γH2AX were quantified. HCT116 p53+/+ cells treated with RITA, nutlin, and etoposide showed significant decreases in cell numbers compared to control wells indicating induction of cell death (Figure 3.5.3A). Changes in cell number were not observed in HCT116 p53-/- cells following treatment. As expected, average p53 nuclear intensity was significantly increased in cells treated with all p53-inducing agents, compared to controls and this was only observed in HCT116 p53+/+ cells (Figure 3.5.3B). Interestingly, unlike nutlin, RITA significantly induced γH2AX nuclear intensity in a p53-dependent manner. Although γH2AX was also induced in cells treated with etoposide, and to a greater degree compared to RITA treated cells, γH2AX induction by etoposide was not p53-dependent (Figure 3.5.3C).

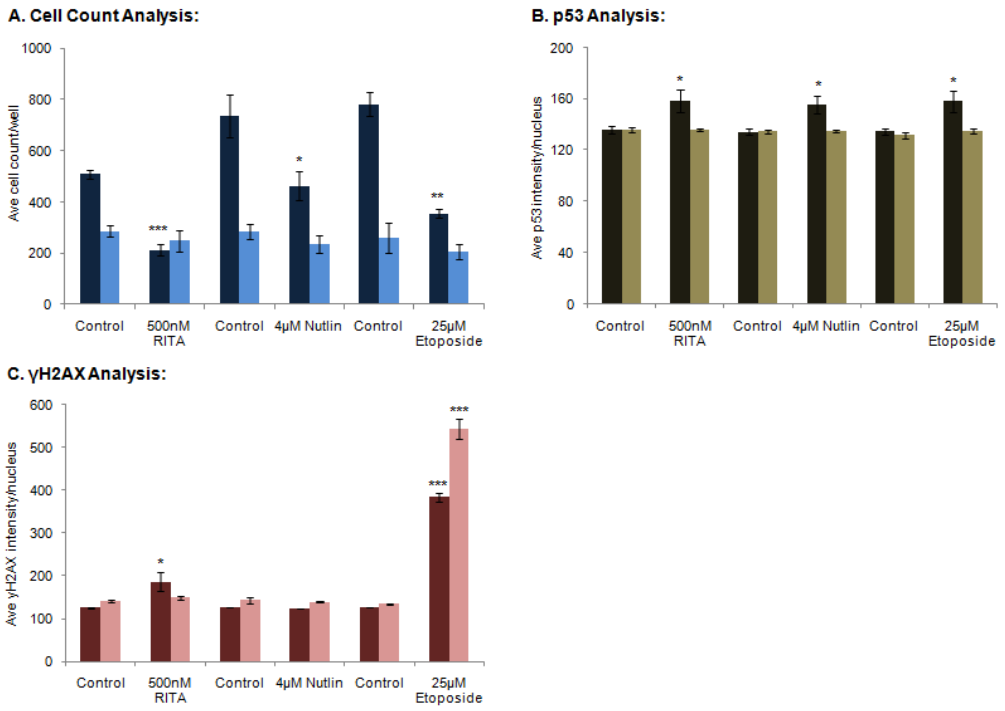


Figure 3.5.3: Quantification of p53 stabilisation and γH2AX in response to RITA, nutlin and etoposide

Graphs show HCT116 p53+/+ (darker shades) and HCT116 p53-/- (lighter shades) cells treated with either DMSO controls, 500nM RITA, 4μM nutlin, or 25μM etoposide for 24 hours in normoxia. Cells were fixed and stained using DAPI (to indicate nuclei), p53 (followed by an Alexa Fluor 488 conjugated secondary antibody), and phosphorylated γH2AX (followed by an Alexa Fluor 568 conjugated secondary antibody). Staining was visualised using the INCell 1000 and images were captured for each fluorescent intensity at 20x magnification, over 10 fields of view. Acquired images were quantified and graphs shown indicate (A) average cell count/well, (B) average p53 intensity/nucleus, and (C) average γH2AX intensity/nucleus for each treatment. Data has been averaged from 3 independent experiments. An unpaired t-test was used to compare the means of control and treated cells for each cell line and a p-value <0.05 was considered statistically significant (*<0.05, **<0.005, ***<0.0005).

Phosphorylation of CHK1 and CHK2 was analysed to assess whether the canonical DNA damage response pathway involving ATM and ATR was activated by RITA treatment. HCT116 p53+/+ cells treated with RITA showed increased phosphorylation of CHK1 and CHK2 and this was inhibited following siRNA mediated knockdown of ATR and ATM respectively (Figure 3.5.4). Interestingly, when CHK1 and CHK2 phosphorylation was inhibited by ATR and ATM siRNA, p53 stabilisation in response to

RITA was not significantly affected suggesting that ATM/ATR signalling is not essential for driving p53 induction in response to RITA. This data show that RITA induces a DNA damage response involving CHK1 and CHK2 phosphorylation (Ahmed et al., 2011; Yang et al., 2009a).

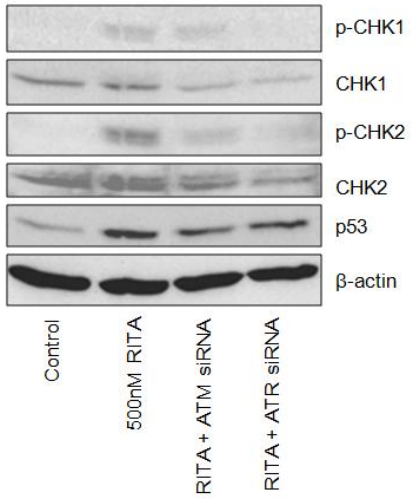


Figure 3.5.4: RITA induced CHK1 and CHK2 phosphorylation is inhibited by ATM and ATR siRNA

Western blot shows HCT116 p53+/+ cells transfected with 100nM siRNA for ATM and ATR for 24 hours prior to a 24 hour dose with 500nM RITA in normoxia. DMSO was used on control cells. Following treatment, HCT116 p53+/+ cells were harvested for western blotting and analysed for phosphorylated CHK1 (p-CHK1), total CHK1, phosphorylated CHK2 (p-CHK2), total CHK2, and p53 protein levels. β-actin was used as a loading control. A representative blot from 2 independent experiments is shown.

Previous data from our laboratory has shown that induction of γH2AX and CHK1 phosphorylation in response to RITA is p53-dependent (Ahmed et al., 2011; Yang et al., 2009a). To reconfirm p53-dependent induction of γH2AX (Figure 3.5.3) and CHK1, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA and assessed for γH2AX, and CHK1 phosphorylation by western analysis (Figure 3.5.5). Phosphorylated CHK1 and γH2AX proteins were induced in response to RITA in HCT116 p53+/+ cells and this was not observed HCT116 p53-/- cells.

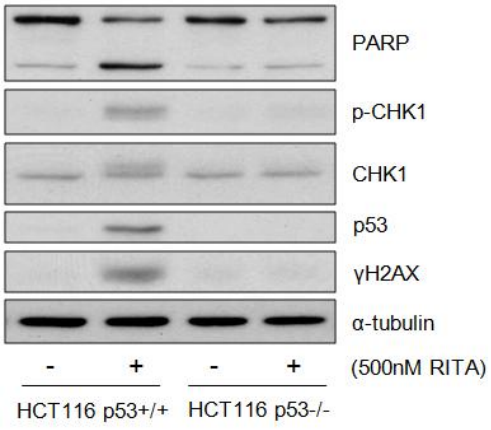


Figure 3.5.5: RITA induces phosphorylated CHK1 and γH2AX in p53 positive cells
Western blot analysis shows HCT116 p53+/+ and HCT116 p53-/- cells treated with either DMSO control (indicated as -), or 500nM RITA (indicated as +) in normoxia for 24 hours. Cells were analysed for cleaved PARP (poly ADP ribose polymerase), phosphorylated CHK1 (p-CHK1), total CHK1, p53 and γH2AX (phosphorylation site ser139) protein levels. α-tubulin was used as a loading control. Representative data from 3 independent experiments is shown.

3.6 RITA induces DNA damage

Cells that suffer from extreme oxygen deprivation (less than 0.5%) elicit cell cycle arrest by activating DNA damage responses involving ATM and ATR (Bencokova et al., 2009). Such responses prevent the formation of lethal DNA strand breaks (Hammond et al., 2002). The mechanisms that hypoxic cells employ against DNA damage contribute significantly to the resistant and aggressive nature of hypoxic tumours. Previous data has demonstrated that RITA can elicit p53-dependent DNA damage responses involving γH2AX, phosphorylation of CHK1 and p53. To assess whether RITA treatment was sufficient to induce DNA damage responses as well as detectable DNA strand breaks, single cell gel electrophoresis assays, also known as the comet assays were performed and DNA damage was analysed in HCT116 p53+/+ and MCF7 cells treated with 500nM RITA. Representative SYBR Green and inverted images used for analysis are shown in Figure 3.6.1.

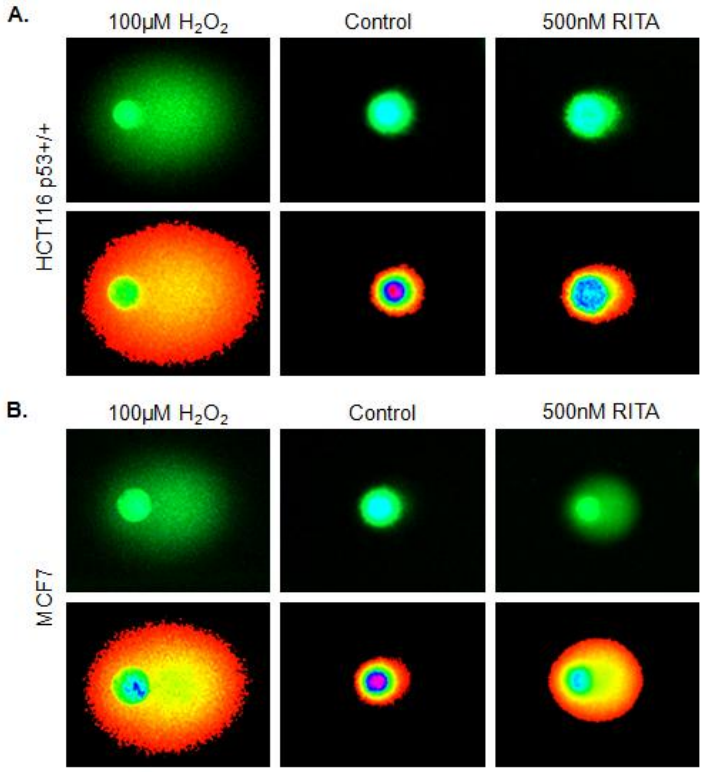


Figure 3.6.1: RITA induces DNA strand breaks

The comet assay was used as a method with which to assess the extent of DNA damage induced by RITA in individual cells. An alkaline comet assay was performed to detect single strand DNA breaks following 500nM RITA treatment for 24 hours. A 20 minute dose of 100μM hydrogen peroxide (H_2O_2) was used as the positive control for DNA damage. Treated cells were mixed with agarose, lysed, and placed on slides for electrophoresis. Following electrophoresis, cell nuclei were fixed and stained using SYBR Green. Images of individual nuclei (or comets) were captured using an inverted fluorescent microscope at 10x magnification. Approximately 100 comets were imaged for each treatment and quantified using TriTek CometScore^{TM} software (see materials and methods). Images show: (A) HCT116 p53+/+ and (B) MCF7 cells treated with either H_2O_2 (hydrogen peroxide), DMSO control, or 500nM RITA. Top panel shows a representative SYBR Green image of a single comet and the inverted image used for quantification is shown below.

Olive moment was used to quantify DNA damage in each comet as an assessment of the quantity of fragmented DNA and the migration distance of DNA from the comet head to the tail (Olive et al., 1990). As shown in Figure 3.6.2, although not statistically significant, HCT116 p53+/+ and MCF7 cells treated with RITA had a slight and

measurable increase in DNA strand breaks that was reproducible over numerous experiments.

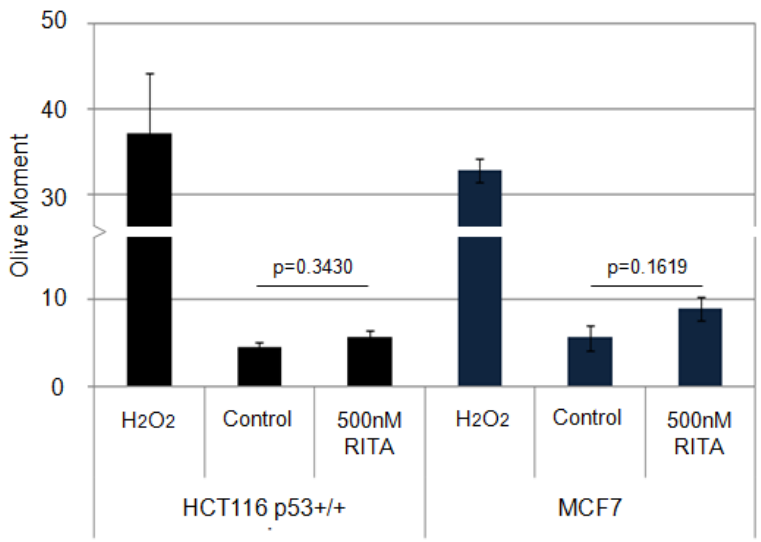


Figure 3.6.2: RITA induces DNA strand breaks

Graph shows olive moment of HCT116 p53+/+ and MCF7 cells treated with either DMSO (as an untreated control), or 500nM RITA for 24 hours in normoxia. Hydrogen peroxide (H_2O_2) was used as a positive control for DNA damage and dosed at 100µM for 20 minutes at 4°C. Following treatment, cells were mixed with agarose and spread onto comet assay slides for lysing and electrophoresis (see materials and methods). Once electrophoresed, cells were fixed and stained for DNA using SYBR Green. Stained cells, referred to as comets were visualised on an inverted fluorescent microscope at 10x magnification. Approximately 100 comets were imaged for each treatment and DNA damage was quantified using TriTek CometScore^{TM} software. Olive moment was used to measure extent of DNA damage in each nucleus (or comet) by calculating the quantity of fragmented DNA that migrates from the comet head to the comet tail, as well as the distance that the DNA travels through the comet tail. Data shown is taken from mean olive moments over 3 independent experiments. An unpaired t-test was used to test statistical significance and a p-value of less than 0.05 was considered significant.

Cells treated with RITA for DNA damage analysis by the comet assay were harvested in parallel for western blotting. As shown in Figure 3.6.3, DNA strand breaks elicited by RITA in HCT116 p53+/+ and MCF7 cells correlate with induction of γH2AX, and with cell death as assessed by cleaved PARP. In summary, RITA induces detectable DNA strand breaks as well as p53-dependent DNA damage responses involving

phosphorylation of p53, γH2AX and the canonical CHK1 and CHK2 DNA damage signalling pathway as previously shown.

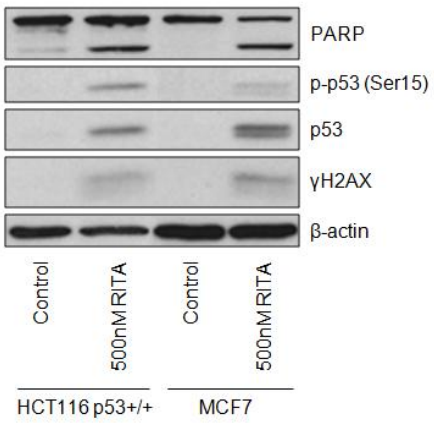


Figure 3.6.3: DNA damage induced by RITA correlates with p53 stabilisation, γH2AX induction, and cleaved PARP

Western blot shows cleaved PARP (poly ADP ribose polymerase), phosphorylated p53 at serine 15 (p-p53), total p53, and γH2AX protein levels of HCT116 p53+/+ and MCF7 cells treated with either DMSO control, or 500nM RITA for 24 hours in normoxia. β-actin was used as a loading control. A representative blot from 3 independent experiments is shown.

3.7 RITA does not induce DNA damage and cell death in Saos-2 cells

As discussed, RITA-induced cell death and DNA damage responses are only elicited in tumour cells with wildtype p53 (Yang et al., 2009a). In previous studies, MCF7 p53 wildtype cells, HCT116 p53 wildtype cells, and HCT116 p53 null cells have been used to confirm the p53-dependent activity of RITA. To extend these studies to other cell lines which differ in p53 status, Saos-2 p53 null cells were also treated with RITA and did not show significant cell death in both normoxia and hypoxia (Figure 3.7.1).

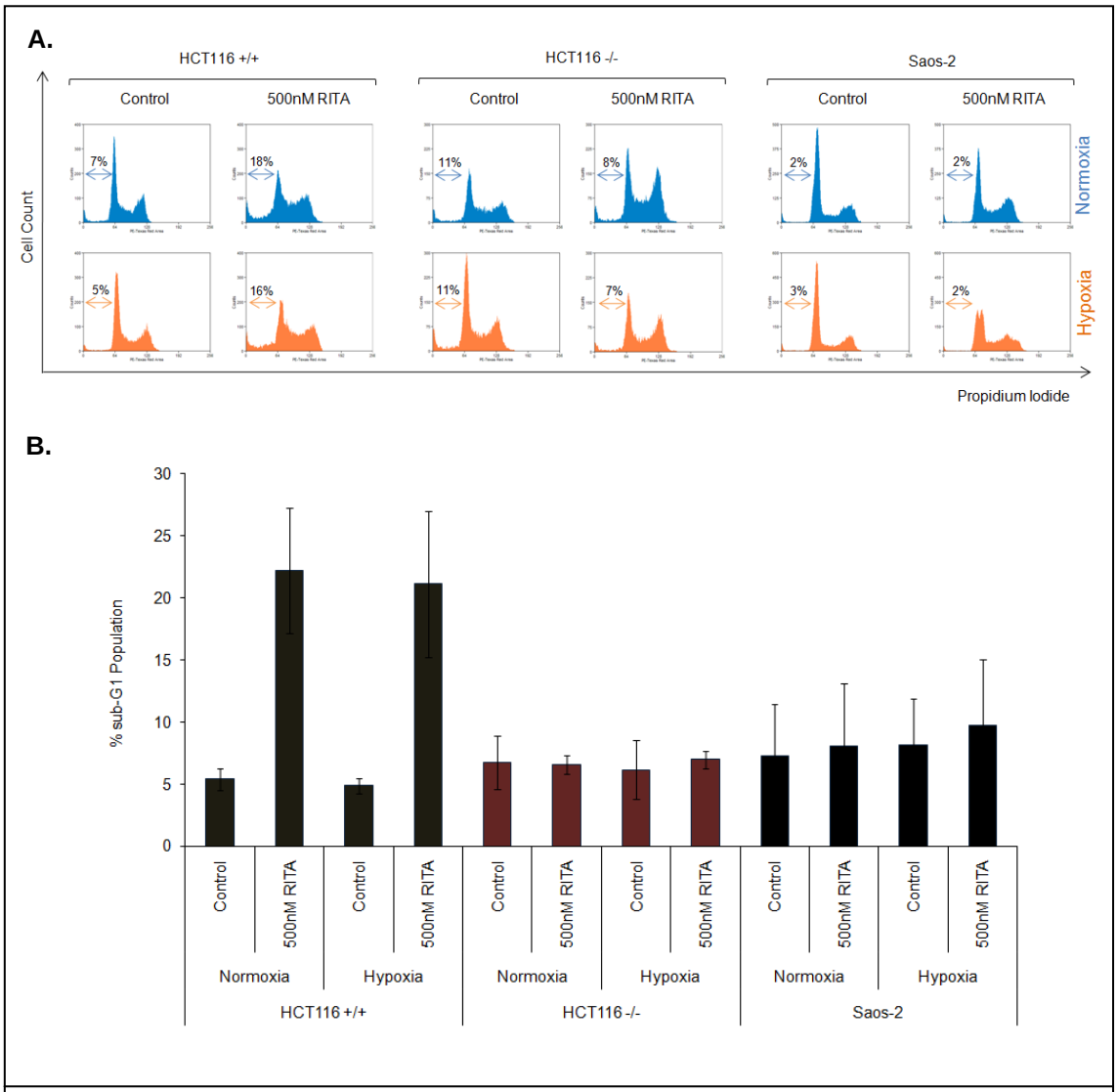


Figure 3.7.1: RITA does not induce apoptosis in HCT116 p53-/- or Saos-2 cells

HCT116 p53+/+, HCT116 p53-/-, and Saos-2 cells were treated for 24 hours with either DMSO as control, or 500nM RITA in normoxia (20% O_2) and in hypoxia (1% O_2). Following treatment cells were harvested for flow cytometry and stained with propidium iodide to analyse DNA content: (A) DNA cell cycle histograms show cell count (y-axis) against propidium iodide intensity (x-axis). Top panel (blue) shows profiles of cells treated in normoxia, and bottom panel (orange) shows cells treated in hypoxia. Cell cycle histograms were gated for sub-G1 populations, and representative quantifications of the percentage of cells in sub-G1 are indicated on each histogram, (B) Graph shows percentage sub-G1 population for each treatment that has been averaged over 3 independent experiments.

Western analysis was used to assess Saos-2 cells for RITA induced cell death as indicated by cleaved PARP. Compared to HCT116 p53+/+ cells, Saos-2 cells were not affected by RITA treatment and showed no change in cleaved PARP (Figure, 3.7.2). These results show that RITA induces cell death only in transformed cells that have wildtype p53.

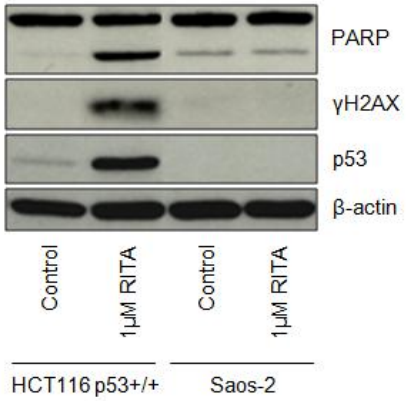


Figure 3.7.2: RITA does not induce cleaved PARP, γH2AX or p53 in Saos-2 cells
Western blot shows cleaved PARP (poly ADP ribose polymerase), phosphorylated γH2AX and p53 protein levels in HCT116 p53+/+ and Saos-2 cells treated with 1μM RITA for 24 hours in normoxia. β-actin was used as a loading control. Data shown is representative of 3 independent experiments.

In addition to this, while HCT116 p53+/+ cells showed a small, but detectable increase in olive moment by RITA treatment, similar changes in DNA damage were not observed in Saos-2 cells (Figure 3.7.3). This data confirm that RITA mediated cell death, DNA damage responses involving γH2AX, and CHK1, and formation of DNA strand breaks are dependent on wildtype p53.

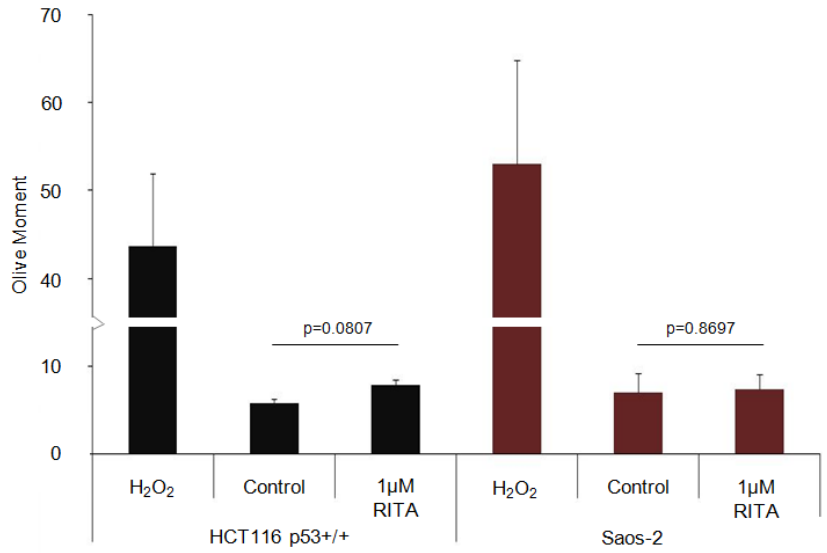


Figure 3.7.3: RITA does not induce DNA strand breaks in Saos-2 cells

Graph shows olive moment of HCT116 p53+/+ and Saos-2 cells treated with 1μM RITA for 24 hours in normoxia. Following treatment, cells were mixed with agarose and spread onto comet assay slides for lysis, and electrophoresis. Cells were then fixed and stained for SYBR Green to detect DNA. Cell nuclei (referred to as comets) were visualised using a fluorescent microscope. Images were taken over 20 fields of view at 10x magnification, and approximately 100 comets were quantified for each treatment using TriTeck^{TM} software (see materials and methods). Olive moment was used as a measure of DNA damage. An unpaired t-test was used to calculate statistical signifance and a p-value of less than 0.05 was considered significant. Data shown has been averaged from 3 independent repeats.

3.8 Discussion

RITA was originally isolated from a cell based screen using the National Cancer Institute (NCI) compound library and was shown to induce p53 and mediate p53-dependent anti-tumour effects (Issaeva et al., 2004). Previous data from our laboratory have shown that RITA can induce significant tumour cell death in both normoxia and hypoxia, by a p53-dependent pathway (Yang et al., 2009a; Yang et al., 2009b). These findings were extended in this chapter to assess responses of other p53-activating agents in normoxia and in response to hypoxia. RITA mediated cell death responses were of particular interest because they were found to be distinct from other DNA damaging agents that activate p53 and induce cell cycle arrest.

Because tumour cell death by RITA is p53-dependent, the mechanism by which p53 is induced by this agent was explored. RITA was originally proposed to activate p53 by disrupting the p53-HDM2 interaction (Issaeva et al., 2004) however subsequent studies indicated that RITA did not inhibit this interaction (Yang et al., 2009a; Yang et al., 2009b). Instead, RITA was found to induce p53-dependent DNA damage responses (Yang et al., 2009a). The p53-dependent DNA damage response elicited by RITA was defined by phosphorylation of p53, phosphorylation of CHK1 and CHK2 to indicate ATM and ATR activation, and induction of γH2AX (Yang et al., 2009b). The comet assay was used to assess whether RITA induced DNA strand breaks. Although not statistically significant, RITA was found to induce detectable DNA damage that was p53-dependent and reproducible over numerous independent experiments. The proposed model for these responses is summarised in Figure 3.8.1.

This is the first study in which p53-dependent DNA damage response pathways have been described. In the canonical DNA damage response, p53 is induced following activation of ATM and ATR in response to genotoxic stress. Due to the fact that DNA damage and DNA damage responses involving γH2AX induction and CHK1 phosphorylation are only observed in cells that are p53 wildtype we propose that RITA functions directly at the level of DNA and that wildtype p53 is not only an effector signalling node, but also acts itself as an essential sensor of DNA damage that mediates downstream RITA induced DNA damage responses.

This model is supported by studies where p53 has been shown to be involved in regulating homologous recombination directly, an important DNA repair process that regulates genomic integrity during DNA replication (Hoeijmakers, 2001b; Lee et al., 1997). Homologous recombination is regulated by numerous proteins and enzymes. Strand exchange reactions are induced by RAD51 which is part of a large complex of repair enzymes, recombination proteins and helicases (Wang et al., 2000). Bloom syndrome protein (BLM) is a RecQ helicase that accumulates in S-phase (Bischof et al., 2001) and is involved in replication fork repair (Chakraverty and Hickson, 1999). By binding directly to BLM, p53 has been shown to co-localise with RAD51 at sites of stalled replication forks induced by hydroxyurea treatment and in doing so, p53 is proposed to have a direct function in modulating homologous recombination (Sengupta et al., 2003). Importantly, binding and relocalisation of p53 to sites of DNA strand breaks during replication is independent of its transactivation functions providing another level of control whereby p53 maintains cell cycle responses by directly inducing repair mechanisms

A role for p53 in sensing DNA damage has also been suggested by Rashid et al, whereby phosphorylated p53 is recruited to sites of DNA strand breaks in an ATM-dependent manner (Al Rashid et al., 2005). Furthermore, p53 has been shown to reside at specific target gene promoters in the absence of genotoxic stress (Kaeser and Iggo, 2002). Localisation of p53 at the DNA not only allows p53 to function as a molecular sensor, but also induces rapid transcriptional responses upon binding of specific co-factors when stress signals are elicited (Aylon and Oren, 2007). Clearly the cellular localisation of p53 is an important determinant of its immediate responses to stress, and localisation of p53 at the DNA may provide p53 with an important opportunity to sense and transmit DNA damage signals to downstream effector pathways. Collectively, these studies and the data which has been presented in this chapter provide an important rationale with which to investigate the novel p53-dependent responses described in greater detail.

3.9 Conclusions for this chapter

- RITA elicits cell death in normoxia and in response to hypoxia.
- RITA inhibits HIF-1α protein expression in hypoxia.
- RITA induces a DNA damage response involving phosphorylation of p53, induction of γH2AX, and phosphorylation of CHK1 and CHK2.
- DNA damage responses involving γH2AX and phosphorylated CHK1 induced by RITA are p53-dependent.
- RITA induces DNA strand breaks in cells that are p53 positive.

3.10 Impact of these findings

Hypoxic tumours are resistant to radio- and chemotherapy, and demonstrate poor p53 activity (Ashcroft et al., 2000; Koumenis et al., 2001). RITA is a small molecule activator of p53 that induces p53-dependent cell death responses in normoxia, and in response to hypoxia. Our studies have demonstrated that RITA mediated DNA damage and DNA damage responses are dependent on wildtype p53, findings that have not been shown in response to other p53 activating agents. Using p53 activation in tumours with deregulated HIF pathways is an important strategy with which to sensitise radio- and chemoresistant tumours to cell death. Furthermore, use of pharmacological activators of p53 will strengthen our understanding of the interactions between HIF and p53 in hypoxia, as well as reveal novel pathways that can be used to improve the selective targeting of hypoxic tumours.

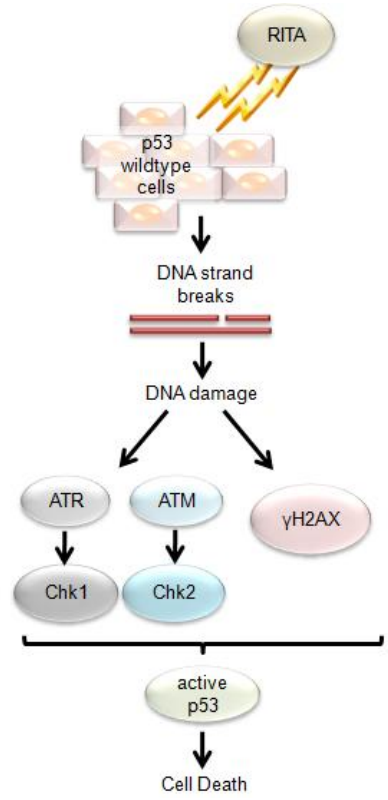


Figure 3.8.1: Induction of wildtype p53 by RITA elicits p53-dependent DNA damage and DNA damage responses that mediate cell death responses

The proposed model for targeting wildtype p53 using RITA involves induction of DNA strand breaks and DNA damage response involving ATM-CHK2, ATR-CHK1, and γH2AX. These processes are dependent on p53, lead to p53 phosphorylation, and induce cell death in normoxia, and in hypoxia.

Chapter 4

RITA activates p53-dependent cell cycle checkpoints

4.1 Introduction

In studies by Isseavia et al. in which the anti-tumour effects of RITA were first described, RITA was proposed to activate p53 by binding to the N-terminal domain of p53 and disrupting its interaction with HDM2 (Issaeva et al., 2004). In initial studies by Yang et al. in our laboratory, when both p53 and HDM2 were stabilised in the cell by the proteosome inhibitor MG132, RITA was unable to disrupt p53-HDM2 complexes (Yang et al., 2009b). In contrast, nutlin-3 a small molecule inhibitor of HDM2 impaired the ability of HDM2 to immunoprecipitate with p53 (Yang et al., 2009b). RITA therefore does not activate p53 by targeting the p53-HDM2 interaction suggesting that RITA has alternative mechanisms for stabilising and activating p53.

In chapter 3 the p53-dependent activity of RITA was assessed (Yang et al., 2009a; Yang et al., 2009b). RITA induced a distinct DNA damage response in cells that had wildtype p53, and this involved phosphorylation of CHK1, CHK2, p53 and induction of γH2AX (Yang et al., 2009a). The DNA damage response studied in response to RITA is important because it requires wildtype p53 and is not observed with other agents that activate p53. In this chapter, the mechanisms of RITA induced p53-dependent DNA damage responses will continue to be addressed.

Previous data in our laboratory using confocal immunoflourescent microscopy demonstrated that cells treated with RITA and hydroxyurea have pan-nuclear staining of γH2AX (Yang et al., 2009a). The γH2AX staining observed in response to RITA and hydroxyurea was similar to that of UV irradiated cells and distinct from other types of stresses such as γ-irradiation where more defined γH2AX foci form in the nucleus (Marti et al., 2006; Yang et al., 2009a). Marti et al. showed that pan nuclear γH2AX co-localised with bromodeoxyuridine (BrdU) staining, a DNA precursor analogue that incorporates into DNA during synthesis. These studies indicated that γH2AX localises to sites of DNA replication during S-phase in response to stress induced by UV treatment. Although γH2AX was detected in cells during all phases of the cell cycle in response to UV treatment, γH2AX intensity was greatest during S-phase (Marti et al., 2006). As both UV irradiation and RITA treatment lead to distinct localisation patterns for γH2AX it will be of interest to assess whether, like UV treatment, DNA damage induced by RITA also has significant effects in cells during S-phase.

4.2 Hypothesis

RITA activates p53-dependent cell cycle checkpoints.

4.3 Aims

- Investigate the mechanism by which RITA activates p53 to induce tumour cell death in more detail.
- Characterise cell cycle checkpoints elicited by RITA in the context of the DNA damage response described so far.

4.4 RITA induces p53 and γH2AX in specific cell cycle phases

RITA not only forms DNA strand breaks, but also elicits DNA damage responses involving phoshorylation of CHK1, CHK2 and induction of γH2AX. Interestingly, phosphorylation of CHK1 and induction of γH2AX was primarily observed in cells with wildtype p53, and because of this, it was concluded that p53 stabilisation by RITA was upstream of CHK1, and γH2AX induction (Ahmed et al., 2011). The RITA induced DNA damage response described is therefore a p53-dependent DNA damage response.

Previous immunohistochemical analyses from our laboratory have shown that both RITA and hydroxyurea induce pan-nuclear localisation of γH2AX rather than localisation of γH2AX at discrete foci (Yang et al., 2009a), a phenotype that is indicative of replication fork stalling, and inhibition of replication fork elongation (Marti et al., 2006). DNA damage induced during DNA replication often leads to the activation of S-phase checkpoints (Merrick et al., 2004) involving γH2AX localisation at DNA strand breaks, as well as activation of ATR and CHK1 signalling (Chen and Sanchez, 2004; Liu et al., 2000). By regulating DNA damage responses and repair in response to DNA strand breaks that occur during replication, S-phase checkpoints prevent cells with damaged DNA from progressing into the next phase of the cell cycle. Due to similarities in γH2AX staining between RITA, hydroxyurea, and UV treated cells, I hypothesised that DNA damage induced by RITA also activates cell cycle checkpoints, and that these checkpoints are dependent on wildtype p53.

To address this hypothesis, RITA's affects on the replication fork machinery and S-phase progression was investigated. A DNA fibre assay was performed by our collaborators Apolinar Maya-Mendoza and Dean Jackson (University of Manchester, UK) as previously described (Jackson and Pombo, 1998). In this assay, DNA is

labelled with BrdU and DNA spreads are prepared to visualise the number of replication forks present in the DNA. RITA treatment increased the percentage of replication forks in HCT116 p53+/+ cells but not in HCT116 p53-/- cells, indicating stalling of the replication fork machinery. A delay in replication fork progression was also observed in cells treated with RITA, indicating prolonged S-phase. This was further confirmed by Apolinar Maya-Mendoza and Dean Jackson using BrdU staining to visualise DNA foci. Immunoflourescent analysis of BrdU foci showed that S-phase was delayed during mid-late stages in RITA treated cells with wildtype p53 (Ahmed et al., 2011).

Data from Apolinar Maya-Mendoza and Dean Jackson suggested that RITA affects cells that are in S-phase (Ahmed et al., 2011). I extended these studies using flow cytometry as a method to assess changes during each phase of the cell cycle. HCT116 p53+/+ cells were analysed for both p53, and γH2AX (phosphorylated at serine 139) following RITA treatment in each cell cycle phase. With support from Science Support Services at the Wolfsson Institute (University College London) HCT116 p53+/+ cells were dual stained with p53, using a FITC (fluorescein isothiocyanate) conjugated secondary antibody, and γH2AX, using an APC (allophycocyanin) conjugated secondary antibody. The fluorescent tags used on the secondary antibodies did not have overlapping wavelengths, and could therefore be detected by the flow cyctometer with minimum need for compensation. Specificity of the secondary antibodies used was tested in untreated HCT116 p53+/+ cells. When staining HCT116 p53+/+ cells with secondary antibodies alone, there was no significant shift in fluorescent intensity compared to the unstained population suggesting that the secondary antibodies used do not bind to cells non-specifically. When staining with both primary and secondary antibodies together, a significant shift in fluorescent intensity was detected indicating that the secondary antibodies used are specific for their corresponding primary antibodies (Figure 4.4.1).

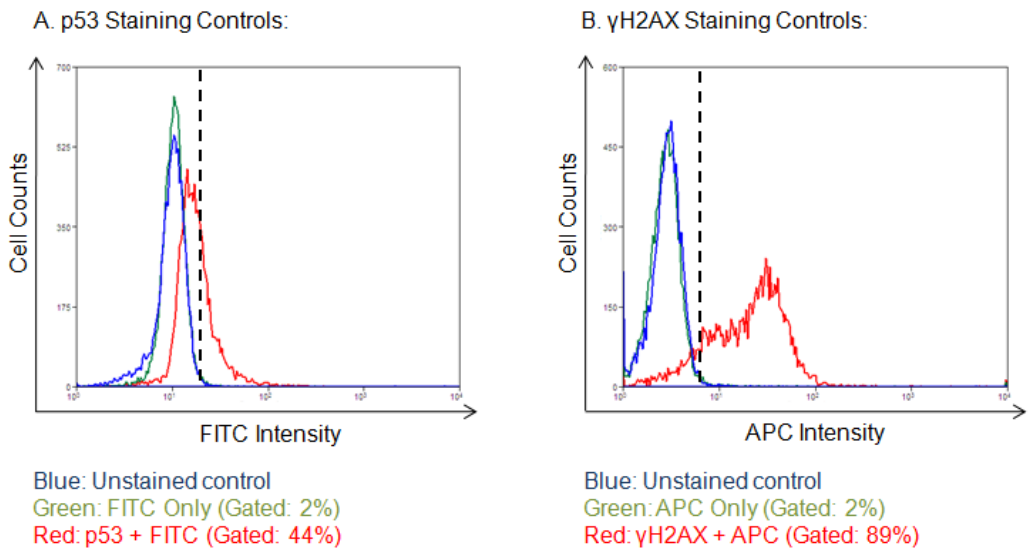


Figure 4.4.1: FACS analysis of FITC and APC antibody controls

Specificity of FITC and APC secondary antibodies was assessed using the CyAn^{TM} ADP flow cytometer (Dako). Graphs show untreated HCT116 p53+/+ cell counts (y-axis) against the fluorescent intensity of either FITC (A), or APC (B) conjugated secondary antibodies (x-axis): (A) HCT116 p53+/+ cells were unstained (blue), stained for FITC conjugated secondary antibody alone (green), or with p53 primary and FITC secondary antibodies together (red), (B) HCT116 p53+/+ cells were unstained (blue), stained for APC conjugated secondary antibody alone (green), or with γH2AX primary and APC secondary antibodies together (red). Graphs were gated against the secondary antibody peak (gate is indicated by a dashed line), and the percentage of cells that have an increase in fluorescent intensity compared to the gated secondary antibody peak alone were quantified. The gated percentages for specific binding have been indicated below each graph.

To assess whether p53 stabilisation and γH2AX induced by RITA could be detected using flow cytometry, HCT116 p53+/+ cells were treated with 500nM RITA for 24 hours in normoxia. As shown in Figure 4.4.2, HCT116 p53+/+ cells treated with RITA had significant increases in p53 fluorescent intensity (Figure 4.4.2A), and in γH2AX intensity (Figure 4.4.2B) compared to the untreated controls.

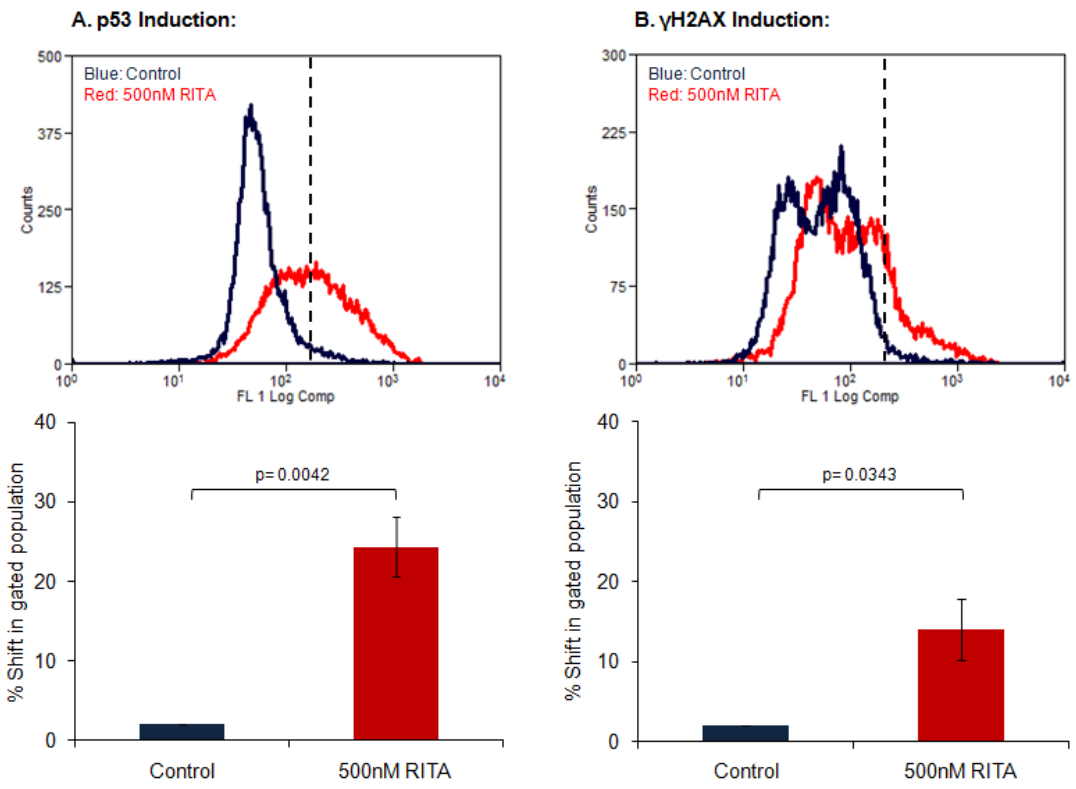


Figure 4.4.2: Cells treated with RITA stain positive for p53 and γH2AX

HCT116 p53+/+ cells were treated with 500nM RITA for 24 hours in normoxia and harvested for flow cytometry. Cells were stained for both p53 and γH2AX (phosphorylated S139). Histograms show the control cell population (blue) and the RITA treated population (red) for (A) p53 staining and (B) γH2AX staining. Cell populations were gated on the control population at a 2% threshold, as indicated by the dashed line. The percentage of cells that shift in fluorescent intensity towards the gated region was calculated and averaged over 3 independent experiments. Representative histograms are shown above, and the corresponding quantified data is shown in the graphs below. Statistical significance was assessed using the unpaired t-test and a two-tailed p-value of <0.05 was considered significant.

Previous data from DNA fibre assays and pan-nuclear staining of γH2AX has indicated that RITA affects cells in S-phase (Ahmed et al., 2011; Yang et al., 2009a). Therefore, propidium iodide stained DNA profiles were gated specifically for sub-G1 and S-phase to assess changes in p53 stabilisation and γH2AX upon RITA treatment in these cell cycle phases. In treated populations there was a significant increase in p53 and γH2AX intensity in sub-G1, and also in S-phase populations (Figure 4.4.3).

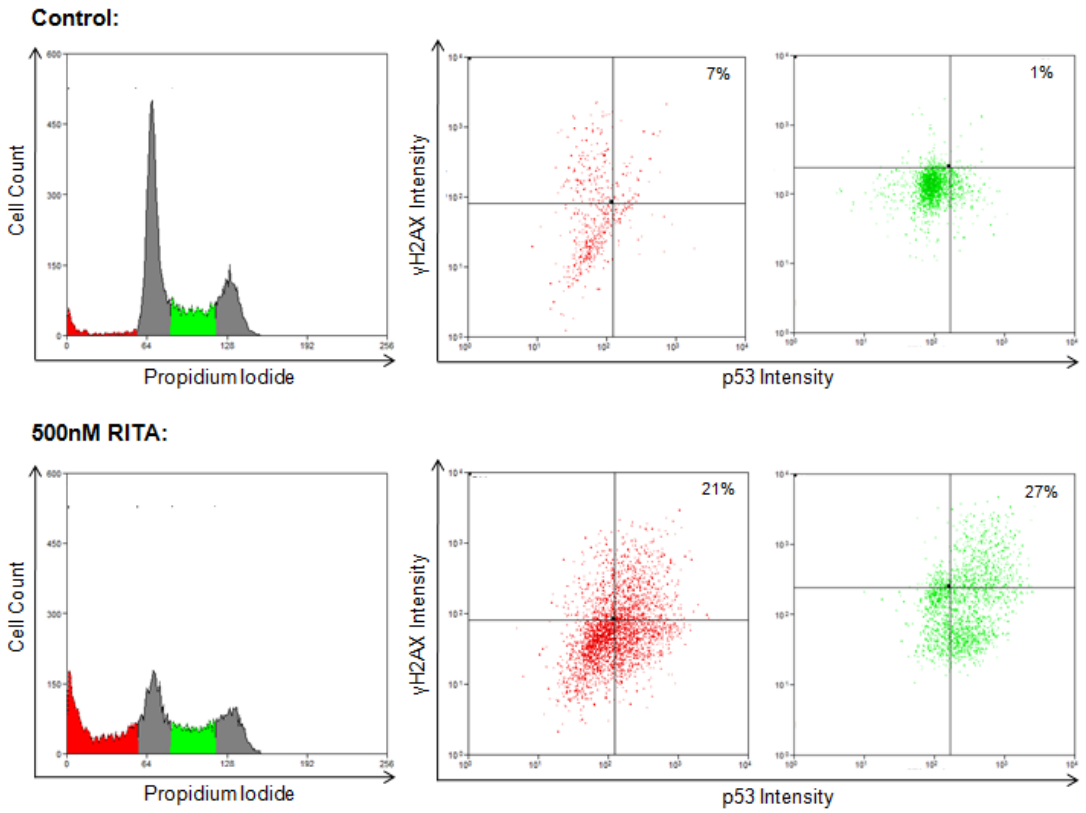


Figure 4.4.3: p53 and γH2AX positive cells are detected in sub-G1 and S-phase populations

Representative flow cytometry plots of cells dosed with RITA for 24 hours and assessed for DNA content, p53 and γH2AX (phosphorylated ser139) staining. Panels on the left show DNA profiles with cell counts on the y-axis, and propidium iodide staining on the x-axis. DNA profiles were gated according to sub-G1 (red) and S-phase (green). Dot plots of cells taken from the gated region within DNA profiles are shown on the right, indicating γH2AX (y-axis) and p53 (x-axis) staining in sub-G1 (red-middle plot), and S-phase (green-right plot) cells. Changes were quantified within the top right quadrant of the dot plots indicating an increase in both p53 and γH2AX staining intensity. Each value on the dot plot represents a single cell with fluorescence for p53 and γH2AX staining. The treated population of cells is represented in the panel below.

Next, because significant changes were observed in p53 and γH2AX in sub-G1 and S-phase cells that had been treated with RITA, the remaining phases of the cell cycle were also analysed. Although increases in p53 and γH2AX in sub-G1, G1, S-phase, and G2 were observed, the changes were most significant in the sub-G1 (47%

increase in staining), and S-phase (34% increase) populations that had been treated with RITA (Figure 4.4.4).

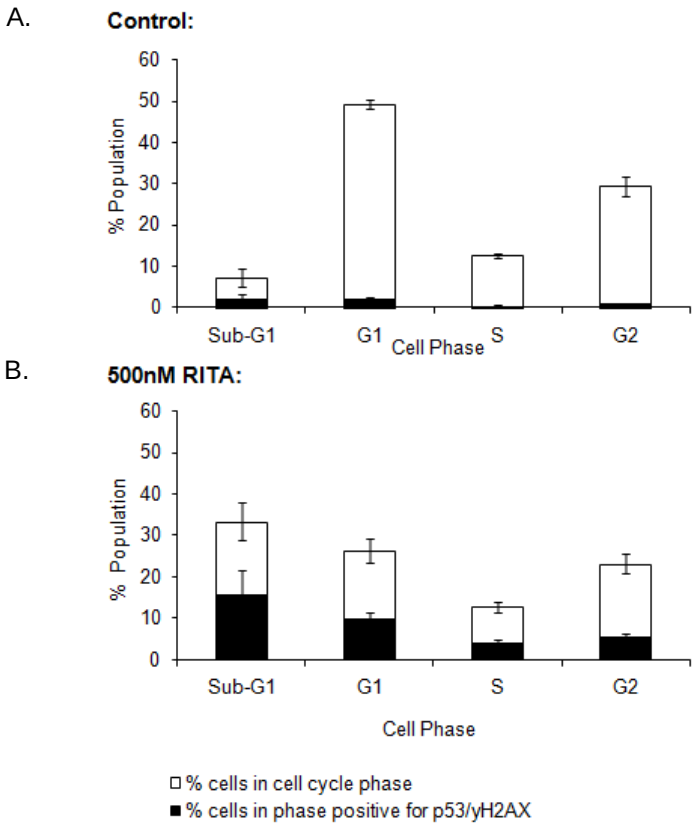


Figure 4.4.4: HCT116 p53+/+ cells stain positive for p53 and γH2AX in cell cycle phases

HCT116 p53+/+ cells were treated with 500nM RITA for 24 hours in normoxia and harvested for FACS analysis. Cells were stained for propidium iodide, p53 and γH2AX (phosphorylated ser139). Propidium iodide DNA profiles were gated according to cell cycle phase. Graphs show: (A) DMSO treated control cells, and (B) cells treated with 500nM RITA. The percentage of cells in each phase of the cell cycle that stain positive for both p53 and γH2AX (black bar) are shown for each cell cycle phase (white bar). All data has been averaged from 3 independent repeat experiments.

As p53 and γH2AX is induced in S-phase cells, and because of supporting data that indicate RITA affects the replication machinery (Ahmed et al., 2011), I hypothesised that p53 stabilisation and γH2AX induction is important for eliciting DNA damage responses as a result of DNA strand breaks induced by RITA during S-phase. To address this hypothesis, HCT116 p53+/+ cells were treated with a dose range of RITA

to assess dose-dependent changes in the sub-G1 and the S-phase populations upon RITA treatment. A dose-dependent increase in sub-G1 cells was found following RITA treatment (Figure 4.4.5A) and this correlated with increases in the percentage of cells that stained positive for p53 and γH2AX in S-phase (Figure 4.4.5B). Interestingly, p53 and γH2AX in S-phase cells were induced at lower doses compared to the accumulation of sub-G1 cells suggesting p53 induction and γH2AX have greater sensitivity to RITA treatment in S-phase cells and may mediate induction of the sub-G1 population at higher doses.

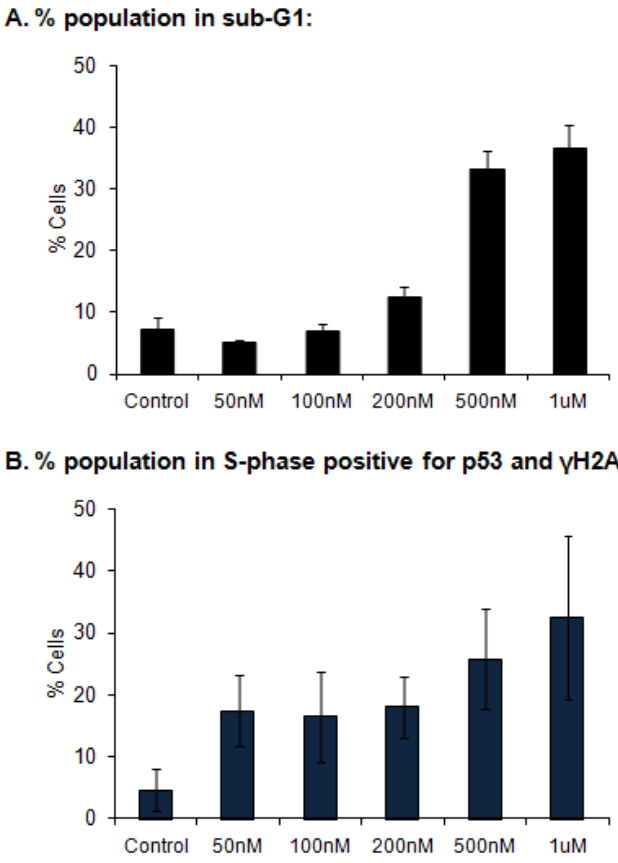


Figure 4.4.5: RITA induces p53, γH2AX and cell death in a dose-dependent manner
HCT116 p53+/+ cells were treated with a dose range of RITA for 24 hours in normoxia and harvested for flow cytometry. Cells were stained for propidium iodide, p53 protein (with a FITC conjugated secondary) and γH2AX (with an APC conjugated secondary). DNA profiles of propidium stained cells were gated and quantified according to the percentage of cells in (A) sub-G1 and (B) the percentage of cells in S-phase that stain positive for both p53 and γH2AX. Graphs represent data averaged from 3 independent repeat experiments.

To assess whether sub-G1 and S-phase effects are time-dependent, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA over a timecourse. The effects of RITA on the sub-G1 population, and on staining of p53 and γH2AX in S-phase cells was assessed to observe the timescale at which these responses to RITA treatment are induced. RITA was found to induce p53 and γH2AX in S-phase cells from 4 hours of treatment (Figure 4.4.6B), while sub-G1 cells were induced following 16 hours of treatment (Figure 4.4.6A). These data suggest that the S-phase population has greater sensitivity to the dose and the length of RITA treatment, and these responses precede the induction of sub-G1 cells. Interestingly, no changes in the sub-G1 population or the S-phase population in HCT116 p53-/- cells were observed. These are the first findings that show p53-dependent DNA damage responses in S-phase cells and are novel because they correlate p53 induction and γH2AX in S-phase cells with p53-dependent cell death responses elicited by RITA.

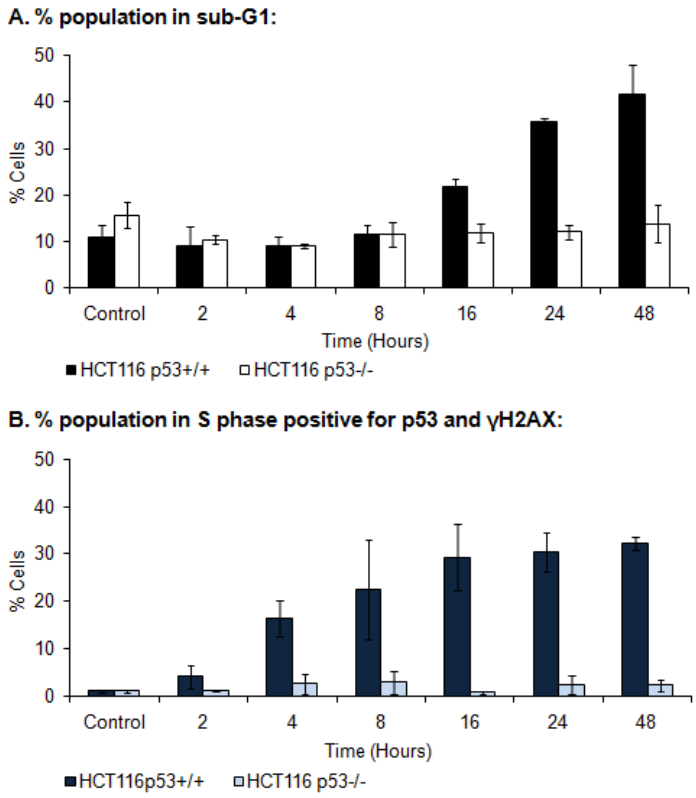


Figure 4.4.6: RITA induces p53, γH2AX and cell death in a time-dependent and p53-dependent manner

HCT116 p53+/+ and HCT116 p53-/- cells treated over a timecourse with 1μM RITA for 24 hours in normoxia, and harvested for flow cytometry at indicated timepoints. Cells were stained with propidium iodide, p53 protein (with a FITC conjugated secondary), and γH2AX protein (with an APC conjugated secondary). DNA profiles of cells stained with propidium iodide were gated and quantified according to (A) the percentage of cells in sub-G1, and (B) the percentage of cells in S-phase that stain positive for both p53 protein and γH2AX. Graphs represent data averaged from 3 independent repeat experiments.

4.5 RITA elicits an S-phase cell cycle checkpoint involving CHK1 phosphorylation

In accordance with previous data published from our laboratory (Yang et al., 2009a) I have shown that RITA can induce phosphorylation of both CHK1 and CHK2 kinases suggesting activation of the canonical ATM/ATR pathway (Figure 3.2.5). As discussed, RITA treatment induced p53 protein and γH2AX in S-phase cells (Figure 4.4.3), and also stalled the replication fork machinery in a p53-dependent manner (Ahmed et al., 2011). Next, I assessed activation of CHK1 by RITA in greater detail. This is because

phosphorylation and activation of CHK1 by ATR is involved in mechanisms that mediate DNA replication origin firing, and the stabilisation of replication forks in response to UV stress and agents that stall DNA replication forks (Chen and Sanchez, 2004; Liu et al., 2000). Therefore I hypothesised that like p53 and γH2AX, CHK1 phosphorylation is also important in eliciting DNA damage responses during S-phase to mediate cell death in response to RITA.

Recent studies whereby CHK1 was shown to mediate p53-dependent transcriptional responses during S-phase arrest elicited by genotoxic stress support this hypothesis (Beckerman et al., 2009). Furthermore, previous work has shown that ATR and CHK1 dysfunction decreases the viability of cells exposed to agents that cause replication stress (Cliby et al., 1998). Similarly, siRNA knockdown of CHK1 sensitises cells that have been treated with replication inhibitors to greater apoptosis (Rodriguez et al., 2008). Due to the established role of CHK1 in regulating checkpoints in response to stress (Liu et al., 2000; Takai et al., 2000), the function of CHK1 in RITA induced cell cycle and DNA damage responses was investigated. To begin, siRNA mediated knockdown of CHK1 was assessed to observe changes in sensitivity to genotoxic stress induced by RITA.

Flow cytometry was used to assess changes in CHK1 phosphorylation when HCT116 p53+/+ cells were treated with RITA, and also investigate changes in cell cycle responses when CHK1 is inhibited by siRNA. In parallel to flow cytometry, cells were harvested for western blotting to confirm loss of CHK1 protein by siRNA. As shown in Figure 4.5.1, appropriate levels of CHK1 protein knockdown was achieved upon siRNA, however no significant changes were observed in cleaved PARP or p53 stabilisation following RITA treatment when CHK1 protein was inhibited by siRNA. Notably knocking down of CHK1 by siRNA induced p53 in the absence of RITA.

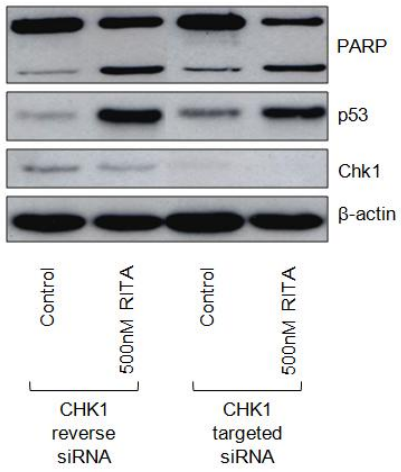


Figure 4.5.1: CHK1 siRNA does not affect RITA induced PARP cleavage
HCT116 p53+/+ cells were transfected with 25nM of either CHK1 reverse control siRNA or CHK1 targeted siRNA. Transfections were incubated for 24 hours. Cells were then treated with 500nM RITA for 24 hours in normoxia and harvested for both flow cytometry and western blotting. Western blots were analysed for cleaved PARP as (an indicator of apoptosis), p53 stabilisation, and total CHK1 protein. β-actin was used as a loading control. Western data shown is representative of 3 independent experiments.

To assess changes in cell cycle profiles, induction of phosphorylated CHK1 protein was first assessed in RITA treated cells using flow cytometry. A significant increase in phosphorylated CHK1 was observed in HCT116 p53+/+ cell that had been treated with RITA, and this was inhibited when CHK1 was targeted by siRNA (Figure 4.5.2).

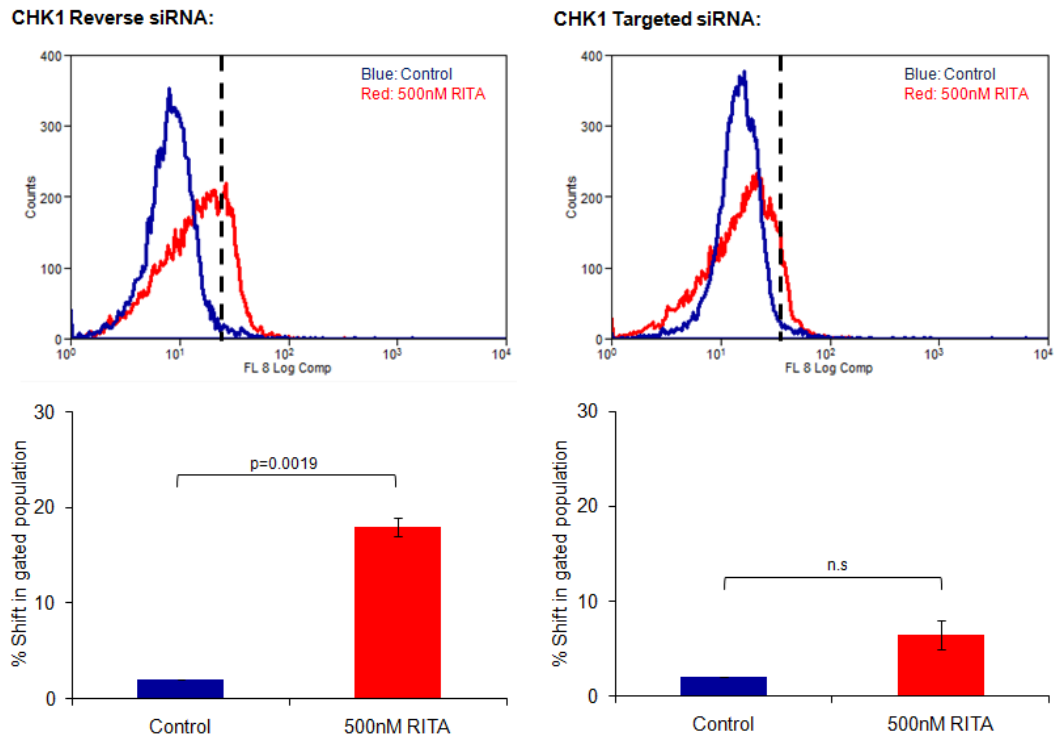


Figure 4.5.2: CHK1 is phosphorylated in response to RITA treatment, and inhibited by CHK1 siRNA

HCT116 p53+/+ cells were transfected with 25nM siRNA for either CHK1 reverse siRNA as a control siRNA, or CHK1 targeted siRNA. Transfections were incubated for 24 hours prior to a 24 hour dose of 500nM RITA in normoxia. Following treatment, cells were harvested for flow cytometry and stained for propidium iodide and phosphorylated CHK1 (p-CHK1, serine 345). Representative histograms are shown, dashed lines indicate where shifts in p-CHK1 intensity were gated and quantified. Quantified data is represented below and has been averaged from 2 independent experiments. Statistical significance was assessed using the unpaired t-test and a p-value <0.05 was considered significant, changes that are not significant have been indicated as n.s.

Because CHK1 is involved in maintaining DNA integrity by mediating cell cycle checkpoints, I predicted that loss of CHK1 may sensitise RITA treated cells to apoptosis. However, as shown in Figure 4.5.1, knockdown of CHK1 by siRNA did not enhance cleaved PARP indicating that CHK1 siRNA does not affect cell death induced by RITA. Similarly, by flow cytometry, knockdown of CHK1 had no significant effect on the sub-G1 population of cells treated with RITA (Figure 4.5.3). In conclusion CHK1 is therefore not essential for mediating cell death in response to RITA.

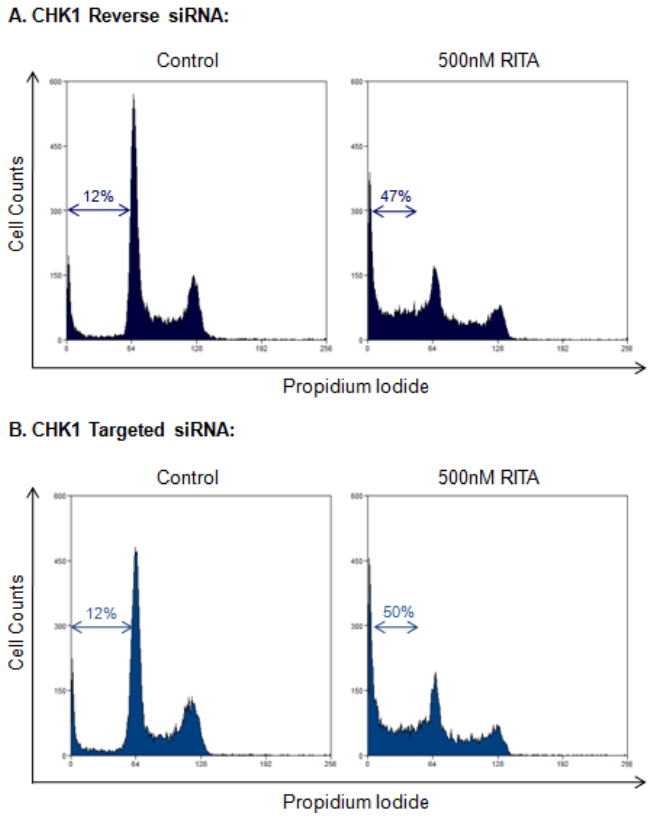


Figure 4.5.3: Loss of CHK1 does not affect RITA induced sub-G1

Graphs show DNA histograms of: (A) HCT116 p53+/+ cells treated with CHK1 reverse siRNA prior to a 24 hour dose of either DMSO control, or 500nM RITA in normoxia, and (B) HCT116 p53+/+ cells treated with CHK1 targeted siRNA prior to dosing with DMSO control or 500nM RITA. Cells were harvested for flow cytometry and stained using propidium iodide to analyse DNA content. DNA histograms show cell count on the y-axis, and propidium iodide intensity on the x-axis. Sub-G1 cells were quantified as an indication of cell death (indicated by arrows). DNA profiles are representative of 3 independent experiments.

In addition to analysing the affects of phosphorylated CHK1 on RITA induced cell death, phosphorylated CHK1 was also analysed in S-phase. The S-phase population was assessed because CHK1 has been shown to induce cell cycle arrest and p53-dependent DNA damage response pathways in S-phase (Beckerman et al., 2009). On this basis the hypothesis was made that although not important for RITA induced apoptosis, CHK1 phosphorylation may affect cells in S-phase. To address this, HCT116 p53+/+ and HCT116 p53-/- cells were dual stained for p53 protein and phosphorylated CHK1 following RITA treatment. The results show that CHK1 is phosphorylated in S-phase, and this correlates with induction of p53 in S-phase cells

that have been treated with RITA. Interestingly, these changes were not observed in HCT116 p53-/- cells (Figure 4.5.4). The data suggest p53-dependent induction of phosphorylated CHK1 in S-phase cells that together with p53 stabilisation may have a role in signalling to the DNA damage response upon genotoxic stress induced by RITA.

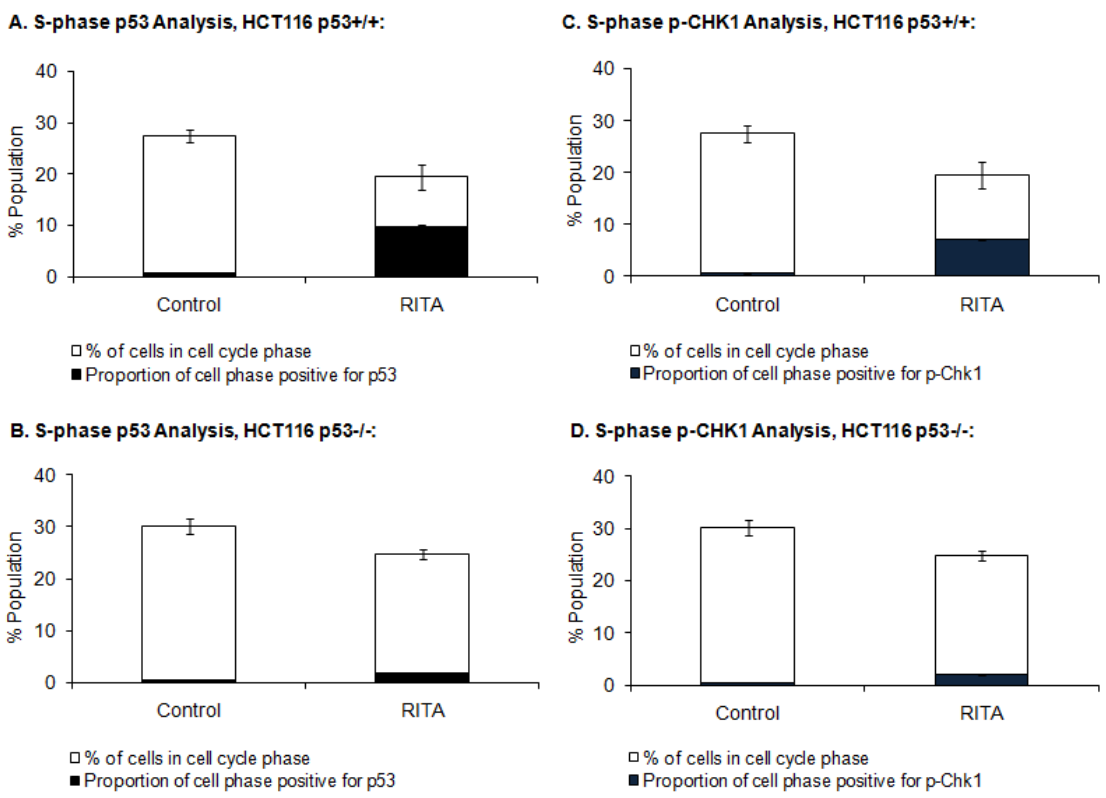


Figure 4.5.4: RITA mediated phosphorylation of CHK1 is observed in the S-phase population of HCT116 p53+/+ cells

HCT116 p53+/+ and HCT116 p53-/- cells were treated with 500nM RITA for 24 hours in normoxia. Cells were harvested for flow cytometry and stained for propidium iodide, phosphorylated CHK1 (p-CHK1), using an APC conjugated secondary antibody, and p53 protein, using a FITC conjugated secondary antibody. DNA profiles were gated for S-phase and assessed for increases in p-CHK1 intensity and p53 intensity. Graphs show (A) Percentage of HCT116 p53+/+ and (B) HCT116 p53-/- cells that stain positive for p53 protein in S-phase, clear bars indicate the total percentage of cells in S-phase, and shaded bars indicate the proportion of cells in S-phase which stain positive for p53 protein, (C) percentage of HCT116 p53+/+ and (D) HCT116 p53-/- cells that stain positive for p-CHK1 in S-phase. Shaded bars indicate the proportion of cells in S-phase which stain positive for p-CHK1. Graphs are representative of data that has been averaged from 3 independent experiments.

Phosphorylation of CHK1 has been shown in response to RITA in S-phase cells that have wildtype p53. Comet analysis was performed in HCT116 p53+/+ cells to assess whether CHK1 phosphorylation affects DNA damage induced by RITA following 24 hour treatment. Changes in RITA-induced DNA damage were investigated in HCT116 p53+/+ cells that had siRNA mediated knockdown of CHK1. DNA damage in response to 24 hour RITA treatment was not affected by CHK1 siRNA (Figure 4.5.5).

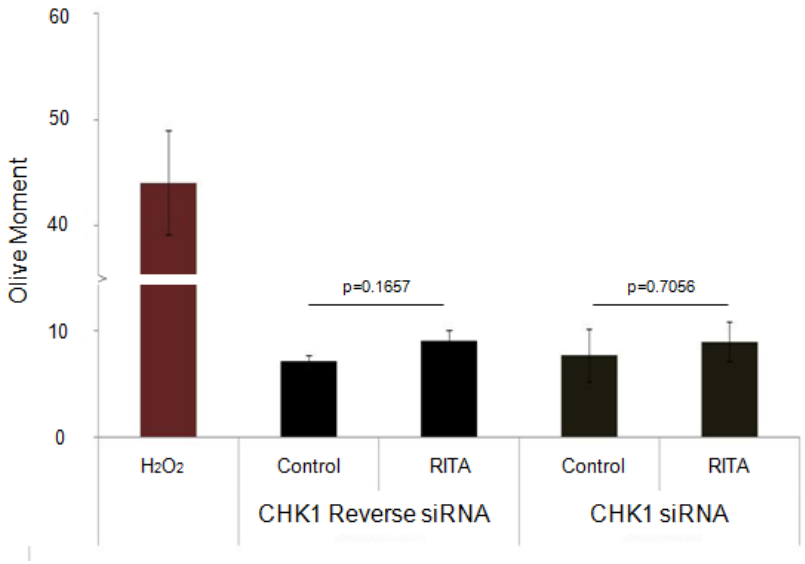


Figure 4.5.5: CHK1 siRNA does not affect RITA induced DNA damage at 24 hours
HCT116 p53+/+ cells were treated with CHK1 reverse siRNA, or CHK1 targeted siRNA, prior to dosing with either DMSO control, or 500nM RITA for 24 hours in normoxia. Following treatment, cells were harvested for comet analysis. Graph shows olive moment as a quantitative measure of DNA damage elicited by 24 hour RITA treatment. Hydrogen peroxide (H_2O_2) exposure at 100µM for 20min in 4°C was used as a positive control for DNA damage. Graph is representative of data averaged form 3 independent experiments. An unpaired t-test was used to assess statistical significance and a p-value of less than 0.05 was considered significant.

Loss of CHK1 by siRNA has been shown to have minimal effects on RITA induced cell death and DNA damage at 24 hours of treatment. Several studies suggest that CHK1 activation is important in regulating immediate responses to DNA damage and hence maintaining genomic integrity (Hirao et al., 2002; Niida et al., 2010; Rodriguez et al., 2008). Therefore, CHK1 may contribute to regulating genomic integrity when cells are exposed to RITA induced stress at early timepoints. To address this DNA strand

breaks were assessed in HCT116 p53+/+ cells with siRNA mediated knockdown of CHK1 following exposure to RITA treatment for 1 hour. DNA damage was found to be significantly induced in RITA treated cells that had loss of CHK1 compared to the untreated controls (Figure 4.5.6, (Ahmed et al., 2011). In conclusion CHK1 induction in response to RITA at early timepoints is important in maintaining genomic integrity.

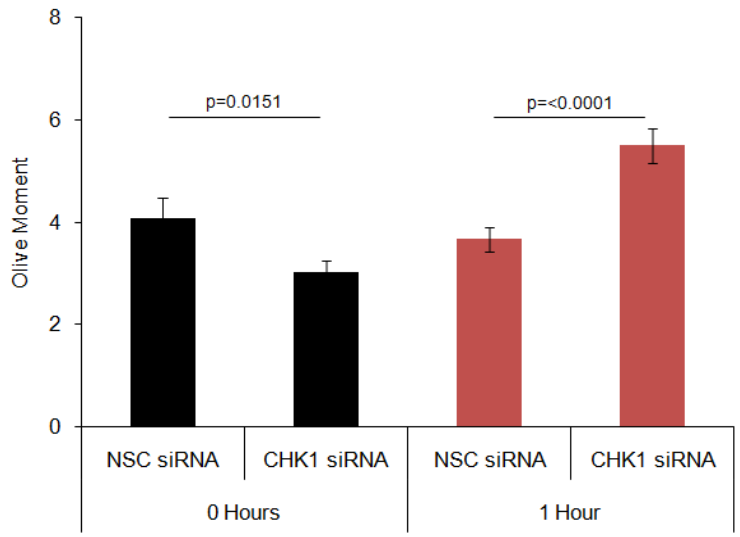


Figure 4.5.6: DNA damage is induced in CHK1 siRNA cells following RITA treatment for 1 hour

Graph shows HCT116 p53+/+ cells transfected with either non-silencing control siRNA (NSC), or siRNA targeted against CHK1 prior to 1 hour exposure of 500nM RITA. Following treatment cells were harvested for comet analysis, mixed with agarose and spread onto comet assay slides for lysis and electrophoresis. Following electrophoresis, slides were fixed and stained for SYBR Green to visualise cell nuclei (referred to as comets). Comets were visualised using an inverted fluorescent microscope at 10x magnification, and images were captured over 20 fields of view for each treatment. Comets were analysed using the TriTeck® comet analysis software (approximately 100 comets were quantified for each treatment). Olive moment was used as an indication of DNA damage. Data shown is representative of 3 independent repeat experiments. An unpaired t-test was used to assess statistical significance and a p-value of less than 0.05 was considered significant.

To assess whether CHK1 was phosphorylated following 1 hour RITA treatment, western analysis was used of HCT116 p53+/+ cells that had been treated with RITA for 1 hour following siRNA knockdown of CHK1. As shown in Figure 4.5.7, CHK1

phosphorylation was induced by RITA as early as 1 hour after treatment. CHK1 is therefore essential in regulating genomic integrity following short term exposure to RITA.

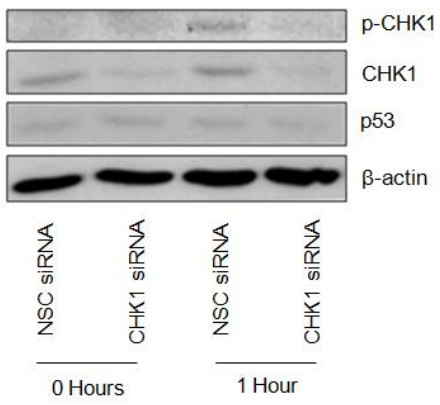


Figure 4.5.7: CHK1 phosphorylation is induced by 1 hour exposure to RITA
Western blot shows HCT116 p53+/+ cells treated with 500nM RITA for 1 hour following siRNA mediated knockdown of either non-silencing control (NSC), or CHK1 targeted siRNA. Western blots were analysed for phosphorylated CHK1 (p-CHK1), total CHK1, p53 and β-actin as the loading control. Data is representative of 3 independent repeat experiments.

In previous experiments, both p53 and γH2AX are induced in all phases of the cell cycle following treatment with RITA, including sub-G1 (Figure 4.4.4). The effects observed in p53 stabilisation, γH2AX induction, and phosphorylated CHK1 may therefore be a result of apoptosis, rather than effects that mediate apoptosis. To test this, the caspase 3 inhibitor Z-DEVD-FMK was used to inhibit apoptosis and phosphorylated CHK1 was investigated by flow cytometry. As expected, sub-G1 cells induced by RITA decreased significantly in the presence of caspase inhibitor, showing inhibition of apoptosis (Figure 4.5.8A). Interestingly, phosphorylated CHK1 was not inhibited when cells were treated with RITA in the presence of caspase inhibitor (Figure 4.5.8B) suggesting that phosphorylation of CHK1 is induced upstream of apoptotic responses elicited by RITA.

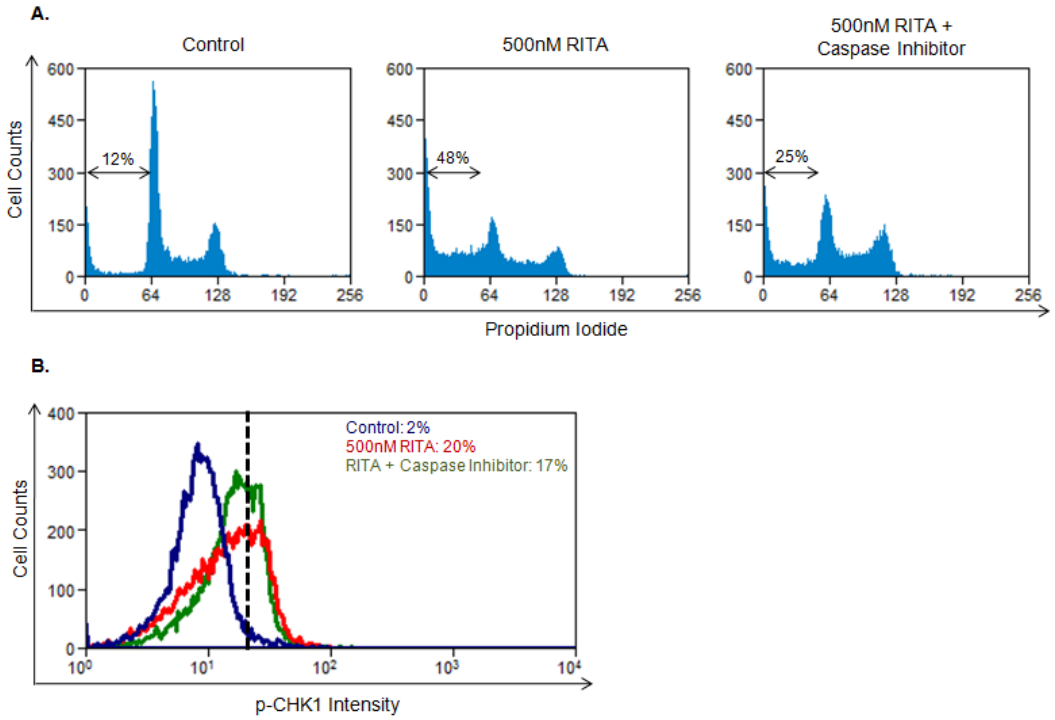


Figure 4.5.8: CHK1 phosphorylation in response to RITA is not affected by caspase inhibition

HCT116 p53+/+ cells were treated with 500nM RITA alone, or in combination with 50μM caspase inhibitor Z-DEVD-FMK for 24 hours in normoxia. Cells were harvested for FACS analysis and stained using propidium iodide and phosphorylated CHK1 (p-CHK1), with an APC conjugated secondary antibody: (A) DNA profiles of cells stained with propidium iodide. Histograms show cell count verses propidium iodide intensity. The sub-G1 population (indicated by arrows) was gated to assess cell death when cells were treated with RITA alone, or in combination with Z-DEVD-FMK, (B) Histogram displaying cell count against phosphorylated CHK1 (p-CHK1) intensity of control (blue), RITA treated (red), or RITA and Z-DEVD-FMK treated cells (green). Histograms were gated according to the control population (as indicated by dashed line) and the percentage of cells that shift in phosphorylated CHK1 intensity towards this gate was quantified. Data shown is representative of 2 independent repeats.

4.6 Discussion

Checkpoint mechanisms are important for inhibiting cell cycle progression in response to stress stimuli (Hartwell and Weinert, 1989). S-phase checkpoints are activated when replication fork progression is inhibited due to DNA strand breaks that form during replication. By delaying S-phase progression in response to DNA strand breaks, replication forks can stabilise, and DNA repair can be initiated (Luciani et al., 2004). In this chapter, it was hypothesised that DNA strand breaks induced by RITA could activate an S-phase checkpoint and DNA fibre experiments were performed to analyse replication fork progression in response to RITA treatment (collaboration with Dean Jackson, University of Manchester). Previous immunoflourescence studies from our laboratory have shown that RITA also induces pan-nuclear staining of γH2AX (Yang et al., 2009a), a distinct pattern of staining compared to other forms of DNA damage that is associated with activation of an S-phase checkpoint (Marti et al., 2006). Consequently RITA treatment in HCT116 p53+/+ cells was shown to result in stalling of the replication fork machinery and a p53-dependent delay in S-phase progression (Ahmed et al., 2011). I have extended these studies throughout this chapter by analysing S-phase cells by flow cytometry and have shown induction of both p53 and γH2AX in response to RITA by a mechanism that is both dose and time-dependent, and correlates with induction of cell death.

Collectively, these findings have led to a model whereby RITA is proposed to induce DNA strand breaks, and stall the replication fork machinery. Stalling of the replication machinery elicits an S-phase checkpoint that involves induction of γH2AX, stabilisation of p53 and phosphorylation of both CHK1 and CHK2. Of interest are the observations that γH2AX induction and CHK1 phosphorylation only occur in cells that have wildtype p53, therefore small molecule activation of p53 by RITA elicits a p53-dependent DNA damage response that is important for mediating both cell cycle checkpoints, and cell death responses. The current proposed model is summarised in Figure 4.6.1.

Tumour suppressor pathways involving checkpoint proteins commonly precede activation of apoptosis in response to stress stimuli (Niida et al., 2010). I assessed CHK1 phosphorylation in response to RITA treatment in detail because CHK1 activation in S-phase cells by replication stress has been shown to elicit cell cycle arrest, and regulate specific DNA repair pathways (Brown and Baltimore, 2003; Liu et al., 2000). Both *in vitro* and *in vivo* models demonstrate that genetic loss of *Chk1* induces proliferation defects, loss of cell cycle checkpoints in response to gamma

irradiation, and early embryonic lethality (Liu et al., 2000; Takai et al., 2000). Defects in cell cycle and cell death pathways caused by loss of *Chk1* can be rescued by a single *Chk1* allele (Niida et al., 2010). Recently, both *Chk1* and *Chk2* deletion were found to promote tumour development *in vivo* due to enhanced genomic instability (Niida et al., 2010).

Due to the importance of CHK1 in regulating DNA integrity in response to replication stress, I hypothesised that CHK1 phosphorylation is important for activating an S-phase checkpoint in response to DNA strand breaks induced by RITA treatment. I addressed this hypothesis by assessing whether siRNA mediated knockdown of CHK1 had effects on cell death responses induced by RITA. However, significant changes in RITA induced p53 stabilisation and cell death were not observed when CHK1 protein was inhibited by siRNA. Analysis of cell death in CHK1 knockdown cells was only performed following 24 hours of RITA treatment at which time it is likely that cell death responses are maximised. It will be of interest to repeat these studies at earlier timepoints following RITA treatment to assess whether loss of CHK1 sensitises cells to immediate forms of cell stress, rather than at later timepoints.

DNA repair was also assessed because several studies implicate both CHK1 and CHK2 activation in early DNA damage repair pathways that regulate the genomic integrity of cells exposed to stress (Hirao et al., 2002). Proliferating cell nuclear antigen (PCNA) is localised at sites of DNA replication during S-phase and recruits DNA polymerases during replication, as well as a range of repair factors at sites of DNA breaks (Tsurimoto, 1999). Shimura et al. have shown that activation of p53 and PCNA in response to low dose radiation is important for suppressing replication fork progression, and initiating DNA repair pathways (Shimura et al., 2006). Formation of replication protein A (RPA) foci that co-localise with PCNA at replication forks and DNA strand breaks are important in signalling to ATR and CHK1 so that appropriate DNA repair pathways can be co-ordinated (Rodriguez et al., 2008).

Furthermore, at timepoints as early as 1 hour after radiation, p53 is stabilised to regulate DNA damage repair and cell death pathways (Niida et al., 2010). Such processes are impaired when both CHK1 and CHK2 function is inhibited, enhancing genomic instability and thereby promoting cell survival and tumour development (Niida et al., 2010). With support from *in vitro* and *in vivo* studies that address a functional role for CHK1 in DNA repair, I hypothesised that checkpoint signalling in response to RITA treatment has an important role in regulating early DNA repair pathways to replication

stress. This hypothesis was addressed by analysing the contribution that CHK1 has in regulating genomic integrity when cells were immediately exposed to RITA induced stress. Data shown confirmed that CHK1 is phosphorylated following 1 hour exposure to RITA treatment and loss of CHK1 by siRNA sensitised RITA treated cells to DNA damage during this time frame (Ahmed et al., 2011).

Finally, although S-phase DNA damage checkpoints are sensitive to low dose radiation and have been studied extensively (Brown et al., 2003), the p53-dependent DNA damage S-phase checkpoint that has been shown in this thesis has only been described in few studies. This checkpoint was originally identified in mouse zygotes (Agarwal et al., 1998) and then studied in mouse embryonic fibroblasts (Shimura et al., 2006). However, the role of p53 in these responses is not well understood. In mouse embryonic fibroblasts that have been treated with radiation doses below 2.5Gy, an ATM mediated, p53-dependent decrease in replication fork progression is observed that is not seen in p53 null cells (Shimura et al., 2006). Therefore low dose radiation could be effective in mediating anti-tumour activity by similar p53-dependent mechanisms to those observed in response to RITA and studies to address this are in progress.

4.7 Conclusions for this chapter

- RITA induces p53 and γH2AX in S-phase.
- RITA inhibits replication fork progression in p53 wildtype cells.
- Stabilisation of p53, induction of γH2AX, and phosphorylation of CHK1 in response to RITA treatment are not observed in p53-null cells.
- Loss of CHK1 by siRNA does not affect RITA induced cell death, or RITA induced DNA damage at 24 hours of treatment. However loss of CHK1 by siRNA does enhance DNA damage induced by immediate exposure to RITA (1 hour) indicating an important role for CHK1 in regulating DNA repair.

4.8 Impact of these findings

As CHK1 is important in initiating repair mechanisms in response to stalled replication forks, many studies have investigated the possibility of using CHK1 inhibitors to inhibit repair pathways and sensitise tumour cells to genotoxic agents. Furthermore, p53 status seems to be essential for the apoptotic outcome of such combinations. For example, CHK1 depletion by siRNA sensitises p53-deficient cells to radiation (Wang et al., 1996) while CHK1 inhibition by the small molecule AZD7762 sensitises a range of

p53-mutated cell lines to radiotherapy, responses that are not observed in p53 wildtype tumour cells or in normal fibroblasts (Mitchell et al., 2010). Depletion of CHK1 by siRNA (Wang et al., 1996) and small molecule inhibition of CHK1 by PD407824 (Arora et al., 2010) also enhances sensitivity to cytotoxics in p53-deficient cells. These combinations are based on the rationale that loss of p53 function inhibits cell cycle arrest in response to genotoxic stress and promotes cell death in cells that are rapidly proliferating.

UCN-01 (7-hydroxystaurosporine) has been studied as a single agent and in combination in several Phase 1 clinical schedules, however poor pharmacokinetics and an unfavourable toxicity profile has limited the progress of UCN-01 through the clinic, and this seems to have been the case for most CHK1 inhibitors in clinical development (Ma et al., 2011). Several redundant pathways maintain cell cycle responses when CHK1 is inhibited. Loss of CHK1 has been shown to increase ATM and ERK1/2 cell survival pathways (Dai et al., 2008; Dent et al., 2011) suggesting that agents targeting the MAPK pathway could potentiate CHK inhibitor function. Crosstalk between CHK1 and CHK2 is well established and studies have shown that CHK1 and CHK2 co-operate to elicit cell cycle checkpoints *in vivo.* Importantly, loss of these checkpoint kinases promotes tumour susceptibility by enhancing genomic instability (Niida et al., 2010). Therefore, targeting CHK1 and or CHK2 may enhance tumour susceptibility in certain models, and may also explain the poor toxicity profile of CHK targeted agents. Clearly, scheduling of combination therapies is critical, and like cyclotherapy, which has been recently shown to improve tumour cell death by targeting cell cycle kinetics, the use of CHK inhibitors will also be critical in ensuring that appropriate scheduling of combinations elicit maximum DNA damage that leads to maximum cell death.

In this study p53-dependent cell cycle checkpoints have been investigated that are important in eliciting DNA damage responses induced by the small molecule RITA. Although loss of CHK1 sensitises tumour cells to cell death by genotoxic agents, enhanced tumour cell death was not achieved in response to RITA when CHK1 was inhibited by siRNA. Activation of wildtype p53 is therefore important in maximising tumour cell death and loss of CHK1 only has implications in regulating DNA repair following early responses to stress. Therapeutically, these studies suggest that CHK1 inhibition may be important in sensitising cells to cell death by p53 activating agents only in combinations that are both time-dependent and dose-dependent. Furthermore, lack of anti-tumour responses in p53 deficient cells suggests that in the tumour models which have been studied in this thesis, p53 signalling is critical in determining cell death responses to genotoxic stress. Signalling through DNA repair pathways is an

important mechanism by which p53-dependent apoptosis is engaged in cells that have lethal, irreparable forms of DNA damage. These findings propose an important level of control for wildtype p53 in signalling to the DNA damage response and repair machinery, and have important implications in determining the anti-tumour activity of agents that regulate p53 activity.

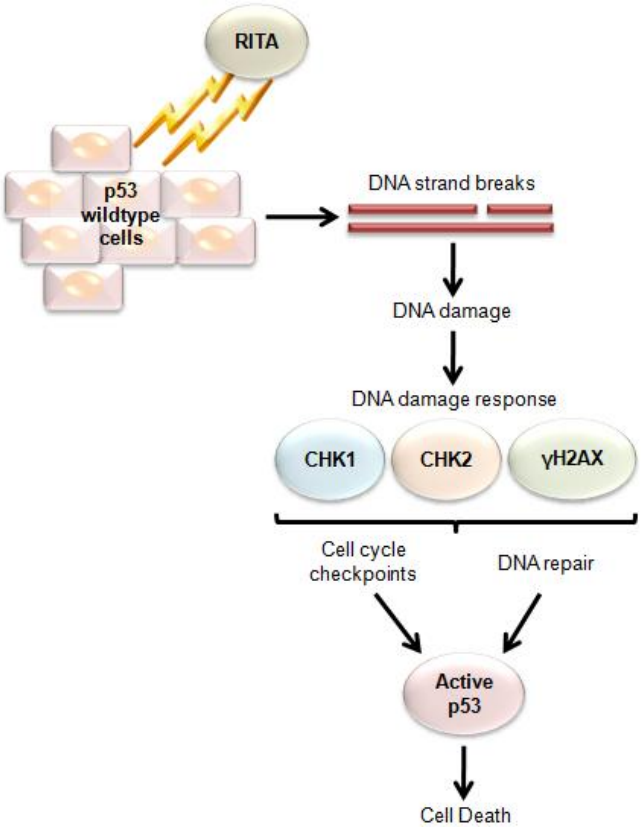


Figure 4.6.1: RITA activates cell cycle checkpoints

RITA induces stalling of the replication fork machinery and the formation of DNA strand breaks leads to p53-dependent phosphorylation of CHK1, induction of γH2AX, and activation of an S-phase cell cycle checkpoint. Although CHK1 is not essential for cell death, activation of this cell cycle checkpoint is important for mediating DNA repair processes. Phosphorylation of CHK2 is also observed in response to RITA treatment however this is not p53-dependent.

Chapter 5

MAPK signalling modulates p53-dependent cell death responses elicited by RITA

5.1 Introduction

The mitogen activated protein kinase (MAPK) family comprise serine threonine specific protein kinases that are activated by a range of extracellular stimuli such as mitogens, growth factors, osmotic stress, heat shock and pro-inflammatory cytokines (Pearson et al., 2001). MAPK signalling is initiated by receptor-mediated events at the cell surface that activate a relay of effector proteins through the cytoplasm and into the nucleus to induce transcriptional changes. These effector proteins comprise the MAPK kinase kinases (MAPKKK) that are activated by cell surface proteins, the MAPK kinases (MAPKK) that transduce signals through the cytoplasm, and the MAPKs that activate transcriptional responses in the nucleus (Raman and Cobb, 2003).

Extracellular signal regulated kinase (ERK) was the first MAPK to be characterised (Boulton et al., 1990). The signalling pathway that leads to ERK activation has been studied in detail, and has been summarised in Figure 5.1.1 (Lewis et al., 1998). Following binding of extracellular ligands such as growth factors and various mitogens, receptor tyrosine kinase (RTK) receptors located on the cell surface membrane undergo dimerisation and autophosphorylation events that initiate complex intracellular signalling cascades involving a series of kinases. Firstly, receptor activation promotes conversion of the G-protein proto-oncogene Ras from its inactive guanosine diphosphate (GDP) bound form, to its active guanosine tripohosphate (GTP) bound form. A conformational change is induced in Ras, leading to recruitment and binding of the MAPKKK c-Raf to the cell membrane. Activated c-Raf phosphorylates the MAPKK's MEK1/MKK1 and MEK2/MKK2 (MAPK and ERK kinases) at specific serine and threonine residues (Dent et al., 1992). MEK1/MKK1 and MEK2/MKK2 then phosphorylate the MAPK's ERK1 (also referred to as $p44^{MAPK}$) and ERK2 (also referred to as $P42^{MAPK}$) at dual threonine and tyrosine residues (Anderson et al., 1990). Activated ERK1 and ERK2 are translocated to the nucleus to phosphorylate transcription factors such as ELK-1 which induce expression of various target genes involved in cell proliferation, cell survival, cell death and differentiation (Marais et al., 1993).

PD98059 [2-(2'amino-3'-methoxyphenyl)-oxanaphthalen-4-one] is an inhibitor of MEK1/MKK1 MAPKK (Alessi et al., 1995), and can reverse the phenotype of RAS transformed cell lines (Dudley et al., 1995). PD98059 was the first synthetic MAPK inhibitor to be identified from a compound library using an *in vitro* kinase screen. Both a constitutively active MEK1 mutant and MEK1 induced by growth factor stimulation was

inhibited by PD98059 with an IC_{50} of 2-7μM (Dudley et al., 1995). PD98059 was shown to be a non-competitive inhibitor of MEK1, and by binding to MEK1, could inhibit growth factor stimulated MEK1 activation by the upstream kinase c-RAF. At an IC_{50} of 50μM, PD98059 also inhibited phosphorylation of MEK2/MKK2 (Alessi et al., 1995; Dudley et al., 1995). PD98059 has been used extensively as a tool for studying the RAS-RAF-MEK-ERK MAPK pathway, and understanding its importance. Constitutive activation of MEK1 and MEK2 is associated with cellular transformation and differentiation in a number of tumour models (Marshall, 1995), and for this reason, many MAPK inhibitors have also been developed for therapeutic targeting of MAPK signalling (Cowley et al., 1994; Sebolt-Leopold et al., 1999).

In certain cases, MAPK activation acts as a tumour suppressor response by regulating cell death induced by oncogenic, and genotoxic stimuli. Phosphorylation and activation of p53 by the PI3K family members ATM and ATR has been described in response to stress stimuli (Banin et al., 1998; Canman et al., 1998). Various stress activated MAPK kinases also phosphorylate and activate p53 directly (Fuchs et al., 1998b; Meek, 1998). For example, NFκB-dependent apoptosis is mediated by ERK1/2 regulation of p53 stability (Ryan et al., 2000). In other studies, ERK1/2 is an upstream regulator of p53 phosphorylation and stability in response to DNA damage induced by cisplatin, and inhibition of ERK1/2 activation by PD98059 represses p53-dependent cell death by this pathway (Persons et al., 2000). Recently, activation of the MKK7 MAPK pathway by oncogenic and genotoxic induced stress was shown to suppress tumour development *in vivo* by eliciting a DNA damage response, and promoting p53 stability (Schramek et al., 2011). In lung cancer and breast cancer models, MKK7 knockout mice developed tumours early and had a poor survival outcome compared to wildtype mice. Schramek et al. showed that p53 activation by DNA damage responses is an important mechanism for tumour suppression, and that MAPK signalling is an important upstream mediator of this response (Schramek et al., 2011). Previous studies in our laboratory had found that RITA-mediated effects were sensitive to MEK-ERK inhibition (Yang, unpublished). Because of the importance of MAPK signalling in p53 mediated apoptosis, I further investigated whether RITA's p53-dependent apoptotic effects were regulated by the MAPK pathway.

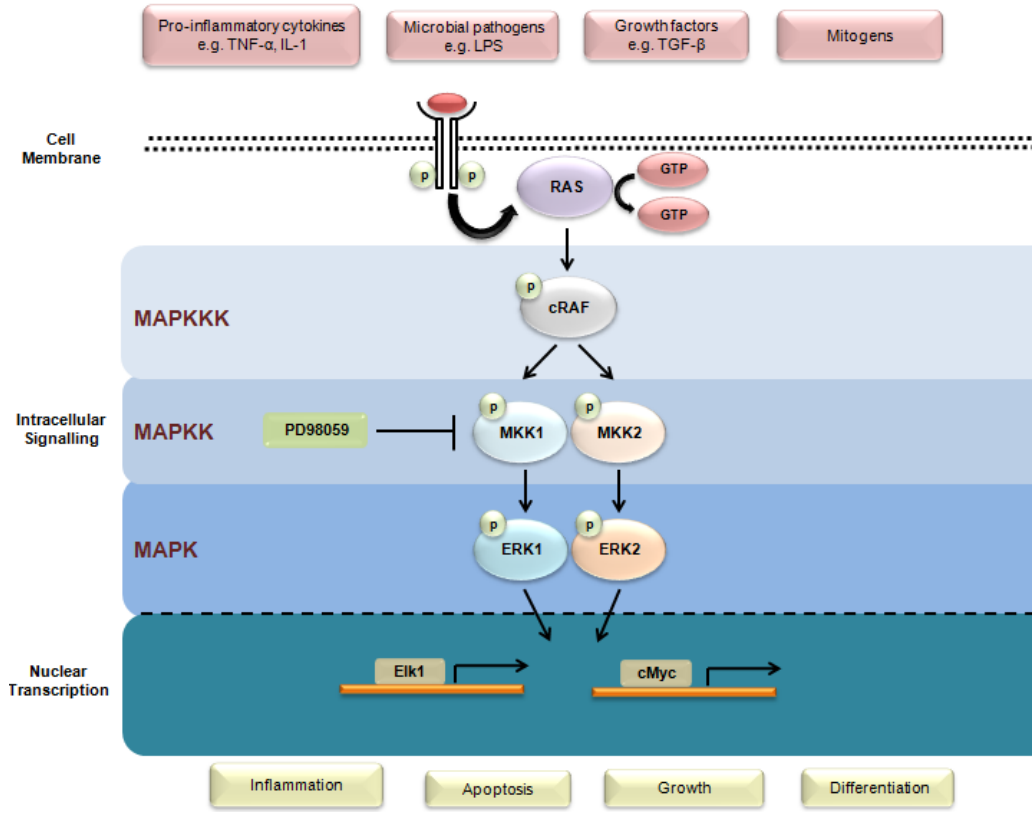


Figure 5.1.1: PD98059 inhibits the RAS-cRAF activated MAPK pathway

Phosphorylation of receptor tyrosine kinase receptors upon binding of specific extracellular ligands on the cell surface membrane leads to activation of the GTPase RAS proto-oncogene. This leads to a sequence of intracellular phosphorylation events involving MAPKKK, MAPKK and MAPK kinases. The MAPKKK c-RAF is phosphorylated by RAS, which in turn phosphorylates the MAPKKs MEK1/MKK1 and MEK2/MKK2 leading to phosphorylation and activation of ERK1 and ERK2 MAPKs, their translocation to the nucleus and activation of many transcription factors involved in the regulation of cell growth, differentiation, and development. PD98059 is an inhibitor of MEK1/MKK1 and MEK2/MKK2 activation and has been used to elucidate the importance of RAS-RAF signalling in response to various extracellular stimuli *in vitro* and *in vivo*.

5.2 Hypothesis

MAPK signalling modulates RITA induced cell death.

5.3 Aims

- Investigate MAPK activation by RITA treatment.
- Understand the importance of MAPK signalling in the regulation of p53-dependent cell death responses using PD98059 and MAPK siRNAs.

5.4 RITA induced cell death is inhibited by PD98059

MAPK activation has been shown to be associated with cisplatin induced apoptosis (Persons et al., 2000). In order to investigate whether ERK1/2 is phosphorylated by RITA, HCT116 p53+/+ cells were treated with RITA over a 24 hour timecourse, and ERK1/2 phosphorylation was assessed by western blotting. PD98059 was also used to investigate whether inhibition of ERK1/2 phosphorylation affected RITA induced cell death, as assessed by cleaved PARP (Figure 5.4.1). Phosphorylation of ERK1/2 occurred at early time points when HCT116 p53+/+ cells were treated with RITA, and this preceded induction of cell death as assessed by cleaved PARP. Treatment of HCT116 p53+/+ cells with RITA in combination with PD98059 inhibited ERK1/2 phosphorylation, cleaved PARP and to some extent, p53 induction. These observations are consistent with previous studies from our laboratory that found PD98059 blocks RITA mediated responses (Yang, unpublished). ERK1/2 signalling is therefore important for mediating cell death responses by RITA.

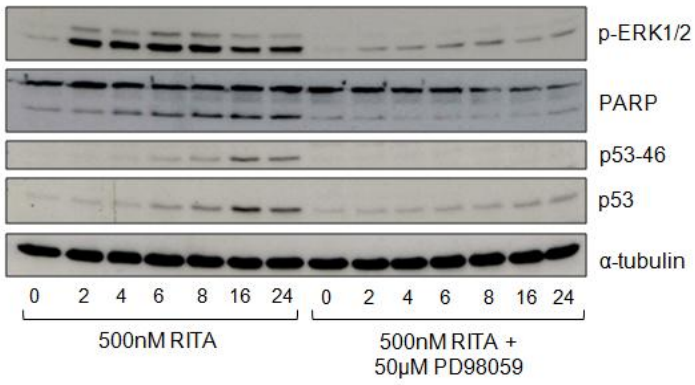


Figure 5.4.1: ERK1/2 is activated in response to RITA treatment and inhibited by PD98059

Western blotting of HCT116 p53+/+ cells treated with 500nM RITA in the absence or presence of 50μM PD98059. Cells were harvested for western blotting over a 24 hour time course and analysed for phosphorylated ERK1/2 at thr202/tyr204 (p-ERK1/2), PARP, phosphorylated p53 at ser46 (p53-46), total p53 protein, and α-tubulin as a loading control. Data shown is representative of 2 experiments.

To assess cell death in greater detail, flow cytometry was used to quantify the sub-G1 population of cells treated with RITA, either alone, or in combination with PD98059. In agreement with Figure 5.4.1, treating cells with PD98059 significantly inhibited cell death induced by RITA, as indicated by the sub-G1 population (Figure 5.4.2).

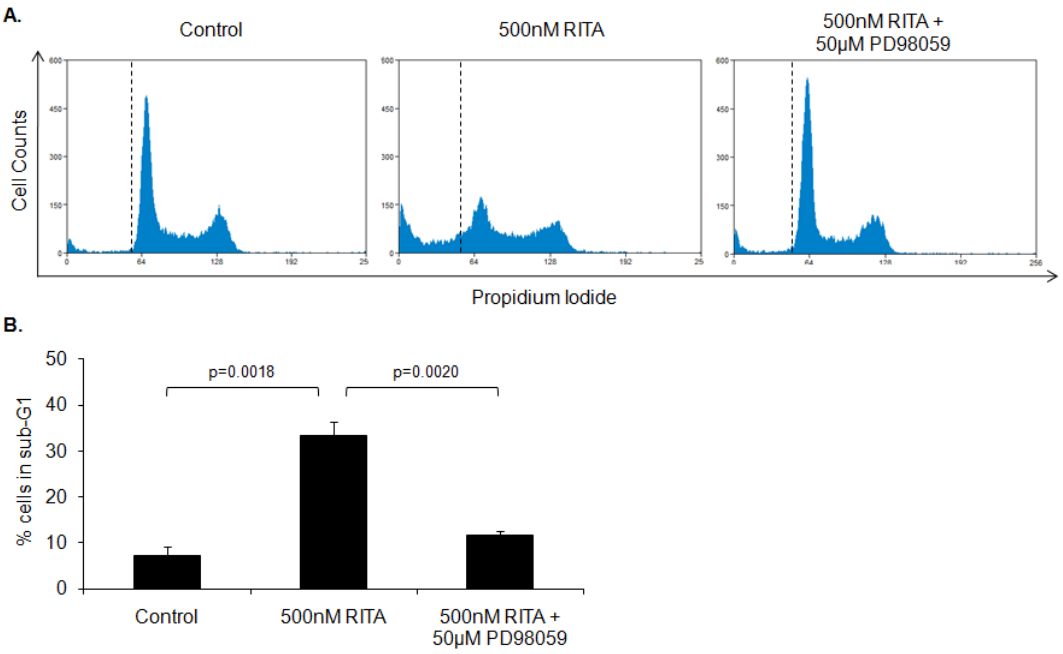


Figure 5.4.2: PD98059 inhibits sub-G1 cells induced by RITA

HCT116 p53+/+ cells were treated with 500nM RITA with or without 50μM PD98059 for 24 hours in normoxia. Cells were harvested for flow cytometry analysis and stained for propidium iodide to analyse DNA content: (A) Cell cycle DNA profiles shown are representative of 3 independent experiments. Profiles show cell count on the y-axis and propidium iodide intensity on the x-axis. Cells were gated for sub-G1 (as indicated by dashed line), (B) Percentage of cells in the population gated for sub-G1 content is quantified, and has been averaged from 3 independent experiments. An unpaired t-test was used to assess statistical significance and a p-value of <0.05 was considered significant.

Flow cytometry was used to quantify changes in p53 and γH2AX induction following PD98059. In agreement with western analysis (Figure 5.4.1), flow cytometric analyses show that both p53 and γH2AX induction are inhibited significantly by PD98059 (Figure 5.4.3).

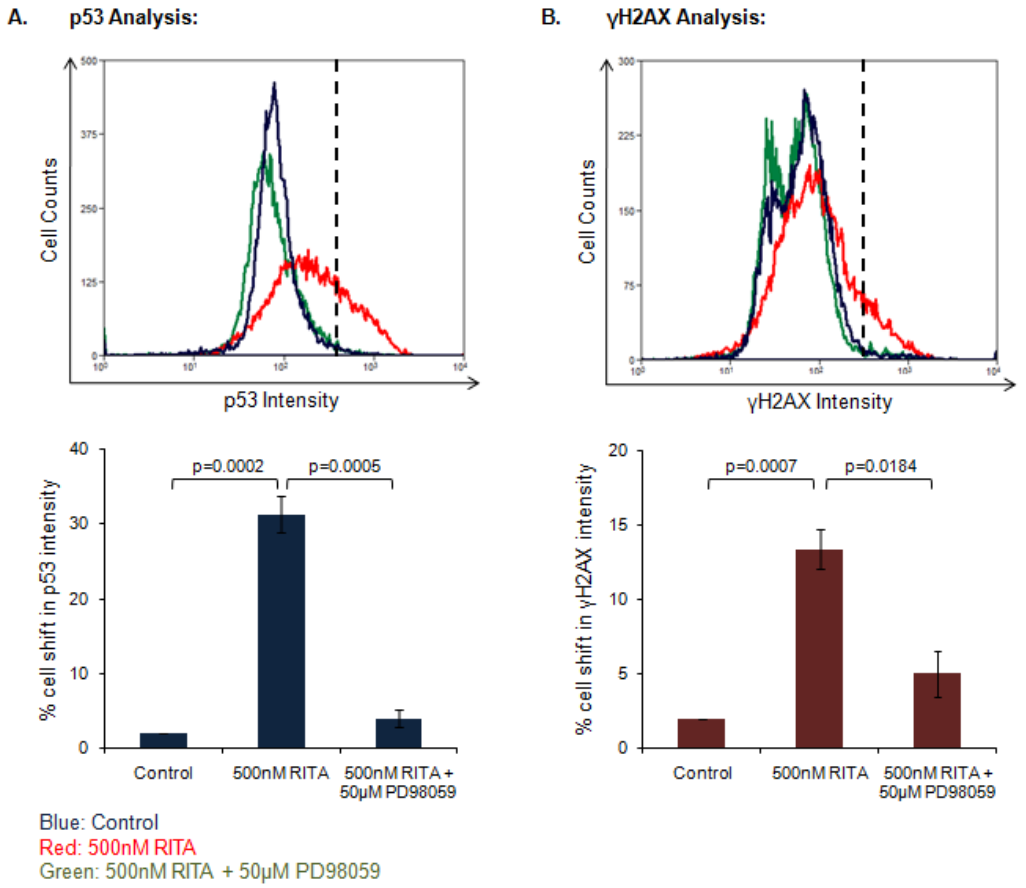


Figure 5.4.3: PD98059 inhibits p53 and γH2AX induced by RITA

HCT116 p53+/+ cells treated with 500nM RITA in the absence or presence of 50μM PD98059 for 24 hours in normoxia. Following treatment, cells were harvested and fixed for flow cytometry, and stained for propidium iodide, p53 (using a secondary FITC conjugated antibody) and γH2AX (using a secondary APC conjugated antibody). Graphs show: (A) analysis of cells stained for p53 induction, and (B) analysis of cells stained for γH2AX (phosphorylated at serine 139). Representative histograms from 3 independent experiments are shown, with cell counts on the y-axis, displayed against p53 fluorescent intensity (left panel) or γH2AX (right panel) on the x-axis. Blue indicates the control population, red indicates cells treated with 500nM RITA, and green represents cells treated with RITA in combination with 50μM PD98059. Histograms were gated against the control population (shown by the dashed line), and the percentage of treated cells that shift in intensity towards the right were quantified. Bar charts below show quantified data of the percentage of cells that shift in either p53 intensity (left), or phosphorylated γH2AX intensity (right), and have been averaged from 3 independent experiments. An unpaired t-test was used to assess statistical significance and a p-value of <0.05 was considered significant.

To evaluate p53 and γH2AX localisation, the INCell 1000 imaging platform was used (GE Healthcare Life Sciences). As for flow cytometry optimisation experiments (Figure 4.4.1), antibody controls were used to assess the specificity of primary p53 antibody binding to its Alexa Fluor 488 conjugated secondary antibody, and primary γH2AX antibody binding to its secondary Alexa Fluor 568 conjugated antibody. These antibodies were optimised so that dual staining of p53 and γH2AX could be performed in a 96 well format. As shown in Figure 5.4.4, Alexa Fluor 488, and Alexa Fluor 568 secondary antibodies that bind p53 and γH2AX respectively did not cause non-specific background staining. At the concentrations used, they gave specific and reproducible staining only in the presence of p53 and γH2AX primary antibodies.

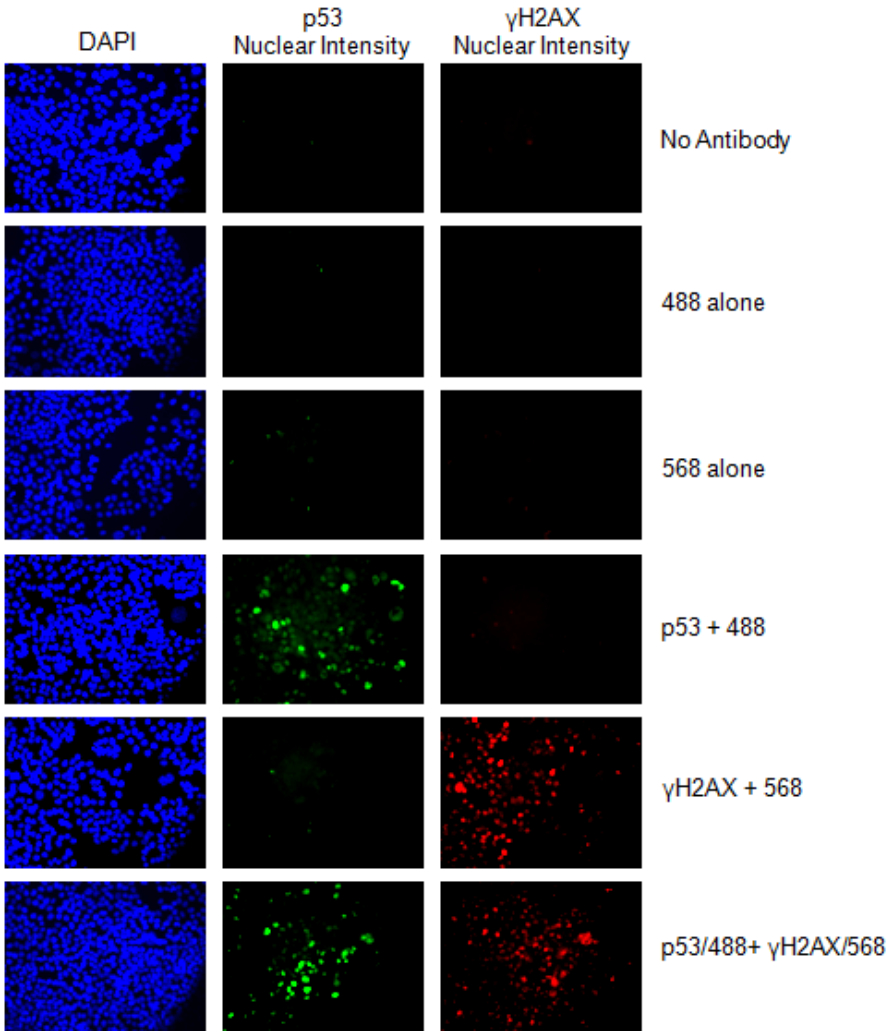


Figure 5.4.4: Immunoflourescent staining of p53 and γH2AX

Images show untreated HCT116 p53+/+ cells stained with DAPI to view nuclei (blue), total p53 primary with Alexa Fluor 488 conjugated secondary antibodies to view p53 intensity in the nucleus (green), and γH2AX primary with Alexa Fluor 568 conjugated secondary antibodies to view γH2AX intensity in the nucleus (red). Staining was performed with either secondary antibodies alone (indicated as 488 alone, or 568 alone), or with primary and secondary antibodies together. Bottom panel of images shows dual staining of cells with total p53 and Alexa Fluor 488, together with γH2AX and Alexa Fluor 568 antibodies, See materials and methods for specific antibody concentrations used. Cells were analysed using the high throughput INCell 1000 screening platform. 10 fields of view were captured for each duplicate well at 20x magnification. Representative images from one field of view are shown.

Using PD98059, MEK-ERK signalling has been shown to be important in regulating p53 stabilisation in response to RITA treatment (Figure 5.4.1 and Figure 5.4.3). I hypothesised that induction of nuclear p53 by RITA would also be affected by PD98059. To address this, HCT116 p53+/+ cells were treated with RITA alone, or in combination with PD98059, and nuclear p53 and γH2AX was analysed using the INCell platform. Increases in p53 and γH2AX nuclear intensity were observed following RITA treatment that was inhibited by PD98059 (Figure 5.4.5). This data supports the requirement of MEK-ERK signalling for stabilising p53 in the nucleus following RITA treatment, and inducing DNA damage responses involving γH2AX.

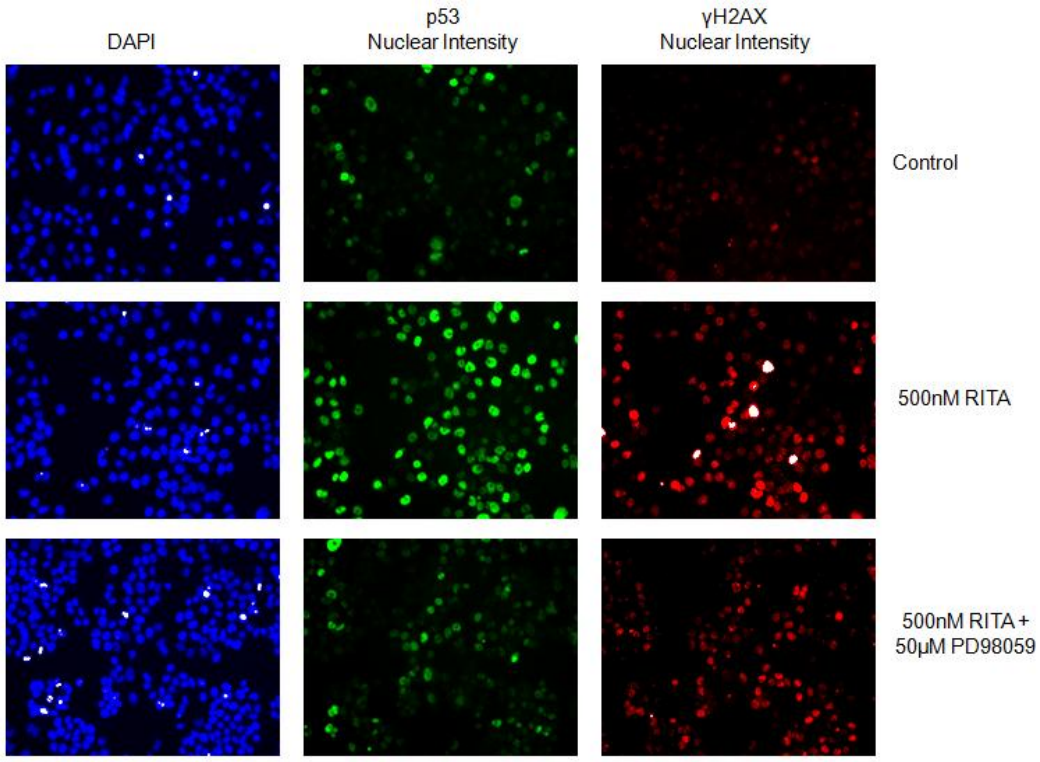


Figure 5.4.5: RITA induced nuclear p53 and γH2AX is inhibited by PD98059
HCT116 p53+/+ cells were treated with 500nM RITA for 24 hours in normoxia in the absence/presence of 50μM PD98059. Cells were fixed and stained for DAPI to indicate nuclei (blue), total p53 staining in the nucleus, as shown by Alexa Fluor 488 staining (green), and γH2AX intensity in the nucleus, shown by Alexa Fluor 568 staining (red). Staining was analysed using the INCell 1000 high content screening platform at 20x magnification. 10 fields of view were captured from each triplicate well. Representative images from 3 independent experiments are shown.

To investigate the affects of PD98059 on HCT116 p53+/+ and HCT116 p53-/- cells treated with RITA, INCell images were quantified and assessed for changes in nuclear p53 and γH2AX. In agreement with Figure 5.4.5, RITA treatment led to significant increases in nuclear p53 which was inhibited in the presence of PD98059. Importantly, PD98059 alone had no effects on p53 stabilisation in the nucleus and as expected, no changes in p53 intensity were observed in HCT116 p53-/- cells (Figure 5.4.6). Cell counts were also greater in cells treated with RITA and PD98059, compared to RITA treatment alone indicating inhibition of RITA induced cell death in the presence of PD98059.

The number of nuclear γH2AX foci was increased in RITA treated cells, and inhibited in the presence of PD98059. However, induction of γH2AX foci in response to RITA was not p53-dependent. The p53 dependency of γH2AX induction in response to RITA has been described in chapter 3 and published previously from our laboratory (Ahmed et al., 2011; Yang et al., 2009a). The sensitivity of quantifying individual cell responses using the INCell analyser allows for more robust analysis of p53-dependent responses to stress. In conclusion, the results presented so far show that PD98059 has significant effects on p53 and γH2AX in cell nuclei following RITA treatment.

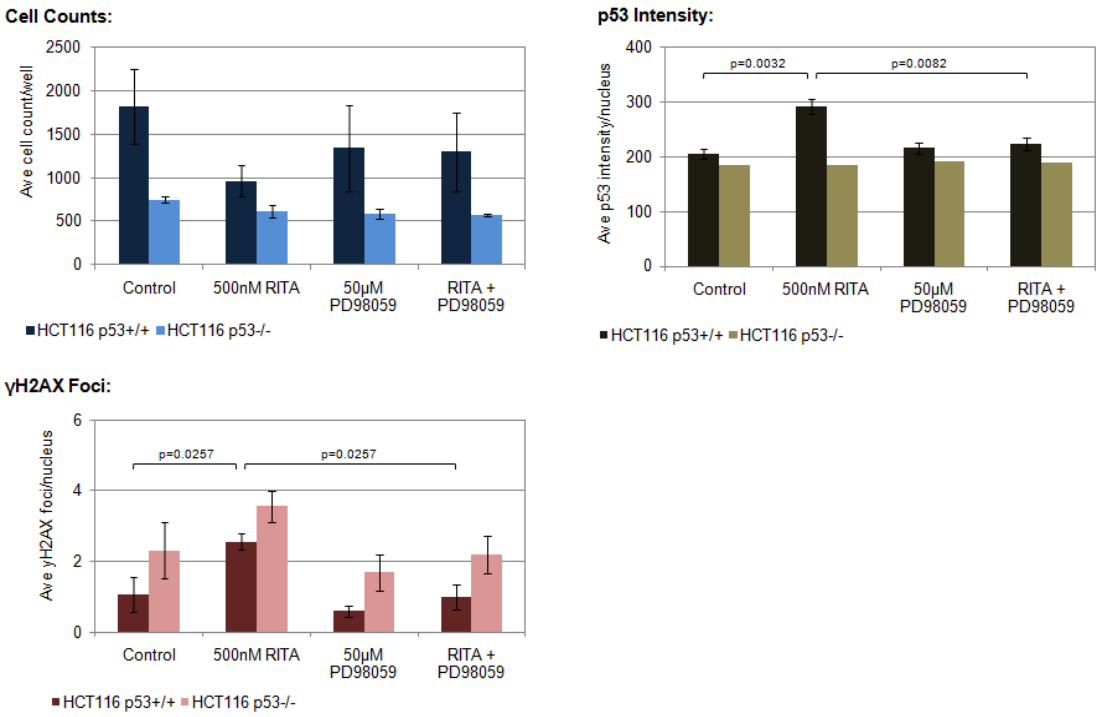


Figure 5.4.6: PD98059 inhibits RITA induced nuclear p53 and γH2AX

HCT116 p53+/+ and HCT116 p53-/- cells were treated with 500nM RITA for 24 hours in normoxia, either alone or in combination with 50μM PD98059. Plates were fixed and stained with immunoflourescent antibodies. DAPI staining was used to assess cell counts by visualising nuclei, p53 nuclear intensity was assessed using a total p53 antibody with an Alexa Fluor 488 conjugated secondary antibody, and γH2AX foci were assessed using a γH2AX antibody (serine 139) with an Alexa Fluor 568 conjugated secondary antibody. Images were captured over 10 fields of view for each duplicate well at 20x magnification and quantified using INCell 1000 Software. Data has been averaged from 3 independent experiments. An unpaired t-test was used to compare statistical significance and a p-value of <0.05 was considered significant.

PD98059 not only inhibits cell death induced by RITA, but also affects the DNA damage response, as shown by inhibition of γH2AX. On this basis, it was therefore hypothesised that PD98059 may also inhibit RITA induced DNA damage. To investigate effects of PD98059 on DNA damage, HCT116 p53+/+ that had been treated with RITA in the absence or presence of PD98059 were assessed by comet analysis. Importantly, PD98059 alone had no significant effect on DNA damage, however, RITA induced DNA damage was inhibited by PD98059 (Figure 5.4.7). The MEK-ERK pathway is therefore an important upstream mediator of RITA-induced DNA damage.

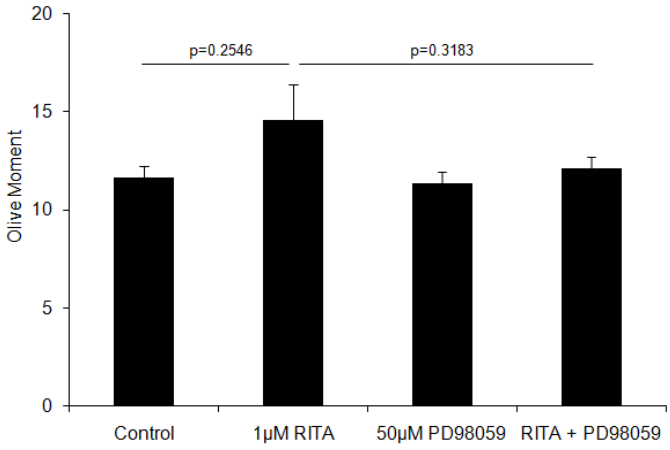


Figure 5.4.7: PD98059 inhibits RITA induced DNA damage

Graph shows comet assay data from HCT116 p53+/+ cells treated with RITA alone, or in combination with PD98059. Following treatment, cells were harvested, counted and mixed with agarose for electrophoresis. Comets (cell nuclei) were visualised using SYBR Green at 10x magnification. Images were captured over 20 fields of view and approximately 100 comets were quantified for each treatment using the TriTek™ comet score analysis software. Olive moment was used as a measure of DNA damage. Data has been averaged from 2 independent repeat experiments.

5.5 PD98059 inhibits cell cycle responses in RITA treated cells

Flow cytometric analysis has previously been used to show that RITA induces p53-dependent cell death. Interestingly, although cell death was not observed in HCT116 p53-/- cells treated with RITA, preliminary experiments showed increases in the G2 population of cells treated with RITA. I hypothesised that because PD98059 inhibits RITA induced cell death in HCT116 p53+/+ cells, PD98059 could also affect cell cycle effects elicited by RITA in HCT116 p53-/- cells. To address this, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA alone or in combination with PD98059, and flow cytometric analysis was used to assess changes in cell cycle phases. Both RITA induced cell death in HCT116 p53+/+ cells and RITA induced effects on the G2 population in HCT116 p53-/- cells were inhibited by PD98059 (Figure 5.5.1).

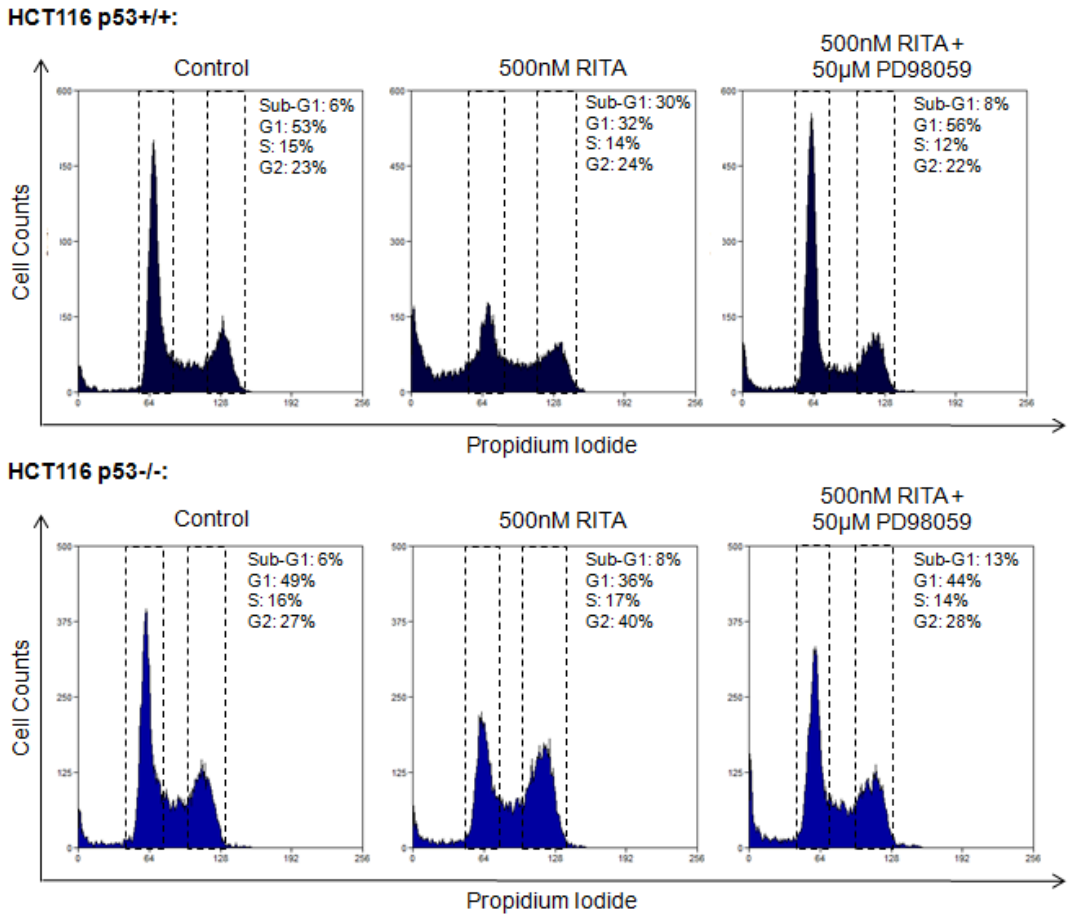


Figure 5.5.1: RITA induced cell cycle effects are inhibited by PD98059 in HCT116 p53+/+ and HCT116 p53-/- cells

HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA with or without 50μM PD98059 for 24 hours in normoxia and harvested for flow cytometry. Profiles shown are of DNA content stained using propidium iodide. Cell counts are represented on the y-axis, and propidium iodide fluorescent intensity on the x-axis. DNA profiles for HCT116 p53+/+ cells are shown in the panel above, and DNA profiles for HCT116 p53-/- treated cells are shown below. Each DNA profile was gated for cell cycle phase (indicated by dashed lines) and the percentage of cells in each phase was calculated. Profiles and quantifications shown are representative of 3 independent experiments.

To quantify HCT116 p53+/+ and HCT116 p53-/- cells treated with RITA and PD98059, propidium iodide flow cytometry profiles were gated and the percentage of cells in each cell cycle phase was calculated. As shown in Figure 5.5.2, cell death was induced by RITA in HCT116 p53+/+ cells (indicated by an increase in the sub-G1 population), and was inhibited in cells treated with RITA and PD98059 (Figure 5.5.2A). Effects on sub-

G1 were not observed in HCT116 p53-/- cells. Both HCT116 p53+/+ and HCT116 p53-/- cells showed decreases in the G1 population upon RITA treatment, which was inhibited by PD98059 (Figure 5.5.2B). Changes in S-phase cells were not observed for both HCT116 p53+/+ and HCT116 p53-/- populations (Figure 5.5.2C). In agreement with Figure 5.5.1, a G2 arrest was only induced in HCT116 p53-/- cells treated with RITA and inhibited in the presence of PD98059 (Figure 5.5.2D). The data shown indicate that MEK-ERK signalling is required for p53-dependent cell death in HCT116 p53+/+ cells, and also has an important role to play in eliciting cell cycle arrest responses in HCT116 p53-/- cells treated with RITA.

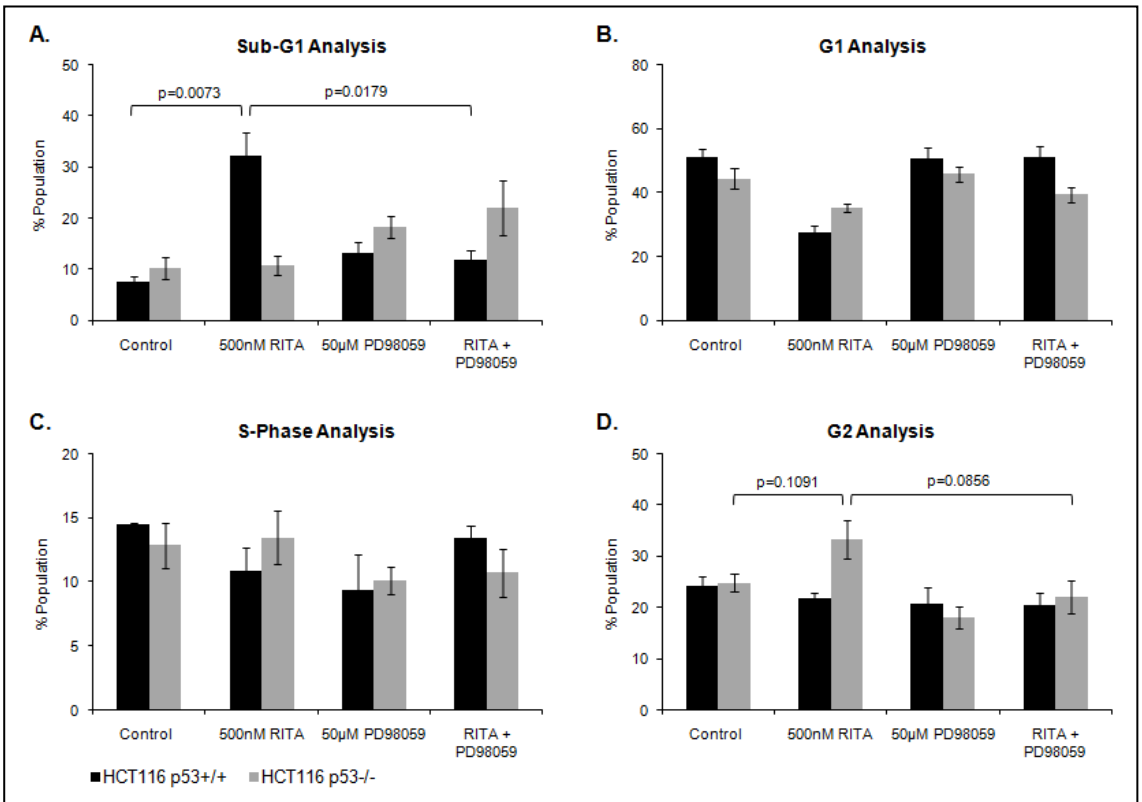


Figure 5.5.2: PD98059 blocks cell cycle effects induced by RITA in HCT116 p53+/+ and HCT116 p53-/- cells

Graphs show HCT116 p53+/+ (black bars) and HCT116 p53-/- (grey bars) cells treated with 500nM RITA with or without 50μM PD98059 for 24 hours in normoxia. Cells were harvested for flow cytometry and stained using propidium iodide to analyse DNA content. DNA profiles for each treatment were gated according to each cell cycle phase and the percentage of cells in each phase was quantified. Bar charts show the percentage of cells in each cell cycle phase: (A) sub-G1, (B) G1, (C) S-phase, (D) G2 analysis. Data shown has been averaged from 3 independent experiments.

In Chapter 4 cell cycle checkpoints elicited by RITA were investigated and affects of RITA on the replication fork machinery, and on S-phase progression were described. Significant changes in the percentage of HCT116 p53+/+ cells in S-phase that express p53, γH2AX, and phosphorylated CHK1 were also observed (Figures 4.4.3 and 4.5.4). It was concluded that p53-dependent cell cycle checkpoints induced by RITA are involved in signalling DNA damage and maintaining genomic integrity during early responses to stress. So far, MEK-ERK inhibition by PD98059 has been shown to inhibit cell cycle responses in HCT116 p53+/+, and HCT116 p53-/- cells. Therefore PD98059 may also affect DNA damage responses that are observed during S-phase in RITA treated cells.

To address this, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA and PD98059, and assessed for changes in p53 and γH2AX by flow cytometric analysis. Both p53 and γH2AX induction in S-phase cells treated with RITA were inhibited by PD98059 (Figure 5.5.3). In agreement with previous conclusions from chapter 4 whereby RITA induced DNA damage responses are p53-dependent, changes in p53 and γH2AX induction following RITA and PD98059 treatment were not observed in HCT116 p53-/- cells. Overall the data presented suggest that RITA can affect the cell cycle in both HCT116 p53+/+ and HCT116 p53-/- cells and these outcomes are modulated by MEK-ERK signalling.

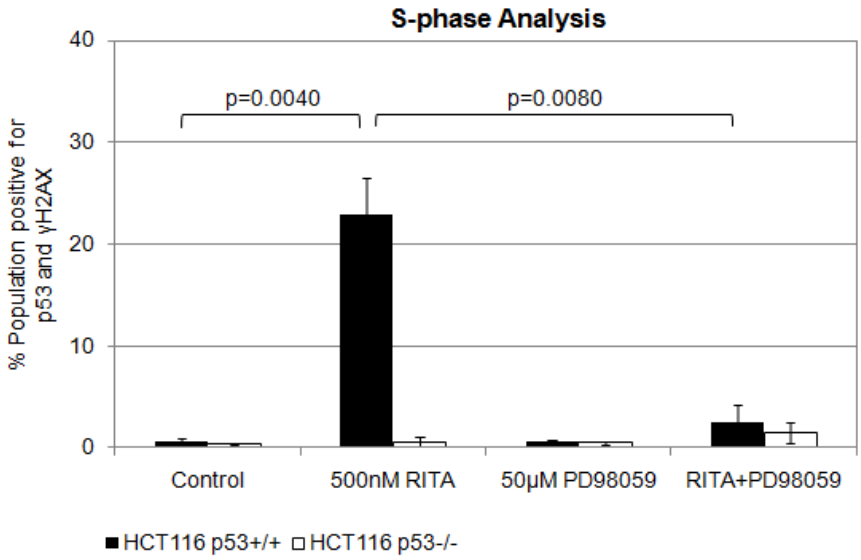


Figure 5.5.3: PD98059 inhibits RITA induced p53 and γH2AX in the S-phase population of HCT116 p53+/+

Graph shows HCT116 p53+/+ (black bars) and HCT116 p53-/- cells (white bars) treated with 500nM RITA with or without 50μM PD98059 for 24 hours in normoxia. Cells were harvested for flow cytometry and stained for propidium iodide to visualise DNA content, total p53 and γH2AX. DNA cell cycle profiles for each treatment were gated for S-phase and the percentage of cells in each S-phase population that were positive for both p53 and γH2AX was quantified. Data shown has been averaged from 3 repeat experiments. An unpaired t-test was used to assess statistical significance and a p-value <0.05 was considered significant.

Finally, because PD98059 inhibited RITA mediated responses in both HCT116 p53+/+ and HCT116 p53-/- cells, I hypothesised that phosphorylation of ERK1/2 by RITA is not dependent on p53 status. To assess ERK1/2 phosphorylation in HCT116 p53+/+, and HCT116 p53-/- cells following RITA treatment, western analysis was used and the induction of ERK1/2 phosphorylation by RITA was found in both HCT116 p53+/+ and HCT116 p53-/- cells (Figure 5.5.4).

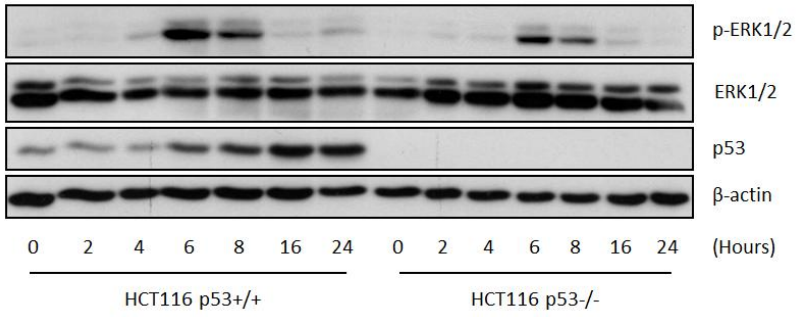


Figure 5.5.4: ERK1/2 phosphorylation induced by RITA is not p53-dependent
HCT116 p53+/+ and HCT116 p53-/- cells were treated with 500nM RITA over a timecourse in normoxia and harvested for western blot analysis. Western blots were analysed for phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, p53, and β-actin as a load control. Data shown is representative of 3 independent repeat experiments.

5.6 PD184352 does not inhibit RITA induced cell death

Studies using PD98059 have shown that MEK-ERK signalling is important for regulating cell cycle responses and cell death in response to RITA. Next, I aimed to assess whether other MAPK inhibitors have similar effects to PD98059 in cells that have been treated with RITA. PD184352 [2-(-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluoro-benzamide], a potent inhibitor of MEK1 with an IC_{50} of 300nM *in vitro* was investigated (Sebolt-Leopold et al., 1999). Compared to PD98059, PD184352 was shown to be more potent, and more selective for MEK1 inhibition (Sebolt-Leopold et al., 1999). Therefore I assessed whether PD184352, like PD98059, could also inhibit RITA induced cell death.

To confirm that the concentrations of PD98059 and PD184352 used inhibited ERK1/2 phosphorylation, HCT116 p53+/+ cells and HCT116 p53-/- cells were treated with 50μM PD98059, and 2μM PD184352, doses at which both inhibitors have been shown to inhibit MEK1/2 phosphorylation. Western analysis confirmed that both 50μM PD98059 and 2μM PD184352 are sufficient to block ERK1/2 phosphorylation (Figure 5.6.1)

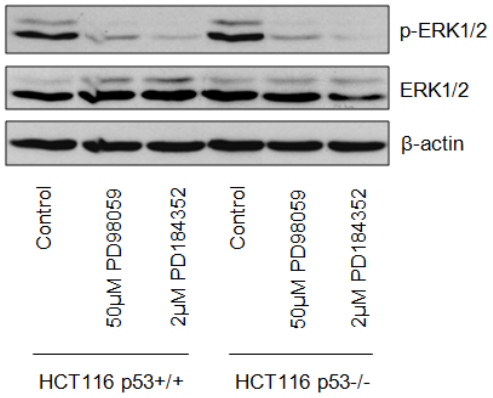


Figure 5.6.1: PD98059 and PD184352 inhibit ERK1/2 phosphorylation
HCT116 p53+/+ and HCT116 p53-/- cells were treated with either 50μM PD98059 or 2μM PD184352 for 24 hours and harvested for western blot analysis. Western blot analysis shows phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, and β-actin as a loading control. Western blots are representative of 3 independent repeat experiments.

Flow cytometric analysis was used to assess the effects of PD184352 on RITA induced cell death. Treatment of HCT116 p53+/+ cells with either PD98059 or PD184352 did not affect the cell cycle (Figure 5.6.2A). Interestingly, unlike PD98059, PD184352 did not inhibit RITA induced cell death in HCT116 p53+/+ cells, as indicated by analysing sub-G1 cells (Figure 5.6.2B). These observations are consistent with previous studies from our laboratory that have found PD184352 to have no significant affects on RITA mediated responses (Yang, unpublished).

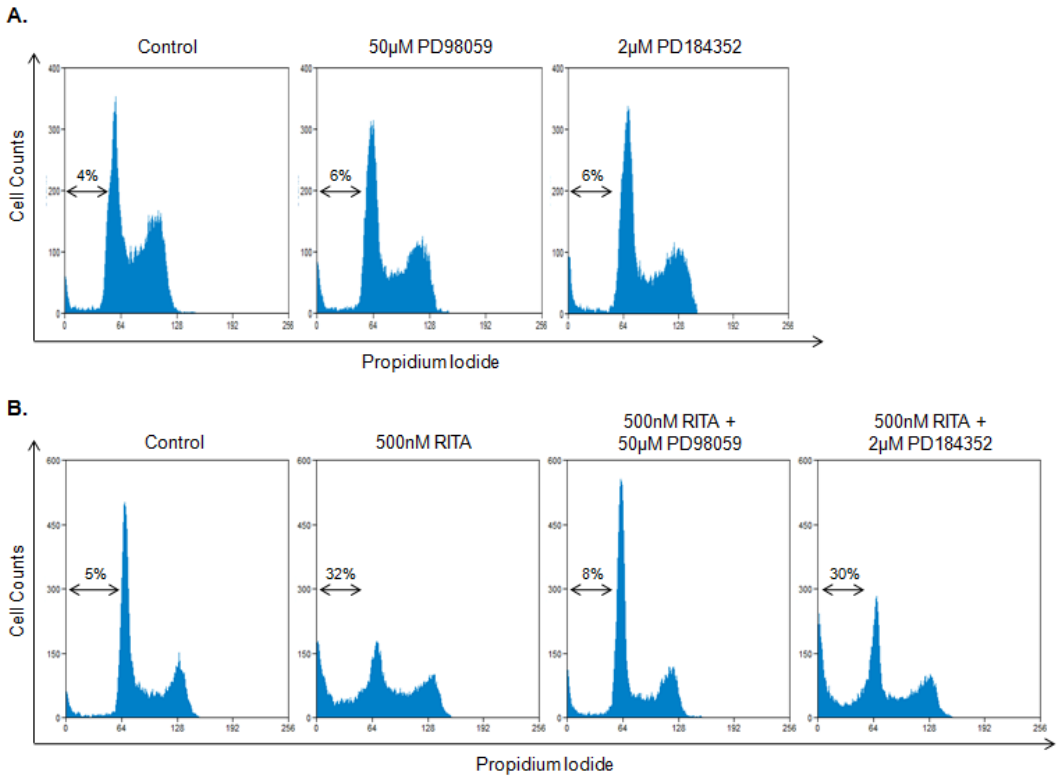


Figure 5.6.2: PD184352 does not affect RITA induced cell death

Flow cytometry analysis of: (A) HCT116 p53+/+ cells treated with DMSO control, 50μM PD98059, and 2μM PD184352, (B) HCT116 p53+/+ treated with 500nM RITA alone or in combination with either 50μM PD98059 or 2μM PD184352. Following 24 hour treatment, cells were stained for propidium iodide and analysed for DNA content by flow cytometry. Graphs show cell count against propidium iodide fluorescent intensity and have been gated for sub-G1 as an indicator of cell death. The percentage of cells in each treated population that are in sub-G1 is shown. Graphs are representative of 3 independent experiments.

To continue assessing whether cell death induced by RITA is affected by MEK1/2 inhibition in response to PD98059 and PD184352 treatment, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA alone or in combination with either PD98059 or PD184352. DNA profiles from flow cytometry were quantified for sub-G1 as an indication of cell death (Figure 5.6.3). As anticipated, RITA treatment increased the percentage of HCT116 p53+/+ cells in sub-G1 to greater than 30% and this increase was not observed in HCT116 p53+/+ cells that had been treated with RITA in combination with PD98509. When HCT116 p53+/+ cells were treated with RITA and PD184352, the sub-G1 population increased to greater than 40%. There was little effect on sub-G1 when HCT116 p53+/+ cells were treated with PD98059 or PD184352

alone. As expected, the sub-G1 population of HCT116 p53-/- cells was not affected to the same extent as in HCT116 p53+/+ cells. In summary, PD98059 but not PD184352 blocks RITA induced cell death in HCT116 p53+/+ cells.

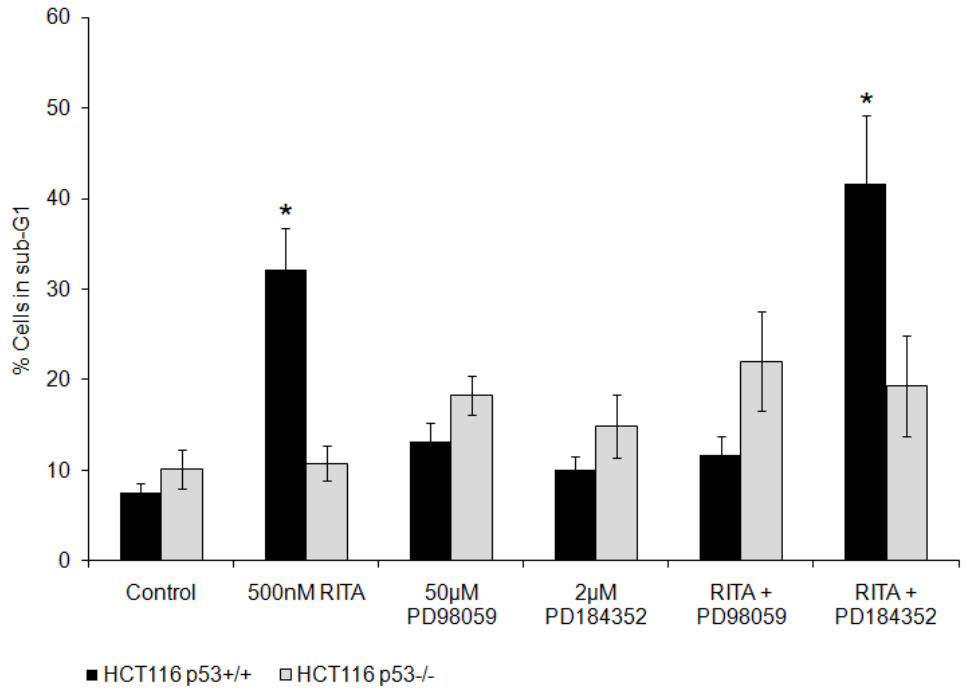


Figure 5.6.3: PD184352 does not inhibit RITA induced sub-G1 in HCT116 p53+/+ cells
Graph shows HCT116 p53+/+ (black bars) and HCT116 p53-/- (grey bars) treated with 500nM RITA for 24 hours alone or in combination with either 50μM PD98059 or 2μM PD184352. Following treatment, cells were stained for propidium iodide and cell cycle DNA profiles were analysed by flow cytometry. DNA profiles were gated and quantified for sub-G1. The percentage of cells in sub-G1 is plotted against each population of treated cells. Data shown has been averaged from 3 independent experiments. An unpaired t-test was used to assess statistical significance between control and treated populations and a p-value of <0.05 was considered significant (indicated by *)

Previous data have shown that PD98059 does not only inhibit p53 stabilisation, and cell death by RITA, but PD98059 also affects γH2AX induction in S-phase cells, and DNA damage (Figure 5.6.3). To investigate whether PD184352 had similar effects to PD98059 in mediating RITA induced DNA damage responses, flow cytometry was used to assess p53 and γH2AX induction in HCT116 p53+/+ and HCT116 p53-/- cells treated with RITA and either PD98059 or PD184352. PD184352 did not inhibit RITA-induced p53 and γH2AX in S-phase cells (Figure 5.6.4) suggesting that because

PD184352 has greater selectivity for MEK1/2 inhibition compared to PD98059, a non-specific MAPK may be inhibited by PD98059 that mediates induction of p53-dependent responses by RITA.

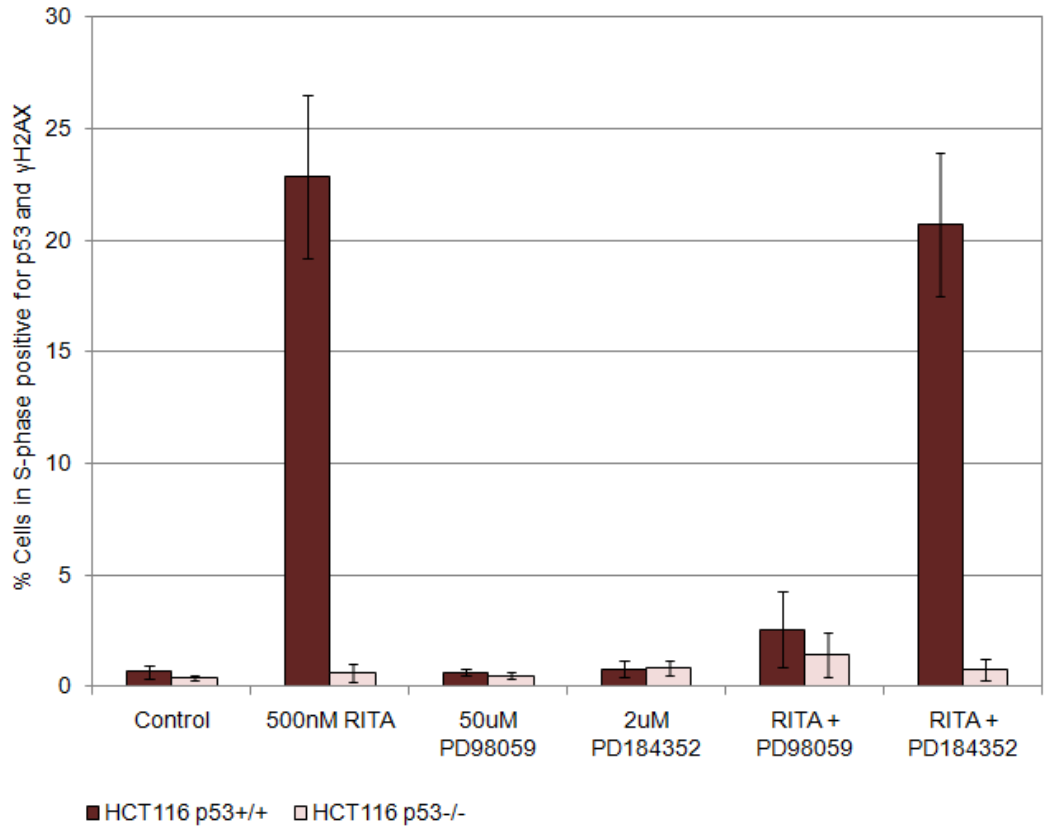


Figure 5.6.4: PD184352 does not inhibit RITA induced p53 and γH2AX in S-phase cells
HCT116 p53+/+ cells were treated with 500nM RITA for 24 hours alone or in combination with either PD98059 or PD184352. Following treatment, cells were fixed for flow cytometric analysis and stained using propidium iodide to assess DNA cell cycle profiles, total p53 protein (using a secondary FITC conjugated antibody), and γH2AX (using a secondary APC conjugated antibody). Cell cycle profiles were gated for the S-phase population, and the percentage of cells in S-phase that stained positive for p53 and γH2AX was quantified. Graph shows percentage of cells in S-phase from each treated population that have a positive shift in both p53 (viewed by a shift in FITC fluorescent intensity), and in γH2AX (viewed by a shift in APC fluorescent intensity) compared to the control S-phase population. Data shown has been averaged from 3 independent experiments.

PD98059 was used at a final concentration of 50μM for all studies so far because this is the screening concentration that has been used to achieve greater than 50% inhibition of MEK1/2 and also assess the specificity profile of PD98059 compared to other MAPK inhibitors (Davies et al., 2000). Previously, 50μM PD98059 was shown to inhibit ERK1/2 protein phosphorylation (Figure 5.6.1). However, 50μM PD98059 may also have several off target effects compared to lower concentrations. Therefore, studies were repeated with the PD98059 IC_{50} dose for MEK1 inhibition of 10μM (Dudley et al., 1995) and effects were compared with 50μM PD98059 used previously. As shown in Figure 5.6.5, 10μM PD98059 also suppressed RITA induced ERK1/2 phosphorylation, p53 induction, and cell death, as indicated by cleaved PARP.

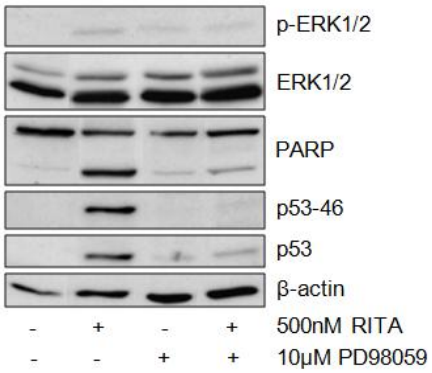


Figure 5.6.5: 10μM PD98059 inhibits RITA induced ERK1/2 activation, cleaved PARP and p53 induction

HCT116 p53+/+ cells were treated with 500nM RITA alone or in combination with 10μM PD98059 for 16 hours in normoxia. Cells were harvested for western blot analysis and analysed for phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, cleaved PARP, phosphorylated p53 at serine 46 (p53-46), and total p53 protein. β-actin was used as a loading control. Western data shown is representative of 3 independent repeat experiments.

In Figure 5.4.3 50μM PD98059 was shown to inhibit RITA induced DNA damage responses involving γH2AX. As with 50μM PD98059, 10μM PD98059 was found to have inhibitory effects on RITA induced cell death. Therefore, I hypothesised that 10μM PD98059 would also inhibit RITA induced DNA damage responses. To address this HCT116 p53+/+ cells were treated with RITA alone or in combination with 10μM PD98059, and changes in γH2AX and phosphorylated CHK1 and CHK2 proteins was assessed by western analysis. DNA damage responses involving induction of γH2AX

and phosphorylation of both CHK1 and CHK2 were found to be inhibited by 10μM PD98059 (Figure 5.6.6).

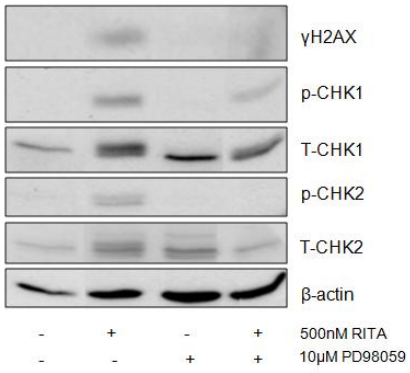


Figure 5.6.6: 10μM PD98059 inhibits RITA induced DNA damage responses
HCT116 p53+/+ cells were treated with 500nM RITA alone or in combination with 10μM PD98059 for 24 hours in normoxia. Cells were harvested for western blotting and analysed for γH2AX, phosphorylated CHK1 (p-CHK1), total CHK1 (T-CHK1), phosphorylated CHK2 (p-CHK2), and total CHK2 (T-CHK2), β-actin was used as a loading control. Western data is representative of 3 independent repeat experiments.

To assess effects of 10μM PD98059 on the cell cycle, HCT116 p53+/+ cells were treated with RITA in combination with 10μM PD98059, and analysed for DNA content by flow cytometry. In agreement with Figure 5.6.2, RITA induced sub-G1 cells were inhibited by 10μM PD98059 in HCT116 p53+/+ (Figure 5.6.7).

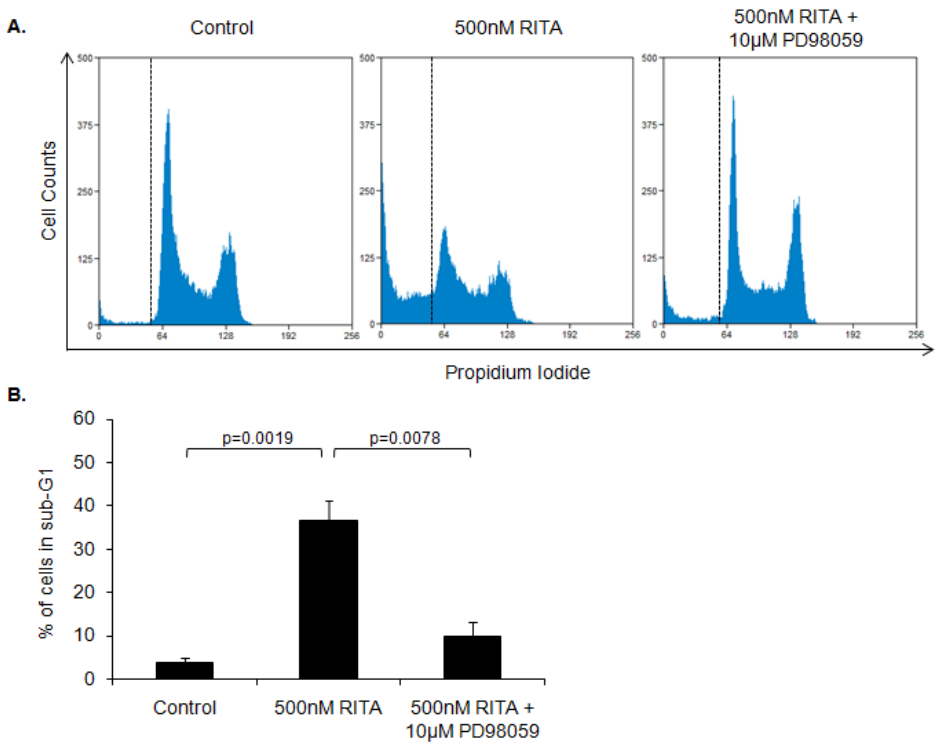


Figure 5.6.7: 10μM PD98059 inhibits RITA induced cell death

HCT116 p53+/+ cells were treated with 500nM RITA alone, or in combination with 10μM PD98059 for 24 hours in normoxia. Following treatment, cells were harvested and fixed for flow cytometry analysis. Propidium iodide was used to stain for DNA content: (A) DNA cell cycle profiles are shown with cell counts displayed on the y-axis against propidium iodide intensity on the x-axis. Profiles were gated for sub-G1 content to indicate cell death (shown by dashed line) and quantified. DNA profiles are representative of 3 independent experiments, (B) Graph shows percentage of cells in each treated population that are in sub-G1. Quantified data has been averaged from 3 repeat experiments. A t-test was used to assess statistical significance and a p-value of <0.05 was considered significant.

INCell analyses was previously used to quantify and assess p53 protein and γH2AX localisation in single cells (Figure 5.3.6). I continued to use the INCell screening platform to assess changes in nuclear p53 and γH2AX protein levels following treatment of HCT116 p53+/+ cells with RITA and either PD98059 or PD184352. As shown in Figure 5.6.8, both 10μM and 50μM PD98059 inhibited loss of cell number induced by RITA and this was not observed in the presence of PD184352 indicating that PD184352 does not affect RITA induced cell death (Figure 5.6.8A). Similarly, unlike PD98059, PD184352 did not inhibit RITA induced nuclear p53 (Figure 5.6.8B) and γH2AX (Figure 5.6.8C).

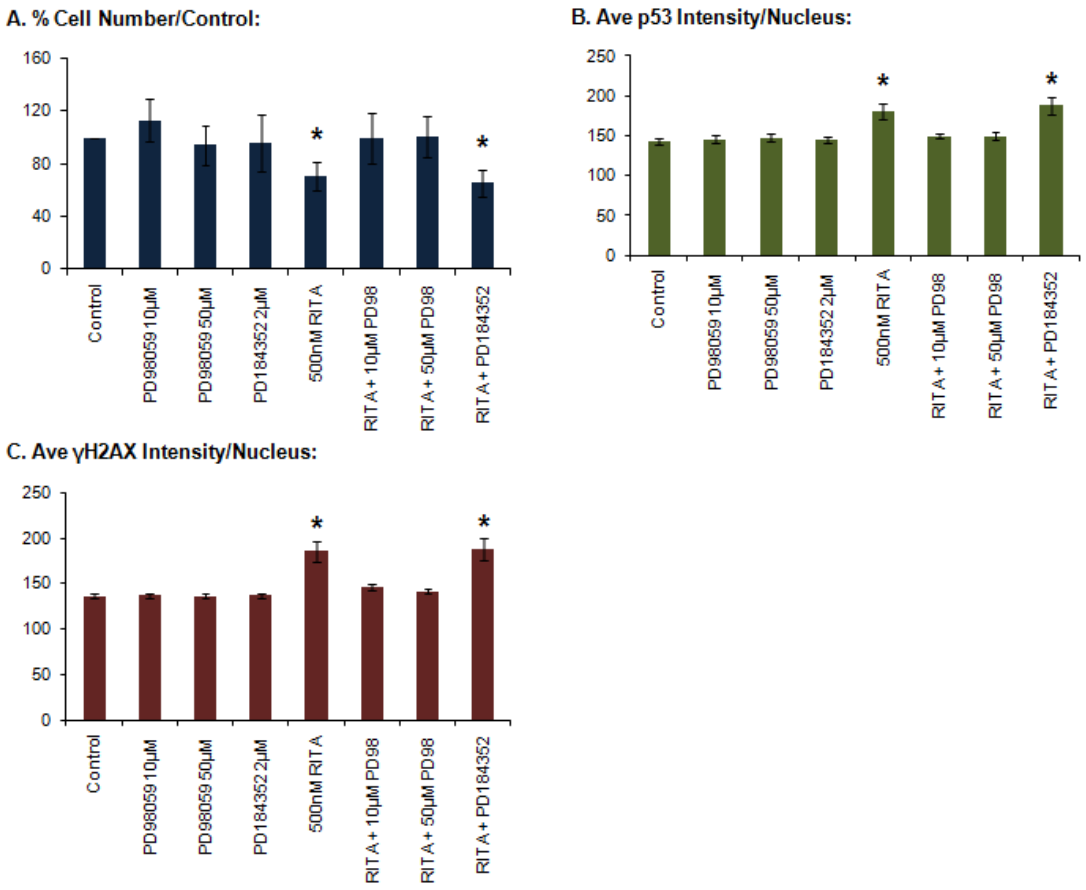


Figure 5.6.8: Unlike PD184352, PD98059 inhibits cell death, p53 and γH2AX nuclear intensity in RITA treated cells

HCT116 p53+/+ cells were treated with 500nM RITA alone, or in combination with 10µM, 50µM PD98059 or 2µM PD184352, either alone or in combination. Cells were dosed for 24 hours in normoxia, fixed and stained using DAPI (to indicate nuclei), total p53 antibody (with an Alexa Fluor 488 conjugated secondary antibody), and γH2AX (with an Alexa Fluor 568 conjugated secondary antibody). Cells were visualised using the INCell 1000 analyser at 20x magnification and images over 10 fields of view were captured for each well. INCell software was used to quantify images for cell number, p53 nuclear intensity, and γH2AX nuclear intensity. Graphs show: (A) % cell number/well, (B) average p53 intensity/nucleus, and (C) average γH2AX intensity/nucleus. All data shown has been averaged from 3 independent experiments. A t-test was used to compare the means of control and treated cells and a p-value of <0.05 was considered statistically significant, as indicated by *.

Nutlin-3 is a small molecule activator of wildtype p53 and stabilises p53 by binding to HDM2, but unlike RITA does not activate a DNA damage response. I hypothesised that unlike RITA, p53 stabilisation by nutlin-3 would not be affected by PD98059. To address this, HCT116 p53+/+ cells were treated with nutlin-3 alone, or in combination with either PD98059 or PD184352, and assessed for changes in p53 and γH2AX nuclear intensity using the INCell analyser. As shown in Figure 5.6.9, neither PD98059 nor PD184352 inhibited loss of cell number (Figure 5.6.9A) and induction of p53 nuclear intensity (Figure 5.6.9B) in response to nutlin-3. As anticipated for the mechanism of action for nutlin-3, no significant changes were observed in γH2AX nuclear intensity following treatment with nutlin-3 and PD98059, or PD184352 (Figure 5.6.9C). Therefore, in conclusion MEK-ERK signalling does not affect nutlin-3 responses.

In chapter 3, p53 induction and cell death in response to RITA was compared to other DNA damaging agents that activate p53. RITA was found to be the only agent used that induced significant cell death in HCT116 p53+/+ cells, both in normoxia and in hypoxia. Studies have shown that cisplatin induced apoptosis can be inhibited with PD98059 (Persons et al., 2000). Like cisplatin, etoposide is a common DNA damaging agent that mediates its cellular effects through p53 activation. I hypothesised that like RITA, etoposide-induced p53 activation and cell death responses may also be affected by MEK-ERK inhibition.

To address this, INCell analysis was used to assess the effects of PD98059 and PD184352 in HCT116 p53+/+ cells that had been treated with etoposide. As shown in Figure 5.6.10, cell number (Figure 5.6.10A), p53 nuclear intensity (Figure 5.6.10B) and γH2AX nuclear intensity (Figure 5.6.10C) induced by etoposide was not affected by either PD98059 or PD184352. The mechanism by which RITA induces p53 and cell death responses is therefore distinct compared to nutlin-3, etoposide and other p53 activating agents.

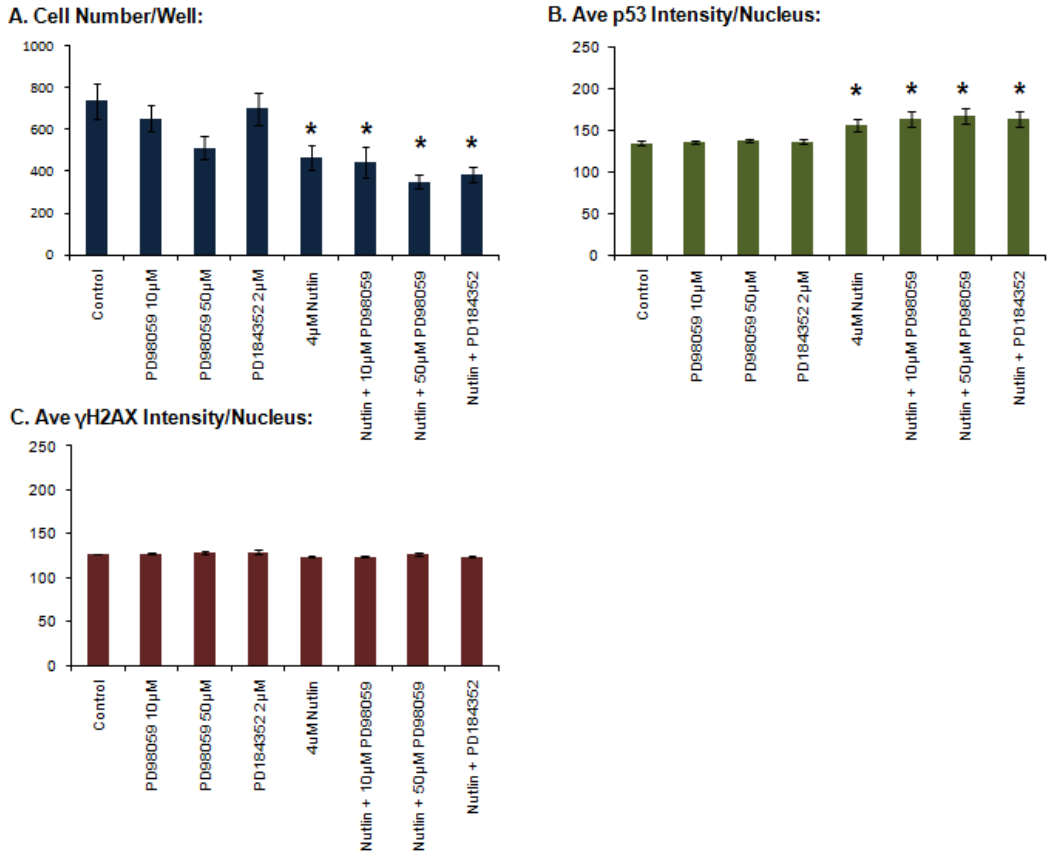


Figure 5.6.9: PD98059 and PD184352 do not affect p53 induced by nutlin-3

HCT116 p53+/+ cells were treated with 4µM nutlin-3 alone, or in combination with 10µM, 50µM PD98059 or 2µM PD184352. Cells were dosed for 24 hours in normoxia, fixed and stained for DAPI, total p53 protein with an Alexa Fluor 488 conjugated secondary antibody, and γH2AX with an Alexa Fluor 568 conjugated secondary antibody. Cells were visualised on the INCell 1000 analyser at 20x magnification and images were captured over 10 fields of view for each treated well. INCell software was used for quantification. Graphs show (A) Cell number/well, (B) Ave p53 intensity/nucleus, (C) Ave γH2AX intensity/nucleus. All data has been averaged from 3 independent experiments. A t-test was used to assess statistical significance between control and treated samples and a p-value of <0.05 was considered significant, as indicated by *.

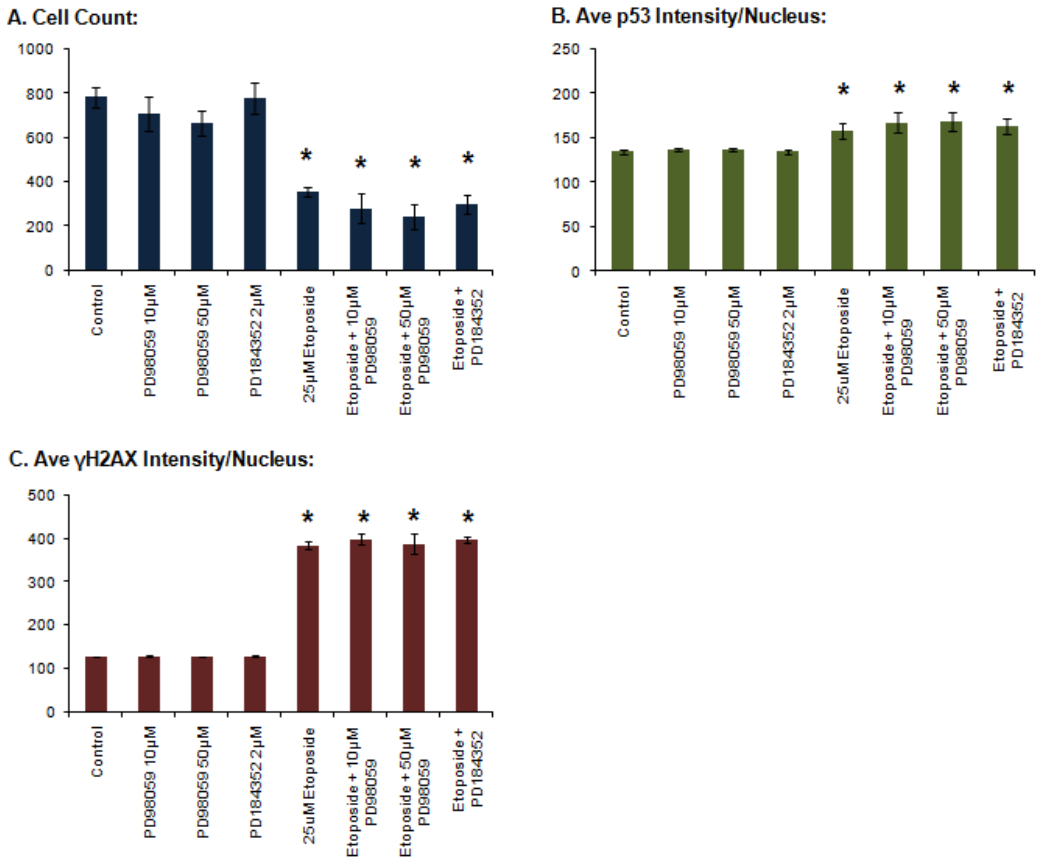


Figure 5.6.10: PD98059 and PD184352 do not affect nuclear p53 and γH2AX induced in response to etoposide

HCT116 p53+/+ cells were treated with 25µM etoposide alone, or in combination with either 10µM, 50µM PD98059 or 2µM PD184352. Cells were dosed for 24 hours in normoxia, fixed and stained using DAPI, p53 with an Alexa Fluor 488 conjugated secondary antibody, and phosphorylated γH2AX with an Alexa Fluor 568 conjugated antibody. Following staining, cells were visualised on the INCell 1000 analyser and images were captured over 10 fields of view for each well at 20x magnification. INCell software was used for quantification. Graphs show: (A) cell number, (B) average p53 intensity/nucleus and (C) average phosphorylated γH2AX intensity/nucleus. Data has been averaged from 3 independent experiments. A t-test was used to assess statistical significance between control and treated samples and a p-value of <0.05 was considered significant, as indicated by *.

In earlier experiments (Figures 5.4.2-7) the effects of PD98059 on RITA treated cells were investigated at 24 hour timepoints. However, DNA damage responses and cell death induced by RITA are both dose, and time-dependent. To assess the effects of PD98059 on RITA responses over a timecourse, HCT116 p53+/+ cells were treated with RITA alone or in combination with 10μM PD98059. Changes in sub-G1 and S-phase cells were assessed over 24 hours using flow cytometry. RITA induced sub-G1 was inhibited when HCT116 p53+/+ cells were treated with RITA in the presence of 10μM PD980059, over a timecourse (Figure 5.6.11A), as was p53 and γH2AX induction in S-phase cells (Figure 5.6.11B).

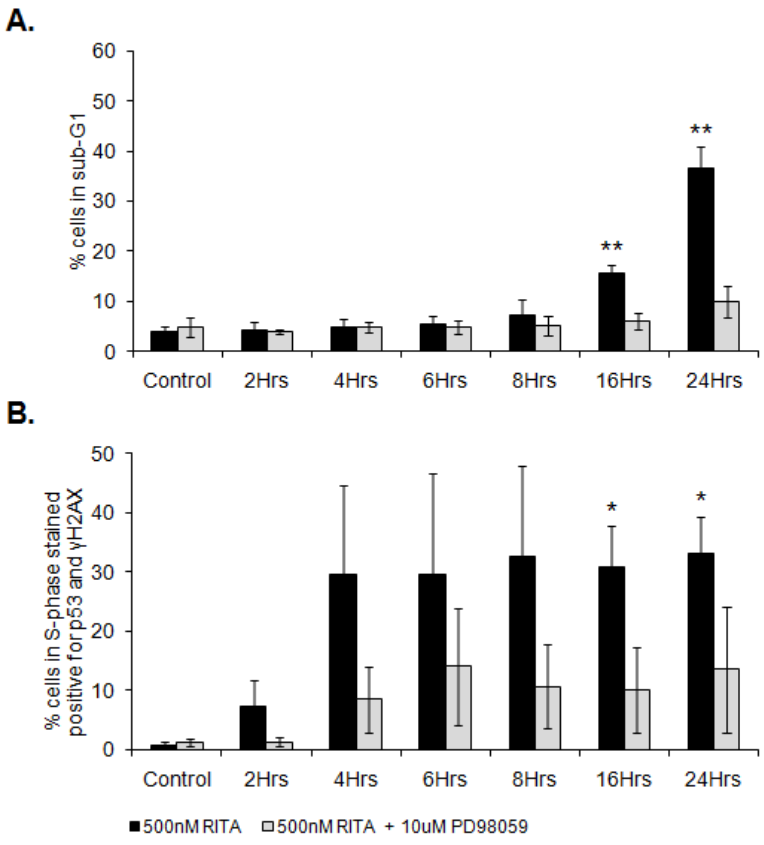


Figure 5.6.11: RITA induced sub-G1, and induction of p53 and γH2AX during S-phase is inhibited by 10μM PD98059

HCT116 p53+/+ cells were treated with 500nM RITA alone or in combination with 10μM PD98059 in normoxia. Cells were harvested for flow cytometric analysis over a 24 hour timecourse, fixed, and stained for propidium iodide, total p53 (using a FITC conjugated secondary antibody), and γH2AX (using an APC conjugated secondary antibody). DNA cell cycle profiles were gated and quantified for sub-G1 and S-phase. Graphs show: (A) sub-G1 analysis of RITA treated cells (black bars) and RITA treated cells in combination with PD98059 (grey bars), and (B) analysis of the percentage of cells in S-phase that stain positive for p53 and γH2AX, as before RITA treated cells (black bars), and RITA treated cells in combination with PD98059 (grey bars) are shown over a 24 hour timecourse. All data has been averaged from 3 independent experiments. An unpaired t-test was used to compare the mean of DMSO controls against RITA treated samples, and a p-value of less than 0.05 was considered statistically significant (as indicated **<0.005, *<0.05).

Several studies have addressed differences in specificity between PD98059 and PD184352. In 2000, Davies et al. screened a range of commonly used protein kinase inhibitors against a large panel of protein kinases to assess their target profiles.

Although both PD98059 and PD184352 inhibited ERK1/2 phosphorylation, PD98059 also inhibited numerous other MAPKs. This was not observed with PD184352 which was concluded to be the most specific MEK1/2 inhibitor in the screen (Davies et al., 2000). Later studies showed that the ERK5/BMK1 pathway was inhibited by PD98059 while remaining unaffected by PD184352, and suggested that the anti-proliferative and anti-tumour effects mediated by PD184352 were dependent on ERK1/2 signalling alone (Squires et al., 2002).

Also of interest were studies showing that PD184352 could only inhibit HIF-1 activity in response to growth factor stimulation, while PD98059 did so in response to hypoxia and growth factors (Sutton et al., 2007). Therefore, ERK1/2 signalling was concluded to be important in regulating HIF activation in response to growth factors, while HIF-1 activity in response to hypoxic stress was suggested to be regulated by additional MAPK signalling pathways (Sutton et al., 2007). Interestingly, PD98059 has also been shown to inhibit pathways that are not involved in MAPK signalling. For example, PD98059 targets cyclo-oxygenase activity directly leading to inhibition of arachidonic acid metabolism, decreased platelet aggregation and significant anti-inflammatory effects (Borsch-Haubold et al., 1998).

Many MAPK signalling pathways are induced by genotoxic stress to regulate cell death responses. To investigate likely MAPKs that are targeted by PD98059, and could be involved in activating p53-dependent cell death by RITA, a candidate kinase approach was used. I investigated MAPKs that are known to be involved in p53-dependent apoptosis in response to genotoxic stress to assess whether they are important in eliciting cell death by RITA.

5.7 The p38 MAPK is phosphorylated by RITA and targeted by PD98059

The Jun N-terminal kinase (JNK) and the p38 stress activated protein kinase are members of the MAPK family. Pathways regulated by JNK and p38 are activated by a number of environmental and genotoxic stimuli, and are involved in critical cell survival, and inflammatory responses, as well as those involved in proliferation and differentiation (Wagner and Nebreda, 2009). JNK and p38 signalling pathways are often deregulated in many cancers and have been shown to correlate with poor patient survival (Demuth et al., 2007). The p38 kinase is important in mediating tumour suppression by inhibiting proliferation, and activating apoptosis in response to cellular stress (Brancho et al., 2003; Bulavin and Fornace, 2004). Mechanisms by which p38 inhibits cell growth involve regulation of cell cycle checkpoints (Thornton and Rincon,

2009), inhibitory effects on cyclin D (Lavoie et al., 1996) and CDC25 phosphatase (Manke et al., 2005), and activation of the Rb and p53 tumour suppressor pathways (Bulavin et al., 2002). The p38 MAPKs are encoded by four genes, MAPK14 encodes p38α (also known as SAPK2), MAPK11 encodes p38β (SAPK2b), MAPK12 encodes p38γ (SAPK3) and MAPK13 encodes p38δ (SAPK4), (Wagner and Nebreda, 2009). The most extensively studied p38 MAPK is p38α. Although several studies describe p38α's role as a tumour suppressor, in certain cell types, p38α has also been associated with oncogenic functions such as migration, invasion (Dolado and Nebreda, 2008), and angiogenesis (Emerling et al., 2005).

RAS induced tumourigenesis is often accompanied by activation of a number of biochemical pathways including the MAPK and PI3K pathways (Downward, 2003a). The importance of p38 signalling in tumour suppression is demonstrated in studies which show p38 to negatively regulate malignant transformation induced by oncogenic H-RAS. In response to reactive oxygen species (ros) induced by oncogenic H-RAS, p38 activation has been shown to induce apoptosis and inhibit formation of subcutaneous tumours, suggesting a role for p38 as an important sensor of oxidative stress (Dolado et al., 2007). The p38 pathway may also inhibit oncogenic mediated tumour progression by suppressing the ERK1/2 pathway (Li et al., 2003), activating premature senescence (Wang et al., 2002), cell cycle inhibitors such as $p16^{INK4a}$ (Bulavin and Fornace, 2004) and p21 (Nicke et al., 2005), and activating p53-dependent cell cycle arrest (Bulavin et al., 2002).

Of interest to this thesis, loss of p38 can inhibit apoptosis and promote cell survival following exposure to chemotherapeutic agents (Sanchez-Prieto et al., 2000). Phosphorylation of p53 at the N-terminus is important for p53 stability (Shieh et al., 1997), and p38 has been shown to localise to the nucleus, complex with p53 and phosphorylate p53 directly on serine 33 and serine 46 in response to genotoxic stress (Bulavin et al., 1999; Sanchez-Prieto et al., 2000). In addition, Cuadrado et al. have identified $p18^{HAMLET}$ as a novel p38 MAPK regulated protein that acts as a transcriptional co-activator for p53. By interacting with p53 in response to genotoxic stress induced by UV irradiation or cisplatin, $p18^{HAMLET}$ stimulates transcription of many p53 regulated pro-apoptotic genes to activate apoptosis (Cuadrado et al., 2007). Clearly, p38 signalling has an integral role to play in p53 mediated cell death responses and is important in maintaining cellular homeostasis in response to genotoxic stress and oncogenic stimuli.

I have shown that p53-dependent DNA damage and cell death responses elicited by RITA are inhibited by PD98059. Due to the role of p38 MAPK in activating p53-induced apoptosis (Bulavin and Fornace, 2004), I hypothesised that p38 could be targeted by PD98059 to inhibit RITA-induced cell death. To address this, western analysis was used to assess p38 phosphorylation in HCT116 p53+/+ and HCT116 p53-/- cells treated with RITA. RITA treatment in HCT116 p53+/+ induced p38 phosphorylation over a timecourse, and this was not observed to the same extent in HCT116 p53-/- cells (Figure 5.7.1). Induction of p38 phosphorylation also correlated with p53 phosphorylation at serine 46, a site that has been shown to be phosphorylated directly by p38 in previous studies (Bulavin et al., 1999; Sanchez-Prieto et al., 2000).

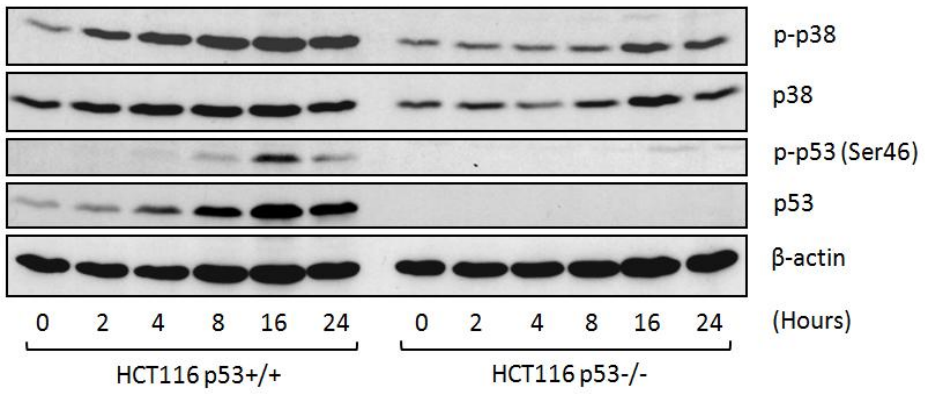


Figure 5.7.1: RITA induces p38 MAPK phosphorylation
HCT116 p53+/+ and HCT116 p53-/- cells were treated with 1μM RITA in normoxia over a 24 hour timecourse. Cells were harvested for western blotting and analysed for phosphorylated p38 (p-p38), total p38, phosphorylated p53 at serine 46 (p-p53), total p53, and β-actin as a loading control. Data shown is representative of 3 independent repeats.

To assess whether phosphorylation of p38 by RITA was inhibited by PD98059, HCT116 p53+/+ cells were treated with RITA, either alone or in combination with PD98059. PD98059 significantly inhibited ERK1/2 phosphorylation and also inhibited p38 phosphorylation in response to RITA. These data are consistent with previous studies from our laboratory showing that RITA induces p38 phosphorylation (at doses as low as 200nM) and this is blocked by PD98059 at 10μM and 50μM concentrations (Yang, unpublished). These studies suggest that p38 is a non-specific target of PD98059.

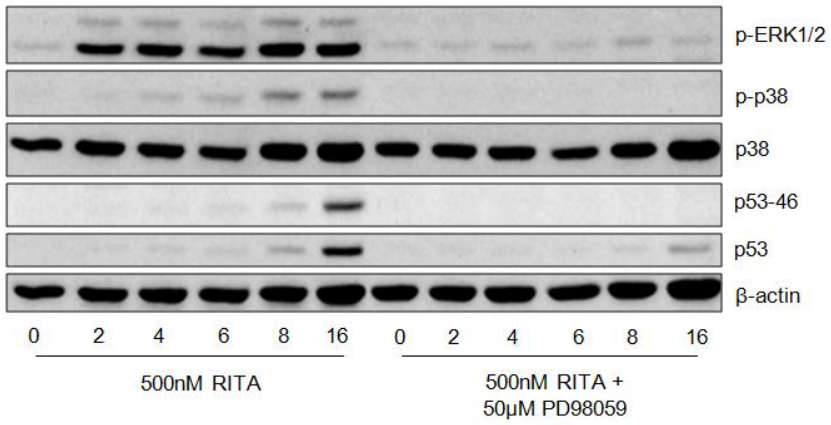


Figure 5.7.2: RITA induced phosphorylation of p38 is inhibited by PD98059
HCT116 p53+/+ cells were treated with 500nM RITA with or without 50μM PD98059 and harvested for western analysis over a 24 hour timecourse. Western blot analysis shows phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-38), total p38, phosphorylated p53 at serine 46 (p53-46), and total p53. β-actin was used as a loading control. Data shown is representative of 3 independent repeats.

Compared to PD98059, PD184352 does not affect RITA mediated DNA damage responses and cell death. To assess the effects of PD184352 on RITA induced p38 phosphorylation, HCT116 p53+/+ and HCT116 p53-/- cells were treated with RITA, either alone, or in combination with PD98059 and PD184352. Phosphorylation of p38 in response to RITA was inhibited by PD98059, and not with PD184352 (Figure 5.7.3). These findings confirm that p38 is a non-specific target of PD98059.

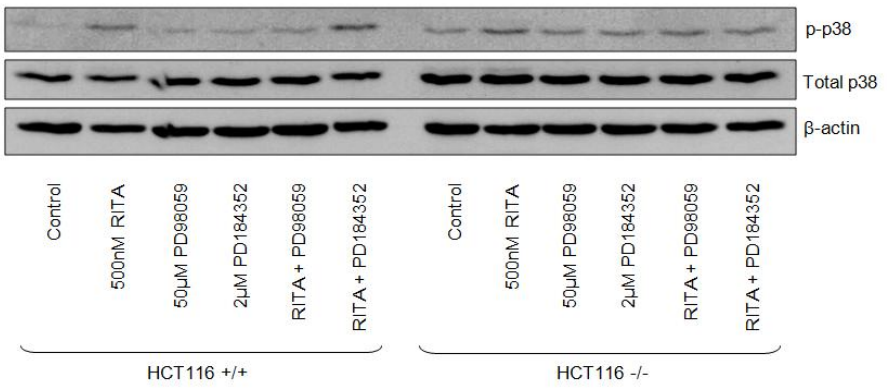


Figure 5.7.3: Phosphorylation of p38 MAPK by RITA is not affected by PD184352
Western blot analysis shows HCT116 p53+/+ and HCT116 p53-/- cells treated with 500nM RITA alone, or in combination with either 50μM PD98059, or 2μM PD184352. Cells were harvested after 24 hours and analysed by western blotting for phosphorylated p38 (p-p38), total p38, and β-actin as a loading control. Data shown is representative of 3 independent repeats.

So far, p38 phosphorylation has been demonstrated in HCT116 p53+/+ cells treated with RITA, and these responses are inhibited in the presence of PD98059. Based on data previously showing that PD98059 inhibits RITA induced cell death, I hypothesised that p38 phosphorylation is essential for eliciting cell death by RITA. To address this, p38α protein expression was blocked by siRNA in HCT116 p53+/+ cells, and RITA-induced cell death was assessed by flow cytometry. Western analysis was used to confirm that p38 was efficiently knocked down by siRNA. As shown in Figure 5.7.4, p38 was inhibited by siRNA and no significant changes were observed in RITA induced p53 or γH2AX (Figure 5.7.4)

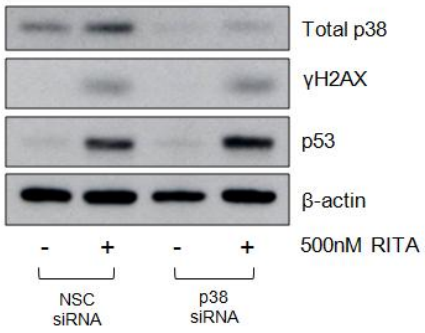


Figure 5.7.4: p38α siRNA does not inhibit RITA induced p53 stabilisation, γH2AX, or cleaved PARP

Western blot shows HCT116 p53+/+ cells transfected for 24 hours with either 50nM non-silencing control (NSC) siRNA, or 50nM p38 siRNA using HiPerfect (as in materials and methods). Following transfection, cells were treated with either DMSO control (indicated by -) or 500nM RITA (indicated by +) and incubated in normoxia for 24 hours. Western blots were analysed for total p38, γH2AX, and total p53 protein expression. β-actin was used as a loading control. Data shown is representative of 3 repeats.

In order to quantify cell death responses in parallel with the above experiments, flow cytometry cell cycle profiles were analysed of HCT116 p53+/+ cells treated with RITA following p38 siRNA knockdown. The sub-G1 population of cells was quantified as an indication of cell death (Figure 5.7.5). Changes in RITA induced cell death were not observed in cells with p38α knockdown.

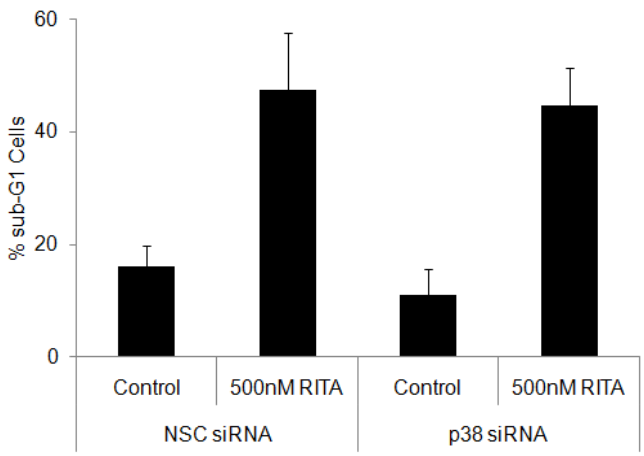


Figure 5.7.5: p38 siRNA does not affect RITA induced cell death
HCT116 p53+/+ cells transfected for 24 hours with either 50nM non-silencing control (NSC) siRNA, or 50nM p38 siRNA prior to 24 hour dosing with 500nM RITA in normoxia. Following treatment, cells were harvested for flow cytometry, fixed and stained using propidium iodide. Cell cycle DNA profiles were gated and quantified for sub-G1 as an indication of cell death. Graph shows the percentage of each treated population in sub-G1. Data shown has been averaged from 2 independent repeat experiments.

PD98059 inhibits RITA induced DNA damage (Figure 5.6.7). Phosphorylation of p38 by RITA is also inhibited by PD98059, I therefore hypothesised that p38 signalling is important for inducing DNA damage by RITA. To address this, HCT116 p53+/+ cells were treated with RITA following siRNA mediated knockdown of p38, and DNA strand breaks were assessed using the comet assay. Loss of p38 was found to have no affect on DNA damage elicited by RITA. In conclusion, although p38 phosphorylation is induced by RITA and targeted by PD98509, p38 is not essential for eliciting RITA induced DNA damage or cell death responses.

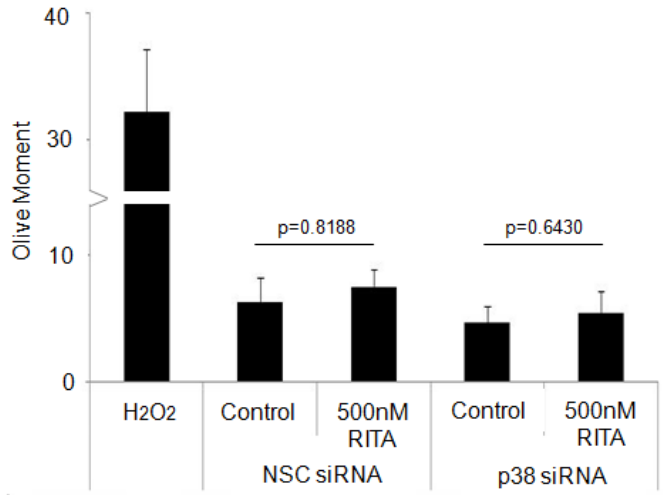


Figure 5.7.6: p38 siRNA does not affect RITA induced DNA damage

HCT116 p53+/+ cells harvested for western blotting to confirm siRNA induced p38 knockdown (Figure 5.7.4), and for flow cytometry to assess sub-G1 (Figure 5.7.5), were also harvested for comet analysis. Following 50nM non-silencing control (NSC) or 50nM p38 siRNA transfection, HCT116 p53+/+ cells were dosed with 500nM RITA for 24 hours in normoxia. Hydrogen peroxide (H_2O_2) was used as a positive control for DNA damage at 100µM for 20 mins, prior to comet preparation. For comet analysis, cells were counted, mixed with agarose, and spread onto comet slides for electrophoresis (see materials and methods). Following electrophoresis, slides were fixed and cells were stained for SYBR Green. Cell nuclei (described as comets) were visualised on an inverted fluorescent microscope at 10x magnification, and images over 20 fields of view for each treatment were captured using ImagePro® software. The TriTek® comet score software programme was used to calculate olive moment as an indication of DNA damage in each cell nucleus (approx 100 comets/treatment). Graph shows olive moment for each treatment and has been averaged from 2 independent repeat experiments. An unpaired t-test was used to calculate statistical significance and a p-value of less than 0.05 was considered significant.

The JNK kinase is activated in response to genotoxic stress elicited by UV irradiation, γ-irradiation and chemotherapeutic drugs (Ip and Davis, 1998). Like p38, JNK kinase also suppresses RAS transformation *in vivo* (Kennedy and Davis, 2003; Kennedy et al., 2003), and promotes p53 stability by either interfering with p53-HDM2 interactions with HDM2, or by direct binding (Adler et al., 1997; Fuchs et al., 1998a). Both JNK and p38 MAPK pathways share several upstream regulators (Cuevas et al., 2007) and a complex level of crosstalk exists between these two pathways (Cheung et al., 2003;

Hui et al., 2007). At the level of p53, it is likely that JNK and p38 co-operate closely to elicit p53-mediated cell death responses.

Previously, p38 kinase was shown to be phosphorylated by RITA, however p38 was not essential for eliciting cell death responses by RITA. I extended these studies to include JNK signalling. As JNK kinase is known to activate p53 in response to genotoxic stress, I hypothesised that RITA also induces phosphorylation of JNK, and this contributes to p53-induced cell death. To address this hypothesis, western analysis was used to assess HCT116 p53+/+ cells treated with 500nM RITA over a timecourse, either alone or in combination with PD98059. Like p38, JNK was also phosphorylated in response to RITA, and these responses were inhibited when cells were treated with RITA in the presence of PD98059 (Figure 5.7.7).

In summary, induction of DNA damage responses and cell death by RITA involves phosphorylation of p38 and JNK stress activated kinases. Because p38 and JNK MAPK's are non-specific targets of the MEK1/2 inhibitor PD98059, I hypothesised that p38/JNK signalling may mediate DNA damage and cell death pathways in response to RITA. However, closer analysis using siRNA mediated knockdown of p38α has shown that although p38 signalling may have an important role in p53-dependent responses activated by RITA, p38 is not essential for p53-dependent cell death. It is possible that p53-dependent cell death responses involve co-ordinated phosphorylation of p53 by several upstream kinases. The possibility that both p38 and JNK may have a co-dependent function in RITA activated cell death requires further investigation.

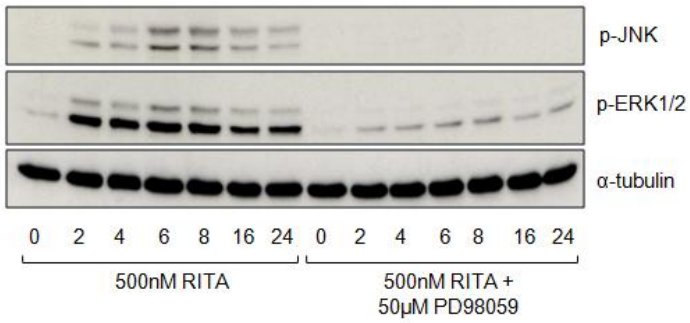


Figure 5.7.7: JNK kinase is phosphorylated in response to RITA and inhibited by PD98059

Western blot shows HCT116 p53+/+ cells treated with 500nM RITA either alone or in combination with 50μM PD98059. Cells were harvested over a 24 hour timecourse and analysed by western blotting for phosphorylated JNK (p-JNK), phosphorylated ERK1/2 (p-ERK1/2), and α-tubulin as a loading control. Data shown is representative of 3 independent repeats.

5.8 Performing a high-throughput siRNA screen

In section 5.7, a candidate kinase approach was used to assess MAPKs that are involved in eliciting p53-dependent cell death responses. Although p38 was induced by RITA, and inhibited by PD98059, closer analysis showed that p38 is not essential in eliciting cell death responses by RITA. Therefore PD98059 may target other MAPKs that are essential in activating p53-dependent responses. To address this, I performed a MAPK siRNA screen.

The INCell 1000 high content screening platform was used in previous experiments to assess changes in nuclear p53 and γH2AX intensity. Using INCell technology enables robust and quantitative analyses of multiple phenotypes within individual cells that are exposed to stress stimuli. I optimised a 96 well high throughput immunoflourescence based assay that could be used to screen multiple targets using the INCell platform (see chapter 2 section 2.7, materials and methods). This assay was previously used to simultaneously analyse both nuclear p53 and γH2AX localisation when cells were treated with RITA alone, or in combination with PD98059 (Figure 5.4.5). The data obtained from these experiments provided a reliable basis with which to screen a MAPK siRNA library.

To begin optimising the immunoflourescent INCell assay for the MAPK siRNA screen, the standard siRNA protocol was optimised to a high throughput 96 well format. RITA induced cell death and DNA damage responses are p53-dependent. Therefore, p53 siRNA was used to assess whether efficient knockdown of p53 could be quantified using INCell technology, and if this correlated with loss of RITA induced cell death, p53 stabilisation, and γH2AX induction. As a comparison, HCT116 p53-/- cells were also treated in parallel to assess the efficiency of p53 knockdown in HCT116 p53+/+ cells (Figure 5.8.1).

As shown in Figure 5.8.1, inhibition of RITA induced p53 stabilisation was achieved in HCT116 p53+/+ cells that had been transfected with p53 siRNA compared to the non-silencing control (NSC). Knockdown of p53 by siRNA in HCT116 p53+/+ cells treated with RITA also inhibited nuclear γH2AX intensity. Induction of nuclear p53 and γH2AX following RITA treatment was not observed in HCT116 p53-/- cells. In conclusion, the INCell screening platform was successfully used to assess nuclear p53 and γH2AX following RITA treatment in cells that had been transfected with siRNA in a 96 well format.

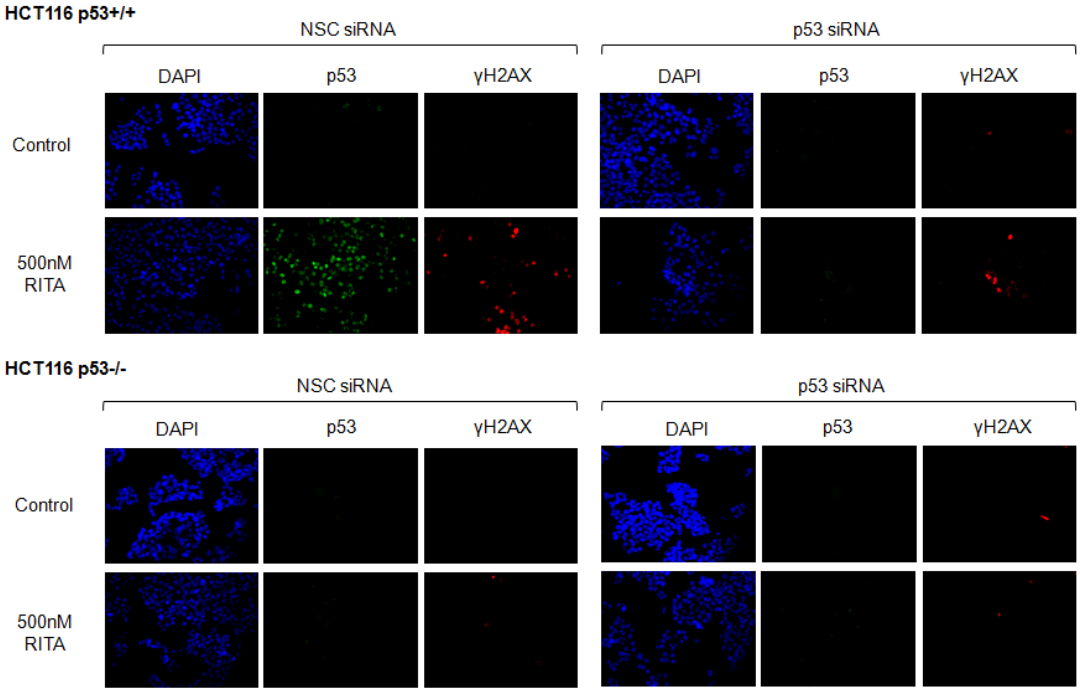


Figure 5.8.1: Induction of nuclear p53 and γH2AX in RITA treated cells is inhibited by p53 siRNA

Images show HCT116 p53+/+ (top panel) and HCT116 p53-/- (bottom panel) cells transfected with either non-silencing control (NSC) siRNA or p53 siRNA for 24 hours using HiPerfect (see materials and methods). Following 24 hour transfection, cells were treated with 500nM RITA and incubated for a further 24 hours in normoxia. Plates were then fixed and stained using specific antibodies against p53 (with an Alexa Fluor 488 conjugated secondary antibody), and γH2AX (with an Alexa Fluor 568 conjugated secondary antibody), DAPI was used to stain nuclei. The INCell 1000 was used to capture 10 images for every well, at each fluorescent intensity using 20x magnification. Representative images from one field and 3 independent experiments are shown.

The INCell 1000 software was used to quantify immunoflourescent images taken by the INCell. RITA induced cell death (indicated by loss of cell counts) was inhibited following knockdown of p53 by siRNA (Figure 5.8.2A). Consistently, changes in cell number were not observed in HCT116 p53-/- cells treated with RITA. Induction of nuclear p53 following RITA treatment was only detected in HCT116 p53+/+ cells, and not in HCT116 p53-/- cells, or HCT116 p53+/+ cells that had loss of p53 by siRNA (Figure 5.8.2B). Notably, nuclear γH2AX induced by RITA was detected in HCT116 p53+/+ cells with siRNA mediated knockdown of p53, and not in HCT116 p53-/- cells, suggesting that p53 siRNA was not sufficient to inhibit nuclear γH2AX localisation

induced in response to RITA (Figure 5.8.2C). Overall, siRNA knockdown can be optimised for a 96 well format and quantified in a reproducible and reliable way according to defined phenotypes established in cells treated with RITA. Following preliminary siRNA experiments, a reverse transfection siRNA library comprising 58 MAPK siRNA oligo's was purchased from Dharmacon. To complete the siRNA screen, the following protocol was recommended by Dharmacon (Figure 5.8.3).

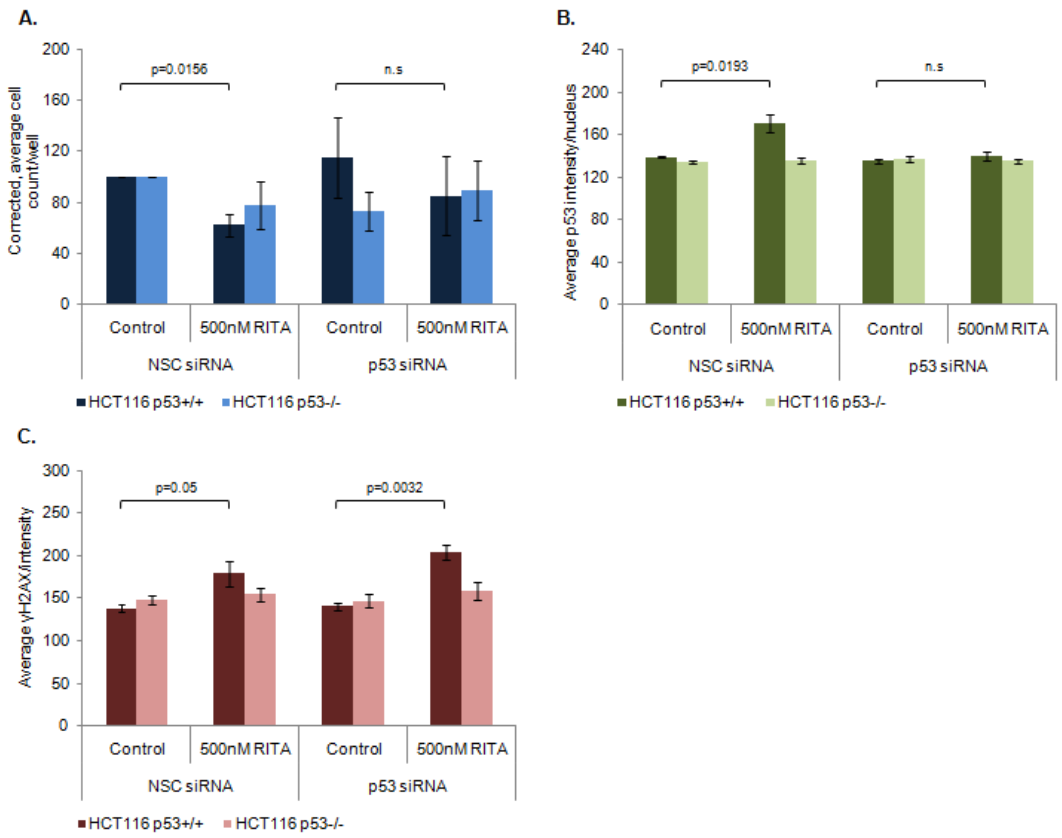


Figure 5.8.2: RITA induced p53 and γH2AX is inhibited by p53 siRNA

Graphs show INCell quantification of HCT116 p53+/+ and HCT116 p53-/- cells transfected with either non-silencing control siRNA (NSC) or p53 siRNA prior to 500nM RITA treatment for 24 hours in normoxia. Cells were stained for: (A) DAPI to visualise nuclei and quantify cell number, (B) total p53 (with an Alexa Fluor 488 conjugated secondary antibody) to quantify p53 nuclear intensity, and (C) phosphorylated γH2AX (with an Alexa Fluor 568 secondary antibody) to assess γH2AX phosphorylation in the nucleus. Images were captured for each fluorescent intensity over 10 fields of view using the INCell analyser at 20x magnification. All images were quantified using INCell software analysis. Data shown has been averaged from 3 independent experiments. An unpaired t-test was used to assess statistical significance between control and treated samples, and a p-value of less than 0.05 was considered significant.

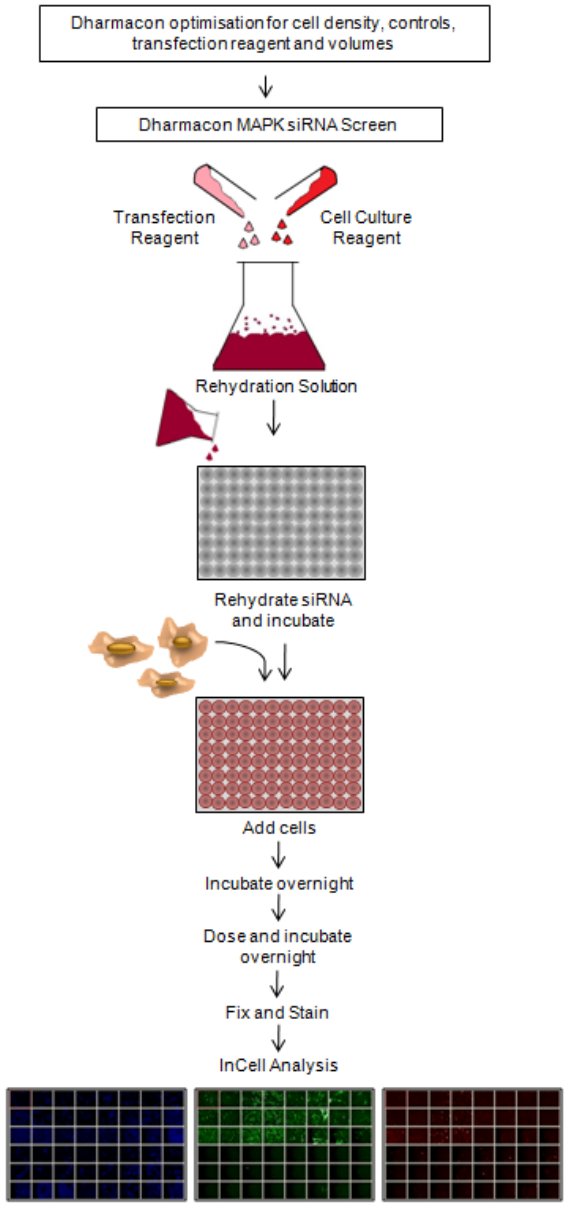


Figure 5.8.3: Performing a high throughput siRNA screen

A Dharmacon siRNA library of 58 MAPK's was purchased. Optimisation plates were supplied with the library to optimise seeding densities and Dharmacon specific transfection reagents. The screening protocol involves combining optimised volumes of transfection reagent with cell culture reagent to form a rehydration solution that is added to every well containing pre-seeded siRNA. The siRNA oligo's are rehydrated and complexes form during an incubation period. Cells are then seeded directly onto the siRNA mix. Following an overnight transfection incubation, the plate is dosed with 500nM RITA and incubated for a further 24 hours before fixing and staining for nuclei, p53 induction and γH2AX. The plate is read on an INCell 1000 analyser for image acquisition and data analysis.

Before performing the MAPK siRNA screen, optimisation plates were used to test the knockdown efficiency of a standard siRNA, in this case p53, against transfection reagents provided specifically by Dharmacon for the screening format. HCT116 p53+/+ cells were treated with RITA in the absence of transfection reagent, and with either a non-silencing control (NSC) siRNA, or p53 targeted siRNA using Dharmacon transfection reagents. As shown in Figure 5.8.4A, the transfection reagent used caused a significant decrease in cell viability compared to untreated cells. However, nuclear p53 induced in response to RITA was inhibited in HCT116 p53+/+ cells following p53 siRNA transfection (Figure 5.8.4B). Inhibition of nuclear γH2AX foci induced by RITA was also observed when cells were treated with p53 siRNA (Figure 5.8.4C). The optimisation data presented is consistent with that in Figure 5.8.2. Therefore siRNA knockdown can be achieved using both standard and screen specific transfection protocols to quantify and evaluate RITA responses.

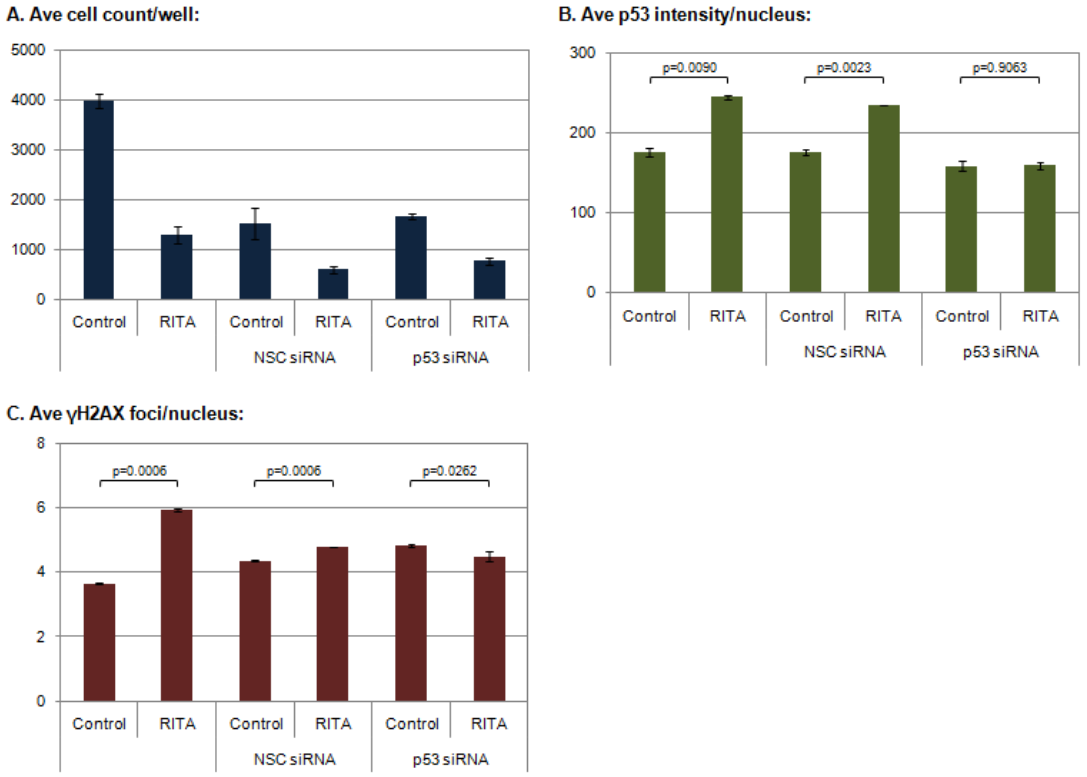


Figure 5.8.4: Optimising p53 siRNA transfection using Dharmacon reagents
HCT116 p53+/+ cells were seeded into 96 well plates containing either non-silencing control (NSC) siRNA, or p53 targeted siRNA. Following 24 hour transfection, cells were treated with 500nM RITA for 24 hours in normoxia, fixed and stained with DAPI, total p53 (with an Alexa Fluor 488 secondary antibody), and phosphorylated γH2AX (with an Alexa Fluor 568 antibody). Images at each fluorescent intensity were captured using the INCell analyser at 20x magnification, and quantified using INCell software. Graphs show: (A) average cell count/well (quantified using DAPI staining), (B) average p53 intensity/nucleus, (C) average phosphorylated γH2AX foci/nucleus. Data has been averaged from 2 independent repeat experiments. An unpaired t-test was used to assess statistical significance between control and treated samples, and a p-value of <0.05 was considered significant.

To identify MAPK siRNAs that inhibit RITA mediated responses, p53 and γH2AX induction in cells treated with RITA was compared in cells with either non-silencing control siRNA, or p53 siRNA (Figure 5.8.5). RITA induced cell death (indicated by cell number/well) was inhibited in p53 siRNA wells compared to NSC siRNA controls (Figure 5.8.5A). Unfortunately, significant loss in cell viability was observed following MAPK siRNA transfection. An unpaired t-test was used to compare the mean statistical difference between RITA induced nuclear p53 in NSC control wells, compared with

MAPK siRNA's. RITA treated cells transfected with MAPK siRNA's that showed a statistically significant decrease in nuclear p53 (with a two tailed p-value of <0.05) are highlighted in black (Figure 5.8.5B). Out of 58 siRNA oligos screened, 9 oligos were shown to significantly reduce nuclear p53 induction upon RITA treatment compared to the non-silencing control. Similar criteria were used to assess nuclear γH2AX foci (Figure 5.8.5C). Out of 58 oligos, 37 oligos showed significant inhibition in RITA-induced nuclear γH2AX compared to the non-silencing controls.

To highlight statistically significant data from the siRNA screen (indicated previously as black bars in Figure 5.8.5) graphs were redrawn to indicate the specific MAPKs that show significant decreases in RITA induced nuclear p53 (Figure 5.8.6A) and γH2AX (Figure 5.8.6B). In agreement with previous data using the MEK1/2 inhibitors PD98059, and PD184352, loss of MAP2K1 (MEK1/MKK1) and MAP2K2 (MEK2/MKK2) by siRNA significantly inhibited nuclear p53 and γH2AX in RITA treated cells. Although several MAPK siRNAs were identified in our screen as inhibiting p53 and γH2AX induction, I investigated MAPK2K3 (MKK3) in further detail as MKK3 is an upstream regulator of the p38 kinase which has been studied previously in mediating p53-dependent cell death responses by RITA.

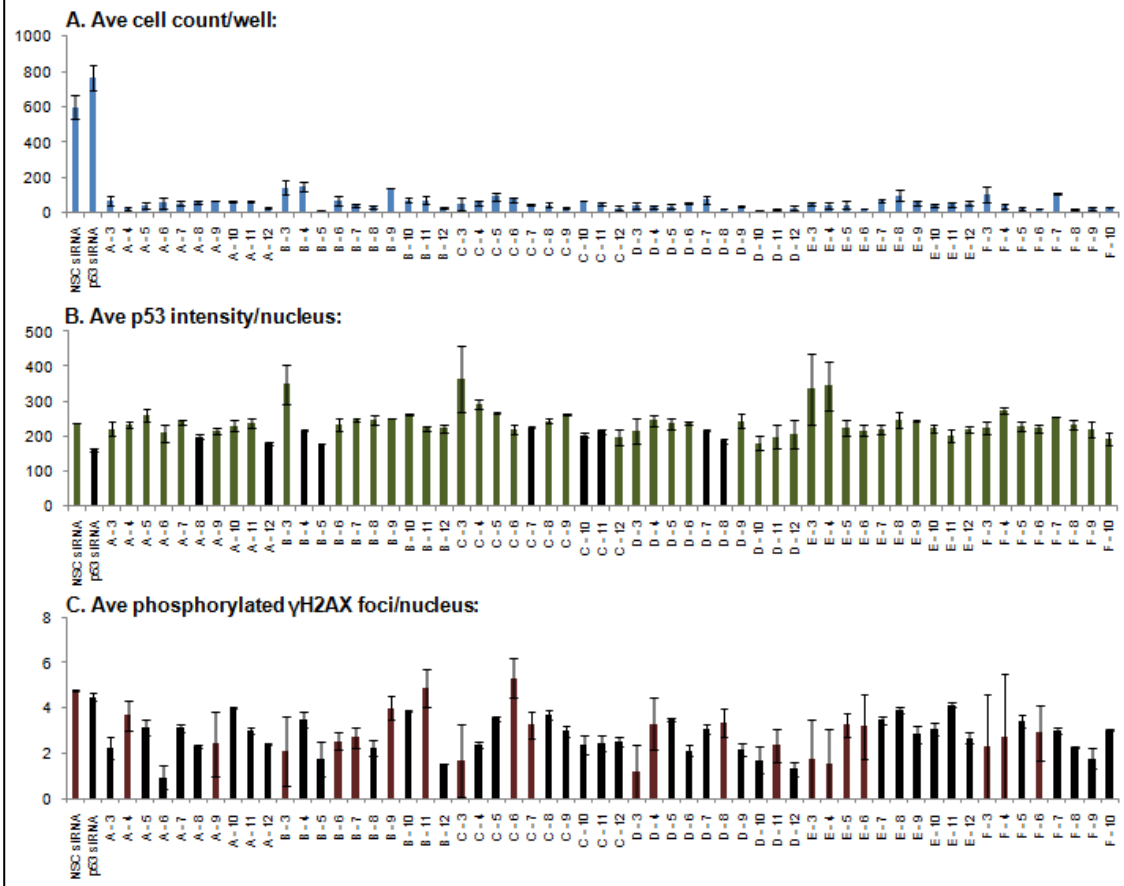


Figure 5.8.5: siRNA screening data

HCT116 p53+/+ cells were transfected with non-silencing control siRNA (NSC), p53 siRNA, or MAPK siRNAs prior to 500nM RITA treatment for 24 hours in normoxia. Following treatment cells were fixed and stained using DAPI, total p53 protein (with an Alexa Fluor 488 secondary antibody), and γH2AX (with an Alexa Fluor 568 secondary antibody). Cells were imaged using the INCell analyser at 20x magnification for each fluorescent intensity. Up to 10 fields of view were captured for each treated well. Images were quantified using INCell software. Graphs show: (A) average cell count/well, (B) average p53 intensity/nucleus and (C) average γH2AX foci/nucleus. An unpaired t-test was used to assess statistical significance and a two-tailed p-value <0.05 was considered significant. Treated wells in which a statistically significant decrease in protein expression is seen compared to the non-silencing control are highlighted in black. Corresponding well numbers for each MAPK siRNA are labelled on the x-axis. All data shown has been calculated from 2 independent repeat screens.

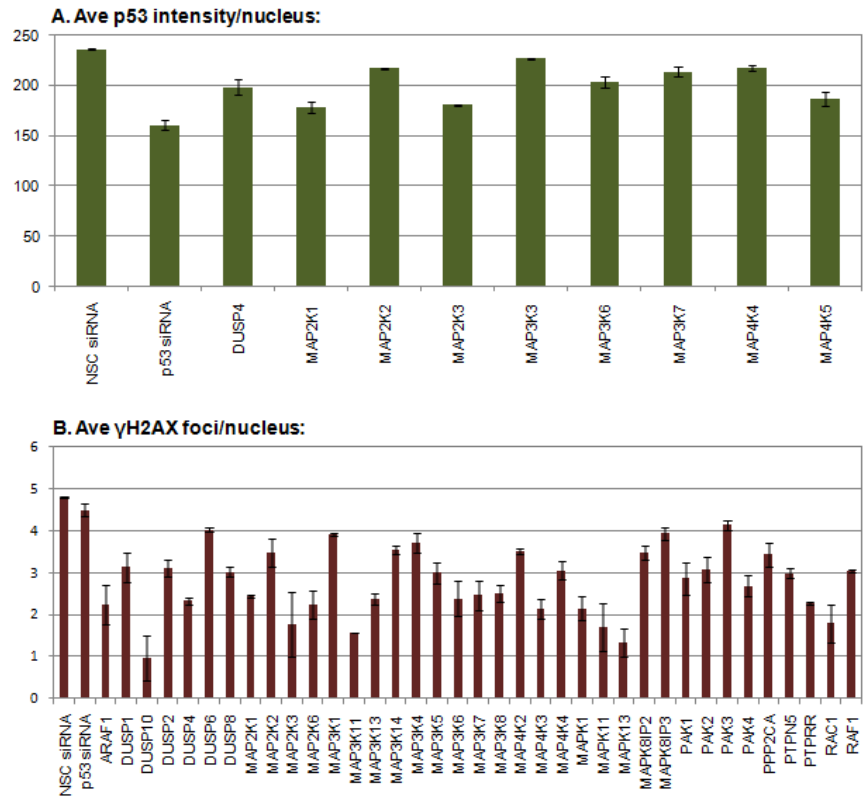


Figure 5.8.6: Statistically significant MAPK screening data

Graphs show HCT116 p53+/+ cells transfected with either non-silencing control siRNA (NSC), p53 siRNA or MAPK siRNA's prior to 24 hour, 500nM RITA treatment in normoxia. Following treatment, HCT116 p53+/+ cells were fixed and stained using DAPI, total p53 protein (with an Alexa Fluor 488 conjugated secondary antibody), and γH2AX (with an Alexa Fluor 568 conjugated secondary antibody). Images were captured using the INCell analyser at 20x magnification over 10 fields of view, and quantified using INCell software. Graphs show: (A) average p53 intensity/nucleus, and (B) average γH2AX foci/nucleus for HCT116 p53+/+ cells treated with RITA following MAPK siRNA tranfection. Data has been taken from the screen shown in Figure 5.8.6, and has been redrawn to indicate RITA treated siRNA wells in which there is a statistically significant decrease in nuclear p53, and γH2AX compared to non-silencing control (NSC) cells. An unpaired t-test was used to assess significance (where two-tailed p-value <0.05). All data has been calculated from 2 independent repeat screens.

5.9 Investigating MKK3 as a target kinase for PD98059

The MAPK siRNA screen identified several siRNAs which showed a decrease in RITA mediated induction of nuclear p53 and γH2AX. The MAP2K3 MAPK kinase (MKK3) was of particular interest because MKK3 has been shown to phosphorylate and activate p38 MAPK specifically, without having any affects on the JNK and ERK signalling pathways (Derijard et al., 1995). Phosphorylation of p38 has already been demonstrated in cells treated with RITA. Consistently, RITA mediated responses are not reversed when p38α is targeted by siRNA, suggesting that p38 is not essential for inducing cell death in response to RITA (Figure 5.9.4).

As MKK3 kinase is an upstream activator of p38 signalling, I hypothesised that MKK3 may be involved in regulating RITA-induced cell death through the p38 signalling pathway. To address this, MKK3 data was isolated from the MAPK siRNA screen data set (Figure 5.8.5). As shown in Figure 5.9.1, RITA induced stabilisation of nuclear p53 (Figure 5.9.1A) and γH2AX (Figure 5.9.1B) was significantly inhibited in the presence of MKK3 siRNA. These responses were also observed in the presence of MAP2K1 (MKK1/MEK1) and MAP2K2 (MKK2/MEK2), the target kinases for PD98059.

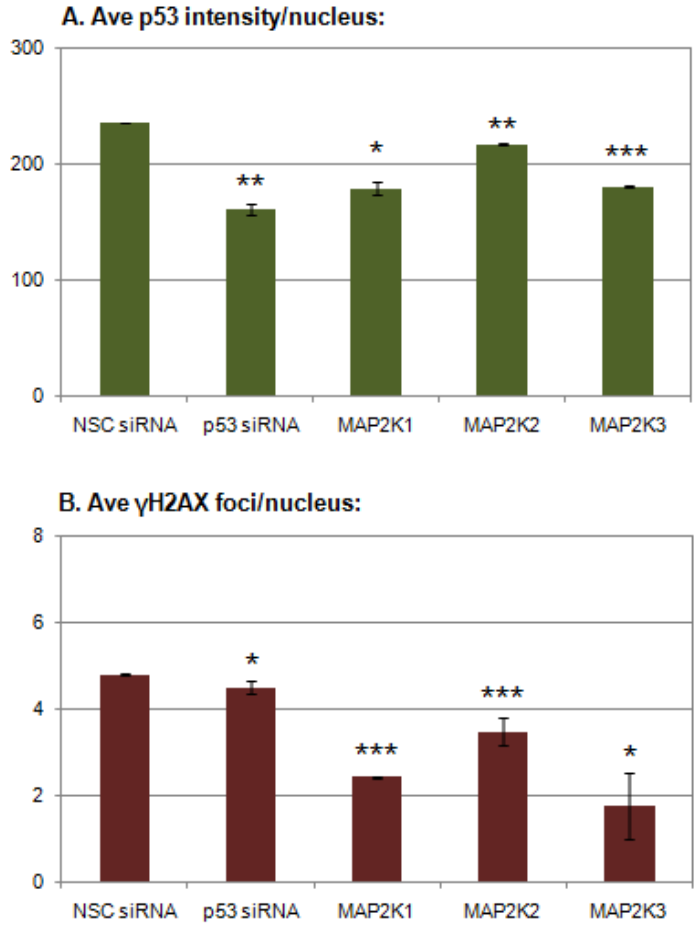


Figure 5.9.1: RITA induced p53 and γH2AX is inhibited by MKK3 siRNA

Graphs show HCT116 p53+/+ cells treated with 50nM of either non-silencing control (NSC) siRNA, p53 siRNA, MAPK2K1 (MKK1/MEK1) siRNA, MAP2K2 (MKK2/MEK2) siRNA, or MAP2K3 (MKK3/MEK3) siRNA using the Dharmacon screening protocol. Following 24 hour transfection, cells were treated with 500nM RITA for another 24 hours in normoxia. Cells were fixed following treatment and stained for DAPI, total p53 protein (with an Alexa Fluor 488 secondary antibody), and γH2AX (with an Alexa Fluor 568 secondary antibody). The INCell analyser was used to capture images for each treatment over 10 fields of view, at 20x magnification and INCell software was used for quantification. Graphs show: (A) average p53 intensity/nucleus and (B) average γH2AX foci/nucleus for specific siRNA oligos indicated on the x-axis. Statistical significance was assessed between RITA treated non-silencing control (NSC) siRNA, and RITA treated MAPK siRNA cells. An unpaired t-test (where two-tailed p-value <0.05) was used to calculate significance, (*p<0.05, **p<0.005, ***p<0.0005). All data has been averaged from 2 independent repeat screens.

PD98059 can inhibit p53-dependent DNA damage and cell death responses elicited by RITA and this is not observed in cells that are treated with RITA in combination with PD184352. Because PD98059 has a broader target profile compared to PD184352, a MAPK siRNA screen was performed and MKK3 was isolated as a potential non-specific target of PD98059 which, when targeted by siRNA mediated knockdown, could inhibit RITA induced p53 and γH2AX (Figure 5.9.1).

To confirm whether MKK3 is targeted by PD98059, HCT116 p53+/+ cells were treated with 500nM RITA over a timecourse, either alone, or in combination with 50μM PD98059. Phosphorylation of MKK3 was induced in cells that had been treated with RITA over a timecourse, and inhibited in cells that had been treated with RITA in combination with PD98059 (Figure 5.9.2). The data presented suggest that like p38 kinase, MKK3 is also a non-specific target of PD98059.

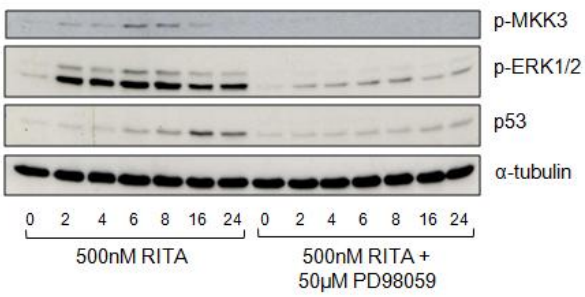


Figure 5.9.2: Phosphorylation of MKK3 is inhibited by PD98059
Western blot shows HCT116 p53+/+ cells treated with 500nM RITA for 24 hours in normoxia, either alone or in combination with 50μM PD98059. Cells were harvested for western analysis over a 24 hour timecourse and assessed for phosphorylated MKK3 (p-MKK3), phosphorylated ERK1/2 (p-ERK1/2), total p53 protein, and α-tubulin as a loading control. Data shown is representative of 3 independent repeats.

5.10 Discussion

Previous studies with PD98059 have shown that MAPK signalling is important in regulating p53-dependent cell death in response to genotoxic stress (Persons et al., 2000; Ryan et al., 2000). The studies presented in this chapter are consistent with these findings and show that PD98059 inhibits many of RITA's distinct p53-dependent effects on cells including induction of DNA damage, activation of DNA damage responses involving γH2AX, CHK1, and CHK2 phosphorylation, stabilisation of p53, and induction of p53-dependent cell death.

A candidate kinase approach was taken, and a MAPK siRNA screen (Dharmacon) was performed to identify MAPKs that are targeted by PD98059 and are not affected by other MAPK inhibitors. The p38 and JNK MAPK signalling pathways were assessed as candidates for their contribution to RITA induced cell death. The upstream activator of p38 signalling, MKK3 was also identified in the MAPK siRNA screen. MKK3 was both phosphorylated in response to RITA, and inhibited in the presence of PD98059. Although p38 was also phosphorylated by RITA and inhibited by PD98059, siRNA mediated knockdown of p38α did not inhibit RITA induced cell death. Therefore, although p38 phosphorylation is induced, it is not essential for eliciting cell death and DNA damage responses by RITA.

Studying the MKK3-p38 pathway has led us to question why MKK3 siRNA inhibits RITA induced p53 and γH2AX responses while p38α siRNA, a downstream target of MKK3 has no significant effects on RITA responses. If not through p38, how then is MKK3 mediating its effects on RITA induced cell death? Independent functions of MKK3 and p38 are shown by *in vivo* models whereby *Mkk3* null mice are viable and fertile (Lu et al., 1999; Tanaka et al., 2002), while inactivation of *p38α* results in early embryonic lethality (Adams et al., 2000) suggesting a more significant role for p38 in cell growth and survival during development. The disconnect between MKK3 and p38 *in vivo* phenotypes has not been addressed directly but suggests a level of redundancy for some upstream MAPKKs. Thus the case may be that MKK3 interacts with other pathways not yet identified which are independent of p38 signalling. Furthermore although we knocked down p38α by siRNA, four other isoforms of p38 have been described which may have independent roles in regulating p53-dependent cell death responses (Freshney et al., 1994). It will be of interest to assess other p38 isoforms in greater detail.

The physiological functions of both JNK and p38 stress pathways may be overlapping and several studies support this hypothesis (Ip and Davis, 1998). The apoptosis signal regulating kinase 1 (ASK1) MAPKKK activates two subgroups of MAPKKs, the MKK4/7 kinases that lead to JNK activation, and the MKK3/6 kinases that activate p38 signalling in response to TNF-α induced apoptosis (Ichijo et al., 1997), and in response to chemotherapeutic agents (Chen et al., 1999). Such studies which show that upstream MAPKKKs link multiple stress pathways suggest again that MAPKs have overlapping and redundant functions depending on cell type and stress stimuli.

PD98059 targets several MAPKs that are not inhibited by PD184352, therefore combined inhibition of numerous MAPKs may be essential for blocking p53-dependent cell death in response to RITA. Further clues arise from the siRNA screen presented in this thesis whereby several MAPK siRNAs were shown to significantly inhibit RITA induced p53 and γH2AX. It is difficult to speculate at this stage exactly which MAPKs have a critical role to play in RITA induced cell death responses, and further studies will be required, taking clues from both siRNA screening data, and from the literature whereby multiple MAPK pathways have been studied in response to stress stimuli both with individual and co-operative effects.

In this study, PD184352 was used as a potent and selective inhibitor of MKK1 compared to PD98059 based on previous screens conducted by Davies et al. (Davies et al., 2000). As PD184352 had no significant effects on RITA mediated responses, it was concluded that PD98059 may target non-specific kinases that are essential for eliciting RITA induced cell death responses. On this basis, a MAPK siRNA screen was conducted. However, data from the screen showed that knockdown of MKK1 and MKK2 significantly inhibited induction of p53, and γH2AX in response to RITA, suggesting that in fact, MKK1 and MKK2 are important in eliciting RITA mediated responses. Why then did the selective MKK1/MKK2 inhibitor PD184352 not affect RITA mediated responses? The answer lies within understanding the importance of comparing the selectivity profiles of related small molecules before making definitive conclusions of the pathways that they target. Firstly, although PD184352 is a selective inhibitor of MKK1, PD184352 does target other structurally related kinases that are not affected by more potent inhibitors such as PD0325901 (Bain et al., 2007). Furthermore, the selective inhibition of MKK1/MKK2 by PD184352 may upregulate other pathways that compensate for promoting tumour cell death in response to p53 activating agents such as RITA. PD98059 may not induce such responses due to broader targeting of kinases. Collectively, these data highlight the importance of using siRNA mediated knockdown of

specific kinases in parallel to small molecule inhibitors when assessing specific cellular pathways. The contribution that MKK1 and MKK2 have in regulating p53-dependent cell death in response to RITA cannot be ignored and I therefore propose that targeted inhibition of MKK1/MKK2 as well as other kinases by PD98059 promotes the cell survival phenotype that has been observed in the experiments presented throughout this chapter.

Using PD98059, I have also shown that ERK1/2 signalling is important in regulating cell cycle responses in both HCT116 p53+/+ and HCT116 p53-/- cells. In HCT116 p53+/+ cells, ERK1/2 phosphorylation mediates RITA induced cell death responses, while in HCT116 p53-/- cells, ERK1/2 phosphorylation promotes RITA induced G2 arrest, which is inhibited in the presence of PD98059. Interestingly, p38 MAPK has an important function in eliciting G2 arrest in p53 null cells during early timepoints after UV irradiation (Bulavin et al., 2001). By regulating cell cycle checkpoints in p53 null cells, p38 signalling promotes cell survival (Reinhardt et al., 2007). Furthermore, Reinhardt et al. demonstrate a requirement for ATM and ATR in activating p38 MAPK and MAPK kinase 2 (MK2), a downstream target kinase of p38 required for UV induced cell cycle checkpoints (Manke et al., 2005). MK2 in turn regulates Cdc25 protein phosphatases that control G2/M transitions following DNA damage (van Vugt et al., 2004). Loss of MK2 function promotes premature mitotic entry and apoptosis in response to DNA damaging agents, and has significant anti-tumour activity *in vivo* (Reinhardt et al., 2007). For this current study, it will be of interest to assess cell cycle effects of p38 or MK2 knockdown in HCT116 p53-/- cells that have been treated with RITA. Collectively, these studies describe an important function for MAPK signalling in regulating cell death, and cell cycle responses induced by stress stimuli and demonstrate how DNA damage responses can rewire complex signalling networks to induce specific cell cycle and cell death effects that are dependent on p53 status.

5.11 Conclusions for this chapter

- PD98059 inhibits RITA induced cell death, DNA damage, and DNA damage responses involving γH2AX, and phosphorylation of both CHK1 and CHK2.
- RITA elicits G2 arrest in HCT116 p53-/- cells that is inhibited when cells are treated with RITA in the presence of PD98059.
- PD184352 does not affect RITA treated cells.
- The MAPK pathway involving MKK3 and p38 phosphorylation is induced by RITA, and inhibited by PD98059.

5.12 Impact of these findings

Sorafenib was developed as a specific inhibitor of RAF-MEK-ERK signalling, however during clinical progression, sorafenib was shown to have activity against various RAF isoforms and against VEGFR-2, VEGFR-3, PDGFR-β, c-Kit and Flt-3 tyrosine kinases (Wilhelm et al., 2004). For this reason, sorafenib is now classed as a multiple kinase inhibitor. Deregulation of VEGF, PDGF-β, and TGF-β is implicated in the malignant phenotype of renal cell carcinoma due to loss of pVHL function, and sorafenib has therefore been used widely in the clinic to treat patients with renal cell carcinoma. The favoured toxicity profile of sorafenib, together with marked tumour regression and improved survival of subjects also suggests that sorafenib could be used in combination with other targeted agents. Bevacizumab for example inhibits multiple VEGF isoforms (Presta et al., 1997) however activity is limited in the clinic due to acquired resistance strategies such as compensatory increases in serum VEGF levels (Yang et al., 2003). Use of sorafenib in combination with bevacizumab may enhance anti-tumour effects by maximising VEGF inhibition. Furthermore, therapeutic potential of the mTOR inhibitor RAD001 has been shown to be limited by the consequent increases in MAPK activity (Carracedo et al., 2008). Use of RAD001 in combination with the MEK-ERK inhibitor PD0325901 has been shown to improve anti-tumour responses by targeting both signalling pathways (Carracedo et al., 2008).

Studies presented in this chapter show that MAPK signalling is important for stabilising wildtype p53 in response to RITA, and activating DNA damage responses involved in cell cycle and cell death responses that determine anti-tumour outcomes. These key findings are summarised in Figure 5.10.1. Therefore, in the tumour models that have been studied so far in this thesis, MAPK inhibition would not be a desirable therapeutic strategy in combination with p53-activating agents. Due to the importance of MAPK signalling in regulating p53-dependent apoptosis, it would be essential to base MAPK combination therapies according to p53 status, and such therapeutic combinations have not been investigated to date. In renal cell carcinoma, which has elevated levels of HIF signalling, as well as deregulated p53 activity, it will be of interest to assess the effects of sorafenib which already has efficacy in this tumour model, in combination with p53 targeted agents like RITA. We would predict that combined inhibition of HIF signalling, as well as induction of p53-dependent cell death in this model would have significant anti-tumour affects.

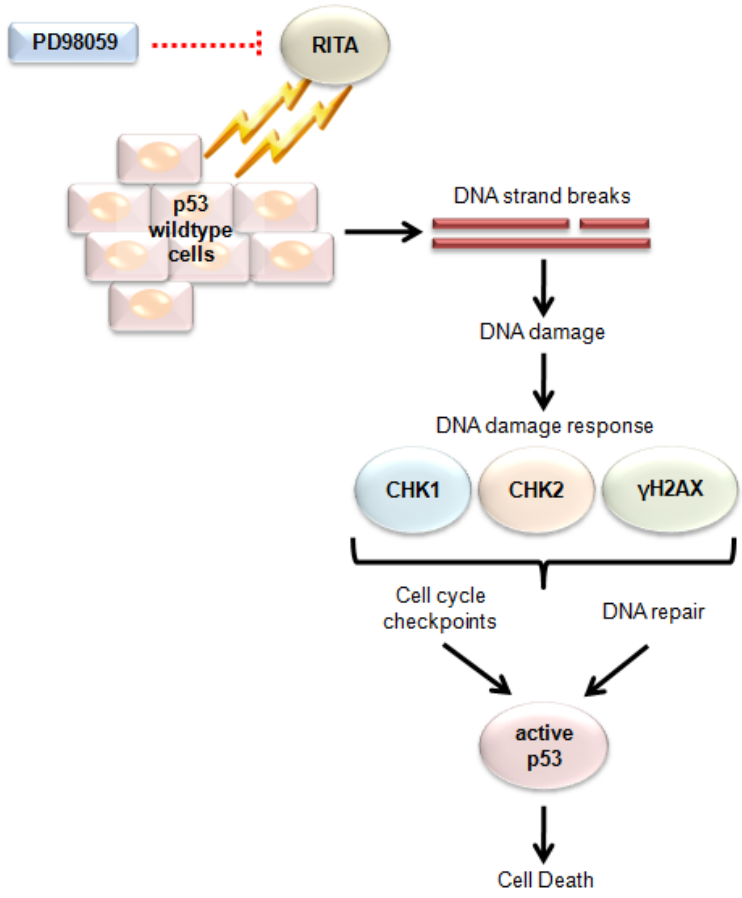


Figure 5.10.1: PD98059 inhibits the anti-tumour activity of RITA

MAPK signalling is important for stabilising wildtype p53 in response to RITA and eliciting DNA damage responses that mediate cell cycle and cell death effects. Overall, MAPK signalling is important for determining the apoptotic outcome of p53 wildtype cells that have been exposed to RITA.

Chapter 6

RITA inhibits p53 targets p21 and HDM2, and blocks the induction of HIF-1α in hypoxia

6.1 Introduction

Previous data from our laboratory has shown that RITA not only stabilises p53, but also inhibits HIF-1α protein induction in hypoxia (Yang et al., 2009a; Yang et al., 2009b), and I have demonstrated that this response is distinct compared to other DNA damaging agents that activate p53 (Figure 3.1.4). Downregulation of HIF-1α protein expression in response to p53 induction by RITA is supported by studies whereby p53 activity has been shown to negatively regulate HIF-1α protein and angiogenesis (Ravi et al., 2000). Although the relationship between HIF and p53 has been described in several studies, the mechanism for these interactions remain poorly understood.

RITA induces p53 in hypoxic tumours and elicits p53-dependent cell death. These studies are important because they not only utilise small molecule activation of p53 to elicit cell death and anti-angiogenic responses, but they also provide us with a tool that can be used to study mechanisms that are deregulated in hypoxic cells. Indeed, the use of RITA as a molecular tool in this thesis has unveiled novel p53-dependent mechanisms that regulate cell cycle, and DNA damage responses which have not been described in previous studies (Ahmed et al., 2011).

It is of interest to study RITA induced cell death in tumours that have deregulated pVHL function and overexpress HIF-1α and HIF-2α protein levels. Tumour models such as renal cell carcinoma and neuroblastoma are important because they rarely express mutant p53, and often have deregulated wildtype p53 activity making them angiogenic due to loss of pVHL function, and resistant to conventional forms of chemo- and radiotherapy. By inducing p53, and inhibiting the HIF pathway, RITA provides an opportunity with which to sensitise such tumours to p53-dependent cell death. However, these studies need greater understanding of the mechanisms by which HIF and p53 pathways are deregulated. In this chapter, I will investigate how HIF-1α and HIF-2α protein expression affects p53-induced cell death in various tumour models.

So far, by showing that p53 induction can inhibit HIF-1α protein expression and induce significant tumour cell apoptosis, I have confirmed the negative relationship that exists between p53 activity and HIF-1α status, while also revealing the presence of novel mechanisms by which small molecule activation of p53 targets HIF signalling in hypoxia. To investigate the effects of p53 activation by RITA in greater detail, p53 target proteins will be analysed, as changes in p53 target gene expression in response to genotoxic stress have significant effects on the apoptotic phenotype of cells. Therefore, RITA induced DNA damage and cell cycle responses may also be

influenced by p53 induced target gene expression. In this chapter, I will explore the mechanism by which RITA regulates p53 activity, and p53 target proteins, as well as the mechanisms by which p53 induction by RITA affects HIF-1α protein expression.

6.2 Hypothesis

RITA affects p53 targets and HIF-1α protein expression.

6.3 Aims

- Investigate the p53 targets p21 and HDM2 at both the protein and mRNA levels when cells are treated with RITA.
- Study translational pathways that inhibit HIF-1α in response to RITA.
- Investigate the importance of HIF status in p53-dependent cell death using various tumour models that have deregulated HIF signalling.

6.4 RITA downregulates the expression of p53 targets p21 and HDM2

The tumour suppressor response controlled by p53 involves induction of numerous p53 target genes that regulate cell cycle arrest, DNA repair, and cell death pathways when cells are exposed to stress. Regulation of HDM2 gene expression by p53 is important because HDM2 is an E3 ligase for p53, and forms a negative feedback loop by targeting p53 for proteosomal degradation. Expression of p21 by p53 is also important because p21 is involved in regulating cell cycle arrest pathways that mediate DNA repair. Activation of p21 favours cell cycle arrest in response to specific forms of stress rather than activation of apoptosis (Gartel and Tyner, 2002). I investigated HDM2 and p21 protein expression in response to RITA, as both HDM2 and p21 are important in determining the apoptotic outcome of cells induced to stress. To begin these studies, I hypothesised that p53 stabilisation would correlate with induction of both HDM2, and p21 protein expression in response to RITA. Changes in p21 and HDM2 protein expression following increasing doses of RITA were assessed.

The results show that RITA induces expression of both p21 and HDM2 in a dose-dependent manner (Figure 6.4.1). Interestingly, as p53 levels remained stabilised at 500nM RITA treatment, decreased expression of HDM2 and p21 protein levels was observed. Contrary to reports which suggest that RITA treatment upregulates HDM2 expression by binding directly to p53 and interfering with the p53-HDM2 interaction (Enge et al., 2009; Issaeva et al., 2004), the data presented here indicate that at higher

doses where RITA stabilises p53 protein (at doses of 500nM and above), HDM2 protein expression is inhibited.

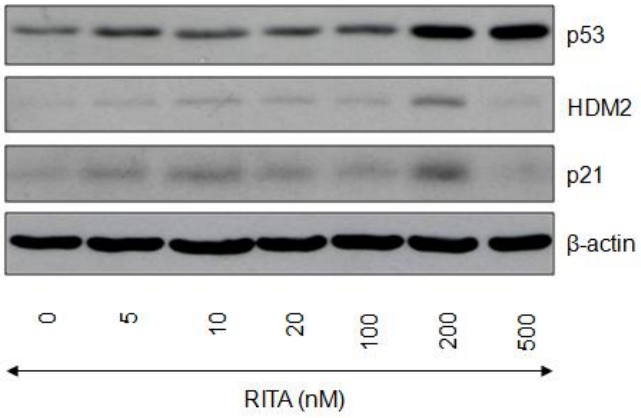


Figure 6.4.1: 500nM RITA inhibits p21 and HDM2 protein expression
Western blot showing HCT116 p53 +/+ cells treated with 0-500nM RITA over 24 hours in normoxia. Cells were harvested for western analysis and blotted for total p53, HDM2, p21 and β-actin as the loading control. Data shown is representative of 3 independent repeat experiments.

To assess whether inhibition of p21 and HDM2 protein levels was due to changes in HDM2 and p21 mRNA, RT-PCR was used. As shown in Figure 6.4.2, changes in HDM2 and p21 mRNA were not detected following RITA treatment suggesting that RITA does not affect HDM2 and p21 protein expression at the level of mRNA.

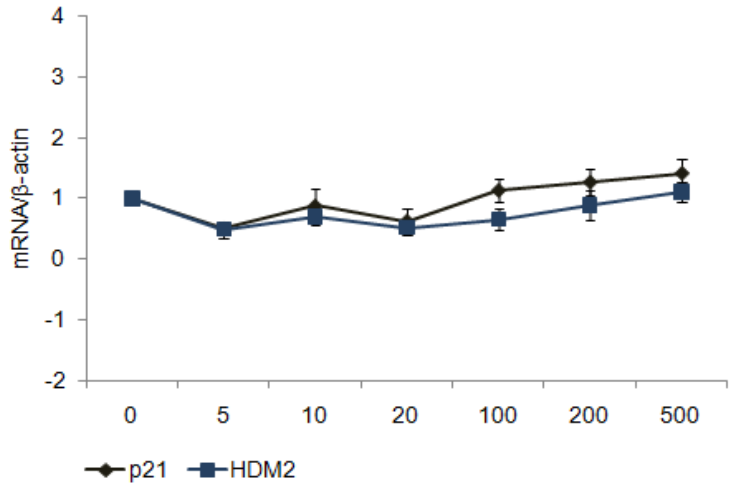


Figure 6.4.2: RITA does not affect p21 and HDM2 mRNA levels
Graph shows RT-PCR analysis of p21 and HDM2 mRNA levels in HCT116 p53+/+ cells treated with 0-500nM RITA (as indicated) for 24 hours in normoxia. Following RT-PCR, mRNA abundance was adjusted relative to the β-actin control. Data shown has been averaged from the means of 3 independent experiments.

6.5 RITA inhibits protein synthesis

HIF-1α is regulated either at the level of protein synthesis, in response to HER2 receptor tyrosine kinase activity (Laughner et al., 2001), and in response to growth factor stimulation (Bardos et al., 2004), or at the level of protein stability by pVHL mediated degradation (Cockman et al., 2000; Jaakkola et al., 2001). To investigate whether RITA affects HIF-1α at the level of protein stability, HIF-1α protein expression in RCC4 cells that have constitutively high HIF-1α protein expression due to genetic loss of pVHL function was assessed. As shown in Figure 6.5.1, RITA inhibited HIF-1α protein in RCC4 cells that have deregulated pVHL, suggesting that RITA does not affect HIF-1α in these cells by regulating pVHL mediated protein stability. Previous data from our laboratory has shown that the proteosome inhibitor MG132 could not recover HIF-1α levels inhibited by RITA (Yang et al., 2009b). I therefore proposed that RITA could inhibit HIF-1α at the level of protein synthesis.

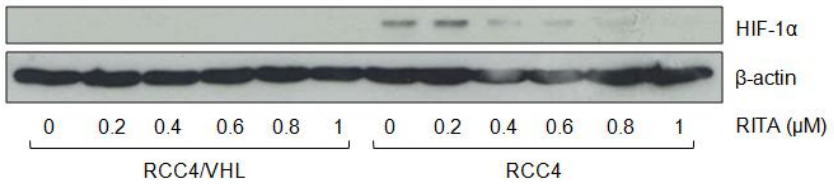


Figure 6.5.1: RITA inhibits constitutively expressed HIF-1α
Western blot showing HIF-1α protein expression in RCC4/VHL cells and RCC4 cells treated with 0-1μM RITA for 24 hours in normoxia. β-actin was used as a loading control. Data shown is representative of 3 repeats.

To explore loss of HIF-1α protein by RITA at the level of protein synthesis, components of the translational machinery were evaluated. The mTOR and the unfolded protein response (UPR) pathways are important pathways that determine transcriptional and translational responses to hypoxic stress, nutrient deprivation, and DNA damage (Wouters and Koritzinsky, 2008). Activation of p53 by stress stimuli has been associated with inhibition of mTOR mediated protein translation (Budanov and Karin, 2008; Levine et al., 2006), and changes to cell growth and survival by both mTOR and UPR translational pathways are important in determining tumour development (Zetterberg et al., 1995).

The mammalian target of rapamycin (mTOR) pathway was assessed first. Growth factors positively regulate mTOR through the PI3K-AKT/PKB pathway (Wullschleger et al., 2006). The AMP-activated protein kinase (AMPK) regulates mTOR and when activated, the mTOR complex phosphorylates various proteins including the ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), that are involved in the initiation step of mRNA translation (Browne and Proud, 2004; Wouters and Koritzinsky, 2008). Due to the role of mTOR signalling in regulating protein synthesis and cell survival in response to stress, I hypothesised that RITA treatment could affect mTOR signalling. To address this, changes in key components of the mTOR pathway were evaluated following RITA treatment. Levels of mTOR protein expression and phosphorylation of AMPK, and 4E-BP1 were not significantly affected in HCT116 p53+/+ cells that had been treated with RITA. The data suggest that mTOR signalling is not involved in regulating protein synthesis in response to RITA (Figure 6.5.2).

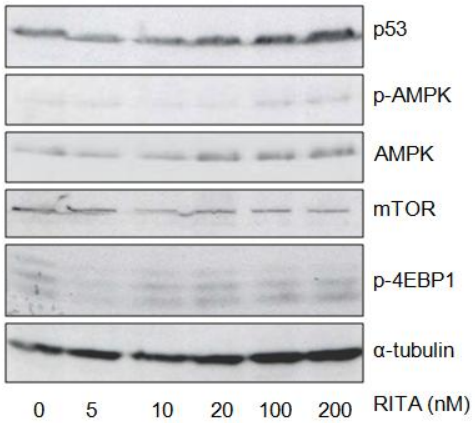


Figure 6.5.2: RITA treatment does not significantly affect the mTOR stress response pathway

HCT116 p53+/+ cells were treated with 0-500nM RITA for 16 hours in hypoxia (1% O_2). Cells were harvested for western analysis following treatment. Western blot shows p53, phosphorylated AMPK (p-AMPK), total AMPK, mTOR, phosphorylated 4EBP1 (p-4EBP1) and α-tubulin protein levels as loading controls. All data shown is representative of 3 repeat experiments.

The unfolded protein response (UPR) occurs when endoplasmic reticulum (ER) stress is induced during changes in glycosylation, redox status, glucose availability and protein load (Zhang and Kaufman, 2006). Inhibition in protein synthesis that results as a consequence of ER stress promotes cell survival by allowing sufficient time for the endoplasmic reticulum to resolve its protein load and improve function (Cullinan and Diehl, 2004). Sensors of the UPR pathway include the PKR-like ER kinase (PERK), the inositol-requiring protein-1 (IRE1) and the activating transcription factor 6 (ATF6) which signal to members of the translation initiation machinery known as the eukaryotic initiation factors (eIF) (Koumenis et al., 2002). The eIF-2 initiation complex consists of the eukaryotic initiation factor-2α (eIF-2α) subunit that binds GTP and the initiator tRNA (Met) to form a complex on the 80S ribosome and initiate mRNA translation (Asano et al., 2000). In response to hypoxia and other forms of ER stress, the eIF-2α subunit is phosphorylated at ser51 by PERK leading to inhibition of global mRNA translation (Koumenis et al., 2002).

Since RITA treatment has been shown to have no significant effect on the mTOR pathway, regulation of protein translation by the UPR pathway was investigated in response to RITA. The phosphorylation status of eIF-2α in HCT116 p53+/+ cells that

had been treated with RITA over increasing timepoints was assessed (Figure 6.5.3). Phosphorylation of eIF-2α was induced in response to RITA treatment, and this correlated with loss of HIF-1α protein expression, and stabilisation of p53. Therefore, it is proposed that RITA induces an ER stress response which inhibits protein translation by activating the UPR pathway.

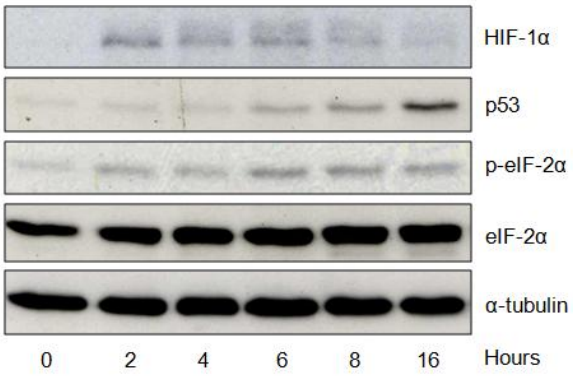


Figure 6.5.3: RITA induces eIF-2α phosphorylation

Western blot shows HCT116 p53+/+ cells treated with 500nM RITA over a 24 hour timecourse in hypoxia (1% O_2). Following treatment cells were harvested for western blotting and analysed for HIF-1α, p53, phosphorylated eIF-2α (p-eIF-2α), total eIF-2α, and α-tubulin as a loading control. All data shown is representative of 3 repeat experiments.

As discussed, PERK is an important sensor of the UPR pathway. RITA responses were assessed in HCT116 p53+/+ cells that had been stably transfected with a dominant negative form of PERK (PERKΔC), (a gift from Brad Wouters, Ontario Cancer Institute, Canada). Compared to the parental pCDNA5 cell line, HCT116 p53+/+ PERKΔC cells had a decrease in basal eIF-2α levels, as expected, as well as loss of eIf-2α phosphorylation induced by RITA, Figure 6.5.4, (Yang et al., 2009b). PERK activity may therefore be important in phosphorylation of eIF-2α by RITA.

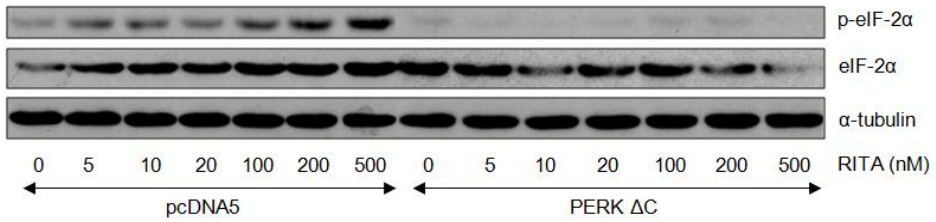


Figure 6.5.4: PERK is important for phosphorylating eIF-2α in response to RITA
Western blot analysis of HCT116 cells that have been stably transfected with either pcDNA5 as a vector control, or a dominant negative form of PERK (PERKΔC). Cells were treated with 0-500nM RITA for 16 hours in hypoxia (1% O_2). Cells were harvested for western blotting and analysed for phosphorylated eIF-2α (p-eIF-2α), total eIF-2α protein levels, and α-tubulin as a loading control. All data shown is representative of 3 independent experiments.

6.7 Loss of HIF-1α sensitises cells to RITA mediated cell death

HIF-1α can promote p53 activation and p53 induced apoptosis in response to gamma irradiation (Moeller et al., 2005), and has been shown to complex directly with p53 to promote p53 stability (An et al., 1998). However, activated p53 can also have significant inhibitory effects on the HIF pathway and on tumour angiogenesis (Ravi et al., 2000). Similarly, studies from our laboratory have indicated that activation of p53 by RITA can inhibit the HIF pathway and achieve cell death and anti-angiogenic effects in hypoxia (Yang et al., 2009b). However, the mechanism by which p53 and HIF co-operate in hypoxia-related radio- and chemoresistance is unclear.

In renal cell carcinoma, the HIF pathway is deregulated due to loss of pVHL function, while wildtype p53 is deregulated by unknown mechanisms (Gurova et al., 2004). I hypothesised that RITA could be used to induce wildtype p53 and activate p53-dependent apoptosis in renal cell carcinoma cells. RITA activity was investigated in RCC4 cells that have constitutively high HIF-1α expression due to loss of pVHL (RCC4), and in RCC4 cells which have functional pVHL, and therefore do not express HIF-1α (RCC4/VHL). As indicated by cleaved PARP, RCC4/VHL cells with loss of HIF-1α expression were sensitised to cell death at lower concentrations of RITA compared to RCC4 cells (Figure 6.7.1). The data shown suggest that HIF-1α status in RCC4 cells is important for determining the apoptotic outcome of cells treated with RITA.

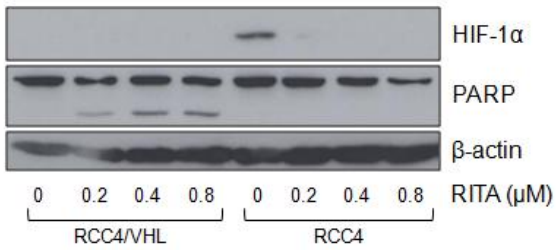


Figure 6.7.1: RITA induces cleaved PARP in RCC4/VHL cells
Western blot showing HIF-1α, and cleaved PARP protein levels in RCC4/VHL and RCC4 cells treated with 0-0.8μM RITA for 24 hours in normoxia. β-actin was used as a loading control. Data is representative of 3 repeats.

As well as renal carcinoma cells, HCT116 p53+/+ cells were also used to assess whether HIF-α status is important in determining apoptosis in colon carcinoma cells treated with RITA. The affects of RITA treatment was investigated following siRNA mediated knockdown of HIF-1α in hypoxia (Figure 6.7.2). HCT116 p53+/+ cells were found to have greater sensitivity to RITA induced apoptosis following knockdown of HIF-1α protein in hypoxia. Interestingly, basal p53 levels were also greater in cells that had lost HIF-1α protein expression, suggesting that wildtype p53 is stabilised in the absence of HIF-1α expression. In conclusion, the data presented show that HIF-α status is important in regulating basal levels of p53 protein, as well as the extent of p53-dependent cell death induced in response to RITA treatment.

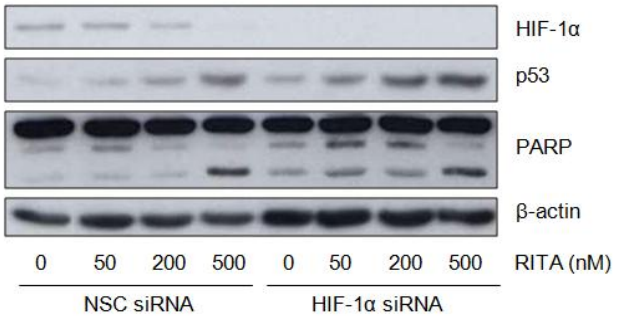


Figure 6.7.2: Loss of HIF-1α by siRNA sensitises HCT116 p53+/+ cells to cell death in response to RITA

Western blot of HCT116 p53+/+ cells transfected with 20nM of either non-silencing control siRNA (NSC), or HIF-1α targeted siRNA for 24 hours. Following transfection, cells were treated with 0-500nM RITA for 16 hours in hypoxia (1% O_2) and analysed for HIF-1α protein, p53, cleaved PARP and β-actin as a loading control.

We have shown that HIF-α status in renal and colon carcinoma cells affects p53-dependent cell death in response to RITA (as indicated by cleaved PARP). Studies using mouse xenograft models have shown that the HIF-2α subunit is important for promoting the development of clear cell renal carcinoma (Kondo et al., 2003), and neuroblastoma (Holmquist-Mengelbier et al., 2006). Recently, Bertout et al. assessed the relationship between HIF-2α and p53 mediated apoptosis in renal carcinoma cells that only express HIF-2α (Bertout et al., 2009). Studies showed that siRNA mediated knockdown of HIF-2α in renal cell carcinoma cells enhances radiation induced cell death by activating p53 transcriptional responses (Bertout et al., 2009). Since I have shown that loss of HIF-1α sensitises renal carcinoma cells to cell death by RITA, I hypothesised that HIF-2α expression alone could also affect p53-dependent cell death in response to RITA.

The 786-O renal carcinoma cell line only expresses HIF-2α protein when pVHL function is deregulated. Cell death induced in response to RITA was assessed in 786-O cells that express HIF-2α (786-O EV), and in 786-O cells that do not express HIF-2α (786-O VHL). Although p53 protein was induced in 786-O EV cells, cleaved PARP was not observed suggesting that 786-O EV cells are resistant to RITA induced apoptosis (Figure 6.7.3). Interestingly, basal p53 protein levels were significantly increased in 786-O VHL cells, compared to 786-O EV cells, and following RITA treatment, stabilised p53 was phosphorylated, and cell death was induced as indicated by cleaved PARP.

Phosphorylation of γH2AX was also observed following treatment with RITA in 786-O VHL cells, indicating induction of DNA damage (Figure 6.7.3). The data shown indicate that HIF-2α stabilisation contributes to the chemo-resistant phenotype of renal cell carcinoma, and loss of HIF-2α protein expression sensitises cells to RITA induced cell death.

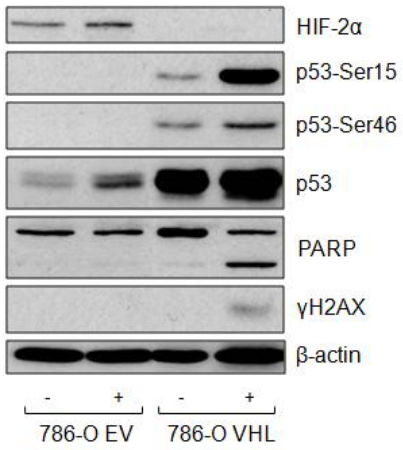


Figure 6.7.3: RITA induces p53 phosphorylation and cleaved PARP in 786-O cells with functional pVHL

Western blotting of 786-O EV and 786-O VHL cells treated with 500nM RITA for 24 hours in normoxia (- indicates DMSO treatment, and + indicates 500nM RITA treatment). Western blots show analysis of HIF-2α protein levels, phosphorylated p53 at serine 15 and serine 46 (p53-Ser15, p53-Ser46), total p53 protein, cleaved PARP, and phosphorylated γH2AX. β-actin was used as a loading control. Data shown is representative of 3 repeat experiments.

6.8 Discussion

6.8.1 RITA affects p53 targets

The studies I have presented in this chapter have shown that 500nM RITA can inhibit HIF-1α protein expression, and block the expression of p53 target proteins p21 and HDM2 (Yang et al., 2009b). Recently, low dose treatment with RITA was shown to induce HDM2 mediated degradation of hnRNP K, a specific co-factor for p53 involved in activation of p21 expression (Enge et al., 2009). By inhibiting hnRNP K activity, cell cycle responses involving p21 were repressed, and RITA could preferentially induce activation of apoptotic pathways. RITA's mechanism of action was distinct compared to nutlin-3 which activated p21 expression and cell cycle effects by inhibiting HDM2 and thereby promoting hnRNP K activity (Tovar et al., 2006; Vassilev et al., 2004).

Consistent with this study, I also found that 500nM RITA inhibits p21 protein expression, however this was not dependent on increased HDM2. Although HDM2 was induced by RITA at lower doses, and shorter timepoints, treatment with 500nM RITA where significant tumour cell death was observed correlated with reduced HDM2 protein levels. Clearly, HDM2-dependent effects induced by RITA regulate hnRNP K mediated cell cycle and cell death responses by dose-, and time-dependent mechanisms (Enge et al., 2009).

The effects of RITA on p53 targets are further supported by studies which describe important mechanisms involving transient repression of HDM2 in response to RITA (Rinaldo et al., 2009). Pro-apoptotic HIPK2 phosphorylates p53 at serine 46 to activate apoptosis in response to severe genotoxic stress (Rinaldo et al., 2007). Rinaldo et al. propose that inhibition of HDM2 by RITA treatment prevents HDM2 mediated degradation of HIPK2, and therefore promotes apoptosis by activating p53 (Rinaldo et al., 2009). Collectively the studies which assess RITA mediated activation of p53 targets show that HDM2 status following p53 activation is important in determining cell cycle and cell death responses.

6.8.2 RITA inhibits protein translation

Inhibition of p53 target proteins p21 and HDM2 in response to RITA is not regulated at the level of mRNA, and inhibition of HIF-1α protein expression by RITA is not affected by MG132, or in cells that have deregulated pVHL function (Yang et al., 2009b). Therefore, I hypothesised that RITA regulates protein levels by affecting protein synthesis. I investigated the mechanism by which HIF-1α protein expression and p53 target proteins are inhibited by RITA and explored both the mTOR pathway, and the unfolded protein response (UPR) pathway. Activation of p53 in response to genotoxic stress induces p53 target genes that are important for signalling to mTOR and inhibiting protein synthesis (Budanov and Karin, 2008). Many proteins targeted by mTOR signalling are involved in controlling the translational machinery and include the ribosomal protein S6 kinases that regulate initiation and elongation phases of translation, as well as eIF4E that is often deregulated in many tumours (Averous and Proud, 2006). The data presented in this chapter show that activation of p53 by RITA induced stress does not affect the mTOR pathway suggesting this pathway is not involved in inhibiting HIF and p53 targets in response to RITA.

The unfolded protein response is activated when hypoxia induces endoplasmic reticulum stress (Koumenis et al., 2002). This response involves key sensors such as

PERK which is involved in the initiation events of mRNA translation. By inhibiting protein synthesis in response to stress, the unfolded protein response allows energy consuming processes to be conserved so protein load is decreased on the endoplasmic reticulum, and cellular function is improved. RITA induces phosphorylation of eIf-2α, a downstream target of PERK, and this pathway is important for inhibiting protein translation. These findings have been summarised in Figure 6.8.1.

6.8.3 Loss of HIF-α sensitises cells to RITA induced apoptosis

Overexpression of both HIF-1α and HIF-2α is associated with an increase in tumour vascularisation and poor prognosis of many cancers such as breast, ovarian and non-small cell lung cancer (Bertout et al., 2008). Clear cell renal cell carcinomas account for 75% of kidney cancers, and are very aggressive cancers that show poor responses to both radiotherapy and chemotherapy due to deregulated HIF-α, and loss of functional p53 (Gurova et al., 2004). Both HIF-1α and HIF-2α have differential effects on the progression of renal cell carcinoma. While HIF-1α contributes towards tumour cell death in these tumours, HIF-2α has important tumour promoting functions (Raval et al., 2005). Restoring pVHL function in renal cell carcinoma inhibits HIF-2α protein expression and significantly decreases tumour growth *in vivo* (Kondo and Kaelin, 2001). HIF-1α and HIF-2α therefore have distinct tumourigenic effects according to the tumour type in which they are expressed (Raval et al., 2005).

Renal cell carcinoma cells were used to understand how deregulated HIF-α expression affects p53 function in response to stress. Deregulation of wildtype p53 by unknown mechanisms contributes to the resistant phenotype of renal cell carcinoma cells. Roberts et al. have suggested that HIF-2α suppresses wildtype p53 in renal cell carcinoma cells by promoting HDM2 mediated degradation of p53 (Roberts et al., 2009). Renal carcinoma cells were sensitised to chemotherapy by using nutlin-3 to stabilise p53, and also inhibiting HIF-2α protein expression, by either siRNA, or reconstitution of wildtype pVHL (Roberts et al., 2009). Recently, siRNA mediated knockdown of HIF-2α protein was also shown to activate p53-dependent apoptosis in radioresistant clear cell renal cell carcinomas (Bertout et al., 2009).

I have shown that RCC4/VHL renal carcinoma cells have higher levels of basal p53, and have greater sensitivity to RITA induced apoptosis compared to RCC4 cells that have high basal HIF-1α protein expression due to loss of pVHL function. RITA induced cell death was also evaluated in 786-O renal carcinoma cells that only express HIF-2α. Loss of HIF-2α protein expression by siRNA was recently found to sensitise 786-O

cells to doxorubicin (Roberts et al., 2009), as well as radiation (Bertout et al., 2009). I investigated the effects of RITA in 786-O cells and showed that p53 is stabilised following RITA treatment in 786-O cells that express HIF-2α. However, stabilised p53 does not correlate with cleaved PARP or induction of γH2AX. In 786-O cells with pVHL, basal levels of wildtype p53 were significantly increased, and following RITA treatment, significant cell death was induced in these cells. The use of small molecule activators of p53 in renal cell carcinoma provides a novel therapeutic strategy with which to restore wildtype p53 activity and sensitise resistant tumour cells to apoptotic signals. While nutlin-3 sensitises 786-O EV cells to doxorubicin and etoposide induced cell death (Roberts et al., 2009), I have shown that RITA can both stabilise p53, and induce DNA damage responses in renal carcinoma cells with deregulated HIF-α.

6.9 Conclusions for this chapter

- Treatment of HCT116 p53+/+ cells with 500nM RITA inhibits HIF-1α protein expression in hypoxia, and inhibits the p53 target proteins, p21 and HDM2.
- RITA does not affect protein stability or mRNA levels of HIF-1α and p53 targets p21 and HDM2.
- Phosphorylation of eIF-2α by RITA is dependent on PERK activity.
- Loss of HIF-1α and HIF-2α in renal cell carcinoma sensitises cells to RITA induced cell death.

6.10 Impact of these findings

Activation of p53 by RITA induced stress drives cell death and DNA damage responses in cells with low HIF-1α and HIF-2α expression. HIF-α status is important in determining the apoptotic response elicited by RITA and the data presented in this chapter suggest that inhibition of specific HIF-α subunits in specific tumours could be used in combination with p53 activating molecules to improve therapeutic targeting of tumours with deregulated HIF and p53 pathways.

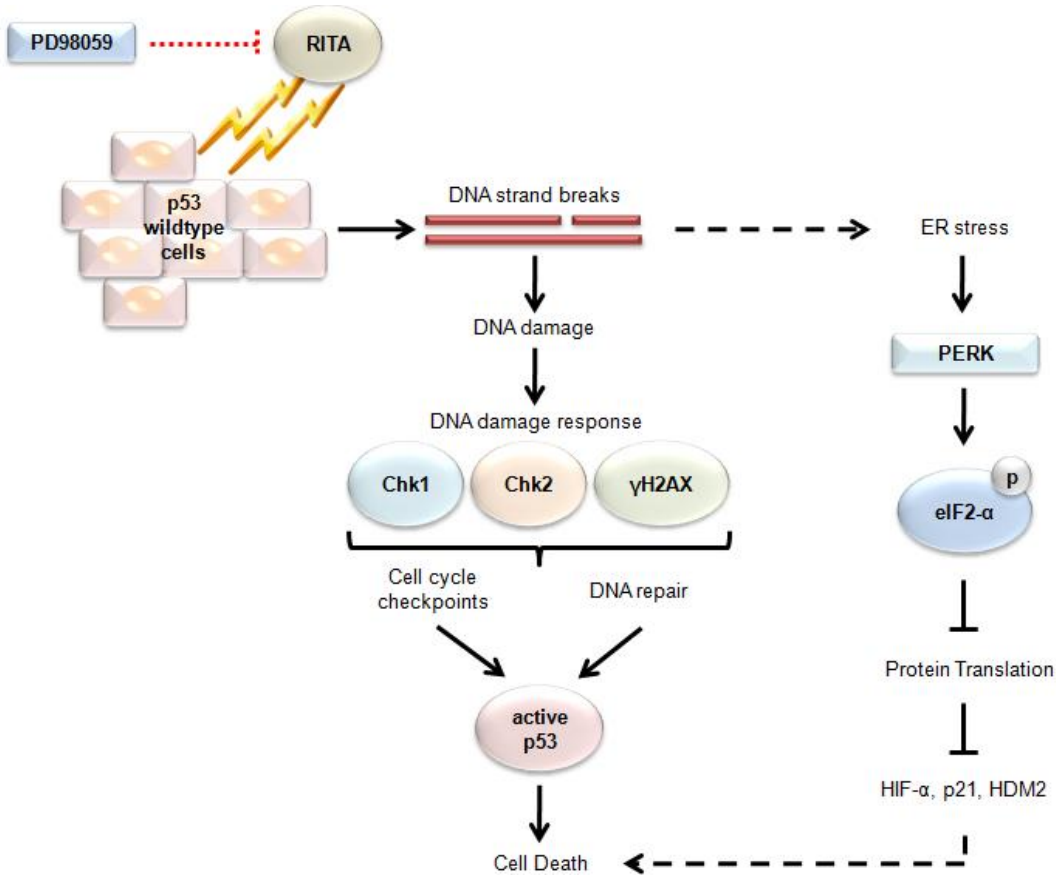


Figure 6.8.1: RITA inhibits HIF-α protein in hypoxia and p53 targets by engaging the PERK translational pathway

RITA targets p53 wildtype cells to induce DNA damage and activate cell cycle and DNA damage responses that mediate anti-tumour activity. Studies with PD98059 demonstrate that the MAPK pathway is important in activating p53 through this pathway. In addition to these findings, RITA also elicits an ER stress response involving activation of PERK and phosphorylation of eIF2-α which lead to inhibition of protein synthesis. Specifically, loss of HIF-1α protein expression and the p53 targets p21 and HDM2 has been observed (Yang et al., 2009b). These responses also contribute significantly to the anti-tumour activity of RITA.

Chapter 7

General Summary

7.1 General Summary

Activation of p53 is important for the antitumor effects of most chemotherapies and loss of p53 function in cancer has been associated with increased resistance to many forms of therapy (El-Deiry, 2003). As with p53, HIF-1α status is also an important determinant for responses to chemo- and radiotherapy, and the severity of hypoxia in tumours correlates with poor patient prognosis (Moeller et al., 2005). Thus, both p53 and HIF-1α have emerged as important therapeutic targets.

Nutlin-3 and RITA have been investigated as small molecule activators of p53-dependent cell death in hypoxic tumours. Nutlin-3, can sensitise hypoxic tumour cells with wildtype p53 when used in combination with radiotherapy (Supiot et al., 2008). The small molecule RITA not only activates p53 by eliciting p53-dependent cell cycle and DNA damage responses, but also targets the HIF pathway, thus achieving both apoptotic and anti-angiogenic effects in hypoxia. The data presented in this thesis strengthens the negative relationship that exists between p53 activity and HIF status in a number of tumour models, while also revealing the presence of novel mechanisms by which small molecule activation of p53 targets HIF signalling in hypoxia.

Several small molecule activators of p53 have been described with distinct mechanisms of action. In order to understand the importance of the studies presented so far, the clinical progress of p53 targeted agents will be discussed briefly, as well as the benefits and drawbacks of investigating agents like RITA.

7.1.1 Clinical importance of p53 activating agents

To date over 150 clinical trials have been designed using p53 status as a clinical biomarker (Cheok et al., 2011). Initial strategies to target wildtype p53 in tumours were developed with caution due to the possibility that normal cells with wildtype p53 would also be targeted by such agents. Studies by Ringhausen et al. showed that loss of *mdm2* expression *in vivo* resulted in spontaneous cell death and tissue damage in adult mice, raising concerns that HDM2 inhibitors would be excessively cytotoxic (Ringshausen et al., 2006). However subsequent studies showed that moderate decreases in *mdm2* activity were well tolerated and had significant anti-tumour activity *in vivo* (Mendrysa et al., 2006). Small molecules that disrupt the p53-HDM2 interaction such as the nutlins and the spiro-oxindoles have since shown anti-tumour activity that is p53-dependent and tumour selective (Shangary et al., 2008; Vassilev et al., 2004). As a result, the most advanced p53 activating molecules in the clinic are inhibitors of

HDM2 (Shangary and Wang, 2008) and analyses of clinical samples correlate responses to HDM2 inhibition with wildtype p53 status (Saddler et al., 2008).

Despite the progress that p53 activating agents have made in the clinic, there is continuous need to improve the safety profile of targeted agents, both as monotherapies and in strategic combinations. The advantages and disadvantages of p53 activating agents will be discussed further with particular focus on RITA as a small molecule activator of p53-dependent cell death that I have studied.

7.1.2 The advantages and drawbacks of small molecule activators of p53

Conventional cytotoxic therapy is aimed at targeting rapidly proliferating cancer cells. Some cancers such as high grade lymphomas are highly proliferative and undergo maximum tumour cell death following cytotoxic therapy. However the main drawback of many cytotoxic agents is that rapidly proliferating normal cells of the bone marrow, gastrointestinal mucosa, hair follicles and gonads are also targeted resulting in severe unwanted side effects such as mylosuppression, nausea, vomiting, hair loss and reduced fertility (Corrie, 2008). Non-genotoxic small molecule agents were developed with the intention of specifically targeting deregulated signalling pathways to enhance tumour cell specificity while reducing the onset of resistance, and the serious side effects that are associated with conventional cytotoxics. To this end, several p53 activating molecules have been identified and are lead compounds for the development of anticancer drugs (Lane et al., 2010). However, small molecule activators of p53 are also important tools for studying p53 functional activity. For this study, RITA has been used as a pharmacological tool to investigate p53-dependent cell death responses in normoxia and in hypoxia.

The most significant advantage of RITA as a small molecule is that RITA selectively induces apoptosis in cells that are p53 positive, and mediates anti-tumour effects both *in vitro* and *in vivo* by this mechanism. However the studies presented in this thesis have shown that RITA also affects numerous other pathways. RITA not only elicits DNA damage, but also induces DNA damage responses, and affects components of the translational machinery. Multiple target pathways affected by RITA have also been investigated in other reports (Enge et al., 2009; Rivera et al., 1999). As discussed in this thesis, these pathways collectively contribute to the anti-tumour activity of RITA. Although DNA damage induced by RITA is not observed to the same extent as conventional DNA damaging agents like cisplatin, RITA would not be an ideal candidate for clinical development, as it would be regarded as a cytotoxic agent and

would be predicted to elicit numerous side effects in normal cells. Both *in vitro* and *in vivo* data suggest that RITA has tumour cell specificity at low doses, and without significant side effects (Issaeva et al., 2004; Yang et al., 2009b) however these studies would need to be conducted in greater detail if RITA was to be pursued as a clinical agent, both as a monotherapy, and in combination against other targeted agents.

Combination therapy is used to maximise tumour cell apoptosis, and also avoid acquired resistance (Brummelkamp et al., 2006). Recently, cyclotherapy was described whereby low doses of a non-genotoxic p53 inducing agent was used to induce reversible cell cycle arrest in normal cells (Carvajal et al., 2005) prior to dosing with a specific agent that targeted the remaining proliferating cancer cells (Sur et al., 2009). Cyclotherapy proved to be an effective strategy for inducing tumour cell death while inhibiting effects on normal cells that are otherwise associated with the development of unwanted side effects (Sur et al., 2009). In other schedules, nutlin-3 was used in combination with the X-linked inhibitor of apoptosis (XIAP) to improve tumour cell death in patients with acute myeloid leukaemia (Carter et al., 2010). My studies have shown that loss of HIF-α by either siRNA mediated knockdown, or due to restoration of pVHL function, can sensitise resistant renal cell carcinoma cells to cell death in response to RITA. These studies are supported by previous findings whereby loss of HIF-2α can sensitise renal cell carcinoma cells to irradiation (Bertout et al., 2009) and provide an important rationale for combination therapies involving both p53 activating agents and HIF inhibitors in specific tumour models whereby both pathways are deregulated.

Tumourigenesis is now considered a multistep process involving sequential deregulation of multiple signalling pathways. Therefore the rationale for developing a ‘magic bullet’ which could target a single deregulated molecule in cancer cells has now been replaced with strategies whereby ‘multi-targeted’ agents affect several deregulated pathways in the same disease. Such multi-targeted kinase inhibitors have better safety profiles compared to conventional cytotoxics (Arora and Scholar, 2005; Crean et al., 2009). Sorafenib for example inhibits different isoforms of the RAF serine kinase as well as targeting other receptor tyrosine kinases such as VEGFR, EGFR and PDGFR and this broad spectrum target profile improves the inhibitory affects of sorafenib on tumour cell proliferation and angiogeneisis (Strumberg, 2005). Indeed I have shown that RITA targets both deregulated p53 and HIF pathways in specific tumour models, and although I have suggested that RITA’s effects on the DNA may be a disadvantage in terms of RITA’s safety profile, this multi-target profile may also give RITA an advantage over other DNA damaging agents in certain tumour models.

Overall, these studies support the fact that multiple pathways lead to p53 activation and suggest that simultaneous targeting of specific co-operative oncogenic pathways could have significant effects on treatment responses.

In summary, the most significant disadvantage that we have discussed of targeted p53 activating agents such as RITA is the possibility that they may have an undesirable therapeutic window, and for this reason may cause unwanted side effects, as is the case with conventional cytotoxics. Most small molecule activators of p53 have overcome these barriers and have made significant progress in clinical schedules. In conclusion, whether or not RITA is a desirable agent for clinical development due to its DNA damaging effects, the most important advantage of this small molecule is its ability to induce p53-dependent cell death by numerous pathways. For this reason, RITA has been used extensively as a pharmacological tool to understand the mechanisms by which RITA induces p53-dependent cell death responses in normoxia and in hypoxia. The novel mechanisms such as the p53-dependent cell cycle checkpoints that have been described involving CHK1 phosphorylation are important in understanding how tumour cells respond to immediate forms of genotoxic stress, and how abrogation of these pathways at specific timepoints can be used to suppress tumour growth. The key findings from this thesis will now be summarised for the different signalling pathways that have been studied in response to RITA.

7.1.3 Summary of key findings and ongoing studies

7.1.3.1 RITA induces p53-dependent cell death in normoxia and hypoxia

RITA was identified as a small molecule that could induce p53-dependent cell death in tumour models by binding to wildtype p53, inhibiting its interaction with HDM2, and therefore promoting p53 activation (Issaeva et al., 2004). My studies have shown for the first time that RITA mediated cell death is distinct compared to other agents that activate p53, in that RITA can elicit p53-dependent DNA damage responses involving γH2AX and CHK1, and also induce significant cell death both in normoxia and hypoxia.

7.1.3.2 RITA activates p53-dependent cell cycle checkpoints

Further elucidation of the mechanism of action by which RITA elicits p53-dependent cell death responses in normoxia and hypoxia showed that following DNA damage, RITA can induce the canonical DNA damage response involving CHK1 and CHK2. Induction of γH2AX was also shown in response to RITA, another marker of DNA damage due to its localisation at DNA strand breaks. The p53-dependent CHK1

phosphorylation in response to RITA was investigated. Although CHK1 phosphorylation was not essential for p53-dependent cell death by RITA, a novel p53-dependent cell cycle checkpoint was described that has importance in modulating DNA repair and hence maintaining DNA integrity in response to genotoxic stress.

7.1.3.3 RITA induced apoptosis is mediated by MAPK signalling

MAPK signalling regulates cell death responses when cells are exposed to a variety of microenvironmental and external stress stimuli. I investigated whether RITA engages MAPK signalling to promote p53 stability and activity. PD98059, a MEK1/MEK2 inhibitor was used to show that ERK1/2 phoshorylation by RITA is important for eliciting p53-dependent DNA damage and cell death responses described previously. Several MAPKs were identified that are induced in response to RITA including JNK kinase, p38 kinase, and the p38 upstream MAPKK, MKK3. A MAPK siRNA screen was also conducted in which knockdown of numerous kinases was shown to inhibit p53 stabilisation, and γH2AX induction in response to RITA treatment. It will be of interest to reconfirm the MAPKs identified in the screen and evaluate their contribution to RITA mediated responses.

7.1.3.4 HIF status influences RITA induced cell death

Stabilisation of p53 by RITA induces the p53 targets HDM2 and p21, however this response is transient and at doses of 500nM and above where p53 stabilisation is maintained, p21 and HDM2 protein expression is inhibited. Initial studies from our laboratory showed that RITA can also inhibit HIF-1α protein expression in hypoxic cells. I explored the mechanism by which p53 target proteins and HIF-1α protein expression is inhibited in cells treated with RITA. RITA was found to induce an ER stress response, and by activating the PERK and eIF-2α pathway was able to inhibit global protein synthesis and therefore the expression of p21, HDM2 and HIF-1α.

Following the findings that RITA can induce both p53 and inhibit HIF-1α, RITA responses were studied in tumour cells in which HIF signalling and p53 activity is deregulated. Renal cell carcinoma cells were used that express both HIF-1α and HIF-2α, or HIF-2α alone due to loss of pVHL function. I showed that renal cell carcinoma cells are sensitised to RITA induced cell death following inhibition of HIF expression by either reconstitution of pVHL, or by siRNA mediated knockdown. The studies presented show that HIF status is important in influencing p53-dependent apoptosis, and provide novel opportunities for the therapeutic targeting of tumours in which HIF signalling is deregulated.

Inactivation of pVHL is associated with development of hereditary and sporadic clear cell renal carcinoma (Kondo et al., 2003), however cell cycle and apoptosis pathways mediated by pVHL are not always dependent on deregulation of the HIF pathway (Kondo et al., 2002; Roe et al., 2006). In response to DNA damage, pVHL is proposed to bind directly to p53 and stabilises p53 by inhibiting p53 binding to HDM2 and suppressing its nuclear export. It will be of interest to understand whether tumour cell apoptosis induced by RITA in renal carcinoma cells is dependent on pVHL and/or HIF-α activity.

7.2 Future studies

7.2.1 Exploring transcriptional co-factors for p53 in hypoxia

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a transcriptional regulator that interacts with many factors involved in chromatin remodelling and has an important role to play in both transcriptional and translational pathways (Bomsztyk et al., 2004). Previously, hnRNP K has been shown to be induced by the DNA damage response pathway, and is important for maintaining cell cycle checkpoints in response to stress (Moumen et al., 2005). Interestingly, hnRNP K acts as an important cofactor for p53 target gene expression (Moumen et al., 2005). Although little is known about the regulation of hnRNP K itself, its involvement in regulating p53 transcription is established and the dependency of this partnership is clear in studies whereby loss of hnRNP K abrogates p53 mediated checkpoints when cells are exposed to DNA damaging agents (Moumen et al., 2005).

Recently, Enge et al. described mechanisms by which differential responses occur to the small molecules nutlin and RITA, both involved in activating p53. While nutlin primarily induced cell cycle arrest by activating p53, RITA induced significant cell death responses. By inhibiting HDM2, nutlin was shown to promote p53 stabilisation, and its association with the transcriptional cofactor hnRNP K to induce p21 gene expression, and subsequent cell cycle arrest (Enge et al., 2009). Moumen and colleagues have previously shown that hnRNP K is regulated at the level of protein stability by HDM2 mediated degradation (Moumen et al., 2005). By binding directly to p53, RITA induced HDM2 targeting of hnRNP K for proteosomal degradation. Cell death responses were activated by RITA due to loss of hnRNP K and inhibition of cell cycle arrest by p21 (Enge et al., 2009).

Because of the involvement of hnRNP K as a critical component of the p53 DNA damage response (Moumen et al., 2005), and data which suggests that hnRNP K activity is modulated in the presence of RITA (Enge et al., 2009), it would be of interest to assess hnRNP K and whether this could act as a critical co-factor for the p53-dependent responses that we have described in our study.

7.2.2 Investigating low dose radiation

The role of p53 in eliciting G1/S and G2/M checkpoints has been described in many studies (Levine, 1997). DNA damage checkpoints induced during S-phase have been studied frequently as mechanisms by which DNA synthesis is inhibited in response to DNA damage. However the role of p53 in mediating S-phase checkpoints is less established. DNA damage can inhibit DNA synthesis by blocking replication origin firing and delaying replication fork progression. Delaying DNA synthesis by activating the S-phase checkpoint is an important mechanism by which DNA repair pathways are given enough time to resolve DNA strand breaks (Boddy and Russell, 2001).

Low dose radiation (below 2.5 Gray) has previously been used to study a p53-dependent S-phase checkpoint in mouse embryonic fibroblasts (MEFs) (Shimura et al., 2006). Shimura et al. show that p53 null MEFs are resistant to inhibition of replication fork progression that is observed in p53 wildtype cells following exposure to low dose irradiation. Furthermore, ATM activation is essential for eliciting the S-phase checkpoint described in p53 wildtype MEFs (Shimura et al., 2006). To my knowledge, our study is the first to describe a p53-dependent S-phase checkpoint that is mediated by CHK1 phosphorylation.

It will be of interest to elucidate whether CHK1 is essential for the replication fork block that has been observed in p53 wildtype cells that have been treated with RITA. Such insights will provide important information on the levels at which p53 and CHK1 regulate genomic integrity. Furthermore, RITA has been used as a tool for understanding how p53 activation can mediate cell death responses in normoxia and in hypoxia. Like low dose irradiation, RITA elicits DNA damage that is moderate, but appears sufficient to transmit significant cell cycle and cell death responses. Studying low dose irradiation as an alternative means with which to activate p53 in normoxia, and in hypoxia will be worthwhile. By targeting p53-dependent cell cycle checkpoints and DNA repair pathways, low dose radiation may therefore be an alternative therapeutic strategy with which to sensitise hypoxic tumours to cell death.

7.2.3 Investigating effects of RITA on ROS production

Alternative targets for RITA have been investigated in several studies. Recently, as well as targeting p53 to elicit p53-dependent apoptosis (Issaeva et al., 2004), RITA was shown to target Thioredoxin reductase 1 (TrxR1), (Hedstrom et al., 2009). TrxR1 is a critical thioredoxin that regulates redox dependent pathways involved in many cell signalling pathways (Arner and Holmgren, 2006; Nordberg and Arner, 2001). Treatment of HCT116 p53+/+ and HCT116 p53-/- cells with RITA was shown to increase reactive oxygen species (ROS) production due to direct inhibition of TrxR1 activity (Hedstrom et al., 2009). Detection of the fluorescence based conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) using flow cytometry showed that RITA induced ROS production was only observed in HCT116 p53+/+ cells. Furthermore, TrxR1 is often overexpressed in human cancers, and the absence RITA mediated ROS production in normal fibroblast cells supported the tumour selective role for cell death in response to RITA (Hedstrom et al., 2009). Overall, Hedstrom and colleagues suggested that RITA sensitises tumour cells to cell death by promoting both p53 stability, and oxidative stress.

It will be of interest to investigate RITA induced ROS production in HCT116 p53+/+ cells and HCT116 p53-/- cells over the dose range at which p53-dependent DNA damage responses and cell death responses have been described in this thesis. This is because TrxR1 has also been shown to be targeted by common alkylating agents and platinum compounds (Witte et al., 2005). Furthermore, because ROS generation has been shown to be enhanced under hypoxic stress (Chandel et al., 1998), studying the effects of ROS in hypoxia, both in the absence and presence of RITA treatment will provide greater insight into the distinct role that oxidative stress has in eliciting p53-dependent cell death in hypoxia. To strengthen these observations, responses will also be compared to those induced by hydrogen peroxide, and in response to N-acetyl-cysteine (NAC), a commonly used ROS inhibitor.

Finally, the role that RITA induced ROS production has on induction of the unfolded protein response and on global protein translation should also be assessed. The oxidative environment of the endoplasmic reticulum is tightly regulated to ensure correct protein folding. Deregulated protein oxidation and elevated ROS levels can activate the unfolded protein response, hence affecting protein synthesis, and cell survival (Malhotra and Kaufman, 2007). Therefore, activation of ROS in response to RITA may also link the unfolded protein response to the p53-dependent pathways that have been described throughout this thesis.

7.3 Final Conclusions

Hypoxia, defined as low oxygen tension, is a common characteristic of growing tumours. Tumour cells adapt to their hypoxic microenvironment by inducing angiogenesis and escaping cell death. In doing so, tumour cells become resistant to radiotherapy and many forms of chemotherapy. Hypoxia signalling and angiogenesis is mediated by the hypoxia-inducible factor (HIF) transcriptional complex. HIF can cross-talk to the p53 tumour suppressor protein, a critical regulator of cellular responses to stress. This study has aimed to understand how cell death responses are regulated in tumour cells by HIF and p53, in normoxia and in hypoxia. These findings are summarised below, and in Figure 7.3.1.

1. *Chapter 3:* I have investigated activation of p53 by the small molecule RITA. Flow cytometry, comet assays and western blot analysis was used to reveal a novel p53-dependent DNA damage response that occurs as a result of DNA strand breaks, and induces significant cell death of tumour cells with wildtype p53.

2. *Chapter 4:* In addition, I have assessed S-phase cells and describe a novel p53-dependent cell cycle checkpoint that involves phosphorylation of CHK1 in response to replication stress induced by RITA. Although not essential for RITA induced cell death, I show that activation of this cell cycle checkpoint is important for DNA repair and genomic integrity.

3. *Chapter 5:* I have also investigated the MAPK pathway as a critical mediator of RITA induced p53-dependent cell death. Inhibition of MEK-ERK phosphorylation by the MAPK signalling inhibitor PD98059 inhibits p53-mediated apoptotic responses by RITA.

4. *Chapter 6:* RITA inhibits global protein synthesis, resulting in significant inhibitory effects on HIF-1α in hypoxia, and the p53 targets p21 and HDM2.

Novel mechanisms have been revealed for p53 activation involving MAPK signalling and p53-dependent pathways that elicit DNA damage, cell cycle and cell death responses. Understanding the mechanisms by which the small molecule RITA achieves anti-tumour effects provides novel insights into the numerous signalling pathways that integrate at the level of stress induced p53 activation, both in normoxia and in hypoxia. These pathways have significance in tumour models like renal cell carcinoma whereby both HIF and p53 pathways are deregulated rendering such

tumours resistant to radiotherapy and many forms of chemotherapy. By harnessing p53 stress induced pathways strategically and in combination with other targeted agents, the therapeutic profile of highly resistant tumours with deregulated HIF and p53 can be significantly improved.

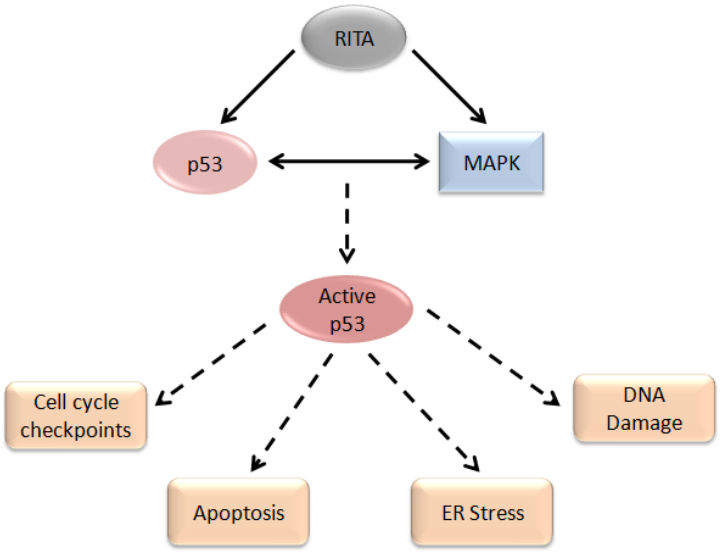


Figure 7.3.1: RITA induced p53 targets multiple signalling pathways that promote tumour cell death in normoxia and hypoxia

RITA activates the MAPK pathway and targets wildtype p53 to elicit p53-dependent cell death responses. Several signalling pathways are involved in these responses. I have investigated DNA damage responses induced by RITA, cell cycle checkpoints, and ER stress responses that inhibit protein synthesis. p53 is an integral mediator of all these signalling pathways that together contribute significantly to the anti-tumour activity of RITA.

References

Acker, T., Diez-Juan, A., Aragones, J., Tjwa, M., Brusselmans, K., Moons, L., Fukumura, D., Moreno-Murciano, M.P., Herbert, J.M., Burger, A.*, et al.* (2005). Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell *8*, 131-141.

Adams, R.H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., and Nebreda, A.R. (2000). Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell *6*, 109-116.

Adler, V., Pincus, M.R., Minamoto, T., Fuchs, S.Y., Bluth, M.J., Brandt-Rauf, P.W., Friedman, F.K., Robinson, R.C., Chen, J.M., Wang, X.W.*, et al.* (1997). Conformation-dependent phosphorylation of p53. Proc Natl Acad Sci U S A *94*, 1686-1691.

Agarwal, M.L., Agarwal, A., Taylor, W.R., Chernova, O., Sharma, Y., and Stark, G.R. (1998). A p53-dependent S-phase checkpoint helps to protect cells from DNA damage in response to starvation for pyrimidine nucleotides. Proc Natl Acad Sci U S A *95*, 14775-14780.

Ahmed, A., and Ashcroft, M. (2010). TRP-ing off the p53 apoptotic switch. Pigment Cell Melanoma Res *23*, 581-582.

Ahmed, A., Yang, J., Maya-Mendoza, A., Jackson, D.A., and Ashcroft, M. (2011). Pharmacological activation of a novel p53-dependent S-phase checkpoint involving CHK-1. Cell Death Dis *2*, e160.

Akyuz, N., Boehden, G.S., Susse, S., Rimek, A., Preuss, U., Scheidtmann, K.H., and Wiesmuller, L. (2002). DNA substrate dependence of p53-mediated regulation of double-strand break repair. Mol Cell Biol *22*, 6306-6317.

Al Rashid, S.T., Dellaire, G., Cuddihy, A., Jalali, F., Vaid, M., Coackley, C., Folkard, M., Xu, Y., Chen, B.P., Chen, D.J.*, et al.* (2005). Evidence for the direct binding of phosphorylated p53 to sites of DNA breaks in vivo. Cancer Res *65*, 10810-10821.

Albain, K.S., Swann, R.S., Rusch, V.W., Turrisi, A.T., 3rd, Shepherd, F.A., Smith, C., Chen, Y., Livingston, R.B., Feins, R.H., Gandara, D.R.*, et al.* (2009). Radiotherapy plus chemotherapy with or without surgical resection for stage III non-small-cell lung cancer: a phase III randomised controlled trial. Lancet *374*, 379-386.

Albrechtsen, N., Dornreiter, I., Grosse, F., Kim, E., Wiesmuller, L., and Deppert, W. (1999). Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53. Oncogene *18*, 7706-7717.

Alderden, R.A., Mellor, H.R., Modok, S., Hambley, T.W., and Callaghan, R. (2006). Cytotoxic efficacy of an anthraquinone linked platinum anticancer drug. Biochem Pharmacol *71*, 1136-1145.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem *270*, 27489-27494.

An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., and Neckers, L.M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature *392*, 405-408.

Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B., and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. Pharm Res *25*, 2097-2116.

Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature *343*, 651-653.

Arner, E.S., and Holmgren, A. (2006). The thioredoxin system in cancer. Semin Cancer Biol *16*, 420-426.

Arora, A., and Scholar, E.M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther *315*, 971-979.

Arora, S., Bisanz, K.M., Peralta, L.A., Basu, G.D., Choudhary, A., Tibes, R., and Azorsa, D.O. (2010). RNAi screening of the kinome identifies modulators of cisplatin response in ovarian cancer cells. Gynecol Oncol *118*, 220-227.

Arum, O., and Johnson, T.E. (2007). Reduced expression of the Caenorhabditis elegans p53 ortholog cep-1 results in increased longevity. J Gerontol A Biol Sci Med Sci *62*, 951-959.

Asano, K., Clayton, J., Shalev, A., and Hinnebusch, A.G. (2000). A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNA(Met) is an important translation initiation intermediate in vivo. Genes Dev *14*, 2534-2546.

Ashcroft, M., Kubbutat, M.H., and Vousden, K.H. (1999). Regulation of p53 function and stability by phosphorylation. Mol Cell Biol *19*, 1751-1758.

Ashcroft, M., Ludwig, R.L., Woods, D.B., Copeland, T.D., Weber, H.O., MacRae, E.J., and Vousden, K.H. (2002). Phosphorylation of HDM2 by Akt. Oncogene *21*, 1955-1962.

Ashcroft, M., Taya, Y., and Vousden, K.H. (2000). Stress signals utilize multiple pathways to stabilize p53. Mol Cell Biol *20*, 3224-3233.

Ashley, D.J. (1969). The two "hit" and multiple "hit" theories of carcinogenesis. Br J Cancer *23*, 313-328.

Avantaggiati, M.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S., and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175-1184.

Averous, J., Fonseca, B.D., and Proud, C.G. (2008). Regulation of cyclin D1 expression by mTORC1 signaling requires eukaryotic initiation factor 4E-binding protein 1. Oncogene *27*, 1106-1113.

Averous, J., and Proud, C.G. (2006). When translation meets transformation: the mTOR story. Oncogene *25*, 6423-6435.

Aylon, Y., and Oren, M. (2007). Living with p53, dying of p53. Cell *130*, 597-600.

Bae, B.I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S.D., Moran, T.H., Montell, C., Ross, C.A.*, et al.* (2005). p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. Neuron *47*, 29-41.

Baehrecke, E.H. (2005). Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol *6*, 505-510.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J *408*, 297-315.

Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K.P., Szekely, L., Klein, G., Terenius, L., and Wiman, K.G. (1995). p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. Nucleic Acids Res *23*, 362-369.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K., and Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y.*, et al.* (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.

Bardos, J.I., and Ashcroft, M. (2005). Negative and positive regulation of HIF-1: a complex network. Biochim Biophys Acta *1755*, 107-120.

Bardos, J.I., Chau, N.M., and Ashcroft, M. (2004). Growth factor-mediated induction of HDM2 positively regulates hypoxia-inducible factor 1alpha expression. Mol Cell Biol *24*, 2905-2914.

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

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C.*, et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Batchelor, E., Mock, C.S., Bhan, I., Loewer, A., and Lahav, G. (2008). Recurrent initiation: a mechanism for triggering p53 pulses in response to DNA damage. Mol Cell *30*, 277-289.

Beall, C.M., Cavalleri, G.L., Deng, L., Elston, R.C., Gao, Y., Knight, J., Li, C., Li, J.C., Liang, Y., McCormack, M.*, et al.* (2010). Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan highlanders. Proc Natl Acad Sci U S A *107*, 11459-11464.

Beckerman, R., Donner, A.J., Mattia, M., Peart, M.J., Manley, J.L., Espinosa, J.M., and Prives, C. (2009). A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. Genes Dev *23*, 1364-1377.

Bencokova, Z., Kaufmann, M.R., Pires, I.M., Lecane, P.S., Giaccia, A.J., and Hammond, E.M. (2009). ATM activation and signaling under hypoxic conditions. Mol Cell Biol *29*, 526-537.

Bergamaschi, D., Samuels, Y., Jin, B., Duraisingham, S., Crook, T., and Lu, X. (2004). ASPP1 and ASPP2: common activators of p53 family members. Mol Cell Biol *24*, 1341-1350.

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer *8*, 592-603.

Bertout, J.A., Majmundar, A.J., Gordan, J.D., Lam, J.C., Ditsworth, D., Keith, B., Brown, E.J., Nathanson, K.L., and Simon, M.C. (2009). HIF2alpha inhibition promotes p53 pathway

activity, tumor cell death, and radiation responses. Proc Natl Acad Sci U S A *106*, 14391-14396.

Bertout, J.A., Patel, S.A., and Simon, M.C. (2008). The impact of O2 availability on human cancer. Nat Rev Cancer *8*, 967-975.

Bischof, O., Kim, S.H., Irving, J., Beresten, S., Ellis, N.A., and Campisi, J. (2001). Regulation and localization of the Bloom syndrome protein in response to DNA damage. J Cell Biol *153*, 367-380.

Boddy, M.N., and Russell, P. (2001). DNA replication checkpoint. Curr Biol *11*, R953-956.

Bohensky, J., Leshinsky, S., Srinivas, V., and Shapiro, I.M. (2010). Chondrocyte autophagy is stimulated by HIF-1 dependent AMPK activation and mTOR suppression. Pediatr Nephrol *25*, 633-642.

Bomsztyk, K., Denisenko, O., and Ostrowski, J. (2004). hnRNP K: one protein multiple processes. Bioessays *26*, 629-638.

Bonner, W.M., Redon, C.E., Dickey, J.S., Nakamura, A.J., Sedelnikova, O.A., Solier, S., and Pommier, Y. (2008). GammaH2AX and cancer. Nat Rev Cancer *8*, 957-967.

Borghaei, H., Smith, M.R., and Campbell, K.S. (2009). Immunotherapy of cancer. Eur J Pharmacol *625*, 41-54.

Borsch-Haubold, A.G., Pasquet, S., and Watson, S.P. (1998). Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059. SB 203580 also inhibits thromboxane synthase. J Biol Chem *273*, 28766-28772.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science *249*, 64-67.

Boya, P., Gonzalez-Polo, R.A., Casares, N., Perfettini, J.L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T.*, et al.* (2005). Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol *25*, 1025-1040.

Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J.J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R.A., and Davis, R.J. (2003). Mechanism of p38 MAP kinase activation in vivo. Genes Dev *17*, 1969-1978.

Bretaud, S., Allen, C., Ingham, P.W., and Bandmann, O. (2007). p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. J Neurochem *100*, 1626-1635.

Brown, E.J., and Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev *17*, 615-628.

Brown, J.M., and Wilson, W.R. (2004). Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer *4*, 437-447.

Brown, K.D., Rathi, A., Kamath, R., Beardsley, D.I., Zhan, Q., Mannino, J.L., and Baskaran, R. (2003). The mismatch repair system is required for S-phase checkpoint activation. Nat Genet *33*, 80-84.

Browne, G.J., and Proud, C.G. (2004). A novel mTOR-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin. Mol Cell Biol *24*, 2986-2997.

Bruick, R.K. (2000). Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A *97*, 9082-9087.

Brummelkamp, T.R., Fabius, A.W., Mullenders, J., Madiredjo, M., Velds, A., Kerkhoven, R.M., Bernards, R., and Beijersbergen, R.L. (2006). An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. Nat Chem Biol *2*, 202-206.

Budanov, A.V., and Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell *134*, 451-460.

Bulavin, D.V., Demidov, O.N., Saito, S., Kauraniemi, P., Phillips, C., Amundson, S.A., Ambrosino, C., Sauter, G., Nebreda, A.R., Anderson, C.W.*, et al.* (2002). Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet *31*, 210-215.

Bulavin, D.V., and Fornace, A.J., Jr. (2004). p38 MAP kinase's emerging role as a tumor suppressor. Adv Cancer Res *92*, 95-118.

Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E., and Fornace, A.J., Jr. (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature *411*, 102-107.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J., Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. Embo J *18*, 6845-6854.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., and Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009-1017.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282*, 1497-1501.

Butt, A.J., Firth, S.M., King, M.A., and Baxter, R.C. (2000). Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J Biol Chem *275*, 39174-39181.

Bykov, V.J., Zache, N., Stridh, H., Westman, J., Bergman, J., Selivanova, G., and Wiman, K.G. (2005). PRIMA-1(MET) synergizes with cisplatin to induce tumor cell apoptosis. Oncogene *24*, 3484-3491.

Calzado, M.A., Renner, F., Roscic, A., and Schmitz, M.L. (2007). HIPK2: a versatile switchboard regulating the transcription machinery and cell death. Cell Cycle *6*, 139-143.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nat Med *6*, 389-395.

Carmeliet, P., and Jain, R.K. (2000). Angiogenesis in cancer and other diseases. Nature *407*, 249-257.

Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A.T., Thomas, G., Kozma, S.C.*, et al.* (2008). Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest *118*, 3065-3074.

Carroll, V.A., and Ashcroft, M. (2006). Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res *66*, 6264-6270.

Carroll, V.A., and Ashcroft, M. (2008). Regulation of angiogenic factors by HDM2 in renal cell carcinoma. Cancer Res *68*, 545-552.

Carter, B.Z., Mak, D.H., Schober, W.D., Koller, E., Pinilla, C., Vassilev, L.T., Reed, J.C., and Andreeff, M. (2010). Simultaneous activation of p53 and inhibition of XIAP enhance the activation of apoptosis signaling pathways in AML. Blood *115*, 306-314.

Carvajal, D., Tovar, C., Yang, H., Vu, B.T., Heimbrook, D.C., and Vassilev, L.T. (2005). Activation of p53 by MDM2 antagonists can protect proliferating cells from mitotic inhibitors. Cancer Res *65*, 1918-1924.

Celeste, A., Petersen, S., Romanienko, P.J., Fernandez-Capetillo, O., Chen, H.T., Sedelnikova, O.A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M.J.*, et al.* (2002). Genomic instability in mice lacking histone H2AX. Science *296*, 922-927.

Chakraverty, R.K., and Hickson, I.D. (1999). Defending genome integrity during DNA replication: a proposed role for RecQ family helicases. Bioessays *21*, 286-294.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C., and Schumacker, P.T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A *95*, 11715-11720.

Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev *14*, 278-288.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. (2001). Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem *276*, 9519-9525.

Chen, D., Li, M., Luo, J., and Gu, W. (2003). Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. J Biol Chem *278*, 13595-13598.

Chen, J., Marechal, V., and Levine, A.J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol *13*, 4107-4114.

Chen, Y., and Sanchez, Y. (2004). Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair (Amst) *3*, 1025-1032.

Chen, Z., Seimiya, H., Naito, M., Mashima, T., Kizaki, A., Dan, S., Imaizumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999). ASK1 mediates apoptotic cell death induced by genotoxic stress. Oncogene *18*, 173-180.

Chene, P. (2003). Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer *3*, 102-109.

Cheok, C.F., Verma, C.S., Baselga, J., and Lane, D.P. (2010). Translating p53 into the clinic. Nat Rev Clin Oncol *8*, 25-37.

Cheok, C.F., Verma, C.S., Baselga, J., and Lane, D.P. (2011). Translating p53 into the clinic. Nat Rev Clin Oncol *8*, 25-37.

Cheung, P.C., Campbell, D.G., Nebreda, A.R., and Cohen, P. (2003). Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha. Embo J *22*, 5793-5805.

Chipuk, J.E., and Green, D.R. (2006). Dissecting p53-dependent apoptosis. Cell Death Differ *13*, 994-1002.

Chun, Y.S., Choi, E., Yeo, E.J., Lee, J.H., Kim, M.S., and Park, J.W. (2001). A new HIF-1 alpha variant induced by zinc ion suppresses HIF-1-mediated hypoxic responses. J Cell Sci *114*, 4051-4061.

Cicalese, A., Bonizzi, G., Pasi, C.E., Faretta, M., Ronzoni, S., Giulini, B., Brisken, C., Minucci, S., Di Fiore, P.P., and Pelicci, P.G. (2009). The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell *138*, 1083-1095.

Cliby, W.A., Roberts, C.J., Cimprich, K.A., Stringer, C.M., Lamb, J.R., Schreiber, S.L., and Friend, S.H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J *17*, 159-169.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem *275*, 25733-25741.

Comerford, K.M., Wallace, T.J., Karhausen, J., Louis, N.A., Montalto, M.C., and Colgan, S.P. (2002). Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res *62*, 3387-3394.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F.*, et al.* (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med *8*, 702-710.

Conacci-Sorrell, M., Zhurinsky, J., and Ben-Ze'ev, A. (2002). The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest *109*, 987-991.

Corrie, P. (2008). Cytotoxic chemotherapy: clinical aspects. Medicine *36*, 24-28.

Cortez, D., Guntuku, S., Qin, J., and Elledge, S.J. (2001). ATR and ATRIP: partners in checkpoint signaling. Science *294*, 1713-1716.

Costanzo, R., Piccirillo, M.C., Sandomenico, C., Carillio, G., Montanino, A., Daniele, G., Giordano, P., Bryce, J., De Feo, G., Di Maio, M.*, et al.* (2011). Gefitinib in non small cell lung cancer. J Biomed Biotechnol *2011*, 815269.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell *77*, 841-852.

Crean, S., Boyd, D.M., Sercus, B., and Lahn, M. (2009). Safety of multi-targeted kinase inhibitors as monotherapy treatment of cancer: a systematic review of the literature. Curr Drug Saf *4*, 143-154.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121-134.

Crook, T., Marston, N.J., Sara, E.A., and Vousden, K.H. (1994). Transcriptional activation by p53 correlates with suppression of growth but not transformation. Cell *79*, 817-827.

Cuadrado, A., Lafarga, V., Cheung, P.C., Dolado, I., Llanos, S., Cohen, P., and Nebreda, A.R. (2007). A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. Embo J *26*, 2115-2126.

Cuevas, B.D., Abell, A.N., and Johnson, G.L. (2007). Role of mitogen-activated protein kinase kinase kinases in signal integration. Oncogene *26*, 3159-3171.

Cullinan, S.B., and Diehl, J.A. (2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. J Biol Chem *279*, 20108-20117.

Culmsee, C., and Landshamer, S. (2006). Molecular insights into mechanisms of the cell death program: role in the progression of neurodegenerative disorders. Curr Alzheimer Res *3*, 269-283.

Dai, Y., Chen, S., Pei, X.Y., Almenara, J.A., Kramer, L.B., Venditti, C.A., Dent, P., and Grant, S. (2008). Interruption of the Ras/MEK/ERK signaling cascade enhances Chk1 inhibitor-induced DNA damage in vitro and in vivo in human multiple myeloma cells. Blood *112*, 2439-2449.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W.*, et al.* (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949-954.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J *351*, 95-105.

Debnath, J., Baehrecke, E.H., and Kroemer, G. (2005). Does autophagy contribute to cell death? Autophagy *1*, 66-74.

Demuth, T., Reavie, L.B., Rennert, J.L., Nakada, M., Nakada, S., Hoelzinger, D.B., Beaudry, C.E., Henrichs, A.N., Anderson, E.M., and Berens, M.E. (2007). MAP-ing glioma invasion: mitogen-activated protein kinase kinase 3 and p38 drive glioma invasion and progression and predict patient survival. Mol Cancer Ther *6*, 1212-1222.

Dent, P., Haser, W., Haystead, T.A., Vincent, L.A., Roberts, T.M., and Sturgill, T.W. (1992). Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science *257*, 1404-1407.

Dent, P., Tang, Y., Yacoub, A., Dai, Y., Fisher, P.B., and Grant, S. (2011). CHK1 inhibitors in combination chemotherapy: thinking beyond the cell cycle. Mol Interv *11*, 133-140.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.H., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science *267*, 682-685.

Di Micco, R., Cicalese, A., Fumagalli, M., Dobreva, M., Verrecchia, A., Pelicci, P.G., and di Fagagna, F. (2008). DNA damage response activation in mouse embryonic fibroblasts undergoing replicative senescence and following spontaneous immortalization. Cell Cycle *7*, 3601-3606.

Ding, K., Lu, Y., Nikolovska-Coleska, Z., Wang, G., Qiu, S., Shangary, S., Gao, W., Qin, D., Stuckey, J., Krajewski, K.*, et al.* (2006). Structure-based design of spiro-oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. J Med Chem *49*, 3432-3435.

Dolado, I., and Nebreda, A.R. (2008). AKT and oxidative stress team up to kill cancer cells. Cancer Cell *14*, 427-429.

Dolado, I., Swat, A., Ajenjo, N., De Vita, G., Cuadrado, A., and Nebreda, A.R. (2007). p38alpha MAP kinase as a sensor of reactive oxygen species in tumorigenesis. Cancer Cell *11*, 191-205.

Downward, J. (2003a). Role of receptor tyrosine kinases in G-protein-coupled receptor regulation of Ras: transactivation or parallel pathways? Biochem J *376*, e9-10.

Downward, J. (2003b). Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer *3*, 11-22.

Dudenhoffer, C., Rohaly, G., Will, K., Deppert, W., and Wiesmuller, L. (1998). Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination. Mol Cell Biol *18*, 5332-5342.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A *92*, 7686-7689.

Dumont, P., Leu, J.I., Della Pietra, A.C., 3rd, George, D.L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet *33*, 357-365.

Economopoulou, M., Langer, H.F., Celeste, A., Orlova, V.V., Choi, E.Y., Ma, M., Vassilopoulos, A., Callen, E., Deng, C., Bassing, C.H.*, et al.* (2009). Histone H2AX is integral to hypoxia-driven neovascularization. Nat Med *15*, 553-558.

Edinger, A.L., and Thompson, C.B. (2004). Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol *16*, 663-669.

El-Deiry, W.S. (2003). The role of p53 in chemosensitivity and radiosensitivity. Oncogene *22*, 7486-7495.

Emerling, B.M., Platanias, L.C., Black, E., Nebreda, A.R., Davis, R.J., and Chandel, N.S. (2005). Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. Mol Cell Biol *25*, 4853-4862.

Enge, M., Bao, W., Hedstrom, E., Jackson, S.P., Moumen, A., and Selivanova, G. (2009). MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. Cancer Cell *15*, 171-183.

Erler, J.T., Bennewith, K.L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q.T., Chi, J.T., Jeffrey, S.S., and Giaccia, A.J. (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. Nature *440*, 1222-1226.

Erster, S., Mihara, M., Kim, R.H., Petrenko, O., and Moll, U.M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol Cell Biol *24*, 6728-6741.

Erster, S., and Moll, U.M. (2004). Stress-induced p53 runs a direct mitochondrial death program: its role in physiologic and pathophysiologic stress responses in vivo. Cell Cycle *3*, 1492-1495.

Escudier, B. (2007). Anti-VEGF therapy for renal cell carcinoma. Clin Adv Hematol Oncol *5*, 530-531.

Esteban, M.A., Tran, M.G., Harten, S.K., Hill, P., Castellanos, M.C., Chandra, A., Raval, R., O'Brien T, S., and Maxwell, P.H. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. Cancer Res *66*, 3567-3575.

Evan, G., and Littlewood, T. (1998). A matter of life and cell death. Science *281*, 1317-1322.

Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature *410*, 842-847.

Feng, Z., Hu, W., Teresky, A.K., Hernando, E., Cordon-Cardo, C., and Levine, A.J. (2007). Declining p53 function in the aging process: a possible mechanism for the increased tumor incidence in older populations. Proc Natl Acad Sci U S A *104*, 16633-16638.

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. Proc Natl Acad Sci U S A *102*, 8204-8209.

Fernando, N.H., and Hurwitz, H.I. (2004). Targeted therapy of colorectal cancer: clinical experience with bevacizumab. Oncologist *9 Suppl 1*, 11-18.

Ferrara, N., Hillan, K.J., Gerber, H.P., and Novotny, W. (2004). Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov *3*, 391-400.

Fitch, M.E., Cross, I.V., and Ford, J.M. (2003). p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, in vivo. Carcinogenesis *24*, 843-850.

Fornari, F.A., Randolph, J.K., Yalowich, J.C., Ritke, M.K., and Gewirtz, D.A. (1994). Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. Mol Pharmacol *45*, 649-656.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell *78*, 1039-1049.

Fritsche, M., Haessler, C., and Brandner, G. (1993). Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene *8*, 307-318.

Fuchs, S.Y., Adler, V., Buschmann, T., Wu, X., and Ronai, Z. (1998a). Mdm2 association with p53 targets its ubiquitination. Oncogene *17*, 2543-2547.

Fuchs, S.Y., Adler, V., Pincus, M.R., and Ronai, Z. (1998b). MEKK1/JNK signaling stabilizes and activates p53. Proc Natl Acad Sci U S A *95*, 10541-10546.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G.F. (1996). Abnormal centrosome amplification in the absence of p53. Science *271*, 1744-1747.

Futreal, P.A., Coin, L., Marshall, M., Down, T., Hubbard, T., Wooster, R., Rahman, N., and Stratton, M.R. (2004). A census of human cancer genes. Nat Rev Cancer *4*, 177-183.

Galban, S., Martindale, J.L., Mazan-Mamczarz, K., Lopez de Silanes, I., Fan, J., Wang, W., Decker, J., and Gorospe, M. (2003). Influence of the RNA-binding protein HuR in pVHL-regulated p53 expression in renal carcinoma cells. Mol Cell Biol *23*, 7083-7095.

Gale, D.P., Harten, S.K., Reid, C.D., Tuddenham, E.G., and Maxwell, P.H. (2008). Autosomal dominant erythrocytosis and pulmonary arterial hypertension associated with an activating HIF2 alpha mutation. Blood *112*, 919-921.

Gao, Y., Ferguson, D.O., Xie, W., Manis, J.P., Sekiguchi, J., Frank, K.M., Chaudhuri, J., Horner, J., DePinho, R.A., and Alt, F.W. (2000). Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. Nature *404*, 897-900.

Garber, J.E., Goldstein, A.M., Kantor, A.F., Dreyfus, M.G., Fraumeni, J.F., Jr., and Li, F.P. (1991). Follow-up study of twenty-four families with Li-Fraumeni syndrome. Cancer Res *51*, 6094-6097.

Gartel, A.L., and Tyner, A.L. (2002). The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther *1*, 639-649.

Giaccone, G., Gonzalez-Larriba, J.L., van Oosterom, A.T., Alfonso, R., Smit, E.F., Martens, M., Peters, G.J., van der Vijgh, W.J., Smith, R., Averbuch, S.*, et al.* (2004a). Combination therapy with gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, gemcitabine and cisplatin in patients with advanced solid tumors. Ann Oncol *15*, 831-838.

Giaccone, G., Herbst, R.S., Manegold, C., Scagliotti, G., Rosell, R., Miller, V., Natale, R.B., Schiller, J.H., Von Pawel, J., Pluzanska, A.*, et al.* (2004b). Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. J Clin Oncol *22*, 777-784.

Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonny, M.V., and Fojo, T. (2000). p53 is associated with cellular microtubules and is transported to the nucleus by dynein. Nat Cell Biol *2*, 709-717.

Gnarra, J.R., Zhou, S., Merrill, M.J., Wagner, J.R., Krumm, A., Papavassiliou, E., Oldfield, E.H., Klausner, R.D., and Linehan, W.M. (1996). Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. Proc Natl Acad Sci U S A *93*, 10589-10594.

Gordan, J.D., Lal, P., Dondeti, V.R., Letrero, R., Parekh, K.N., Oquendo, C.E., Greenberg, R.A., Flaherty, K.T., Rathmell, W.K., Keith, B.*, et al.* (2008). HIF-alpha effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. Cancer Cell *14*, 435-446.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S.E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J *18*, 6462-6471.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature *379*, 88-91.

Greco, O., Joiner, M.C., Doleh, A., Powell, A.D., Hillman, G.G., and Scott, S.D. (2006). Hypoxia- and radiation-activated Cre/loxP 'molecular switch' vectors for gene therapy of cancer. Gene Ther *13*, 206-215.

Grinkevich, V.V., Nikulenkov, F., Shi, Y., Enge, M., Bao, W., Maljukova, A., Gluch, A., Kel, A., Sangfelt, O., and Selivanova, G. (2009). Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis. Cancer Cell *15*, 441-453.

Gruber, M., Hu, C.J., Johnson, R.S., Brown, E.J., Keith, B., and Simon, M.C. (2007). Acute postnatal ablation of Hif-2alpha results in anemia. Proc Natl Acad Sci U S A *104*, 2301-2306.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., and Bradfield, C.A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr *7*, 205-213.

Gurova, K.V., Hill, J.E., Razorenova, O.V., Chumakov, P.M., and Gudkov, A.V. (2004). p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. Cancer Res *64*, 1951-1958.

Gutierrez, A.A., Lemoine, N.R., and Sikora, K. (1992). Gene therapy for cancer. Lancet *339*, 715-721.

Haase, V.H., Glickman, J.N., Socolovsky, M., and Jaenisch, R. (2001). Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A *98*, 1583-1588.

Hakansson, P., Hofer, A., and Thelander, L. (2006). Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. J Biol Chem *281*, 7834-7841.

Halazonetis, T.D., Davis, L.J., and Kandil, A.N. (1993). Wild-type p53 adopts a 'mutant'-like conformation when bound to DNA. EMBO J *12*, 1021-1028.

Hammond, E.M., Denko, N.C., Dorie, M.J., Abraham, R.T., and Giaccia, A.J. (2002). Hypoxia links ATR and p53 through replication arrest. Mol Cell Biol *22*, 1834-1843.

Hammond, E.M., Dorie, M.J., and Giaccia, A.J. (2004). Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. Cancer Res *64*, 6556-6562.

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

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

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

Hao, J., Song, X., Song, B., Liu, Y., Wei, L., Wang, X., and Yu, J. (2008). Effects of lentivirus-mediated HIF-1alpha knockdown on hypoxia-related cisplatin resistance and their dependence on p53 status in fibrosarcoma cells. Cancer Gene Ther *15*, 449-455.

Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., and Imura, N. (2001). Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of

HIF-mediated gene expression by HIF-3alpha. Biochem Biophys Res Commun *287*, 808-813.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol *8*, 774-785.

Harper, J.W., and Adams, P.D. (2001). Cyclin-dependent kinases. Chem Rev *101*, 2511-2526.

Harris, C.C. (1996). p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. Carcinogenesis *17*, 1187-1198.

Harris, S.L., and Levine, A.J. (2005). The p53 pathway: positive and negative feedback loops. Oncogene *24*, 2899-2908.

Hartwell, L.H., and Kastan, M.B. (1994). Cell cycle control and cancer. Science *266*, 1821-1828.

Hartwell, L.H., and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629-634.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Hay, T.J., and Meek, D.W. (2000). Multiple sites of in vivo phosphorylation in the MDM2 oncoprotein cluster within two important functional domains. FEBS Lett *478*, 183-186.

Hedstrom, E., Eriksson, S., Zawacka-Pankau, J., Arner, E.S., and Selivanova, G. (2009). p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA. Cell Cycle *8*, 3576-3583.

Herbst, R.S., Giaccone, G., Schiller, J.H., Natale, R.B., Miller, V., Manegold, C., Scagliotti, G., Rosell, R., Oliff, I., Reeves, J.A.*, et al.* (2004). Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. J Clin Oncol *22*, 785-794.

Hill, M.M., Adrain, C., Duriez, P.J., Creagh, E.M., and Martin, S.J. (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. EMBO J *23*, 2134-2145.

Hirao, A., Cheung, A., Duncan, G., Girard, P.M., Elia, A.J., Wakeham, A., Okada, H., Sarkissian, T., Wong, J.A., Sakai, T.*, et al.* (2002). Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. Mol Cell Biol *22*, 6521-6532.

Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996). cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). Mol Cell Biol *16*, 1706-1713.

Hoeijmakers, J.H. (2001a). DNA repair mechanisms. Maturitas *38*, 17-22; discussion 22-13.

Hoeijmakers, J.H. (2001b). Genome maintenance mechanisms for preventing cancer. Nature *411*, 366-374.

Hogenesch, J.B., Gu, Y.Z., Jain, S., and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci U S A *95*, 5474-5479.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science *253*, 49-53.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K.*, et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell *10*, 413-423.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett *420*, 25-27.

Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J *18*, 22-27.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., and Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol *23*, 9361-9374.

Huang, J., and Berger, S.L. (2008). The emerging field of dynamic lysine methylation of non-histone proteins. Curr Opin Genet Dev *18*, 152-158.

Huang, J., Papadopoulos, N., McKinley, A.J., Farrington, S.M., Curtis, L.J., Wyllie, A.H., Zheng, S., Willson, J.K., Markowitz, S.D., Morin, P.*, et al.* (1996a). APC mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci U S A *93*, 9049-9054.

Huang, L.E., Arany, Z., Livingston, D.M., and Bunn, H.F. (1996b). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem *271*, 32253-32259.

Huarte, M., Guttman, M., Feldser, D., Garber, M., Koziol, M.J., Kenzelmann-Broz, D., Khalil, A.M., Zuk, O., Amit, I., Rabani, M.*, et al.* (2010). A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell *142*, 409-419.

Hui, L., Bakiri, L., Mairhorfer, A., Schweifer, N., Haslinger, C., Kenner, L., Komnenovic, V., Scheuch, H., Beug, H., and Wagner, E.F. (2007). p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. Nat Genet *39*, 741-749.

Hwang, B.J., Ford, J.M., Hanawalt, P.C., and Chu, G. (1999). Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc Natl Acad Sci U S A *96*, 424-428.

Ichii, S., Horii, A., Nakatsuru, S., Furuyama, J., Utsunomiya, J., and Nakamura, Y. (1992). Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). Hum Mol Genet *1*, 387-390.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90-94.

Imai, T., Horiuchi, A., Wang, C., Oka, K., Ohira, S., Nikaido, T., and Konishi, I. (2003). Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. Am J Pathol *163*, 1437-1447.

Ip, Y.T., and Davis, R.J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol *10*, 205-219.

Isaacs, J.S., Jung, Y.J., Mimnaugh, E.G., Martinez, A., Cuttitta, F., and Neckers, L.M. (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem *277*, 29936-29944.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med *10*, 1321-1328.

Iwabuchi, K., Bartel, P.L., Li, B., Marraccino, R., and Fields, S. (1994). Two cellular proteins that bind to wild-type but not mutant p53. Proc Natl Acad Sci U S A *91*, 6098-6102.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y.*, et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev *12*, 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J.*, et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jackson, D.A., and Pombo, A. (1998). Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J Cell Biol *140*, 1285-1295.

Jain, R.K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*, 58-62.

Jang, H., Choi, S.Y., Cho, E.J., and Youn, H.D. (2009). Cabin1 restrains p53 activity on chromatin. Nat Struct Mol Biol *16*, 910-915.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. CA Cancer J Clin *61*, 69-90.

Jen, J., Kim, H., Piantadosi, S., Liu, Z.F., Levitt, R.C., Sistonen, P., Kinzler, K.W., Vogelstein, B., and Hamilton, S.R. (1994a). Allelic loss of chromosome 18q and prognosis in colorectal cancer. N Engl J Med *331*, 213-221.

Jen, J., Powell, S.M., Papadopoulos, N., Smith, K.J., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1994b). Molecular determinants of dysplasia in colorectal lesions. Cancer Res *54*, 5523-5526.

Jiang, B.H., Semenza, G.L., Bauer, C., and Marti, H.H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol *271*, C1172-1180.

Jimenez, G.S., Khan, S.H., Stommel, J.M., and Wahl, G.M. (1999). p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. Oncogene *18*, 7656-7665.

Jordan, V.C. (2006). Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol *147 Suppl 1*, S269-276.

Kaelin, W.G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer *2*, 673-682.

Kaeser, M.D., and Iggo, R.D. (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci U S A *99*, 95-100.

Kaluzova, M., Kaluz, S., Lerman, M.I., and Stanbridge, E.J. (2004). DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1alpha in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. Mol Cell Biol *24*, 5757-5766.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C.*, et al.* (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-661.

Kastan, M.B. (2007). Wild-type p53: tumors can't stand it. Cell *128*, 837-840.

Kastan, M.B., and Lim, D.S. (2000). The many substrates and functions of ATM. Nat Rev Mol Cell Biol *1*, 179-186.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res *51*, 6304-6311.

Keating, G.M., and Santoro, A. (2009). Sorafenib: a review of its use in advanced hepatocellular carcinoma. Drugs *69*, 223-240.

Kennedy, N.J., and Davis, R.J. (2003). Role of JNK in tumor development. Cell Cycle *2*, 199-201.

Kennedy, N.J., Sluss, H.K., Jones, S.N., Bar-Sagi, D., Flavell, R.A., and Davis, R.J. (2003). Suppression of Ras-stimulated transformation by the JNK signal transduction pathway. Genes Dev *17*, 629-637.

Kerbel, R.S. (2001). Clinical trials of antiangiogenic drugs: opportunities, problems, and assessment of initial results. J Clin Oncol *19*, 45S-51S.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y., and Shkedy, D. (1999). Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. Proc Natl Acad Sci U S A *96*, 14973-14977.

Kibel, A., Iliopoulos, O., DeCaprio, J.A., and Kaelin, W.G., Jr. (1995). Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. Science *269*, 1444-1446.

Kim, J.W., Tchernyshyov, I., Semenza, G.L., and Dang, C.V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab *3*, 177-185.

Kim, W.Y., and Kaelin, W.G. (2004). Role of VHL gene mutation in human cancer. J Clin Oncol *22*, 4991-5004.

Kinzler, K.W., and Vogelstein, B. (1996a). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Kinzler, K.W., and Vogelstein, B. (1996b). Life (and death) in a malignant tumour. Nature *379*, 19-20.

Kinzler, K.W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature *386*, 761, 763.

Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D.J., Bohlen, P., and Kerbel, R.S. (2000). Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest *105*, R15-24.

Knudson, A.G. (1993). Antioncogenes and human cancer. Proc Natl Acad Sci U S A *90*, 10914-10921.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A *68*, 820-823.

Koc, A., Wheeler, L.J., Mathews, C.K., and Merrill, G.F. (2004). Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. J Biol Chem *279*, 223-230.

Kojima, K., Burks, J.K., Arts, J., and Andreeff, M. (2010). The novel tryptamine derivative JNJ-26854165 induces wild-type p53- and E2F1-mediated apoptosis in acute myeloid and lymphoid leukemias. Mol Cancer Ther *9*, 2545-2557.

Kondo, K., and Kaelin, W.G., Jr. (2001). The von Hippel-Lindau tumor suppressor gene. Exp Cell Res *264*, 117-125.

Kondo, K., Kim, W.Y., Lechpammer, M., and Kaelin, W.G., Jr. (2003). Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol *1*, E83.

Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W.G., Jr. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell *1*, 237-246.

Koukourakis, M.I., Giatromanolaki, A., Sivridis, E., Gatter, K.C., and Harris, A.L. (2005). Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma. Neoplasia *7*, 1-6.

Koumenis, C., Alarcon, R., Hammond, E., Sutphin, P., Hoffman, W., Murphy, M., Derr, J., Taya, Y., Lowe, S.W., Kastan, M.*, et al.* (2001). Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. Mol Cell Biol *21*, 1297-1310.

Koumenis, C., Naczki, C., Koritzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Koromilas, A., and Wouters, B.G. (2002). Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. Mol Cell Biol *22*, 7405-7416.

Krajewski, M., Ozdowy, P., D'Silva, L., Rothweiler, U., and Holak, T.A. (2005). NMR indicates that the small molecule RITA does not block p53-MDM2 binding in vitro. Nat Med *11*, 1135-1136; author reply 1136-1137.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kung, A.L., Rebel, V.I., Bronson, R.T., Ch'ng, L.E., Sieff, C.A., Livingston, D.M., and Yao, T.P. (2000). Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. Genes Dev *14*, 272-277.

Laderoute, K.R., Amin, K., Calaoagan, J.M., Knapp, M., Le, T., Orduna, J., Foretz, M., and Viollet, B. (2006). 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol *26*, 5336-5347.

Lander, E.S. (2011). Initial impact of the sequencing of the human genome. Nature *470*, 187-197.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W.*, et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Lane, D., and Crawford, L. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lane, D.P., Cheok, C.F., and Lain, S. (2010). p53-based cancer therapy. Cold Spring Harb Perspect Biol *2*, a001222.

LaRusch, G.A., Jackson, M.W., Dunbar, J.D., Warren, R.S., Donner, D.B., and Mayo, L.D. (2007). Nutlin3 blocks vascular endothelial growth factor induction by preventing the interaction between hypoxia inducible factor 1alpha and Hdm2. Cancer Res *67*, 450-454.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C., and Semenza, G.L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol *21*, 3995-4004.

Lavoie, J.N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem *271*, 20608-20616.

Lebel, M., Cardiff, R.D., and Leder, P. (2001). Tumorigenic effect of nonfunctional p53 or p21 in mice mutant in the Werner syndrome helicase. Cancer Res *61*, 1816-1819.

Lee, S., Cavallo, L., and Griffith, J. (1997). Human p53 binds Holliday junctions strongly and facilitates their cleavage. J Biol Chem *272*, 7532-7539.

Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell *81*, 1013-1020.

Lee, Y.M., Lim, J.H., Chun, Y.S., Moon, H.E., Lee, M.K., Huang, L.E., and Park, J.W. (2009). Nutlin-3, an Hdm2 antagonist, inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated inactivation of HIF-1alpha. Carcinogenesis *30*, 1768-1775.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Levine, A.J., Feng, Z., Mak, T.W., You, H., and Jin, S. (2006). Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. Genes Dev *20*, 267-275.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Lewis, T.S., Shapiro, P.S., and Ahn, N.G. (1998). Signal transduction through MAP kinase cascades. Adv Cancer Res *74*, 49-139.

Li, S.P., Junttila, M.R., Han, J., Kahari, V.M., and Westermarck, J. (2003). p38 Mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase1,2. Cancer Res *63*, 3473-3477.

Linke, S.P., Sengupta, S., Khabie, N., Jeffries, B.A., Buchhop, S., Miska, S., Henning, W., Pedeux, R., Wang, X.W., Hofseth, L.J.*, et al.* (2003). p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. Cancer Res *63*, 2596-2605.

Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A.*, et al.* (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev *14*, 1448-1459.

Liu, Y., and Kulesz-Martin, M. (2001). p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis *22*, 851-860.

Ljungman, M. (2000). Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. Neoplasia *2*, 208-225.

Loewer, A., Batchelor, E., Gaglia, G., and Lahav, G. (2010). Basal dynamics of p53 reveal transcriptionally attenuated pulses in cycling cells. Cell *142*, 89-100.

Lokich, J., and Anderson, N. (1998). Paclitaxel hypersensitivity reactions: a role for docetaxel substitution. Ann Oncol *9*, 573.

Lowe, S.W. (1995). Cancer therapy and p53. Curr Opin Oncol *7*, 547-553.

Lowe, S.W., and Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev *7*, 535-545.

Lu, H.T., Yang, D.D., Wysk, M., Gatti, E., Mellman, I., Davis, R.J., and Flavell, R.A. (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. EMBO J *18*, 1845-1857.

Luciani, M.G., Oehlmann, M., and Blow, J.J. (2004). Characterization of a novel ATR-dependent, Chk1-independent, intra-S-phase checkpoint that suppresses initiation of replication in Xenopus. J Cell Sci *117*, 6019-6030.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G.*, et al.* (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350*, 2129-2139.

Ma, C.X., Janetka, J.W., and Piwnica-Worms, H. (2011). Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. Trends Mol Med *17*, 88-96.

Mabjeesh, N.J., Post, D.E., Willard, M.T., Kaur, B., Van Meir, E.G., Simons, J.W., and Zhong, H. (2002). Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. Cancer Res *62*, 2478-2482.

Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev *15*, 2675-2686.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature *414*, 550-554.

Malhotra, J.D., and Kaufman, R.J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal *9*, 2277-2293.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A.*, et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science *250*, 1233-1238.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403-407.

Mandriota, S.J., Turner, K.J., Davies, D.R., Murray, P.G., Morgan, N.V., Sowter, H.M., Wykoff, C.C., Maher, E.R., Harris, A.L., Ratcliffe, P.J.*, et al.* (2002). HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. Cancer Cell *1*, 459-468.

Manke, I.A., Nguyen, A., Lim, D., Stewart, M.Q., Elia, A.E., and Yaffe, M.B. (2005). MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. Mol Cell *17*, 37-48.

Marais, R., Wynne, J., and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell *73*, 381-393.

Marshall, M.S. (1995). Ras target proteins in eukaryotic cells. FASEB J *9*, 1311-1318.

Marti, T.M., Hefner, E., Feeney, L., Natale, V., and Cleaver, J.E. (2006). H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks. Proc Natl Acad Sci U S A *103*, 9891-9896.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J *20*, 5197-5206.

Mastrogiannaki, M., Matak, P., Keith, B., Simon, M.C., Vaulont, S., and Peyssonnaux, C. (2009). HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. J Clin Invest *119*, 1159-1166.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Maya, R., Balass, M., Kim, S.T., Shkedy, D., Leal, J.F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y.*, et al.* (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev *15*, 1067-1077.

Mayo, L.D., and Donner, D.B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A *98*, 11598-11603.

Mayo, L.D., Turchi, J.J., and Berberich, S.J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. Cancer Res *57*, 5013-5016.

Meek, D.W. (1994). Post-translational modification of p53. Semin Cancer Biol *5*, 203-210.

Meek, D.W. (1998). New developments in the multi-site phosphorylation and integration of stress signalling at p53. Int J Radiat Biol *74*, 729-737.

Meletis, K., Wirta, V., Hede, S.M., Nister, M., Lundeberg, J., and Frisen, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. Development *133*, 363-369.

Meley, D., Bauvy, C., Houben-Weerts, J.H., Dubbelhuis, P.F., Helmond, M.T., Codogno, P., and Meijer, A.J. (2006). AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem *281*, 34870-34879.

Mendrysa, S.M., O'Leary, K.A., McElwee, M.K., Michalowski, J., Eisenman, R.N., Powell, D.A., and Perry, M.E. (2006). Tumor suppression and normal aging in mice with constitutively high p53 activity. Genes Dev *20*, 16-21.

Merrick, C.J., Jackson, D., and Diffley, J.F. (2004). Visualization of altered replication dynamics after DNA damage in human cells. J Biol Chem *279*, 20067-20075.

Midgley, C.A., Desterro, J.M., Saville, M.K., Howard, S., Sparks, A., Hay, R.T., and Lane, D.P. (2000). An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene *19*, 2312-2323.

Minet, E., Mottet, D., Michel, G., Roland, I., Raes, M., Remacle, J., and Michiels, C. (1999). Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. FEBS Lett *460*, 251-256.

Minsky, N., and Oren, M. (2004). The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression. Mol Cell *16*, 631-639.

Mitchell, J.B., Choudhuri, R., Fabre, K., Sowers, A.L., Citrin, D., Zabludoff, S.D., and Cook, J.A. (2010). In vitro and in vivo radiation sensitization of human tumor cells by a novel checkpoint kinase inhibitor, AZD7762. Clin Cancer Res *16*, 2076-2084.

Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-299.

Moeller, B.J., Dreher, M.R., Rabbani, Z.N., Schroeder, T., Cao, Y., Li, C.Y., and Dewhirst, M.W. (2005). Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell *8*, 99-110.

Mokbel, K. (2002). The evolving role of aromatase inhibitors in breast cancer. Int J Clin Oncol *7*, 279-283.

Momand, J., Wu, H.H., and Dasgupta, G. (2000). MDM2--master regulator of the p53 tumor suppressor protein. Gene *242*, 15-29.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P*., et al.* (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. Science *314*, 126-129.

Moumen, A., Masterson, P., O'Connor, M.J., and Jackson, S.P. (2005). hnRNP K: an HDM2 target and transcriptional coactivator of p53 in response to DNA damage. Cell *123*, 1065-1078.

Muller, S., Berger, M., Lehembre, F., Seeler, J.S., Haupt, Y., and Dejean, A. (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. J Biol Chem *275*, 13321-13329.

Muppidi, J.R., Tschopp, J., and Siegel, R.M. (2004). Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity *21*, 461-465.

Nakayama, K., Frew, I.J., Hagensen, M., Skals, M., Habelhah, H., Bhoumik, A., Kadoya, T., Erdjument-Bromage, H., Tempst, P., Frappell, P.B.*, et al.* (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. Cell *117*, 941-952.

Nass, S.J., and Dickson, R.B. (1997). Defining a role for c-Myc in breast tumorigenesis. Breast Cancer Res Treat *44*, 1-22.

Nicke, B., Bastien, J., Khanna, S.J., Warne, P.H., Cowling, V., Cook, S.J., Peters, G., Delpuech, O., Schulze, A., Berns, K.*, et al.* (2005). Involvement of MINK, a Ste20 family kinase, in Ras oncogene-induced growth arrest in human ovarian surface epithelial cells. Mol Cell *20*, 673-685.

Nieves-Neira, W., Rivera, M.I., Kohlhagen, G., Hursey, M.L., Pourquier, P., Sausville, E.A., and Pommier, Y. (1999). DNA protein cross-links produced by NSC 652287, a novel thiophene derivative active against human renal cancer cells. Mol Pharmacol *56*, 478-484.

Niida, H., Murata, K., Shimada, M., Ogawa, K., Ohta, K., Suzuki, K., Fujigaki, H., Khaw, A.K., Banerjee, B., Hande, M.P.*, et al.* (2010). Cooperative functions of Chk1 and Chk2 reduce tumour susceptibility in vivo. EMBO J *29*, 3558-3570.

Nordberg, J., and Arner, E.S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free radical biology & medicine *31*, 1287-1312.

Nordling, C.O. (1953). A new theory on cancer-inducing mechanism. Br J Cancer *7*, 68-72.

Nyberg, K.A., Michelson, R.J., Putnam, C.W., and Weinert, T.A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet *36*, 617-656.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000a). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y.*, et al.* (2000b). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell *102*, 849-862.

Offer, H., Wolkowicz, R., Matas, D., Blumenstein, S., Livneh, Z., and Rotter, V. (1999). Direct involvement of p53 in the base excision repair pathway of the DNA repair machinery. FEBS Lett *450*, 197-204.

Olive, P.L., Banath, J.P., and Durand, R.E. (1990). Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells. J Natl Cancer Inst *82*, 779-783.

Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., Inoue, M., Bergers, G., Hanahan, D., and Casanovas, O. (2009). Antiangiogenic therapy elicits malignant

progression of tumors to increased local invasion and distant metastasis. Cancer Cell *15*, 220-231.

Paez, J.G., Janne, P.A., Lee, J.C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F.J., Lindeman, N., Boggon, T.J.*, et al.* (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*, 1497-1500.

Pan, Y., Oprysko, P.R., Asham, A.M., Koch, C.J., and Simon, M.C. (2004). p53 cannot be induced by hypoxia alone but responds to the hypoxic microenvironment. Oncogene *23*, 4975-4983.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., and Denko, N.C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab *3*, 187-197.

Parant, J., Chavez-Reyes, A., Little, N.A., Yan, W., Reinke, V., Jochemsen, A.G., and Lozano, G. (2001). Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet *29*, 92-95.

Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. (1996). Cancer statistics, 1996. CA Cancer J Clin *46*, 5-27.

Patel, S.A., and Simon, M.C. (2008). Biology of hypoxia-inducible factor-2alpha in development and disease. Cell Death Differ *15*, 628-634.

Pause, A., Lee, S., Lonergan, K.M., and Klausner, R.D. (1998). The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. Proc Natl Acad Sci U S A *95*, 993-998.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y., Burgess, W.H., Linehan, W.M., and Klausner, R.D. (1997). The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci U S A *94*, 2156-2161.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev *22*, 153-183.

Peng, J., Zhang, L., Drysdale, L., and Fong, G.H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A *97*, 8386-8391.

Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., and Comoglio, P.M. (2003). Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell *3*, 347-361.

Percy, M.J., Furlow, P.W., Lucas, G.S., Li, X., Lappin, T.R., McMullin, M.F., and Lee, F.S. (2008). A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. N Engl J Med *358*, 162-168.

Persons, D.L., Yazlovitskaya, E.M., and Pelling, J.C. (2000). Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. J Biol Chem *275*, 35778-35785.

Pierce, A.J., and Jasin, M. (2001). NHEJ deficiency and disease. Mol Cell *8*, 1160-1161.

Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., and Ferrara, N. (1997). Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res *57*, 4593-4599.

Prives, C. (1998). Signaling to p53: breaking the MDM2-p53 circuit. Cell *95*, 5-8.

Raman, M., and Cobb, M.H. (2003). MAP kinase modules: many roads home. Curr Biol *13*, R886-888.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol *25*, 5675-5686.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev *14*, 34-44.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev *12*, 162-169.

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M.E., and Tegtmeyer, P. (1995). The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proc Natl Acad Sci U S A *92*, 9455-9459.

Reinhardt, H.C., Aslanian, A.S., Lees, J.A., and Yaffe, M.B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell *11*, 175-189.

Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science *256*, 1193-1195.

Rinaldo, C., Prodosmo, A., Mancini, F., Iacovelli, S., Sacchi, A., Moretti, F., and Soddu, S. (2007). MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis. Mol Cell *25*, 739-750.

Rinaldo, C., Prodosmo, A., Siepi, F., Moncada, A., Sacchi, A., Selivanova, G., and Soddu, S. (2009). HIPK2 regulation by MDM2 determines tumor cell response to the p53-reactivating drugs nutlin-3 and RITA. Cancer Res *69*, 6241-6248.

Ringshausen, I., O'Shea, C.C., Finch, A.J., Swigart, L.B., and Evan, G.I. (2006). Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. Cancer Cell *10*, 501-514.

Risau, W. (1995). Differentiation of endothelium. Faseb J *9*, 926-933.

Rivera, M.I., Stinson, S.F., Vistica, D.T., Jorden, J.L., Kenney, S., and Sausville, E.A. (1999). Selective toxicity of the tricyclic thiophene NSC 652287 in renal carcinoma cell lines: differential accumulation and metabolism. Biochem Pharmacol *57*, 1283-1295.

Roberts, A.M., Watson, I.R., Evans, A.J., Foster, D.A., Irwin, M.S., and Ohh, M. (2009). Suppression of hypoxia-inducible factor 2alpha restores p53 activity via Hdm2 and reverses chemoresistance of renal carcinoma cells. Cancer Res *69*, 9056-9064.

Robinson, B.W., Im, M.M., Ljungman, M., Praz, F., and Shewach, D.S. (2003). Enhanced radiosensitization with gemcitabine in mismatch repair-deficient HCT116 cells. Cancer Res *63*, 6935-6941.

Rodriguez, R., Gagou, M.E., and Meuth, M. (2008). Apoptosis induced by replication inhibitors in Chk1-depleted cells is dependent upon the helicase cofactor Cdc45. Cell Death Differ *15*, 889-898.

Roe, J.S., Kim, H., Lee, S.M., Kim, S.T., Cho, E.J., and Youn, H.D. (2006). p53 stabilization and transactivation by a von Hippel-Lindau protein. Mol Cell *22*, 395-405.

Rofstad, E.K., and Halsor, E.F. (2000). Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. Cancer Res *60*, 4932-4938.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., and Bonner, W.M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem *273*, 5858-5868.

Rosenberg, B., VanCamp, L., Trosko, J.E., and Mansour, V.H. (1969). Platinum compounds: a new class of potent antitumour agents. Nature *222*, 385-386.

Roukos, D.H. (2009). Genome-wide association studies: how predictable is a person's cancer risk? Expert Rev Anticancer Ther *9*, 389-392.

Rubenstein, J.L., Kim, J., Ozawa, T., Zhang, M., Westphal, M., Deen, D.F., and Shuman, M.A. (2000). Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. Neoplasia *2*, 306-314.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J *17*, 3005-3015.

Ryan, K.M., Ernst, M.K., Rice, N.R., and Vousden, K.H. (2000). Role of NF-kappaB in p53-mediated programmed cell death. Nature *404*, 892-897.

Saddler, C., Ouillette, P., Kujawski, L., Shangary, S., Talpaz, M., Kaminski, M., Erba, H., Shedden, K., Wang, S., and Malek, S.N. (2008). Comprehensive biomarker and genomic analysis identifies p53 status as the major determinant of response to MDM2 inhibitors in chronic lymphocytic leukemia. Blood *111*, 1584-1593.

Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell *8*, 781-794.

Sanchez-Prieto, R., Rojas, J.M., Taya, Y., and Gutkind, J.S. (2000). A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Res *60*, 2464-2472.

Schmid, T., Zhou, J., Kohl, R., and Brune, B. (2004). p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). Biochem J *380*, 289-295.

Schramek, D., Kotsinas, A., Meixner, A., Wada, T., Elling, U., Pospisilik, J.A., Neely, G.G., Zwick, R.H., Sigl, V., Forni, G.*, et al.* (2011). The stress kinase MKK7 couples oncogenic stress to p53 stability and tumor suppression. Nat Genet *43*, 212-219.

Schuler, M., and Green, D.R. (2005). Transcription, apoptosis and p53: catch-22. Trends Genet *21*, 182-187.

Scortegagna, M., Ding, K., Zhang, Q., Oktay, Y., Bennett, M.J., Bennett, M., Shelton, J.M., Richardson, J.A., Moe, O., and Garcia, J.A. (2005). HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood *105*, 3133-3140.

Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S.*, et al.* (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med *5*, 810-816.

Secchiero, P., Corallini, F., Gonelli, A., Dell'Eva, R., Vitale, M., Capitani, S., Albini, A., and Zauli, G. (2007). Antiangiogenic activity of the MDM2 antagonist nutlin-3. Circ Res *100*, 61-69.

Semenza, G.L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol *15*, 551-578.

Semenza, G.L. (2001). Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. Pediatr Res *49*, 614-617.

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., and Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem *271*, 32529-32537.

Semenza, G.L., Nejfelt, M.K., Chi, S.M., and Antonarakis, S.E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A *88*, 5680-5684.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol *12*, 5447-5454.

Sendoel, A., Kohler, I., Fellmann, C., Lowe, S.W., and Hengartner, M.O. (2010). HIF-1 antagonizes p53-mediated apoptosis through a secreted neuronal tyrosinase. Nature *465*, 577-583.

Sengupta, S., Linke, S.P., Pedeux, R., Yang, Q., Farnsworth, J., Garfield, S.H., Valerie, K., Shay, J.W., Ellis, N.A., Wasylyk, B.*, et al.* (2003). BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. EMBO J *22*, 1210-1222.

Shangary, S., Qin, D., McEachern, D., Liu, M., Miller, R.S., Qiu, S., Nikolovska-Coleska, Z., Ding, K., Wang, G., Chen, J.*, et al.* (2008). Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci U S A *105*, 3933-3938.

Shangary, S., and Wang, S. (2008). Targeting the MDM2-p53 interaction for cancer therapy. Clin Cancer Res *14*, 5318-5324.

Shechter, D., Costanzo, V., and Gautier, J. (2004). ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol *6*, 648-655.

Sherr, C.J. (1998). Tumor surveillance via the ARF-p53 pathway. Genes Dev *12*, 2984-2991.

Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Shikama, N., Lee, C.W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M., and La Thangue, N.B. (1999). A novel cofactor for p300 that regulates the p53 response. Mol Cell *4*, 365-376.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer *3*, 155-168.

Shimura, T., Toyoshima, M., Adiga, S.K., Kunoh, T., Nagai, H., Shimizu, N., Inoue, M., and Niwa, O. (2006). Suppression of replication fork progression in low-dose-specific p53-dependent S-phase DNA damage checkpoint. Oncogene *25*, 5921-5932.

Shin, D.H., Chun, Y.S., Lee, D.S., Huang, L.E., and Park, J.W. (2008). Bortezomib inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated repression of hypoxia-inducible factor-1. Blood *111*, 3131-3136.

Smith, M.L., Chen, I.T., Zhan, Q., O'Connor, P.M., and Fornace, A.J., Jr. (1995). Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage. Oncogene *10*, 1053-1059.

Soengas, M.S., Alarcon, R.M., Yoshida, H., Giaccia, A.J., Hakem, R., Mak, T.W., and Lowe, S.W. (1999). Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science *284*, 156-159.

Spierings, D., McStay, G., Saleh, M., Bender, C., Chipuk, J., Maurer, U., and Green, D.R. (2005). Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. Science *310*, 66-67.

Squires, M.S., Nixon, P.M., and Cook, S.J. (2002). Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. Biochem J *366*, 673-680.

Stebbins, C.E., Kaelin, W.G., Jr., and Pavletich, N.P. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. Science *284*, 455-461.

Strumberg, D. (2005). Preclinical and clinical development of the oral multikinase inhibitor sorafenib in cancer treatment. Drugs Today (Barc) *41*, 773-784.

Stucki, M., Clapperton, J.A., Mohammad, D., Yaffe, M.B., Smerdon, S.J., and Jackson, S.P. (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell *123*, 1213-1226.

Sun, X., Kanwar, J.R., Leung, E., Lehnert, K., Wang, D., and Krissansen, G.W. (2001). Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy. Gene Ther *8*, 638-645.

Supiot, S., Hill, R.P., and Bristow, R.G. (2008). Nutlin-3 radiosensitizes hypoxic prostate cancer cells independent of p53. Mol Cancer Ther *7*, 993-999.

Sur, S., Pagliarini, R., Bunz, F., Rago, C., Diaz, L.A., Jr., Kinzler, K.W., Vogelstein, B., and Papadopoulos, N. (2009). A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. Proc Natl Acad Sci U S A *106*, 3964-3969.

Sutton, K.M., Hayat, S., Chau, N.M., Cook, S., Pouyssegur, J., Ahmed, A., Perusinghe, N., Le Floch, R., Yang, J., and Ashcroft, M. (2007). Selective inhibition of MEK1/2 reveals a

differential requirement for ERK1/2 signalling in the regulation of HIF-1 in response to hypoxia and IGF-1. Oncogene *26*, 3920-3929.

Taira, N., Nihira, K., Yamaguchi, T., Miki, Y., and Yoshida, K. (2007). DYRK2 is targeted to the nucleus and controls p53 via Ser46 phosphorylation in the apoptotic response to DNA damage. Mol Cell *25*, 725-738.

Takahashi, A., Sasaki, H., Kim, S.J., Tobisu, K., Kakizoe, T., Tsukamoto, T., Kumamoto, Y., Sugimura, T., and Terada, M. (1994). Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. Cancer Res *54*, 4233-4237.

Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F., and Minna, J.D. (1989). p53: a frequent target for genetic abnormalities in lung cancer. Science *246*, 491-494.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M., and Nakayama, K. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes Dev *14*, 1439-1447.

Tanaka, N., Kamanaka, M., Enslen, H., Dong, C., Wysk, M., Davis, R.J., and Flavell, R.A. (2002). Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. EMBO Rep *3*, 785-791.

Tannock, I.F. (1998). Conventional cancer therapy: promise broken or promise delayed? Lancet *351 Suppl 2*, SII9-16.

Taylor, W.R., and Stark, G.R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803-1815.

Tazuke, S.I., Mazure, N.M., Sugawara, J., Carland, G., Faessen, G.H., Suen, L.F., Irwin, J.C., Powell, D.R., Giaccia, A.J., and Giudice, L.C. (1998). Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia. Proc Natl Acad Sci U S A *95*, 10188-10193.

Thornton, T.M., and Rincon, M. (2009). Non-classical p38 map kinase functions: cell cycle checkpoints and survival. Int J Biol Sci *5*, 44-51.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12*, 3320-3324.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*, 72-82.

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C., and Abraham, R.T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-157.

Tlsty, T.D. (1997). Genomic instability and its role in neoplasia. Curr Top Microbiol Immunol *221*, 37-46.

Toft, N.J., and Arends, M.J. (1998). DNA mismatch repair and colorectal cancer. J Pathol *185*, 123-129.

Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B.T., Qing, W., Packman, K.*, et al.* (2006). Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A *103*, 1888-1893.

Treuner, K., Helton, R., and Barlow, C. (2004). Loss of Rad52 partially rescues tumorigenesis and T-cell maturation in Atm-deficient mice. Oncogene *23*, 4655-4661.

Tsurimoto, T. (1999). PCNA binding proteins. Front Biosci *4*, D849-858.

Van Cutsem, E., Kohne, C.H., Hitre, E., Zaluski, J., Chang Chien, C.R., Makhson, A., D'Haens, G., Pinter, T., Lim, R., Bodoky, G.*, et al.* (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med *360*, 1408-1417.

van Maanen, J.M., Retel, J., de Vries, J., and Pinedo, H.M. (1988). Mechanism of action of antitumor drug etoposide: a review. J Natl Cancer Inst *80*, 1526-1533.

van Vugt, M.A., van de Weerdt, B.C., Vader, G., Janssen, H., Calafat, J., Klompmaker, R., Wolthuis, R.M., and Medema, R.H. (2004). Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. J Biol Chem *279*, 36841-36854.

Vassilev, L.T. (2007). MDM2 inhibitors for cancer therapy. Trends Mol Med *13*, 23-31.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C.*, et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.

Vaupel, P., Schlenger, K., Knoop, C., and Hockel, M. (1991). Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res *51*, 3316-3322.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A.*, et al.* (2001). The sequence of the human genome. Science *291*, 1304-1351.

Vermorken, J.B., Mesia, R., Rivera, F., Remenar, E., Kawecki, A., Rottey, S., Erfan, J., Zabolotnyy, D., Kienzer, H.R., Cupissol, D.*, et al.* (2008). Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med *359*, 1116-1127.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. N Engl J Med *319*, 525-532.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

von Pawel, J., von Roemeling, R., Gatzemeier, U., Boyer, M., Elisson, L.O., Clark, P., Talbot, D., Rey, A., Butler, T.W., Hirsh, V.*, et al.* (2000). Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: A report of the international CATAPULT I study group. Cisplatin and Tirapazamine in Subjects with Advanced Previously Untreated Non-Small-Cell Lung Tumors. J Clin Oncol *18*, 1351-1359.

Vousden, K.H., and Lane, D.P. (2007). p53 in health and disease. Nat Rev Mol Cell Biol *8*, 275-283.

Wagner, E.F., and Nebreda, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer *9*, 537-549.

Wang, G.L., and Semenza, G.L. (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*, 1230-1237.

Wang, H., Wang, M., Bocker, W., and Iliakis, G. (2005). Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionizing radiation and treated with kinase inhibitors. J Cell Physiol *202*, 492-502.

Wang, Q., Fan, S., Eastman, A., Worland, P.J., Sausville, E.A., and O'Connor, P.M. (1996). UCN-01: a potent abrogator of G2 checkpoint function in cancer cells with disrupted p53. J Natl Cancer Inst *88*, 956-965.

Wang, W., Chen, J.X., Liao, R., Deng, Q., Zhou, J.J., Huang, S., and Sun, P. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol Cell Biol *22*, 3389-3403.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J., and Qin, J. (2000). BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev *14*, 927-939.

Warburg, O. (1956). On respiratory impairment in cancer cells. Science *124*, 269-270.

Warburg, O., Wind, F., and Negelein, E. (1927). The Metabolism of Tumors in the Body. J Gen Physiol *8*, 519-530.

Warburton, H.E., Brady, M., Vlatkovic, N., Linehan, W.M., Parsons, K., and Boyd, M.T. (2005). p53 regulation and function in renal cell carcinoma. Cancer Res *65*, 6498-6503.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science *297*, 63-64.

Wenger, R.H., Rolfs, A., Marti, H.H., Guenet, J.L., and Gassmann, M. (1996). Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxia-inducible factor-1 alpha. Biochem Biophys Res Commun *223*, 54-59.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W.*, et al.* (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J *17*, 271-273.

Wilhelm, S.M., Adnane, L., Newell, P., Villanueva, A., Llovet, J.M., and Lynch, M. (2008). Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther *7*, 3129-3140.

Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M.*, et al.* (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res *64*, 7099-7109.

Witte, A.B., Anestal, K., Jerremalm, E., Ehrsson, H., and Arner, E.S. (2005). Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds. Free radical biology & medicine *39*, 696-703.

Wouters, B.G., and Koritzinsky, M. (2008). Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat Rev Cancer *8*, 851-864.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Xu, B., Kim, S., and Kastan, M.B. (2001). Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. Mol Cell Biol *21*, 3445-3450.

Xu, B., Kim, S.T., Lim, D.S., and Kastan, M.B. (2002). Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. Mol Cell Biol *22*, 1049-1059.

Yang, J., Ahmed, A., and Ashcroft, M. (2009a). Activation of a unique p53-dependent DNA damage response. Cell Cycle *8*, 1630-1632.

Yang, J., Ahmed, A., Poon, E., Perusinghe, N., de Haven Brandon, A., Box, G., Valenti, M., Eccles, S., Rouschop, K., Wouters, B.*, et al.* (2009b). Small-molecule activation of p53 blocks hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression in vivo and leads to tumor cell apoptosis in normoxia and hypoxia. Mol Cell Biol *29*, 2243-2253.

Yang, J.C., Haworth, L., Sherry, R.M., Hwu, P., Schwartzentruber, D.J., Topalian, S.L., Steinberg, S.M., Chen, H.X., and Rosenberg, S.A. (2003). A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med *349*, 427-434.

Yang, T., Namba, H., Hara, T., Takmura, N., Nagayama, Y., Fukata, S., Ishikawa, N., Kuma, K., Ito, K., and Yamashita, S. (1997). p53 induced by ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells. Oncogene *14*, 1511-1519.

Yang, Y., Ludwig, R.L., Jensen, J.P., Pierre, S.A., Medaglia, M.V., Davydov, I.V., Safiran, Y.J., Oberoi, P., Kenten, J.H., Phillips, A.C.*, et al.* (2005). Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell *7*, 547-559.

Yuan, J., and Glazer, P.M. (1998). Mutagenesis induced by the tumor microenvironment. Mutat Res *400*, 439-446.

Zetterberg, A., Larsson, O., and Wiman, K.G. (1995). What is the restriction point? Curr Opin Cell Biol *7*, 835-842.

Zhang, D., Hedlund, E.M., Lim, S., Chen, F., Zhang, Y., Sun, B., and Cao, Y. (2011). Antiangiogenic agents significantly improve survival in tumor-bearing mice by increasing tolerance to chemotherapy-induced toxicity. Proc Natl Acad Sci U S A *108*, 4117-4122.

Zhang, K., and Kaufman, R.J. (2006). Protein folding in the endoplasmic reticulum and the unfolded protein response. Handb Exp Pharmacol, 69-91.

Zhang, W., Weissfeld, J.L., Romkes, M., Land, S.R., Grandis, J.R., and Siegfried, J.M. (2007). Association of the EGFR intron 1 CA repeat length with lung cancer risk. Mol Carcinog *46*, 372-380.

Zhang, Z., Wang, H., Prasad, G., Li, M., Yu, D., Bonner, J.A., Agrawal, S., and Zhang, R. (2004). Radiosensitization by antisense anti-MDM2 mixed-backbone oligonucleotide in in vitro and in vivo human cancer models. Clin Cancer Res *10*, 1263-1273.

Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L., and Simons, J.W. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res *59*, 5830-5835.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.

Zou, L., and Elledge, S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

Appendix

Published Papers

MOLECULAR AND CELLULAR BIOLOGY, Apr. 2009, p. 2243–2253 Vol. 29, No. 8
0270-7306/09/$08.00+0 doi:10.1128/MCB.00959-08

Small-Molecule Activation of p53 Blocks Hypoxia-Inducible Factor 1α and Vascular Endothelial Growth Factor Expression In Vivo and Leads to Tumor Cell Apoptosis in Normoxia and Hypoxia▽

Jun Yang,[1]† Afshan Ahmed,[2]† Evon Poon,[2] Nina Perusinghe,[1] Alexis de Haven Brandon,[3] Gary Box,[3] Melanie Valenti,[3] Suzanne Eccles,[3] Kasper Rouschop,[4] Brad Wouters,[5] and Margaret Ashcroft[1,2]*

Cell Growth Regulation and Angiogenesis Team, Cancer Research UK Centre for Cancer Therapeutics, the Institute of Cancer Research, 15 Cotswold Rd., SaHon, Surrey SM2 5NG, United Kingdom[1]; Hypoxia Signalling and Angiogenesis Laboratory, Centre for Cell Signalling and Molecular Genetics, Department of Metabolism and Experimental Therapeutics, Division of Medicine, Univerisity College London, Rayne Building, 5 University Street, London WC1E 6JJ, United Kingdom[2]; Tumor Biology and Metastasis Team, Cancer Research UK Centre for Cancer Therapeutics, the Institute of Cancer Research, 15 Cotswold Rd., Sutton, Surrey SM2 5NG, United Kingdom[3]; Maastricht Radiation Oncology (Maastro), University of Maastricht, 6200 Maastricht, The Netherlands[4]; and Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada M5G OA3[5]

Received 17 June 2008/Returned for modification 3 September 2008/Accepted 30 January 2009

The p53 tumor suppressor protein negatively regulates hypoxia-inducible factor 1α (HIF-1α). Here, we show that induction of p53 by the small-molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) [2,5-bis(5-hydroxymethyl-2-thienyl) furan] (NSC-652287) inhibits HIF-1α and vascular endothelial growth factor expression in vivo and induces significant tumor cell apoptosis in normoxia and hypoxia in p53-positive cells. RITA has been proposed to stabilize p53 by inhibiting the p53-HDM2 interaction. However, induction of p53 alone was insufficient to block HIF-1α induced in hypoxia and has previously been shown to require additional stimuli, such as DNA damage. Here, we identify a new mechanism of action for RITA: RITA activates a DNA damage response, resulting in phosphorylation of p53 and γH2AX in vivo. Unlike other DNA damage response-inducing agents, RITA treatment of cells induced a p53-dependent increase in phosphorylation of the α subunit of eukaryotic initiation factor 2, requiring PKR-like endoplasmic reticulum kinase activity, and led to the subsequent downregulation of HIF-1α and p53 target proteins, including HDM2 and p21. Through the identification of a new mechanism of action for RITA, our study uncovers a novel link between the DNA damage response-p53 pathway and the protein translational machinery.

Solid tumors require blood vessels to supply them with oxygen and nutrients in order to grow beyond the macroscopic level or metastasize to other organs. Characteristically, solid tumors contain areas of low oxygen tension (hypoxia). The cellular response to hypoxia is primarily mediated by hypoxia-inducible factors (HIFs) hypoxia-inducible factor 1 (HIF-1) and HIF-2 (12, 42). HIFs are transcription factors that are often deregulated in cancer and play a key role in promoting angiogenesis and tumor progression. Hypoxia usually confers tumor resistance to chemotherapy and radiotherapy (8), and HIF-1 is an important determinant for this resistance (7, 35, 38, 49). Thus, targeting HIF has the potential not only to block tumor angiogenesis but also to improve the efficacy of chemotherapy and radiotherapy within a given cancer setting.

HIF-1 and HIF-2 are heterodimeric complexes consisting of HIF-1α and HIF-2α (HIF-α), respectively, that dimerize to a constitutively expressed β subunit (HIF-1β). HIF transcriptional activity is regulated by the availability of the α subunit which is hydroxylated at conserved prolyl and asparaginyl residues in normoxia. Hydroxylation allows for the binding of von Hippel-Lindau protein (pVHL) E3 ligase that targets HIF-α for ubiquitin-mediated degradation by the proteasome (24, 25). In hypoxia, inhibition of hydroxylation results in stabilization of HIF-α and leads to transcriptional activation of target genes involved in angiogenesis, cell survival, and metabolic adaptation (42).

The p53 tumor suppressor protein is induced and activated in response to a variety of cellular stressors, including DNA damage, oxidative stress, and cellular senescence (3, 48), and is a potent negative regulator of HIF-1α, mediating both apoptotic (20, 43) and antiangiogenic effects when overexpressed (27, 39). p53 plays an important role in apoptosis in hypoxic tumor cells in that only wild-type p53-expressing cells undergo strong apoptosis (20), while apoptosis is significantly reduced when tumors express mutant p53 (20, 43). HIF-1α accumulation is blocked by overexpression (39) or activation (27) of p53. In addition, HIF-1-dependent transcription negatively correlates with p53 status (41). p53 is mutated in about 50% of human cancers, and several agents that can reactivate mutant p53 (11, 19) or activate wild-type p53 (23, 45, 47) in tumor cells have been reported. However, many of these emerging p53-targeted agents have not yet been evaluated for their ability to affect the HIF-1 pathway or assessed for their effectiveness at mediating tumor cell death in hypoxia. Recently, a small-molecule activator of p53, RITA (reactivation of p53 and in-

* Corresponding author. Mailing address: Centre for Cell Signalling and Molecular Genetics, Division of Medicine, University College London, Rayne Building, 5 University Street, London WC1E 6JJ, United Kingdom. Phone: 44 (0) 207 679 0799. Fax: 44 (0) 207 679 6211. E-mail: m.ashcroft@ucl.ac.uk.
† These authors contributed equally to this work.
▽ Published ahead of print on 17 February 2009.

duction of tumor cell apoptosis) [2,5-bis(5-hydroxymethyl-2-thienyl) furan] was described (23). RITA was shown to induce tumor cell apoptosis in a p53-dependent manner and to inhibit tumor growth in vivo (23). RITA was proposed to stabilize and activate p53 by disruption of the p53-HDM2 interaction (23). However, subsequent nuclear magnetic resonance (NMR) studies did not support this proposed mechanism (31).

In this study, we assess the effects of RITA on HIF-1α accumulation and apoptotic responses in normoxia and hypoxia. We demonstrate that RITA induces and activates p53, mediating significant tumor cell apoptosis in both normoxia and hypoxia. Furthermore, we demonstrate that RITA blocks HIF-1α and vascular endothelial growth factor (VEGF) expression in vitro and in vivo. We propose that RITA functions by inducing a DNA damage response indicated by increased p53 and γH2AX phosphorylation in vitro and in vivo. Unlike other DNA-damaging agents, RITA treatment of cells induces phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) and subsequently leads to the downregulation of HIF-1α and p53 target proteins, including HDM2 and p21, in a dose-, time-, and p53-dependent manner. Our study provides new mechanistic insight into p53-dependent antiangiogenic and apoptotic responses mediated by activation of the DNA damage response-p53 pathway.

MATERIALS AND METHODS

Cell culture. All tumor cell lines were maintained in Dulbecco modified Eagle medium. Medium was supplemented with 10% fetal calf serum purchased from Harlan (Oxford, United Kingdom), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (all purchased from Gibco/Life Technologies, Paisley, United Kingdom). The matched colorectal cell lines $p53^{-/-}$ HCT116 and $p53^{+/+}$ HCT116 have been described previously (10). The tetracycline-responsive (TetON) p53-inducible Saos-2 cell line was a gift from Karen Vousden (Beatson Institute for Cancer Research, Glasgow, United Kingdom) and has been described previously (36). The tetracycline-inducible HCT116-pCDNA5 (control) and HCT116-PERKΔC (PKR-like endoplasmic reticulum kinase [PERK] dominant-negative) cell lines were a kind gift from Kasper Rouschop (University of Maastricht, Maastricht, The Netherlands) and Brad Wouters (Ontario Cancer Institute, Toronto, Ontario, Canada). The breast carcinoma cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC).

siRNA duplexes and transient transfection. The small interfering RNA (siRNA) to p53 (human p53, 5′-GCATCTTATCCGAGTGGAA-3′) was obtained as a gel-purified annealed duplex from Dharmacon (Lafayette, CO) and used at a final concentration of 5 nM. The siRNA to p21 was described previously (4) and purchased from Dharmacon (Lafayette, CO). The nonsilencing control siRNA duplex (5′-AATTCTCCGAACGTGTCACGT-3′) was obtained from Qiagen (Crawley, United Kingdom). Transient transfections with siRNA duplexes were carried out using HiPerfect transfection reagent (Qiagen) according to the manufacturer's instructions.

Antibodies. The HIF-1α monoclonal antibody was purchased from BD Transduction Laboratories (Oxford, United Kingdom). The p53 monoclonal antibody (DO-1) was purchased from Calbiochem (Merck Biosciences, Nottingham, United Kingdom). The p53 polyclonal antibody, the monoclonal anti-phospho-S15-p53, polyclonal anti-phospho-S51-eIF-2α, anti-phospho-T172-AMPKα (antibody to AMP-activated protein kinase [AMPK] with T172 phosphorylated), anti-AMPKα, anti-phospho-T37/46-4E-BP1, and anti-phospho-T389-p70S6K were all purchased from Cell Signaling Technologies (Danvers, MA). The anti-phospho-S139-γH2AX monoclonal antibody was purchased from Upstate (Millipore, United Kingdom).

Inductions and drug treatments. Physiological hypoxia was achieved by incubating cells in 1% O_2, 5% CO_2 and 94% nitrogen in a LEEC dual gas incubator (GA-156). The hypoxic mimetic agent deferoxamine mesylate was used at a final concentration of 500 μM. RITA [2,5-bis(5-hydroxymethyl-2-thienyl) furan] was obtained from the National Cancer Centre, Drug Therapeutic Program, Frederick, MD (NSC-652287) and dissolved in dimethyl sulfoxide (DMSO). Nutlin-3 (Sigma, Gillingham, United Kingdom) was used at a final concentration of 4 μM. The proteasome inhibitor MG132 (Calbiochem-Merck Biosciences, Nottingham, United Kingdom) was used at 10 μM unless otherwise stated, and the caspase-3 inhibitor Z-DEVD-FMK [Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK] tone) (Calbiochem) was used at the indicated concentrations.

Western blot analysis and immunoprecipitation. After treatment, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 2× sample buffer (125 mM Tris [pH 6.8], 4% sodium dodecyl sulfate, 0.01% bromophenol blue, 10% β-mercaptoethanol, 10% glycerol). Alternatively, cells were harvested in NP-40 lysis buffer (100 mM Tris [pH 8.0], 100 mM $NaCl_2$, 1% NP-40) containing an EDTA-free protease inhibitor cocktail (Boehringer Mannheim-Roche Diagnostics Ltd., Burgess Hill, United Kingdom) to determine total protein concentration using a standard protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). For immunoprecipitation of p53 complexes, cells were lysed in 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1.5 mM Na_3VO_4, and 1% Triton X-100 containing EDTA-free protease inhibitor cocktail (Boehringer Mannheim). After centrifugation, 5 μg of the p53 monoclonal antibody DO-1 (Calbiochem) was added to the supernatant and rotated for 3 h at 4°C. Then, 50 μl of 50% slurry of protein G-Sepharose (Pierce Biotechnology Inc., Rockford, IL) was added and rotated for another 2 h at 4°C. Immunoprecipitated complexes were washed three times with lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and assessed by Western blotting using standard procedures. Western blots were quantified by densitometric analysis as described recently (12).

VEGF quantification. Following treatment, conditioned medium was removed from cells and analyzed by an enzyme-linked immunosorbent assay for secreted vascular endothelial growth factor (VEGF) (QuantiGlo; R&D Systems, Minneapolis, MN). Samples were assessed in duplicate, and a calibration curve was performed for each experiment.

FACS. Cell death was analyzed by fluorescence-activated cell sorting (FACS) using a Beckman Coulter Diagnostics machine (High Wycombe, United Kingdom). Briefly, total populations of cells, including floating and adherent cells, were fixed in 70% ethanol and stained with propidium iodide (50 μg/ml). RNase was added at 100 μg/ml. The percentage of cells with a sub-G_1 DNA content was taken as a measurement of apoptosis.

Immunofluorescence. HCT116 ($p53^{-/-}$ and $p53^{+/+}$), MCF-7, and MDA-MB-231 cells were cultured on sterile glass coverslips in six-well dishes. Following treatment, cells were fixed for 1 h with 4% paraformaldehyde in 1× PBS and then blocked with IFF buffer (PBS containing 1% bovine serum albumin and 2% fetal calf serum) for 1 h. Cells were permeabilized with PBS containing 0.5% Triton X-100 for 10 min and washed with PBS. The p53 (DO-1; Calbiochem) and phospho-Ser139-γH2AX (Upstate-Millipore, United Kingdom) monoclonal antibodies were used at 2 μg/ml in IFF blocking buffer. The secondary antibody Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G1 (Molecular Probes, Leiden, Netherlands) was used at 1/200 in IFF buffer. Nuclei were visualized by TO-PRO-3 staining (Molecular Probes).

Real-time quantitative PCR. Total RNA was extracted from cells using the RNeasy minikit (Qiagen, Crawley, United Kingdom). Five micrograms of total RNA was used for first-strand cDNA synthesis using the SuperScript II first-strand synthesis system (Invitrogen, Paisley, United Kingdom) and random hexamers according to the manufacturer's instructions. Real-time PCR was performed using DyNAmo Sybr green quantitative PCR kit (Finnzymes, GRI Ltd., Braintree, United Kingdom) and the DNA Engine Opticon2 system (GRI Ltd., Braintree, United Kingdom) as we have previously described (12). Primer sequences used are as follows: 5′-GCAAGCCCTGAAAGCG-3′ (forward) and 5′-GGCTGTCCGACTTTGA-3′(reverse) for HIF-1α, 5′-GTTCCTTGTGGAG CCGGAGC-3′ (forward) and 5′-GGTACAAGACAGTGACAGGTC-3′ (reverse) for p21, and 5′-ATGGGGAAGGTGAAGGTCG (forward) and 5′-TA AAAGCAGCCCTGGTGACC-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Where indicated, PCR samples were separated by 3% agarose gel electrophoresis in the presence of ethidium bromide.

In vivo analysis. All procedures involving animals were performed within guidelines set out by The Institute of Cancer Research's Animal Ethics Committee and the United Kingdom Co-ordinating Committee for Cancer Research Committee on the Welfare of Animals in Experimental Neoplasia (46). To determine the distribution of RITA in mice, RITA was prepared at 2.5 mg/ml in PBS containing 10% DMSO and 5% Tween 20 and administered intraperitoneally (25 mg/kg of body weight) to female BALB/c mice. Mice were terminated by exsanguination under anesthesia at 0.08, 0.25, 0.5, 1, 2, 6, and 24 h after administration of RITA, and plasma, liver, spleen, kidney, and lung samples were removed for pharmacokinetic analysis. Tissue samples were homogenized with 3× (vol/wt) PBS, and 50 μl was extracted by the addition of 150 μl of methanol. Plasma samples (50 μl) were extracted by protein precipitation with 150 μl of methanol. Extracts were analyzed by liquid chromatography-mass spectrometry

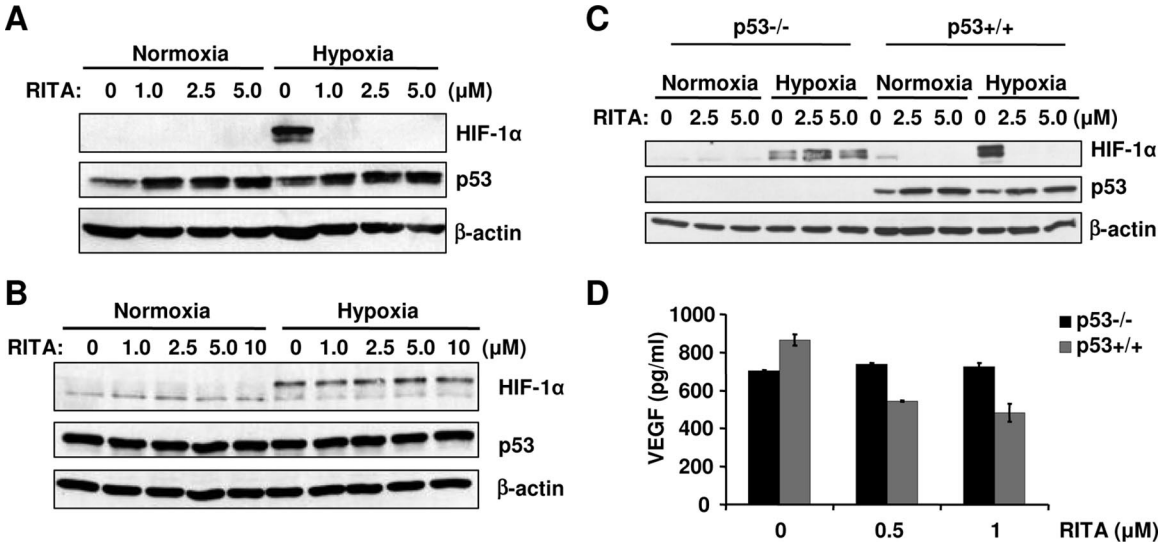


FIG. 1. RITA induces p53-dependent inhibition of HIF-1α and VEGF in normoxia and hypoxia. (A to C) Western blot analysis shows HIF-1α and p53 protein levels in MCF-7 cells (A), MDA-MB-231 (B), and $p53^{-/-}$ HCT116 or $p53^{+/+}$ HCT116 cells (C) treated with RITA over a concentration range for 16 h in normoxia or hypoxia (1% O_2). Actin was used as a load control. (D) The graph shows VEGF (pg/ml) secreted into cell culture medium from $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (0 to 1 μM) for 16 h in hypoxia (1% O_2).

using a reverse-phase Synergi Polar-RP (Phenomenex) analytical column (50 by 2.1 mm) and positive-ion mode electrospray ionization and single-ion monitoring. To assess the effects of RITA on p53, HIF-1α, HDM2, p21, VEGF, and phosphorylated γH2AX proteins, 2 million $p53^{+/+}$ HCT116 cells were injected subcutaneously into bilateral flanks of female NCr athymic mice at 6 to 8 weeks of age and treated intraperitoneally with either solvent control or 10 mg of RITA per kg. Each group contained eight mice. One tumor per mouse was used for pharmacokinetic analysis (as described above), and samples from the second tumor were bisected. Half the samples were snap-frozen using liquid nitrogen for examination of protein expression by Western blotting, and the remaining samples were fixed in 10% formalin for immunohistochemical analysis. For protein extraction, frozen tumors were ground using a bead grinder homogenizer (Precellys; Stretton Scientific, Stretton, United Kingdom) in freshly prepared lysis buffer. Samples were assessed for total protein concentration using a standard protein assay (Bio-Rad) and subjected to Western blot analysis.

RESULTS

RITA induces p53-dependent downregulation of HIF-1α and VEGF expression. RITA has been shown to induce p53, mediate apoptosis, and inhibit tumor growth in vivo (23). Given that active p53 downregulates HIF-1α expression (27, 39), we hypothesized that RITA could potentially mediate antiangiogenic effects by inhibiting HIF activity. To address this, we assessed the effects of RITA on a series of tumor cells that express either wild-type p53 ($p53^{+/+}$ HCT116, MCF-7, U2OS, and RCC4), mutant p53 (MDA-MB-231 and HT29) or are p53 null ($p53^{-/-}$ HCT116 and Saos-2). We found that RITA induced p53 to comparable levels in normoxia and hypoxia and blocked HIF-1α induction in hypoxia in MCF-7 cells (Fig. 1A). In addition, HIF-1α induction in response to the hypoxia mimetic agent deferoxamine mesylate was also blocked by RITA in $p53^{+/+}$ HCT116 cells (data not shown). However, RITA did not induce p53 or affect HIF-1α levels in MDA-MB-231 cells, which express mutant p53 (Fig. 1B). Similar results were obtained for the wild-type p53-expressing U2OS and RCC4 cells, while HIF-1α levels were not affected by RITA in HT29 cells, which express mutant p53, or in p53-null Saos-2 cells (data not shown). These data suggest that the ability of RITA to block HIF-1α was p53 dependent. To assess this further, matched $p53^{-/-}$ HCT116 and $p53^{+/+}$ HCT116 cells (10) were used. We found that RITA blocked HIF-1α induction only in response to hypoxia in $p53^{+/+}$ HCT116 cells (Fig. 1C) and resulted in a significant inhibition of VEGF expression (Fig. 1D). Our data indicate that RITA blocks HIF-1α and VEGF induction in response to hypoxia and that this is p53 dependent.

RITA induces p53-dependent cell death in normoxia and hypoxia. Induction of apoptosis by p53 contributes to the antitumor effect of most conventional chemotherapeutic drugs (14, 33). However, hypoxia confers tumor cell resistance to killing by chemotherapy and radiotherapy (1, 8). Since RITA selectively kills tumor cells bearing wild-type p53 (23) and inhibits HIF-1α expression (Fig. 1A to C), we next determined whether RITA could induce cell death in tumor cells in response to hypoxia. We found that RITA could induce a clear increase in rounding and displacement of cells from monolayers in tissue culture, indicative of apoptosis, to a similar extent in normoxia and hypoxia (Fig. 2A). Using FACS, we also found that there was a marked increase in the sub-G_1 content of cells (28 to 30%) induced by RITA (Fig. 2B) and an increase in poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 2C), indicating that RITA induced apoptosis in cells exposed to normoxia and hypoxia. Consistent with the effects we observed for HIF-1α (Fig. 1A to C), RITA did not mediate cell death in MDA-MB-231 cells in either normoxia or hypoxia (Fig. 2A) or in $p53^{-/-}$ HCT116 cells (Fig. 2B). To further confirm the role of p53 in RITA-induced cell death, we used siRNA to knock down p53. We found that p53 knockdown reduced PARP cleavage induced by RITA in both normoxia and hypoxia, indicating that p53 is important for RITA-induced apoptosis (Fig. 2C). In addition, we found that inhibition of caspase-3 activation by the inhibitor Z-DEVD-FMK could block PARP

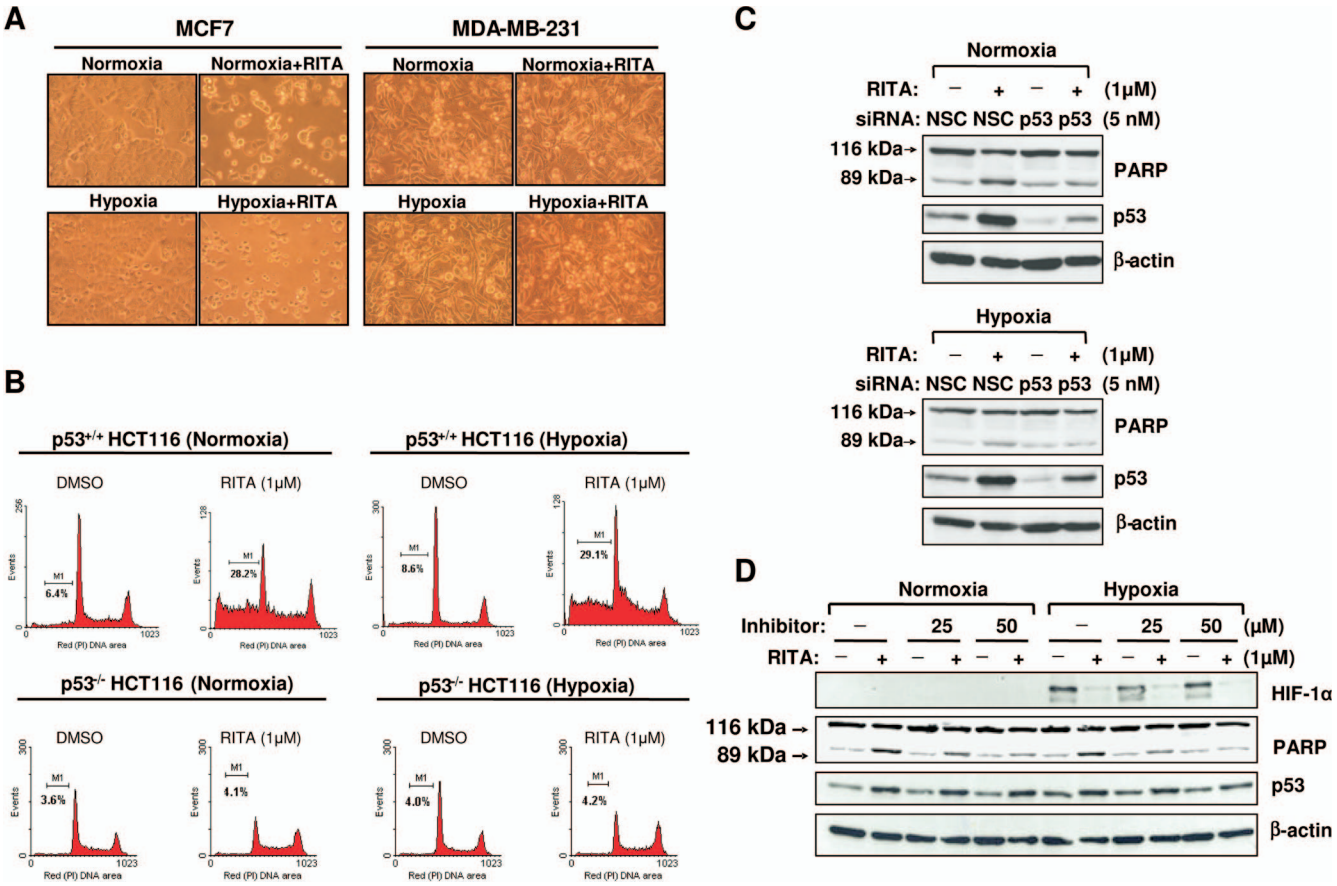


FIG. 2. RITA induces p53-dependent cell death in normoxia and hypoxia. (A) Photographic images of MCF-7 and MDA-MB-231 cells treated with RITA (1 μM) for 24 h in normoxia and hypoxia (1% O_2). Cells treated with RITA have displaced into the culture medium. (B) FACS analysis of $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) for 24 h in normoxia or hypoxia (1% O_2). Graphs show DNA content of cells (events) measured by propidium iodide (PI) stain and FACS analysis. The percentage of sub-G_1 content (the percentage shown under the M1 bar) indicates the apoptotic population of cells. (C) Western blot analysis shows p53 protein and cleaved PARP cleavage as an indicator of apoptosis in $p53^{+/+}$ HCT116 cells transiently transfected with siRNA to p53 or a nonsilencing control (NSC) siRNA duplex. Twenty-four hours after transfection, cells were treated with RITA (+) (1 μM) for 16 h in normoxia or hypoxia (1% O_2). Actin was used as a load control. (D) Western blot analysis shows HIF-1α, cleaved PARP, and p53 proteins in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (+) (1 μM) for 16 h in normoxia and hypoxia (1% O_2) in the presence or absence (−) of the caspase-3 inhibitor Z-DEVD-FMK at 25 μM or 50 μM.

cleavage induced by RITA in normoxia and hypoxia in a dose-dependent manner without affecting the ability of RITA to block HIF-1α accumulation in hypoxia (Fig. 2D). These data suggest that the cell death induced by RITA per se is not responsible for the inhibition of HIF-1α, indicating that these are potentially mechanistically separable responses. Taken together, our data show that RITA blocks HIF-1α and VEGF induced in hypoxia and mediates p53-dependent tumor cell apoptosis in normoxia and hypoxia.

RITA induces p53-dependent eIF-2α phosphorylation and blocks HIF-1α protein. HIF-α expression is usually regulated at the level of protein stability (24, 25) and synthesis (32, 44). To examine the mechanism by which RITA inhibits the expression of HIF-1α, we next assessed the effects of RITA on HIF-1α expression in the presence of the MG132 proteasome inhibitor. We found that MG132 did not affect the ability of RITA to block HIF-1α expression in either normoxia or hypoxia (Fig. 3A). Again, these effects were dependent on p53 (Fig. 3A). In addition, RITA could significantly block HIF-1α protein constitutively stabilized in RCC4 cells in normoxia due to loss of pVHL function (Fig. 3B). Furthermore, quantitative PCR analysis showed that RITA had no significant effect on HIF-1α mRNA expression either in the presence or absence of p53 (Fig. 3C). Collectively, these data indicate that RITA potentially affects HIF-1α at the level of protein synthesis. Recent studies have shown that activation of p53 by genotoxic stress blocks protein translation via inhibition of mammalian target of rapamycin (mTOR) signaling (9, 15, 17). To address this here, components of the translational machinery were evaluated. We found that phosphorylation of the downstream effector of mTOR, p70S6K was moderately increased in response to RITA in a p53-dependent manner (Fig. 3D), while phosphorylated 4E-BP1 was not significantly altered in RITA-treated cells (data not shown and Fig. 4B), suggesting that the mTOR pathway may not be involved here. In contrast, we found that RITA induced a marked phosphorylation of eIF-2α on Ser51 in a p53-dependent manner (Fig. 3D).

Activation of p53 by genotoxic stress has been shown to induce the phosphorylation of AMP-activated protein kinase (15, 17) and blocks translation via the mTOR pathway (9). To

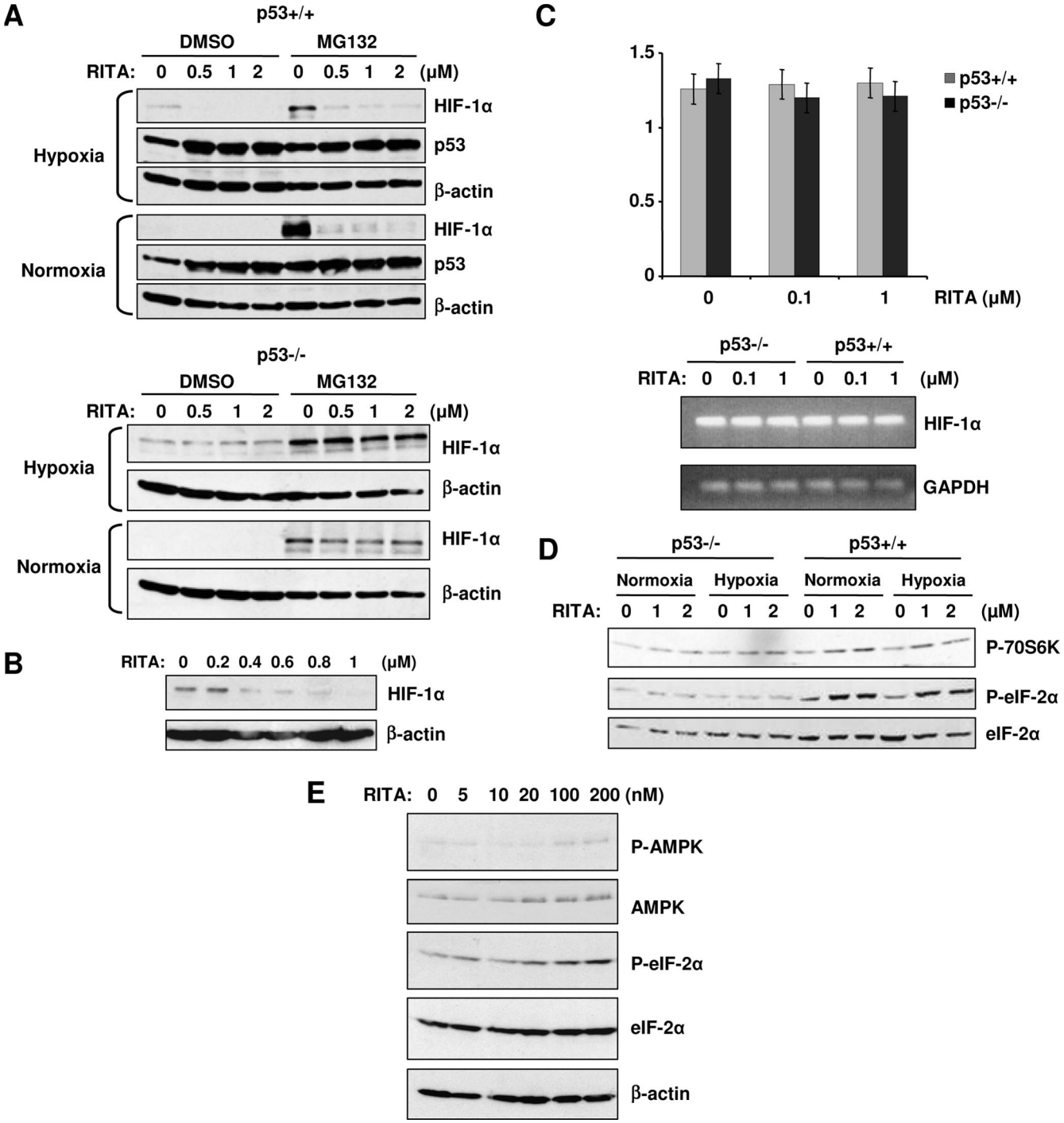


FIG. 3. RITA induces phosphorylation of eIF-2α and blocks HIF-1α protein. (A) Western blot analysis shows HIF-1α and p53 protein levels in $p53^{+/+}$ HCT116 cells or $p53^{-/-}$ HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O_2) in the presence or absence of the proteasome inhibitor MG132 (50 μM). Actin was used as a load control. (B) Western blot analysis shows HIF-1α protein levels in RCC4 cells treated with RITA (1 μM) for 16 h. Actin was used as a load control. (C) Evaluation of HIF-1α transcripts by quantitative real-time PCR. $p53^{+/+}$ HCT116 cells were treated with the indicated concentrations of RITA for 16 h in hypoxia (1% O_2). Total RNA was prepared, and quantitative real-time PCR was performed. The graph shows the HIF-1α transcript levels relative to the level of the GAPDH control. PCR products were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide. GAPDH was used as a load control. (D) Western blot analysis shows phosphorylated eIF-2α (P-eIF-2α), phosphorylated p70S6K (P-70S6K), and eIF-2α proteins in $p53^{-/-}$ HCT116 and $p53^{+/+}$ HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O_2). Actin was used as a load control. (E) Western blot analysis shows phosphorylated AMPK (P-AMPK) and total AMPK protein in $p53^{+/+}$ HCT116 cells treated with the indicated concentrations of RITA for 16 h in normoxia or hypoxia (1% O_2). Actin was used as a load control.

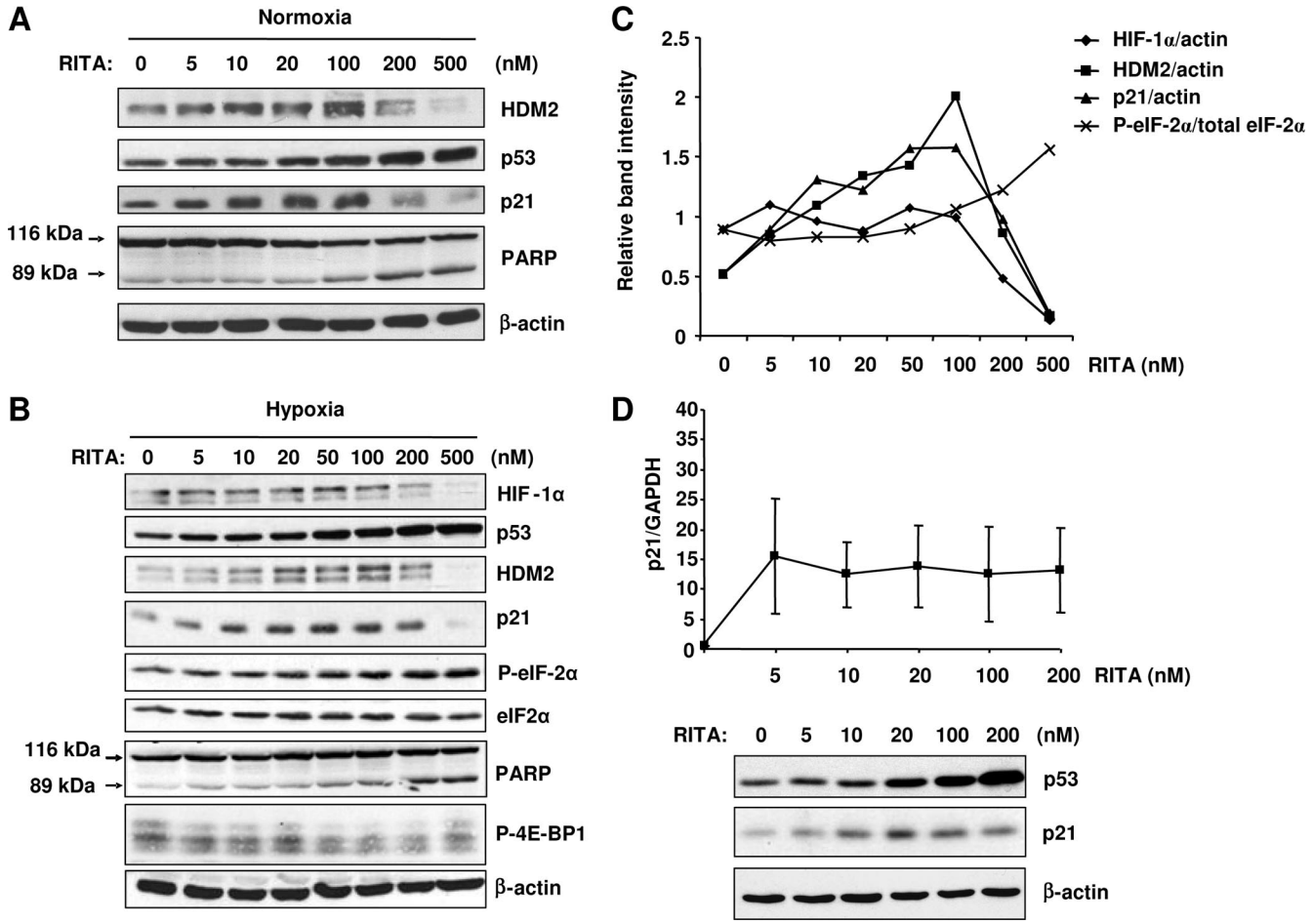


FIG. 4. RITA induces phosphorylation of eIF-2α and downregulates p53 targets. (A and B) Western blot analysis shows HIF-1α, p53, HDM2, p21, phosphorylated eIF-2α (P-eIF-2α), total eIF-2α, cleaved PARP, and phosphorylated 4E-BP1 (P-4E-BP1) in $p53^{+/+}$ HCT116 cells treated with RITA over a concentration range (0 to 500 nM) for 16 h in normoxia (A) and hypoxia (1% O_2). (B) Actin was used as a load control. (C) The graph shows the results of densitometric analysis of the Western blot for hypoxia conditions as in panel B. HIF-1α, HDM2, and p21 protein levels are represented relative to the actin load control, and phosphorylated eIF-2α levels are represented relative to total eIF-2α protein. (D) Total RNA was prepared from $p53^{+/+}$ HCT116 cells treated with RITA and exposed to hypoxia as described above for panel A, and quantitative real-time PCR was performed. The graph shows the p21 transcript levels relative to the level of the GAPDH control and averaged for two independent experiments. Western blot analysis shows p53 and p21 proteins. Actin was used as a load control.

address whether phosphorylation of AMPK was induced in response to RITA, we treated cells with RITA at increasing concentrations curve and assessed phosphorylated AMPK protein. We found that AMPK phosphorylation was not significantly altered in response to RITA treatment of cells, while eIF-2α phosphorylation increased in a dose-dependent manner (Fig. 3E). Taken together, our data indicate that RITA affects protein translation via eIF-2α.

RITA induces p53-dependent eIF-2α phosphorylation and downregulates p53 targets. The alpha subunit of eukaryotic initiation factor 2 is responsible for binding of Met-tRNAi^{Met} to the 40S ribosome for protein translation initiation (2). Phosphorylation of eIF-2α on Ser51 usually results in general protein synthesis inhibition (13). To evaluate this further, $p53^{+/+}$ HCT116 cells were treated with RITA over a concentration range in normoxia and hypoxia. We found that RITA induced p53 and the p53 targeted p21 and HDM2 in a dose-dependent manner in normoxia and hypoxia (Fig. 4A and B). Surprisingly, as p53 levels continued to increase in response to increasing concentrations of RITA, the expression levels of not only HIF-1α but also of HDM2 and p21 proteins were also downregulated (Fig. 4A and B). Concurrently, eIF-2α phosphorylation increased and cell death was induced by PARP cleavage (Fig. 4B and C). Further analysis indicated that the downregulation of p21 protein induced by RITA treatment of cells was not due to a decrease in p21 mRNA (Fig. 4D). These findings are consistent with our findings for HIF-1α (Fig. 3C). Interestingly, knockdown of p21 using siRNA could increase basal PARP cleavage in HCT116 cells and enhance the overall cleaved PARP induced in response to RITA (data not shown), suggesting that knocking down p21 can drive the p53-mediated apoptotic response induced by RITA.

PERK activity is important for RITA-mediated induction of eIF-2α phosphorylation. Recent studies have shown that hypoxia mediates a time-dependent transient induction of eIF-2α phosphorylation, which results in inhibition of protein synthe-

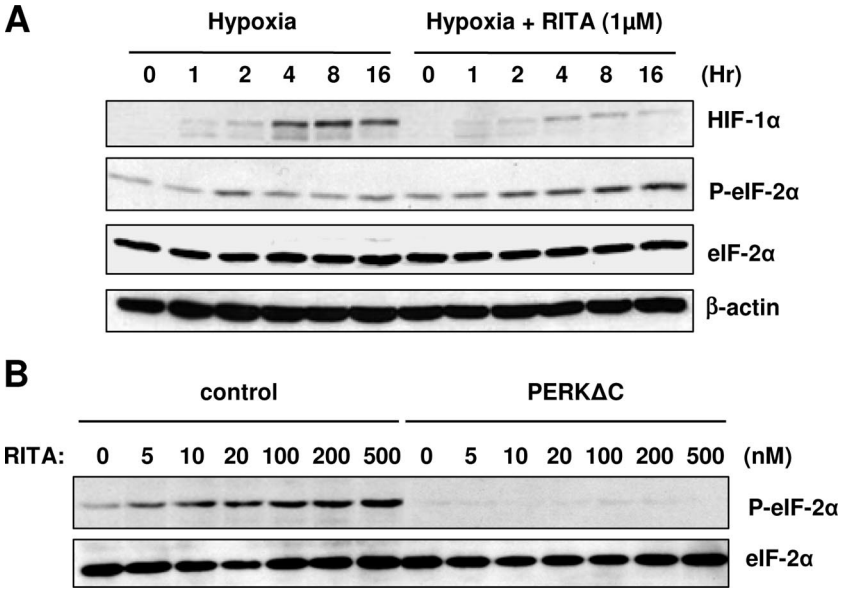


FIG. 5. PERK activity is important for RITA-mediated induction of eIF-2α phosphorylation. (A) Western blot analysis shows HIF-1α, phosphorylated eIF-2α (P-eIF-2α), and total eIF-2α proteins in $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) and exposed to hypoxia (1% O_2) for the times indicated (in hours). Actin was used as a load control. (B) Western blot analysis shows phosphorylated eIF-2α and total eIF-2α proteins in HCT116 cells stably expressing a pCDNA5 control plasmid (control) or a dominant-negative form of PERK (PERKΔC) under the control of the tetracycline promoter. Cells were treated with RITA (1 μM) and exposed to hypoxia (1% O_2) for 16 h. Actin was used as a load control.

sis (29, 30). We found that eIF-2α phosphorylation was induced and maintained in hypoxia by treatment of cells with RITA (Fig. 5A), suggesting that protein synthesis was blocked by RITA. In hypoxia, PKR-like endoplasmic reticulum kinase is activated to control translation via direct phosphorylation of eIF-2α (5, 50). To assess whether PERK activity is important for the induction of eIF-2α phosphorylation in response to RITA treatment of cells, we used a dominant-negative form of PERK (PERKΔC). We found that the expression of PERKΔC blocked basal eIF-2α phosphorylation and significantly inhibited the ability of RITA to induce eIF-2α phosphorylation in cells (Fig. 5B), indicating that PERK activity is important for the p53-mediated eIF-2α phosphorylation induced in response to RITA.

RITA does not disrupt the p53-HDM2 interaction. Since RITA could induce significant cell death in both normoxia and hypoxia, we next addressed its mechanism of action. RITA was originally proposed to stabilize and activate p53 by disruption of p53-HDM2 interaction by binding to the N-terminal domain of p53 (23). Recent studies using NMR techniques have indicated that RITA does not block the p53-HDM2 interaction in vitro (31). In addition, we found that RITA induced a p53-dependent induction of HDM2 at very low concentrations (<100 nM), but at doses which induced apoptosis, RITA actually downregulated HDM2 expression (Fig. 4A and B). To evaluate this further, p53 complexes were immunoprecipitated from $p53^{+/+}$ HCT116 cell lysates, and associated HDM2 was assessed in the presence of RITA. We found that RITA was not able to disrupt immunoprecipitated p53-HDM2 complexes even at submillimolar concentrations in vitro (data not shown). In contrast, we found that the small-molecule nutlin-3 that has been shown to disrupt the p53-HDM2 interaction by binding to HDM2 (47) could significantly impair the ability of HDM2 to coimmunoprecipitate with p53 (Fig. 6A), indicating that RITA may activate p53 via mechanisms that do not target the p53-HDM2 interaction. Interestingly, we found that induction and activation of p53 in the absence of eliciting a stress response either by using a tetracycline-inducible system (Fig. 6B) or by treatment of cells with nutlin-3 (Fig. 6C) was not sufficient to inhibit HIF-1α in response to hypoxia, further supporting the possibility that RITA may induce and activate p53 via mechanisms other than disruption of the p53-HDM2 interaction.

RITA induces a DNA damage response. RITA has previously been reported to cause protein-DNA and DNA-DNA intrastrand cross-links (37, 40), suggesting that it may activate p53 by inducing a DNA damage response. To investigate this, we first assessed the phosphorylation status of p53 in response to RITA, since phosphorylation of p53 within the N terminus is usually induced by genotoxic stress (3, 26). $p53^{+/+}$ HCT116, MCF-7, and U2OS cells were treated with RITA in normoxia or hypoxia and assessed for phosphorylation of p53 at Ser15 (Fig. 7A and D). We found that RITA induced N-terminal phosphorylation of p53, while nutlin-3 was unable to mediate p53 phosphorylation (Fig. 7A). In addition, assessment of γH2AX, a histone protein usually phosphorylated by DNA damage-induced stress (6, 28), showed that RITA induced the phosphorylation of γH2AX at Ser139 and that p53 and phosphorylated γH2AX proteins were induced by RITA and localized to the nucleus in both normoxia and hypoxia (1% O_2) to a similar extent (Fig. 7B and C). Similar data were obtained for other p53-positive cells, such as MCF-7 and U2OS cells, indicating that these responses were not specific to HCT116 cells (data not shown). Previous studies have shown that γH2AX phosphorylation is induced in extreme hypoxia (<0.02% O_2)

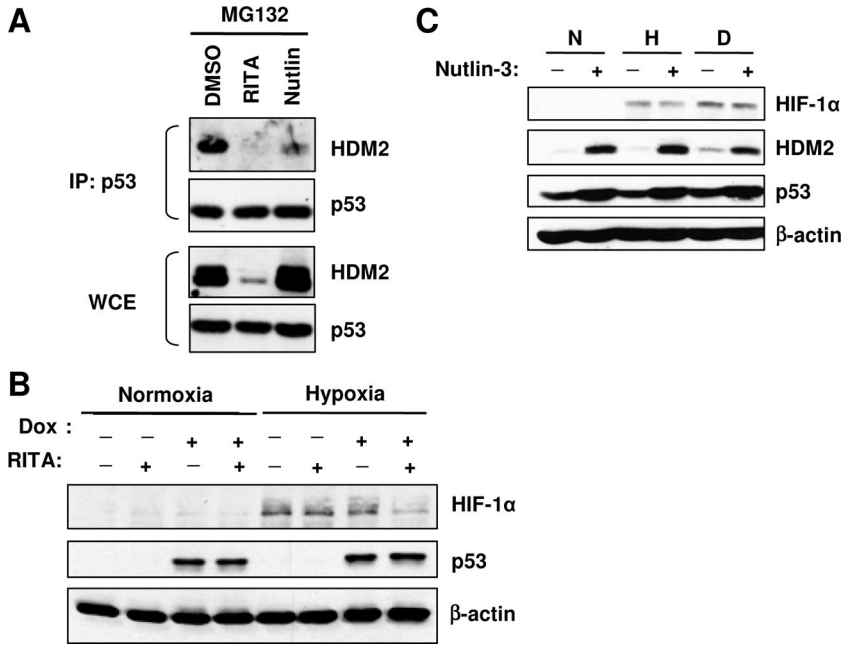


FIG. 6. RITA does not disrupt the p53-HDM2 interaction. (A) Western blot analysis of p53-HDM2 immunoprecipitated (IP) complexes. $p53^{+/+}$ HCT116 cells were treated with RITA (1 μM) or nutlin-3 (4 μM) in the presence of MG132 (10 μM) for 16 h. Cell lysates were immunoprecipitated with a monoclonal antibody to p53 (DO-1), and immunoprecipitated p53 complexes were assessed for associated HDM2 protein by Western blotting. Total immunoprecipitated p53 protein was evaluated using a polyclonal antibody. WCE, whole-cell extracts. (B) Western blot analysis shows HIF-1α and p53 proteins in TetON p53-inducible Saos-2 cells treated with doxycycline (Dox) (800 ng/ml) (+) for 24 h, then washed with PBS, and treated with RITA (1 μM) (+) for a further 16 h in normoxia or hypoxia (1% O_2). (C) Western blot analysis shows HIF-1α, HDM2, and p53 proteins in $p53^{+/+}$ HCT116 cells treated with nutlin-3 (4 μM) (+) in normoxia (N), hypoxia (H), or deferoxamine mesylate (D) for 16 h. Actin was used as a load control.

and is localized to discrete nuclear foci (21). However, we found that exposure of cells to hypoxia at 1% O_2 did not induce γH2AX phosphorylation in cells (Fig. 7C), consistent with a another study (21). Intriguingly, we found that unlike other agents that activate a DNA damage response, RITA induced eIF-2α phosphorylation, which correlated with a significant inhibition of HIF-1α in p53-positive cells (Fig. 7D). Taken together, our study identifies a new mechanism of action for RITA: RITA functions by activating a DNA damage response.

RITA induces p53 and γH2AX phosphorylation and blocks HIF-1α and VEGF expression in vivo. RITA has previously been shown to mediate antitumor activity in a subcutaneous HCT116 colon carcinoma xenograft model (23). We next determined whether RITA induced a DNA damage response and downregulation of HIF-1α and VEGF expression in $p53^{+/+}$ HCT116 cell-derived tumor xenografts. Consistent with a previous study (23), we found that RITA was well-tolerated in mice at 10 to 25 mg/kg daily dosing. In addition, we detected RITA in the plasma and tissues of mice (kidney, liver, lung, and spleen) by liquid chromatography-mass spectroscopy (data not shown), indicating that the compound was distributed in vivo. We found that p53, Ser15 phosphorylation of p53, and Ser139 phosphorylation of γH2AX were induced at 24 h with a reduction in HIF-1α and HDM2 in tumors from the RITA-treated group compared with the solvent (DMSO) control group (Fig. 8A). We also observed a marked decrease in VEGF levels in tumor lysates from the RITA-treated group compared to those of the DMSO-treated control group (Fig. 8B). Immunohistochemical analysis indicated that p53 and phosphorylated γH2AX staining correlated with increased tumor cell apoptosis (data not shown). Our analyses confirm that RITA induces a DNA damage response and mediates a reduction in HIF-1α and VEGF expression in vivo.

DISCUSSION

The p53 tumor suppressor protein is a potent negative regulator of HIF-1α and VEGF in hypoxia (39). In addition, the induction of apoptosis by p53 contributes to the antitumor effects of most conventional chemotherapeutic drugs (14, 33). However, hypoxia and HIF-1α status in tumor cells are important determinants for resistance to chemotherapeutic agents and radiotherapy (7, 35, 38, 49). Therefore, the possibility that small-molecule activation of wild-type p53 could potentially mediate both antiangiogenic effects via blockade of the HIF pathway and apoptosis in hypoxic tumor cells is of particular interest.

In this study, we have explored the mechanistic properties of the small-molecule activator of p53, RITA. RITA was originally identified in a cell-based screen using the National Cancer Institute (NCI) compound library and has been shown recently to mediate p53-dependent antitumor activity in vivo (23). Here, we show that RITA blocks HIF-1α and VEGF expression in vivo and mediates significant cell death responses in hypoxic tumor cells in a p53-dependent manner. Inhibition of HIF-1α was not due to an effect on protein degradation or changes in mRNA levels, indicating an effect on HIF-1α protein synthesis. We discovered that RITA induced p53-depen-

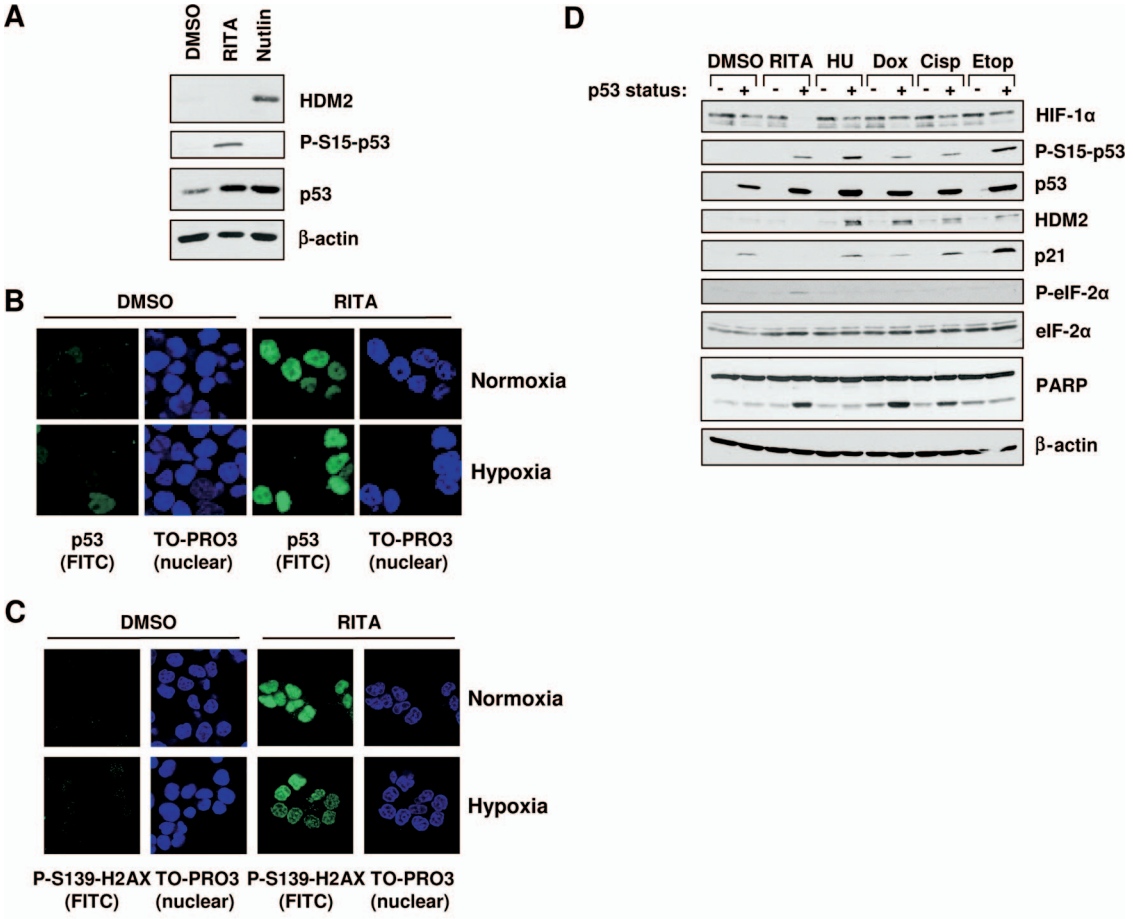


FIG. 7. RITA induces a DNA damage response. (A) Western blot analysis shows HDM2, p53, and phosphorylated-S15-p53 (P-S15-p53) proteins in $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) or nutlin-3 (4 μM) for 16 h. (B and C) Immunofluorescence analysis shows p53 (B) and phosphorylated-S139-γH2AX proteins (fluorescein isothiocyanate [FITC]) (C) in $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) in normoxia or hypoxia (1% O_2) for 16 h. Nuclei (blue) were visualized with TO-PRO-3 staining. Images (×40) were captured using a confocal microscope (Leica). (D) Western blot analysis shows HIF-1α, p53, phosphorylated S15-p53, HDM2, p21, eIF-2α, phosphorylated eIF-2α, and PARP proteins in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with DMSO, 1 μM RITA, 1 mM hydroxyurea (HU), doxorubicin (Dox), cisplatin (Cisp), or 25 μM etoposide (Etop) for 16 h in hypoxia (1% O_2). The status of p53 is shown above the lanes: +, present; −, absent.

dent phosphorylation of eIF-2α, which correlated with its ability to block HIF-1α induction in hypoxia. Concurrently, we found that RITA also blocked expression of the p53 target proteins HDM2 and p21, indicating that RITA may affect protein synthesis via phosphorylation of eIF-2α. Of particular interest is how the p53-dependent response induced by RITA signals to the translational machinery at the level of eIF-2α, since other known activators of p53 used in this study did not induce phosphorylation of eIF-2α. Activation of p53 by RITA did not appear to significantly block components of mTOR signaling, ruling out the possibility that downregulation of HIF-α, p21, and HDM2 were due to p53 cross talk to AMPK-mTOR signaling as reported recently (9, 15–17). Interestingly, we discovered that the activity of PERK, an upstream kinase that phosphorylates eIF-2α in response to hypoxia, was important for the induction of eIF-2α phosphorylation in response to RITA (5), suggesting that p53 signals to the translational machinery by regulating PERK–eIF-2α activation. Indeed, a recent report has linked the unfolded protein response pathway with p53 accumulation via a PERK-dependent mechanism (51).

Further investigation into the mechanism of action of RITA indicated that RITA did not appear to block the p53-HDM2 interaction as originally proposed to be its mechanism of action for inducing p53 (23). In fact, we found that RITA activated a DNA damage response in vitro and in vivo. Previous studies have shown that RITA causes protein-DNA and DNA-DNA intrastrand cross-links (37, 40), supporting the notion that RITA could induce and activate p53 by eliciting a DNA damage response in cells. Indeed, the ability of RITA to mediate both apoptotic and antiangiogenic effects in hypoxia may be due to its ability not only to induce p53 but also to elicit a concurrent DNA damage response. Intriguingly however, unlike other agents that mediate a DNA damage response, RITA-mediated effects are entirely dependent on p53.

Activation of the DNA damage response by RITA was apparent in both normoxia and hypoxia to a similar extent. While transient overexpression of p53 has been reported to block

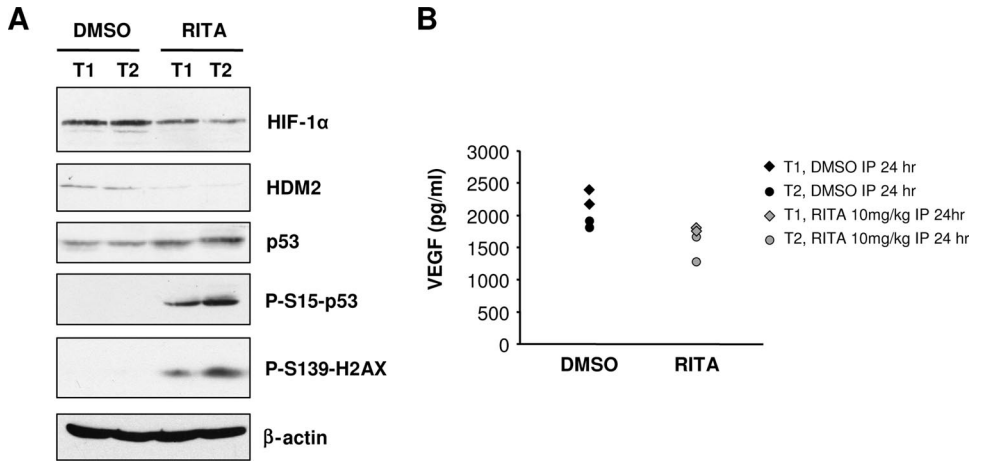


FIG. 8. RITA induces p53 and γH2AX phosphorylation and blocks HIF-1α and VEGF expression in tumor xenografts. (A) Western blot analysis of $p53^{+/+}$ HCT116 tumor xenograft lysates. Mice bearing $p53^{+/+}$ HCT116 cell-derived tumor xenografts were administered with a single intraperitoneal dose of RITA at 10 mg/kg for 24 h. Tumor cell lysates from eight tumors were analyzed per group. Western blots show a representation of two independent tumors (tumor 1 [T1] and T2) from the DMSO- and RITA-treated groups, assessed for HIF-1α, HDM2, p53, phosphorylated-S15-p53 (P-S15-p53), and phosphorylated-S139-γH2AX (P-S139-H2AX) proteins. Actin was used as a load control. (B) The graph shows VEGF levels in tumor lysates. Four data points are shown for each condition. Experiments show two independent tumors (T1 and T2) in two independent experiments (diamonds and circles). Abbreviations: IP, intraperitoneally; hr, hours.

HIF-1α and VEGF induced in hypoxia (39), a previous report has shown that DNA damage is a prerequisite for p53-mediated downregulation of HIF-1α (27). Interestingly, we found that RITA's ability to inhibit HIF-1α was not affected by blockade of apoptosis, indicating that the p53-dependent apoptotic and antiangiogenic responses mediated by RITA are mechanistically separable processes. While the p53-dependent apoptotic function induced by RITA did not appear to influence the observed HIF-1α blockade, conversely we found that in renal carcinoma cells expressing constitutively high basal levels of HIF-α protein due to loss of pVHL function, RITA was at least fivefold less potent in blocking tumor cell growth than in renal carcinoma cells expressing pVHL. In addition, we found that p21 knockdown by siRNA could enhance basal PARP cleavage and increase overall PARP cleaved in response to RITA (data not shown). These observations are consistent with the mTOR inhibitor RAD001 (4). Collectively, these findings indicate that the HIF-α status in cells contributes to RITA's apoptotic outcome and that downregulation of p21 by RITA may help drive the apoptotic response induced by p53.

The ability to both induce apoptosis and suppress tumor angiogenesis by targeting p53 is of significant interest therapeutically. Clinical studies have shown that the combined administration of chemotherapeutic agent 5-fluorouracil and bevacizumab, a monoclonal antibody targeted against VEGF, produced a dramatic increase in survival in colorectal cancer patients (18, 22). However, bevacizumab did not yield long-term survival benefits as a single agent (34). This suggests that combination of drugs that lead to both tumor cell death and suppression of angiogenesis would be more clinically desirable, even when one agent is a classical cytotoxin. Indeed, RITA appears to be able to mediate both processes as a single agent: RITA induces a DNA damage response, resulting in p53-mediated apoptosis, and also blocks HIF-1α expression, resulting in a significant reduction in VEGF expression.

In conclusion, our study utilizes the small-molecule activator of p53 RITA to evaluate mechanisms by which p53 can both induce apoptosis and suppress tumor angiogenesis. By identifying a new mechanism of action for RITA, we have uncovered the existence of a DNA damage response that when activated leads to the death of hypoxic tumor cells and blockade of the HIF-1α/VEGF pathway in vivo. Further evaluation of this response and the identification of other agents or activators of this response will be of particular interest and may lead to improved targeting of HIF/hypoxia signaling in tumor cells.

ACKNOWLEDGMENTS

We thank Galina Selivanova (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden) for initial discussions and the Developmental Therapeutics Program, National Cancer Institute, Maryland for supplying RITA (NSC-652287). We thank Karen Vousden (Beatson Institute for Cancer Research, Glasgow, United Kingdom) for TetON p53-inducible cells and Bert Vogelstein (Johns Hopkins School of Medicine, Baltimore, MD) for the $p53^{-/-}$ HCT116 and $p53^{+/+}$ HCT116 cells. We thank Nicola Wilsher and Amin Mizra (Cancer Research-United Kingdom Centre for Cancer Therapeutics, The Institute of Cancer Research, United Kingdom) for mass spectroscopy analysis and NMR confirmation of the RITA structure. For input regarding the measurement of DNA damage, we thank Neville Ashcroft, Penny Jeggo, Keith Caldecott (Genome Damage and Stability Centre, University of Sussex, United Kingdom), and Apolinar Maya-Mendoza and Dean Jackson (Faculty of Life Sciences, University of Manchester, MIB, Manchester, United Kingdom). We thank all other members of the Ashcroft lab for their input. Finally, we thank Neville Ashcroft for critical review of the manuscript and Patrick Maxwell (University College, London, United Kingdom) for useful discussions.

This work was funded by University College London, The Institute of Cancer Research, United Kingdom and Cancer Research United Kingdom (CUK) program grant number C309/A2187 (M.A., N.P., A.H.B., G.B., M.V., and S.E.). J.Y. and A.A. were funded by Cancer Research United Kingdom studentships C7358/A4420 and C7358/A8020, respectively. E.P. was funded by Cancer Research United Kingdom project grant C7358/A9958.

REFERENCES

1. **Achison, M., and T. R. Hupp.** 2003. Hypoxia attenuates the p53 response to cellular damage. Oncogene **22:**3431–3440.

2. **Asano, K., J. Clayton, A. Shalev, and A. G. Hinnebusch.** 2000. A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator $tRNA^{Met}$ is an important translation initiation intermediate in vivo. Genes Dev. **14:**2534–2546.
3. **Ashcroft, M., Y. Taya, and K. H. Vousden.** 2000. Stress signals utilize multiple pathways to stabilize p53. Mol. Cell. Biol. **20:**3224–3233.
4. **Beuvink, I., A. Boulay, S. Fumagalli, F. Zilbermann, S. Ruetz, T. O'Reilly, F. Natt, J. Hall, H. A. Lane, and G. Thomas.** 2005. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell **120:**747–759.
5. **Blais, J. D., V. Filipenko, M. Bi, H. P. Harding, D. Ron, C. Koumenis, B. G. Wouters, and J. C. Bell.** 2004. Activating transcription factor 4 is translationally regulated by hypoxic stress. Mol. Cell. Biol. **24:**7469–7482.
6. **Bonner, W. M., C. E. Redon, J. S. Dickey, A. J. Nakamura, O. A. Sedelnikova, S. Solier, and Y. Pommier.** 2008. γH2AX and cancer. Nat. Rev. Cancer **8:**957–967.
7. **Brizel, D. M., G. S. Sibley, L. R. Prosnitz, R. L. Scher, and M. W. Dewhirst.** 1997. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int. J. Radiat. Oncol. Biol. Phys. **38:**285–289.
8. **Brown, J. M., and W. R. Wilson.** 2004. Exploiting tumour hypoxia in cancer treatment. Nat. Rev. Cancer **4:**437–447.
9. **Budanov, A. V., and M. Karin.** 2008. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell **134:**451–460.
10. **Bunz, F., A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J. P. Brown, J. M. Sedivy, K. W. Kinzler, and B. Vogelstein.** 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science **282:**1497–1501.
11. **Bykov, V. J., and K. G. Wiman.** 2003. Novel cancer therapy by reactivation of the p53 apoptosis pathway. Ann. Med. **35:**458–465.
12. **Carroll, V. A., and M. Ashcroft.** 2006. Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res. **66:**6264–6270.
13. **Dever, T. E.** 2002. Gene-specific regulation by general translation factors. Cell **108:**545–556.
14. **El-Deiry, W. S.** 2003. The role of p53 in chemosensitivity and radiosensitivity. Oncogene **22:**7486–7495.
15. **Feng, Z., W. Hu, E. de Stanchina, A. K. Teresky, S. Jin, S. Lowe, and A. J. Levine.** 2007. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. **67:**3043–3053.
16. **Feng, Z., W. Hu, G. Rajagopal, and A. J. Levine.** 2008. The tumor suppressor p53: cancer and aging. Cell Cycle **7:**842–847.
17. **Feng, Z., H. Zhang, A. J. Levine, and S. Jin.** 2005. The coordinate regulation of the p53 and mTOR pathways in cells. Proc. Natl. Acad. Sci. USA **102:** 8204–8209.
18. **Fernando, N. H., and H. I. Hurwitz.** 2004. Targeted therapy of colorectal cancer: clinical experience with bevacizumab. Oncologist **9**(Suppl. 1)**:**11–18.
19. **Foster, B. A., H. A. Coffey, M. J. Morin, and F. Rastinejad.** 1999. Pharmacological rescue of mutant p53 conformation and function. Science **286:** 2507–2510.
20. **Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia.** 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature **379:**88–91.
21. **Hammond, E. M., M. J. Dorie, and A. J. Giaccia.** 2003. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J. Biol. Chem. **278:**12207–12213.
22. **Hurwitz, H.** 2004. Integrating the anti-VEGF-A humanized monoclonal antibody bevacizumab with chemotherapy in advanced colorectal cancer. Clin. Colorectal Cancer **4**(Suppl. 2)**:**S62–S68.
23. **Issaeva, N., P. Bozko, M. Enge, M. Protopopova, L. G. Verhoef, M. Masucci, A. Pramanik, and G. Selivanova.** 2004. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat. Med. **10:**1321–1328.
24. **Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane, and W. G. Kaelin, Jr.** 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science **292:**464–468.
25. **Jaakkola, P., D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, C. W. Pugh, and P. J. Ratcliffe.** 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science **292:**468–472.
26. **Jimenez, G. S., S. H. Khan, J. M. Stommel, and G. M. Wahl.** 1999. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. Oncogene **18:**7656–7665.
27. **Kaluzová, M., S. Kaluz, M. I. Lerman, and E. J. Stanbridge.** 2004. DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1α in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. Mol. Cell. Biol. **24:**5757–5766.
28. **Kao, J., M. T. Milano, A. Javaheri, M. C. Garofalo, S. J. Chmura, R. R. Weichselbaum, and S. J. Kron.** 2006. γ-H2AX as a therapeutic target for improving the efficacy of radiation therapy. Curr. Cancer Drug Targets **6:**197–205.
29. **Koumenis, C.** 2006. ER stress, hypoxia tolerance and tumor progression. Curr. Mol. Med. **6:**55–69.
30. **Koumenis, C., C. Naczki, M. Koritzinsky, S. Rastani, A. Diehl, N. Sonenberg, A. Koromilas, and B. G. Wouters.** 2002. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2α. Mol. Cell. Biol. **22:**7405–7416.
31. **Krajewski, M., P. Ozdowy, L. D'Silva, U. Rothweiler, and T. A. Holak.** 2005. NMR indicates that the small molecule RITA does not block p53-MDM2 binding in vitro. Nat. Med. **11:**1135–1137.
32. **Laughner, E., P. Taghavi, K. Chiles, P. C. Mahon, and G. L. Semenza.** 2001. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. **21:**3995–4004.
33. **Lowe, S. W., and A. W. Lin.** 2000. Apoptosis in cancer. Carcinogenesis **21:**485–495.
34. **Mayer, E. L., N. U. Lin, and H. J. Burstein.** 2007. Novel approaches to advanced breast cancer: bevacizumab and lapatinib. J. Natl. Compr. Cancer Netw. **5:**314–323.
35. **Moeller, B. J., M. R. Dreher, Z. N. Rabbani, T. Schroeder, Y. Cao, C. Y. Li, and M. W. Dewhirst.** 2005. Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell **8:**99–110.
36. **Nakano, K., E. Balint, M. Ashcroft, and K. H. Vousden.** 2000. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene **19:**4283–4289.
37. **Nieves-Neira, W., M. I. Rivera, G. Kohlhagen, M. L. Hursey, P. Pourquier, E. A. Sausville, and Y. Pommier.** 1999. DNA protein cross-links produced by NSC 652287, a novel thiophene derivative active against human renal cancer cells. Mol. Pharmacol. **56:**478–484.
38. **Nordsmark, M., M. Overgaard, and J. Overgaard.** 1996. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother. Oncol. **41:**31–39.
39. **Ravi, R., B. Mookerjee, Z. M. Bhujwalla, C. H. Sutter, D. Artemov, Q. Zeng, L. E. Dillehay, A. Madan, G. L. Semenza, and A. Bedi.** 2000. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. **14:**34–44.
40. **Rivera, M. I., S. F. Stinson, D. T. Vistica, J. L. Jorden, S. Kenney, and E. A. Sausville.** 1999. Selective toxicity of the tricyclic thiophene NSC 652287 in renal carcinoma cell lines: differential accumulation and metabolism. Biochem. Pharmacol. **57:**1283–1295.
41. **Salnikow, K., M. Costa, W. D. Figg, and M. V. Blagosklonny.** 2000. Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer. Cancer Res. **60:**5630–5634.
42. **Semenza, G. L., L. A. Shimoda, and N. R. Prabhakar.** 2006. Regulation of gene expression by HIF-1. Novartis Found. Symp. **272:**2–8.
43. **Stempien-Otero, A., A. Karsan, C. J. Cornejo, H. Xiang, T. Eunson, R. S. Morrison, M. Kay, R. Winn, and J. Harlan.** 1999. Mechanisms of hypoxia-induced endothelial cell death. Role of p53 in apoptosis. J. Biol. Chem. **274:**8039–8045.
44. **Thomas, G. V., C. Tran, I. K. Mellinghoff, D. S. Welsbie, E. Chan, B. Fueger, J. Czernin, and C. L. Sawyers.** 2006. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat. Med. **12:**122–127.
45. **Tovar, C., J. Rosinski, Z. Filipovic, B. Higgins, K. Kolinsky, H. Hilton, X. Zhao, B. T. Vu, W. Qing, K. Packman, O. Myklebost, D. C. Heimbrook, and L. T. Vassilev.** 2006. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc. Natl. Acad. Sci. USA **103:**1888–1893.
46. **United Kingdom Co-ordinating Committee on Cancer Research.** 1998. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). Br. J. Cancer **77**(Suppl. 1)**:**1–10.
47. **Vassilev, L. T., B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, and E. A. Liu.** 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science **303:**844–848.
48. **Vogelstein, B., and K. W. Kinzler.** 2004. Cancer genes and the pathways they control. Nat. Med. **10:**789–799.
49. **Williams, K. J., B. A. Telfer, D. Xenaki, M. R. Sheridan, I. Desbaillets, H. J. Peters, D. Honess, A. L. Harris, G. U. Dachs, A. van der Kogel, and I. J. Stratford.** 2005. Enhanced response to radiotherapy in tumours deficient in the function of hypoxia-inducible factor-1. Radiother. Oncol. **75:**89–98.
50. **Wouters, B. G., and M. Koritzinsky.** 2008. Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat. Rev. Cancer **8:**851–864.
51. **Zhang, F., R. B. Hamanaka, E. Bobrovnikova-Marjon, J. D. Gordan, M. S. Dai, H. Lu, M. C. Simon, and J. A. Diehl.** 2006. Ribosomal stress couples the unfolded protein response to p53-dependent cell cycle arrest. J. Biol. Chem. **281:**30036–30045.

[Cell Cycle 8:10, 1630-1632; 15 May 2009]; ©2009 Landes Bioscience

Letter to the Editor

Activation of a unique p53-dependent DNA damage response

Jun Yang,[1] Afshan Ahmed[2] and Margaret Ashcroft[1,2,*]

[1]Cell Growth Regulation and Angiogenesis Team; Cancer Research UK Centre for Cancer Therapeutics; The Institute of Cancer Research; Sutton, Surrey UK; [2]Hypoxia Signalling and Angiogenesis Laboratory; Centre for Cell Signalling and Molecular Genetics; University College London; Division of Medicine; London, UK

Key words: small molecule activator, DNA damage response, hypoxia

Hypoxia is a common feature of solid tumors that renders them resistant to conventional chemotherapeutic agents and radiotherapy.[2] Hypoxia inducible factors (HIFs) are essential components of the hypoxic response in cells, linking tumor hypoxia to angiogenesis and tumor progression through the upregulation of key angiogenic factors such as vascular endothelial growth factor (VEGF). The HIF pathway is deregulated in most human cancers and targeting HIF has become an attractive strategy for the development of new therapeutic agents.[6] Indeed, the ability to induce both tumor cell apoptosis and mediate anti-angiogenic effects is of particular interest therapeutically. The p53 tumor suppressor protein is a potent negative regulator of the HIF pathway, mediating both apoptotic and anti-angiogenic effects when induced.[5,9] Several agents have been described that can activate wild-type p53,[4,7,8] or reactivate mutant p53,[1,3] in tumor cells. However, many of these new p53-targeted agents have not yet been evaluated for their ability to inhibit the HIF pathway or assessed for their effectiveness at mediating tumor cell death in hypoxia. Recently, we have explored the mechanistic properties of the small molecule activator of p53, RITA (reactivation of p53 and induction of tumor cell apoptosis).[9] RITA (NSC-652287) was originally identified in a cell-based screen using the National Cancer Institute (NCI) compound library and has been shown previously to mediate p53-dependent anti-tumor activity in vivo.[4] We have found that RITA can mediate significant tumor cell apoptosis in normoxia and hypoxia in a p53-dependent manner (Fig. 1A and reviewed in ref. 9) and promote both apoptotic and anti-angiogenic effects in vivo.[9] In addition, we have found that RITA activates a DNA damage response in vitro and in vivo.[9] Further exploration of the DNA damage response induced by RITA as indicated by phosphorylated p53,[9] γH2AX and CHK1 proteins suggested that this response only occurred in cells expressing wild-type p53 (Fig. 1B) but did not occur in the absence of p53 (Fig. 1B) nor was the DNA damage response induced by RITA observed in cells expressing mutant p53 (Fig. 1C). Moreover, immunohistochemical analysis showed that RITA induced a pan-nuclear localization of phosphorylated γH2AX only in cells expressing wild-type p53 (Fig. 1D). In contrast however, several well known DNA-damage response inducing agents including hydroxyurea, doxorubicin, cisplatin and etoposide could readily induce phosphorylated γH2AX in both $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells (Fig. 1E), and in p53 mutant MDA-MB-231 cells (Fig. 1F). Collectively, our data indicate that unlike the DNA-damaging agents used here, RITA uniquely activates a p53-dependent DNA damage response. Activation of p53 by RITA leads to both apoptotic[4] and anti-angiogenic effects in vivo.[9] Since RITA can induce significant tumor cell apoptosis in normoxia and hypoxia, the identification of components of this p53-dependent DNA damage response may lead to a potential strategy for improving the therapeutic targeting of hypoxic tumor cells.

*Correspondence to: Margaret Ashcroft; Centre for Cell Signalling and Molecular Genetics; University College London; Division of Medicine; Rayne Building; 5 University Street; London WC1E 6JJ UK; Tel.: +44.0.20.7679.6205; Fax: +44.0.20.7679.6211; Email: m.ashcroft@ucl.ac.uk

Submitted: 03/01/09; Accepted: 03/02/09

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/article/8326

Acknowledgements

We thank Galina Selivanova (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden) for helpful discussions. For input regarding the measurement of DNA damage, we thank Neville Ashcroft, Penny Jeggo, Keith Caldecott (Genome Damage and Stability Centre, University of Sussex, UK) and Apolinar Maya-Mendoza and Dean Jackson (Faculty of Life Sciences, University of Manchester, MIB, Manchester, UK). We thank Evon Poon and all other members of the Ashcroft lab for their input and support. J.Y. and A.A. were funded by Cancer Research UK studentships, C7358/A4420 and C7358/A8020 respectively.

References

1. Bykov VJ, et al. Ann Med 2003; 35:458-65.
2. Carroll VA, et al. Expert Rev Mol Med 2005; 7:1-16.
3. Foster BA, et al. Science 1999; 286:2507-10.
4. Issaeva N, et al. Nat Med 2004; 10:1321-8.
5. Ravi R, et al. Genes Dev 2000; 14:34-44.
6. Semenza GL. Nat Rev Cancer 2003; 3:721-32.
7. Tovar C, et al. Proc Natl Acad Sci USA 2006; 103:1888-93.
8. Vassilev LT, et al. Science 2004; 303:844-8.
9. Yang J, et al. Mol Cell Biol 2009; In press.

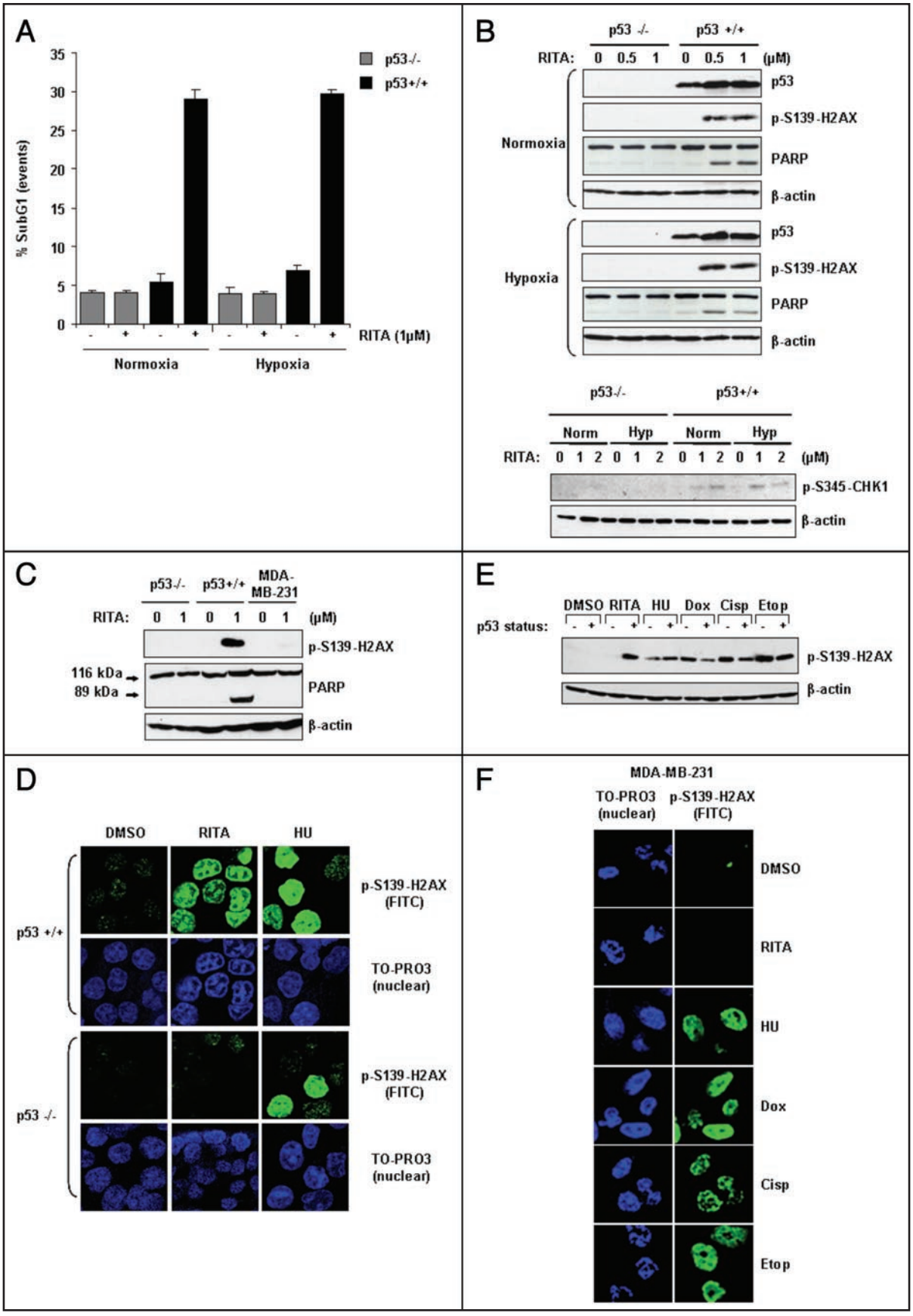

A
% SubG1 (events)
p53-/-
p53+/+
RITA (1μM)
Normoxia
Hypoxia
B
p53 -/-
p53 +/+
RITA: 0 0.5 1 0 0.5 1 (μM)
Normoxia
Hypoxia
p53
p-S139-H2AX
PARP
β-actin
Norm
Hyp
RITA: 0 1 2 0 1 2 0 1 2 0 1 2 (μM)
p-S345-CHK1
C
MDA-MB-231
RITA: 0 1 0 1 0 1 (μM)
116 kDa
89 kDa
D
DMSO
RITA
HU
p53 +/+
p53 -/-
p-S139-H2AX (FITC)
TO-PRO3 (nuclear)
E
p53 status:
DMSO RITA HU Dox Cisp Etop
F
MDA-MB-231
TO-PRO3 (nuclear)
p-S139-H2AX (FITC)
DMSO
RITA
HU
Dox
Cisp
Etop

Figure 1. (See previous page) (A) Flow cytometric analysis of $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) for 24 hr in normoxia or hypoxia (1% O_2). DNA content of cells (events) was measured by propidium iodide (PI) stain. Graph shows the percentage (%) subG_1 population of cells (events) as a measure of the apoptotic population. (B) Western blot analysis (upper) shows p53, phosphorylated-S139-H2AX, and PARP proteins in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA at the indicated concentrations for 16 hr in normoxia or hypoxia (1% O_2). Actin was used as a load control. Western blot analysis (lower) shows phosphorylated-S345-CHK1 protein in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA at the indicated concentrations for 16 hr in normoxia or hypoxia (1% O_2). Actin was used as a load control. (C) Western blot analysis shows phosphorylated-S139-H2AX and PARP proteins in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells, or MDA-MB-231 cells (p53 mutant) treated with RITA (1 μM) for 16 hr. Actin was used as a load control. (D) Immunofluorescence analysis shows phosphorylated-S139-H2AX protein (FITC) in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) or 1 mM of hydroxyurea (HU) for 16 hr in hypoxia (1% O_2). Nuclei (blue) were visualized with TO-PRO-3 staining. Images (x40) were captured using a confocal microscope (Leica). (E) Western blot analysis shows phosphorylated S139-H2AX protein in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with DMSO (control) RITA (1 μM), hydroxyurea (HU, 1 mM), doxorubicin (Dox, 1 μM), cisplatin (Cisp, 10 μg/ml), etoposide (Etop, 25 μM) for 16 hr in hypoxia (1% O_2). Actin was used as a load control. (F) Immunofluorescence analysis of phosphorylated-S139-H2AX protein (FITC) in MDA-MB-231 cells. Cells were treated with DMSO (control) RITA (1 μM), hydroxyurea (HU, 1 mM), doxorubicin (Dox, 1 μM), cisplatin (Cisp, 10 μg/ml), etoposide (Etop, 25 μM) for 16 hr in hypoxia (1% O_2). Nuclei were visualized with TO-PRO-3 staining (blue). Images (x40) were captured using a confocal microscope (Leica).

Citation: Cell Death and Disease (2011) 2, e160; doi:10.1038/cddis.2011.42

www.nature.com/cddis

Pharmacological activation of a novel p53-dependent S-phase checkpoint involving CHK-1

A Ahmed[1], J Yang[1,3], A Maya-Mendoza[2], DA Jackson[2] and M Ashcroft*[,1]

We have recently shown that induction of the p53 tumour suppressor protein by the small-molecule RITA (reactivation of p53 and induction of tumour cell apoptosis; 2,5-bis(5-hydroxymethyl-2-thienyl)furan) inhibits hypoxia-inducible factor-1α and vascular endothelial growth factor expression *in vivo* and induces p53-dependent tumour cell apoptosis in normoxia and hypoxia. Here, we demonstrate that RITA activates the canonical ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related DNA damage response pathway. Interestingly, phosphorylation of checkpoint kinase (CHK)-1 induced in response to RITA was influenced by p53 status. We found that induction of p53, phosphorylated CHK-1 and γH2AX proteins was significantly increased in S-phase. Furthermore, we found that RITA stalled replication fork elongation, prolonged S-phase progression and induced DNA damage in p53 positive cells. Although CHK-1 knockdown did not significantly affect p53-dependent DNA damage or apoptosis induced by RITA, it did block the ability for DNA integrity to be maintained during the immediate response to RITA. These data reveal the existence of a novel p53-dependent S-phase DNA maintenance checkpoint involving CHK-1.
Cell Death and Disease (2011) **2,** e160; doi:10.1038/cddis.2011.42; published online 19 May 2011
Subject Category: Cancer

Solid tumours characteristically contain areas of low oxygen tension (hypoxia). Hypoxia stabilises the expression of the hypoxia-inducible factor (HIF)-α transcription factor. Deregulated overexpression of HIF-α in tumour cells initiates a transcriptional programme that renders tumour cells resistant to chemotherapy and radiotherapy, resulting in a more aggressive and metastatic cancer phenotype.[1] Targeting HIF/hypoxia signalling, therefore, has become an attractive strategy for the development of new cancer treatments.[2]

The p53 tumour suppressor protein is a potent negative regulator of HIF-1α, mediating both apoptotic[3,4] and anti-angiogenic effects when overexpressed.[5,6] HIF-1α accumulation in hypoxia is blocked by overexpression[5] or activation[6] of p53, and HIF-1-dependent transcription negatively correlates with p53 status.[7] p53 is mutated in about 50% of human cancers, and several agents have been described that can reactivate mutant[8,9] or activate wild-type p53[10–12] in tumour cells. However, many of these emerging p53-targeted agents have not yet been evaluated for their effectiveness at mediating tumour cell death in normoxia and hypoxia. We have been exploring the mechanistic properties of the small-molecule activator of p53, RITA (reactivation of p53 and induction of tumour cell apoptosis; 2,5-bis (5-hydroxymethyl-2-thienyl) furan, NSC-652287).[12–14] RITA was originally identified in a cell-based screen using the National Cancer Institute compound library and was shown to mediate p53-dependent antitumour activity *in vivo*.[12] Subsequently, we have found that RITA significantly inhibits HIF-1α induction and elicits p53-dependent apoptotic responses in normoxia and hypoxia, and promotes both apoptotic and antiangiogenic effects *in vivo*.[15] RITA was originally proposed to stabilise and activate p53 by disruption of the p53–human double minute 2 (HDM2) interaction,[12] and mediate p53-dependent apoptosis and other cellular responses via the regulation of p53 transcriptional responses.[16] Recently, we have shown that RITA mediates an effect on the protein translation machinery and downregulates both HDM2 and p21 protein levels in a dose- and time-dependent manner while concurrently inducing apoptosis.[15] Moreover, and consistent with previous reports indicating that RITA causes protein–DNA and DNA–DNA intrastrand crosslinks,[13,14] we have found that RITA activates a DNA damage response.[17] Collectively, these studies indicate that RITA exhibits a complex mechanism of action that leads to p53 activation and apoptotic responses.

Here, we further explore the mechanism of action of RITA, with particular focus on the DNA damage response. We demonstrate that RITA activates the canonical ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related (ATM/ATR) DNA damage cascade and induces DNA damage in p53 positive cells. Interestingly, RITA also mediates checkpoint kinase (CHK)-1 phosphorylation and slows replication fork elongation and S-phase progression in a

[1]Department of Metabolism and Experimental Therapeutics, Division of Medicine, Centre for Cell Signalling and Molecular Genetics, University College London, Rayne Building, 5 University Street, London WC1E 6JJ, UK and [2]Faculty of Life Sciences, University of Manchester, MIB, Manchester, M1 7DN, UK
*Corresponding author: M Ashcroft, Department of Metabolism and Experimental Therapeutics, Division of Medicine, Centre for Cell Signalling and Molecular Genetics, University College London, Rayne Building, 5 University Street, London WC1E 6JJ, UK. Tel: +44 20 7679 0799; Fax: +44 20 7679 6211;
E-mail: m.ashcroft@ucl.ac.uk
[3]Current address: Department of Genetics and Tumour Cell Biology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA
Keywords: p53; hypoxia; HIF-1α; DNA damage; CHK-1
Abbreviations: RITA, reactivation of p53 and induction of tumour cell apoptosis; HIF-1α, hypoxia-inducible factor-1α; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; CHK, checkpoint kinase; HDM2, human double minute 2; PARP, poly ADP ribose polymerase; BrdU, bromodeoxyuridine
Received 08.4.11; accepted 11.4.11; Edited by G Melino

p53-dependent manner. Loss of CHK-1 does not significantly affect p53-mediated apoptotic responses induced by RITA, but does significantly enhance DNA damage upon short term exposure to RITA. Our study identifies a novel p53-dependent S-phase checkpoint involving CHK-1.

Results

RITA induces a DNA damage response. Recently, we have found that RITA can mediate significant tumour cell apoptosis in normoxia and hypoxia in a p53-dependent manner and activate a DNA damage response *in vitro* and *in vivo*.[15] Consistent with our previous studies,[17] here, we found that RITA induced phosphorylation of the histone protein H2AX at Ser139 ((γH2AX); Figure 1a), which is usually associated with DNA damage-induced stress.[18] Concurrently, we found that RITA increased cleaved poly ADP ribose polymerase (PARP; Figure 1a) and the percentage of cells in sub-G1 (Figure 1b), indicative of increased apoptosis. Both DNA damage and apoptotic responses induced by RITA were observed in normoxia and hypoxia, only in p53-positive cells (Figure 1).

To further explore the DNA damage response induced by RITA, we first assessed the phosphorylation status of p53 in response to RITA, as phosphorylation of p53 within the N-terminus is usually induced by genotoxic stress.[19] $p53^{+/+}$ HCT116 cells were treated with RITA over a concentration curve and assessed for Ser15, Ser20 and Ser46 phosphorylation of p53 (Figure 2a). We found that RITA induced N-terminal phosphorylation of p53 (Figure 2a). DNA damage is usually sensed by the PI-3K-related protein kinases ATM and ATR, which activate the transducer checkpoint kinases CHK-2 and CHK-1, respectively.[20] ATM/ATR directly phosphorylates p53 at Ser15, whereas CHK-2/CHK-1 phosphorylates Ser20 on p53.[21] Consistent with activation of the canonical ATM/ATR DNA damage response pathway, we found that RITA also induced phosphorylation of Ser345 on CHK-1 and Thr68 on CHK-2 (Figure 2a), and increased γH2AX in a dose-dependent manner (Figure 2a). These responses correlated with a dose-dependent increase of cells in sub-G1, indicative of apoptosis (Figure 2a, graph). In addition, we found that induction of phosphorylated CHK-1, CHK-2, p53 and γH2AX proteins was time-dependent (Figures 2b and c) and correlated with a time-dependent increase in cells in sub-G1 (Figure 2d). Thus, the DNA damage response induced by RITA is both dose- and time-dependent.

We found that the induction of phosphorylated p53 (Ser15 and Ser20), CHK-1 and CHK-2 proteins in response to RITA was blocked by wortmannin, a kinase inhibitor of ATM/ATR and other phosphatidylinositol 3-kinase family members (Figure 2e), whereas the induction of cleaved PARP was only marginally affected (Figures 2e and f). Interestingly, we found that wortmannin had no significant effect on the induction of eukaryotic initiation factor-2α phosphorylation or the downregulation of HDM2 and p21 proteins induced by RITA (Figure 2f), which we have previously described.[15] These data indicate that the DNA damage and translational responses induced by RITA are potentially separable processes. As we would anticipate from the wortmannin effects observed (Figures 2e and f), in response to RITA, we found that ATM or ATR siRNA blocked the induction of phosphorylated CHK-1 and CHK-2 (Figure 2g), which are downstream targets of ATR and ATM, respectively. Finally, further analysis of the DNA damage response induced by RITA indicated a slight but measurable increase in DNA damage in p53-positive HCT116 and MCF-7 cells (Figures 3a and b), which was not observed in p53-null HCT116 or Saos-2 cells (Figure 3c). Taken together, these data suggest that RITA activates the canonical ATM/ATR DNA damage response pathway and induces DNA damage in p53 positive cells.

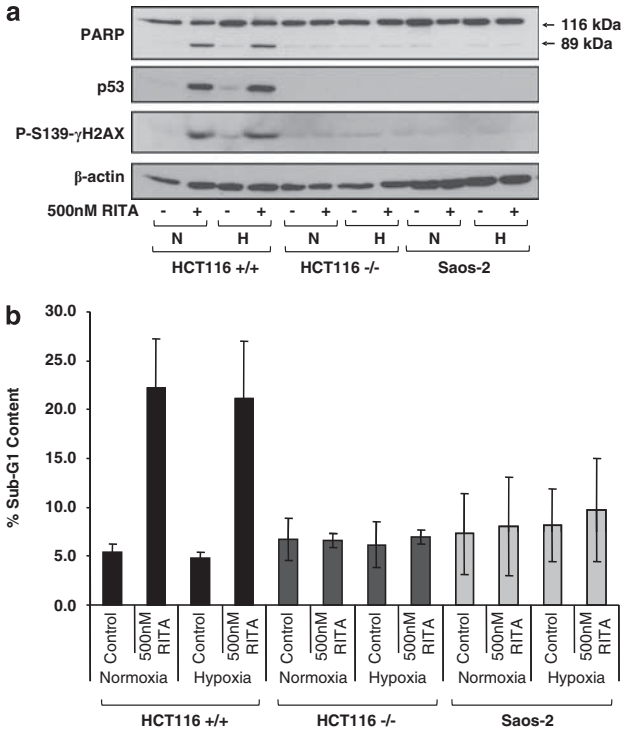


Figure 1 RITA induces p53-dependent apoptosis in normoxia and hypoxia. (**a** and **b**) $p53^{-/-}$HCT116, $p53^{+/+}$HCT116 cells or Saos-2 (p53 null) cells were treated with or without RITA (500 nM) for 16 h in either normoxia or hypoxia (1% O_2). (**a**) Western blots show cleaved PARP (89 kDa), p53 and phosphorylated-S139-γH2AX proteins. Actin was used as a loading control. (**b**) In parallel with the western analysis described in **a**, cells were harvested for flow cytometric analysis. Cells were fixed and stained using propidium iodide to visualise DNA profiles. The graph shows the percentage (%) of cells in sub-G1 in response to the treatments as indicated. Data is averaged from three independent experiments

RITA stalls replication fork elongation and prolongs S-phase progression in p53-positive cells. We have previously observed that most cells treated with RITA showed an intense pan-nuclear staining of γH2AX.[17] A similar pan-nuclear staining pattern was observed in response to hydroxyurea treatment.[17,22] This type of DNA damage response is indicative of potential stalling of the replication fork or inhibition of replication fork elongation, and can be mediated during processing of bulky DNA lesions.[22] During replication, cells usually respond to DNA damage by

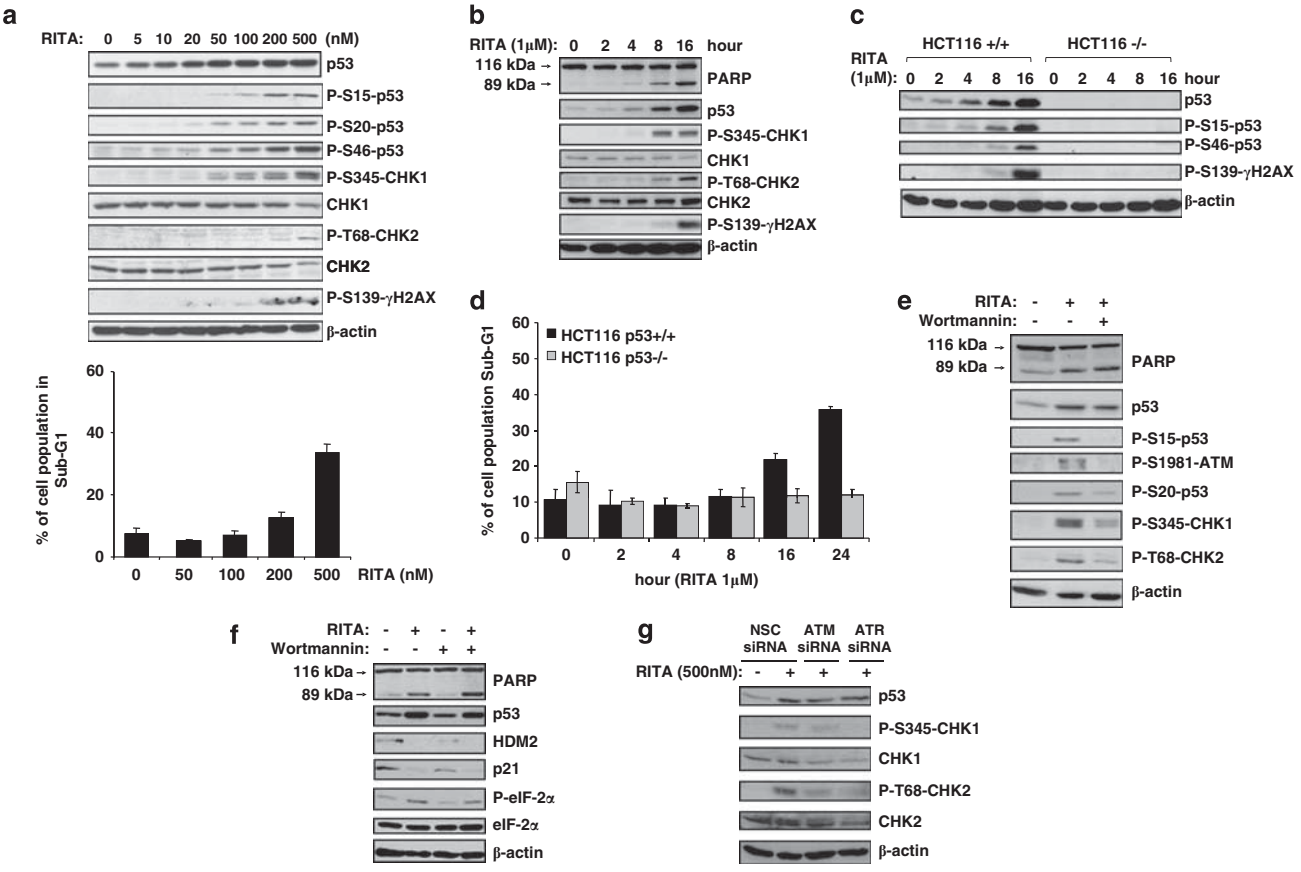


Figure 2 RITA induces a DNA damage response. (**a**) Western blot analysis of DNA damage response markers in $p53^{+/+}$HCT116 cells treated with the indicated concentrations of RITA for 16 h. Western blot analysis shows p53, phosphorylated p53 (S15, S20, S46), phosphorylated-S345-CHK-1, phosphorylated-T68-CHK-2, phosphorylated-S139-γH2AX, CHK-1 and CHK-2 proteins. Actin was used as a loading control. In parallel, cells were harvested for flow cytometric analysis. Cells were fixed and stained using propidium iodide to visualise DNA profiles. The graph shows the percentage (%) of cells in sub-G1 in response to RITA treatment as indicated. Data is averaged from three independent experiments. (**b**) Western blot analysis of DNA damage response markers in $p53^{+/+}$HCT116 cells treated with RITA (1 μM) for the indicated time. Western blot analysis shows p53, phosphorylated-S345-CHK-1, phosphorylated-T68-CHK-2, phosphorylated-S139-γH2AX, CHK-1 and CHK-2 proteins Actin was used as a loading control. (**c**) Western blot analysis shows p53, phosphorylated p53 (S15 and S46) and phosphorylated-S139-γH2AX in $p53^{+/+}$HCT116 cells and $p53^{-/-}$HCT116 cells treated with RITA (1 μM) for the indicated time. Actin was used as a loading control. (**d**) In parallel with the western analysis described in **c**, cells were harvested for flow cytometric analysis. Cells were fixed and stained using propidium iodide to visualise DNA profiles. The graph shows the percentage (%) of cells in sub-G1 in response to RITA treatment as indicated. Data are averaged from three independent experiments. (**e** and **f**) $p53^{+/+}$HCT116 cells were treated with RITA (1 μM) in the presence or absence of wortmannin (10 μM) for 16 h and assessed by western blot for p53, phosphorylated p53 (S15 and S20), phosphorylated-S1981-ATM, phosphorylated-S345-CHK-1, phosphorylated-T68-CHK-2 proteins, HDM2, p21, phosphorylated eukaryotic initiation factor-2α (eIF-2α), total eIF-2α, and cleaved PARP proteins. Actin was used as a loading control. (**g**) $p53^{+/+}$HCT116 cells were transfected with a non-silencing control siRNA (NSC), ATM siRNA or ATR siRNA. Cells were treated with RITA (500 nM) for 24 h then harvested for western analysis. Western blot analysis shows p53, phosphorylated-S345-CHK-1, phosphorylated-T68-CHK-2, CHK-1 and CHK-2 proteins. Actin was used as a loading control

activating an intra-S-phase checkpoint.[23] Therefore, we next assessed whether RITA mediated a p53-dependent DNA damage response by affecting the replication fork and S-phase progression. To do this, we treated $p53^{-/-}$ or $p53^{+/+}$ HCT116 cells with RITA and performed a DNA fibre assay, as previously described.[24,25] We found that RITA induced a marked increase in the percentage of replication forks in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells (Figure 4a, compare $p53^{-/-}$ with $p53^{+/+}$, lanes 2 and 3). Intriguingly, this effect was only observed in a sub-population of replicons, such that the second and third classes were very substantially affected by RITA treatment, whereas the seventh/eight/ninth classes were less affected (Figure 4a, compare untreated *versus* RITA-treated in $p53^{+/+}$ cells, lanes 2 and 3 with lanes 7, 8 and 9). Statistical analysis (*t*-test) showed significance (*P*-value = 4.85E−25) in $p53^{+/+}$ HCT116 cells for untreated *versus* RITA-treated (Figure 4b). Visualisation of DNA foci using bromodeoxyuridine (BrdU) pulse labelling and immunohistochemical analyses was performed to assess S-phase progression in unsynchronised $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells. We found that although the S-phase programme was maintained upon treatment with RITA, our data indicated that S-phase was prolonged at mid-late stages (Figure 4c).

Previous studies have shown that CHK-1 predominantly regulates DNA replication, fork elongation and effects S-phase progression.[25] As we found that RITA induced a p53-dependent increase in replication fork number

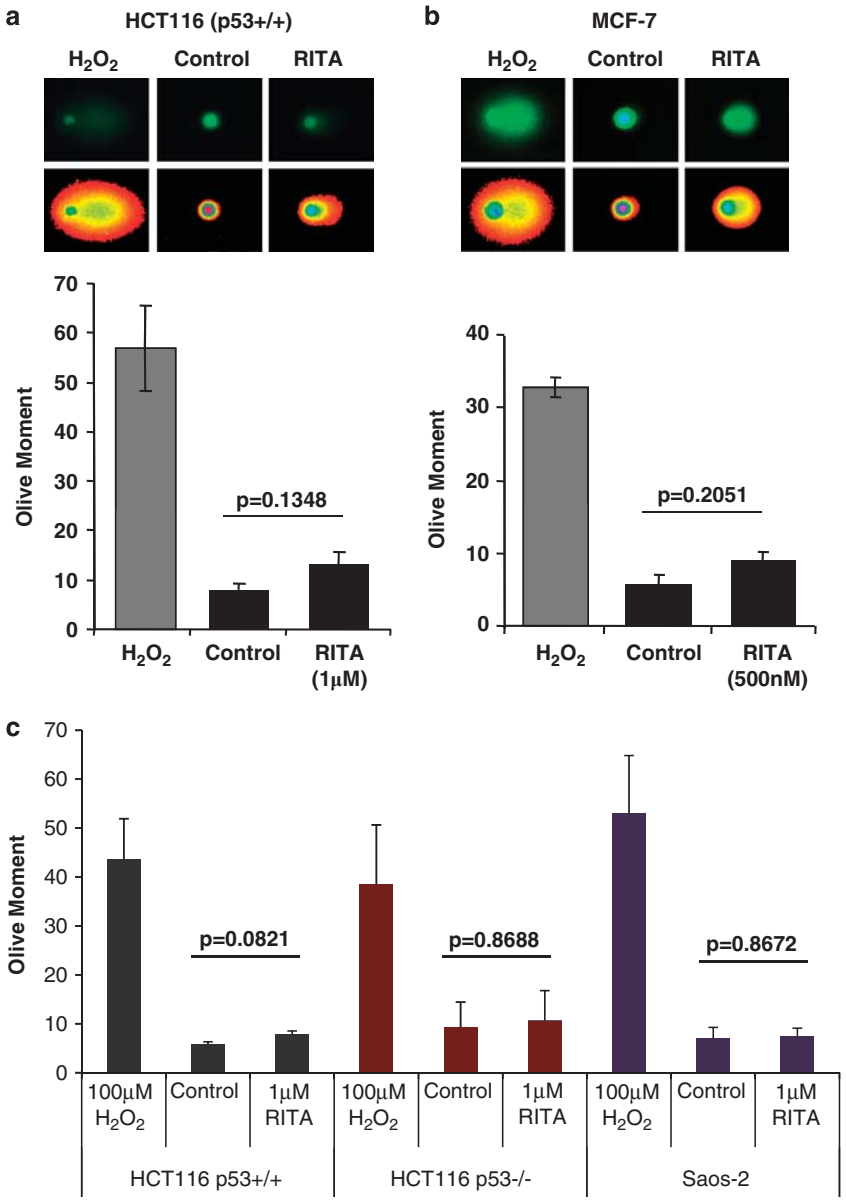
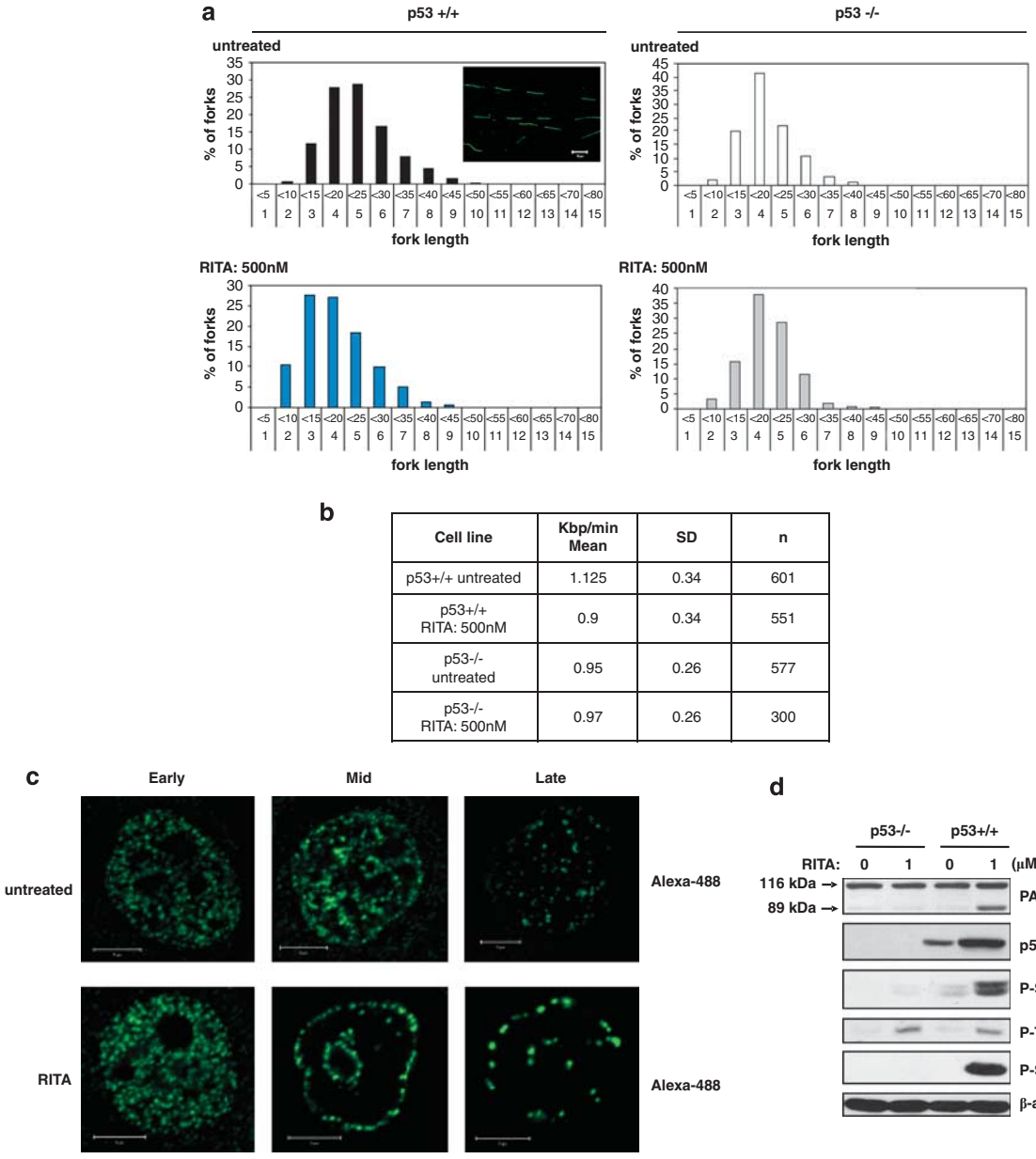


Figure 3 RITA induces DNA damage in p53-positive cells. (**a**) $p53^{+/+}$ HCT116 and (**b**) MCF-7 cells were treated with RITA at the indicated concentrations and assessed for DNA strand breaks using the comet assay (upper panels show representative Sybr green-stained comets (upper row) and the respective digitally converted images (lower row) for each condition as indicated). As a positive control for DNA damage, cells were treated with H_2O_2 (100 μM for 20 min). Olive moment for each condition ($\sim$100 comets/sample) was measured using the Comet Score software (TriTek Corporation, Sumerduck, VA, USA). Graphs (lower panels) show mean olive moment as indicated. (**c**) $p53^{+/+}$ HCT116 $p53^{-/-}$ HCT116 and Saos-2 were treated as described in **a**. Graph shows average olive moment as a measurement of DNA damage. Data is represented from three independent experiments

(Figures 4a and b) and affected S-phase progression (Figure 4c), we next assessed whether CHK-1 phosphorylation was also affected by p53 status. To do this, $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells were treated with RITA. We found that both CHK-1 and CHK-2 were phosphorylated in response to RITA treatment (Figure 4d). However, we found that phosphorylation of CHK-1 at Ser345 induced by RITA was affected by p53 status, whereas RITA-induced phosphorylated CHK-2 was observed in both $p53^{-/-}$ and $p53^{+/+}$ cells to a similar extent (Figure 4d). Taken together, our studies indicate that RITA activates a p53-dependent DNA damage response involving CHK-1 that functions to stall DNA replication fork elongation and prolong S-phase progression.

RITA induces p53 and phosphorylated CHK-1 and γH2AX proteins in S-phase. Given that RITA induces significant p53-dependent apoptotic (Figure 1) and S-phase responses (Figure 4), we next explored the induction of p53 and γH2AX proteins in sub-G1 and S-phase cell populations using fluorescence-activated cell sorting (FACS) analysis



Cell line	Kbp/min Mean	SD	n
p53+/+ untreated	1.125	0.34	601
p53+/+ RITA: 500nM	0.9	0.34	551
p53-/- untreated	0.95	0.26	577
p53-/- RITA: 500nM	0.97	0.26	300

Figure 4 RITA stalls replication fork elongation and slows S-phase progression in p53-positive cells. (**a**) DNA fibre assay of $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (500 nM) for 16 h. Graphs show percentage (%) number of forks *versus* fork length for each condition as indication. (**b**) Table (lower panel) shows a summary of the results from **a** for each condition analysed. Statistical analysis (*t*-test) showed significance (*P*-value = 4.85E−25) for untreated *versus* RITA treated in $p53^{+/+}$ HCT116 cells. (**c**) RITA prolongs S-phase in $p53^{+/+}$ HCT116 cells. Immunofluorescence analysis shows DNA replication foci (Alexa-488, green) in $p53^{+/+}$ HCT116 cells after treatment with RITA (500 nM) for 16 h. BrdU incorporation was used as a measure for S-phase progression as indicated (early, mid and late stages). (**d**) Western blot analysis shows phosphorylated-S345-CHK-1, phosphorylated-T68-CHK-2, phosphorylated-S139-γH2AX and PARP proteins in $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) for 16 h. Actin was used as a loading control

(Figure 5a). We found that p53 protein was induced in response to RITA in all cell cycle phases (G1 = 38%, S = 43% and G2 = 40%). Interestingly, after treatment with RITA, a higher proportion of cells in S-phase were positive for γH2AX protein (G1 = 22%, S = 26% and G2 = 10%). Moreover, we found a dose- and time-dependent increase in both p53 and γH2AX protein levels in cells in S-phase in response to RITA (Figures 5b and c). In addition, we found a significant increase in the proportion of cells in S-phase expressing phosphorylated CHK-1 protein (Figures 5d and e), whereas an increase in cells expressing phosphorylated CHK-1 protein was not observed in cells in sub-G1 (data not shown). Together, these data suggest that RITA activates a p53-dependent S-phase checkpoint involving CHK-1.

CHK-1 knockdown does not affect RITA-induced apoptosis but affects DNA damage in cells treated with RITA. To further assess whether CHK-1 was required for p53-dependent apoptotic and DNA damage responses

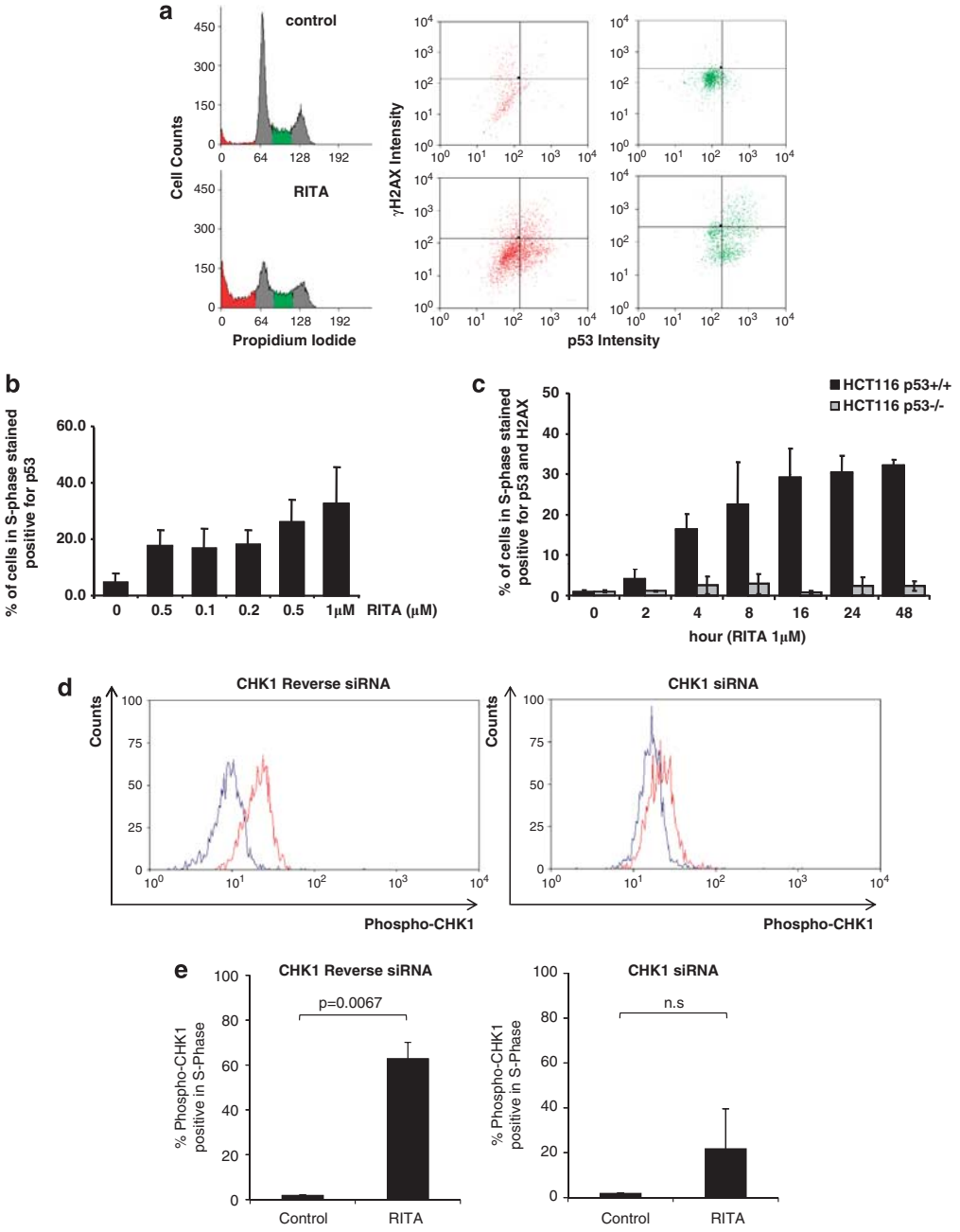


Figure 5 RITA induces p53 and phosphorylated CHK-1 and γH2AX proteins in S-phase. (**a**) Representative flow cytometric analysis plots of $p53^{+/+}$ HCT116 cells treated with RITA (500 nM) for 24 h. Cells were stained for DNA content, using propidium iodide, p53 and phosphorylated-S139-γH2AX proteins. DNA profiles (graphs) were gated for cells in sub-G1 (red) and S-phases (green). Changes were quantified within the top right quadrant of each of dot plot. This quadrant indicates an increase in both p53 and phosphorylated-S139-γH2AX staining intensity. (**b**) $p53^{+/+}$ HCT116 cells were treated with RITA at the concentrations indicated for 24 h and harvested for flow cytometric analysis as described in **a**. Graph shows average percentage of cells in S-phase that stain positive for both p53 and phosphorylated-S139-γH2AX proteins. (**c**) $p53^{+/+}$ HCT116 and $p53^{-/-}$ HCT116 cells were treated with RITA (1 μM) for the indicated time and harvested for flow cytometric analysis as described in **a**. Graph shows average percentage of cells in S-phase that stain positive for both p53 and phosphorylated-S139-γH2AX proteins. Data have been averaged from three independent repeat experiments. (**d** and **e**) CHK-1 is phosphorylated in S-phase cells in response to RITA. $p53^{+/+}$ HCT116 cells were transfected with either CHK-1 reverse siRNA, or CHK-1 targeted siRNA before RITA treatment (500 nM) for 24 h. (**d**) Propidium iodide FACS profiles were gated for the S-phase population of cells, and phosphorylated CHK-1 protein was quantified in the S-phase population (blue = control, red = RITA). The data is representative of two experiments. (**e**) Graphs show data described in **d** as the percentage (%) of cells in S-phase expressing phosphorylated CHK-1. n.s., not significant

induced by RITA, we knocked down CHK-1 using siRNA (Figure 5e). Interestingly, we found that CHK-1 knockdown had no significant effect on p53-dependent apoptotic responses induced by RITA (Figures 6a and b), whereas the inhibition of caspase-3 activation by the inhibitor Z-DEVD-FMK blocked the increase in cells in sub-G1 (Figure 6a) and the increase in cleaved PARP (Figure 6b), but had no significant effect on phosphorylated CHK-1

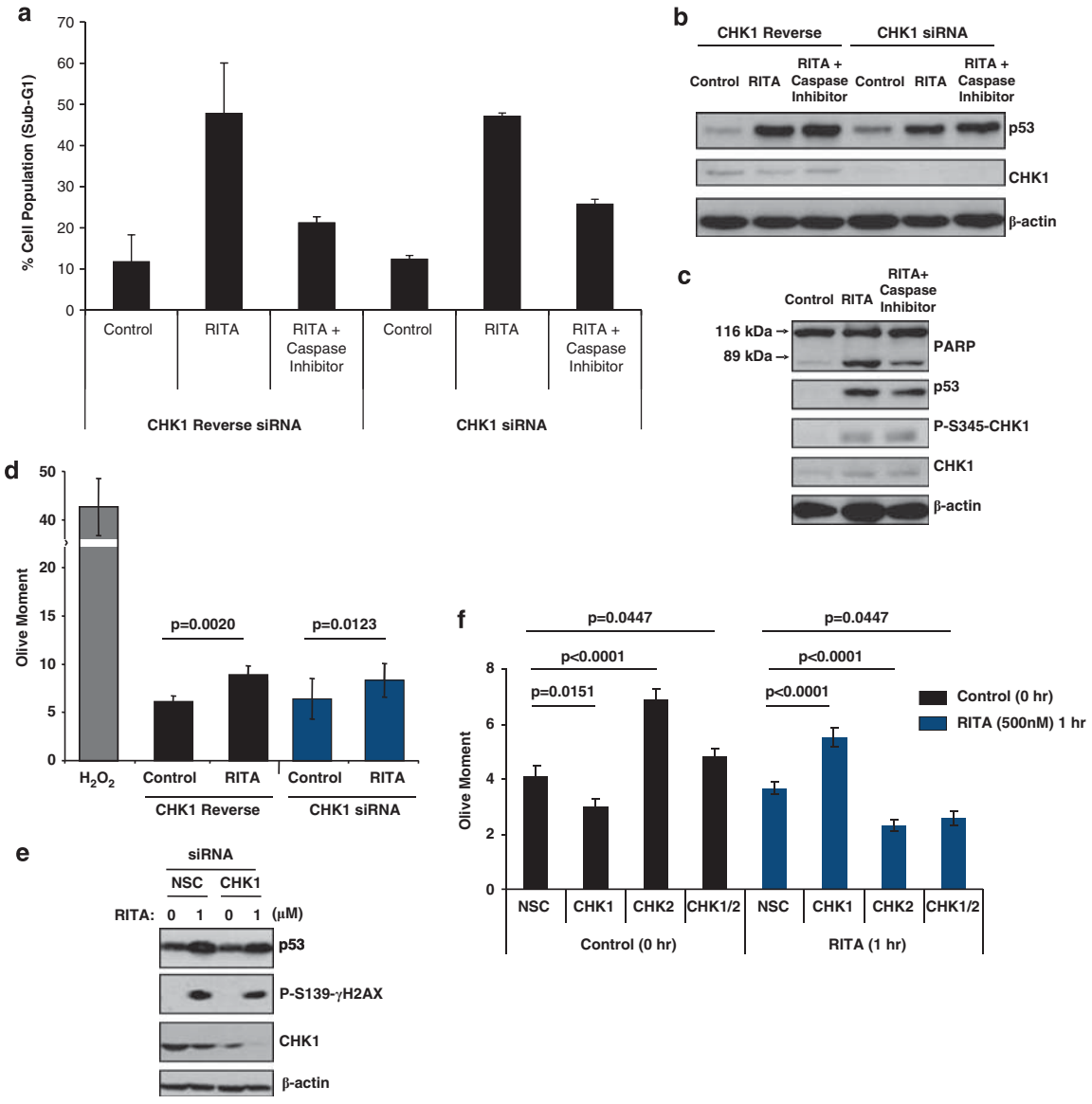


Figure 6 CHK-1 knockdown does not affect RITA-induced apoptosis but significantly enhances DNA damage in cells treated with RITA. (**a** and **b**) $p53^{+/+}$ HCT116 cells transiently transfected with a non-silencing control (NSC) siRNA duplex and siRNA to CHK-1 and treated with RITA (1 μM) for 16 h in the presence and absence of the caspase-3 inhibitor Z-DEVD-FMK. Cells were harvested for flow cytometric analysis (**a**) or western analysis (**b**). (**a**) Graph shows the percentage of cells within sub-G1, as indicated. Data are representative of two independent experiments ($\pm$ S.D.). (**b**) Western blot analysis shows p53 and CHK-1 proteins. Actin was used as a loading control. (**c**) $p53^{+/+}$ HCT116 cells treated with RITA (1 μM) for 16 h in the presence and absence of the caspase-3 inhibitor Z-DEVD-FMK. Western blot analysis shows PARP, p53, CHK-1 and phosphorylated-S139-γH2AX proteins. Actin was used as a loading control. (**d**) $p53^{+/+}$ HCT116 cells transiently transfected with a NSC siRNA duplex and siRNA to CHK-1 and treated with RITA (500 nM) for 16 h and harvested for assessment of DNA damage using the Comet Score software (TriTek Corporation, Sumerduck, VA, USA). Graph shows mean olive moment ($\sim$ 100 comets/sample) for each condition as indicated. As a positive control for DNA damage, cells were treated with H_2O_2 (100 μM for 20 min). (**e**) In parallel with **d**, cells were harvested for western blot analysis. Western blots show p53, CHK-1 and phosphorylated-S139-γH2AX proteins. Actin was used as a loading control. (**f**) $p53^{+/+}$ HCT116 cells transiently transfected with a NSC siRNA duplex, CHK-1 and/or CHK-2 siRNA and treated with RITA (500 nM) for 1 h. The untreated control samples were harvested at 0 h. Cells were harvested for assessment of DNA damage using a comet assay, as described in **d**. Graph shows mean olive moment ($\sim$ 100 comets/sample) for each condition as indicated

induced by RITA (Figure 6c). CHK-1 has an important role in DNA repair[26] and is essential for the maintenance of genomic stability,[27] particularly during DNA replication and replication fork progression.[25,28–30] Thus, we next investigated whether CHK-1 knockdown affected the DNA damage response induced by RITA. We found that CHK-1 knockdown had no significant effect on the small amount of accumulated DNA damage at 24 h of exposure to RITA (Figure 6d), nor was there any significant effect on the γH2AX induced by RITA in CHK-1 knockdown cells at this time point (Figure 6e). Notably, a previous report has shown that phosphorylation at Ser345 on CHK-1 increases its turnover, and thereby reduces total CHK-1 protein levels.[31] Consistently, we found that total CHK-1 protein levels decreased in the presence of RITA (Figure 6e). This effect was more dramatic in the presence of CHK-1 siRNA (Figure 6e).

Finally, consistent with a role for CHK-1 in DNA repair and maintenance,[26,27] we found that CHK-1 but not CHK-2 knockdown significantly enhanced DNA damage induced at only 1 h of RITA treatment (Figure 6f), indicating that CHK-1 is essential for maintaining DNA integrity upon short term exposure of cells to RITA. Consistently, we found that CHK-1 phosphorylation was induced by RITA during this time frame (data not shown).

Discussion

HIF is upregulated in most human cancers due to changes in tumour microenvironmental stimuli and genetic abnormalities.[1] Of particular interest, is the small-molecule approaches that have been taken recently to target the HIF pathway as a basis for the development of new therapeutics in the treatment of cancer.[2,32] The p53 tumour suppressor protein is a potent negative regulator of HIF signalling in tumours.[5] We have recently shown that small-molecule activator of p53, RITA, can mediate both antiangiogenic effects via blockade of the HIF pathway and elicit apoptosis in hypoxic tumour cells *in vitro* and *in vivo*.[15] Intriguingly, unlike other p53-activating agents, RITA causes significant tumour cell apoptosis in normoxia and hypoxia (1% O_2), without eliciting either a measurable G1 and/or G2 arrest.[15] Given that hypoxic tumour cells expressing high basal levels of HIF-α are usually resistant to killing by conventional radio and chemotherapeutic agents, in this study, we further investigated the mechanistic properties of RITA, with particular focus on exploring the DNA damage response.

Here, we found that RITA activated the canonical ATM/ATR DNA damage response pathway that leads to activation of CHK-1 and CHK-2 phosphorylation. Intriguingly, however, and confirming our recent findings,[17] we found that the induction of phosphorylated CHK-1 and γH2AX proteins observed in response to RITA was dependent on p53 status.

Previous studies have reported a p53-dependent DNA damage checkpoint.[33,34] Activation of a p53-dependent S-phase DNA damage checkpoint occurs to delay DNA synthesis and to allow time to resolve a potential replication block.[33,35] Our earlier immunohistochemical analyses showed that RITA induced a pan-nuclear localisation of γH2AX opposed to localisation to discrete nuclear foci.[17] This type of DNA damage response indicates potential stalling of the replication fork or is mediated during processing of bulky DNA lesions.[22] Indeed, we found that RITA induced a p53-dependent increase in replication fork number in a subpopulation of replicons. Notably, a decline in replication fork rate is known to be consistent with increased rates of local origin activation and higher replication fork densities,[25] indicating that RITA stalled DNA replication elongation and affected replication fork rate.

Consistent with these observations, we found that treatment of cells with RITA also prolonged mid-late S-phase progression in p53-positive cells. Our findings indicate that RITA activates a p53-dependent checkpoint that may involve CHK-1.

CHK-1 has been shown to have an important role in regulating DNA replication fork elongation and S-phase progression.[25] In response to RITA, we found that the relative percentage of cells in S-phase expressing either p53 or γH2AX protein was increased compared with other cell cycle phases. Concurrently, we observed a significantly high percentage of cells in S-phase expressing phosphorylated CHK-1 upon RITA treatment. Activation of CHK-1 is crucial for eliciting replication checkpoints in response to DNA-damaging agents, providing protection to cells by allowing a slowing of S-phase progression,[28,29] and appears to be critically involved in stabilising stalled replication forks.[30] In addition, CHK-1 is important in DNA repair upon exposure to hydroxyurea.[26,36] Interestingly, we found that knockdown of CHK-1 by siRNA had only a minimal effect on the induction of p53-dependent apoptosis and γH2AX in response to RITA. However, knockdown of CHK-1 but not CHK-2 significantly increased DNA damage induced within only 1 h of RITA treatment, indicating that the activation of CHK-1 mediated by RITA is important for maintaining DNA integrity.

RITA was originally identified using a cell-based screen[12] and proposed to bind to the N terminus of p53 and induce p53 stabilisation by disruption of the p53–HDM2 interaction.[12] Other studies have shown that RITA can also cause protein–DNA and DNA–DNA intrastrand crosslinks,[13,14] thereby suggesting that RITA may also act to stabilise p53 by interchelating with DNA. This latter possibility is supported by the observations that RITA activates the canonical DNA damage response. Of significance here is our observation that induction of CHK-1 phosphorylation by RITA is affected by p53 status. These findings suggest that RITA functions mechanistically both at the level of DNA and at the level of p53. Along with its role in G1/S- and G1/M-dependent cell cycle checkpoints, p53 has also been implicated in S-phase processes and DNA repair. Specifically, p53 is transported to sites of stalled DNA replication forks and binds to RAD51,[37] thus p53 provides an S-phase-specific role that is independent of its known transcriptional activities.[37] In addition, CHK-1 binds to and phosphorylates RAD51, providing a vital role in DNA repair upon exposure to hydroxyurea.[36] Collectively, these events ensure that DNA integrity is maintained, and it may be that p53 itself provides an important role in a molecular sensor mechanism at the level of DNA.

In conclusion, our study highlights a novel role for p53 in the activation of a p53-dependent S-phase replication checkpoint that involves CHK-1 and functions to protect the integrity of DNA. As we have previously shown that exposure of tumour cells to RITA leads to significant p53-dependent apoptosis in normoxia and hypoxia,[15] it will be of particular interest to further examine the precise molecular mechanisms underlying this p53-dependent S-phase checkpoint in hypoxia.

Materials and Methods

Cell culture. All tumour cell lines were maintained in Dulbecco modified Eagle's medium. Medium was supplemented with 10% fetal calf serum purchased from Harlan (Oxford, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (all purchased from Gibco/Life Technologies, Paisley, UK). The matched colorectal cell lines $p53^{-/-}$HCT116 and $p53^{+/+}$HCT116 have been described previously.[38] Human MCF-7 (breast carcinoma) and Saos-2 (osteosarcoma) cells were purchased from American Type Culture Collection (Manassas, VA, USA).

siRNA duplexes and transient transfection. The siRNA to CHK-1 (5′-GGTGCCTATGGAGAAGTT-3′) or CHK-2 (5′-CTTGAAGAGGTATCCGUGG-3′) was obtained as a gel-purified annealed duplex from Dharmacon (Lafayette, CO,

USA) and used at a final concentration of 25 nM, respectively. The non-silencing control siRNA duplex (5′-AATTCTCCGAACGTGTCACGT-3′) was obtained from QIAGEN (Crawley, UK) and has been used by us previously.[39] Transient transfections with siRNA duplexes were carried out using HiPerfect transfection reagent (QIAGEN) according to the manufacturer's instructions.

Antibodies. The HIF-α monoclonal antibody was purchased from BD Transduction Laboratories (Oxford, UK). The p53 monoclonal antibody (DO-1) was purchased from Calbiochem (Merck Biosciences, Nottingham, UK). The CHK1 monoclonal and CHK2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The p53 polyclonal antibody, polyclonal anti-phospho-S345-CHK1, polyclonal anti-phospho-T68-CHK2, monoclonal anti-phospho-S15-p53, polyclonal anti-phospho-S20-p53 and polyclonal anti-phospho-S46P-p53 were all purchased from Cell Signaling Technologies (Danvers, MA, USA). The anti-phospho-S139-γH2AX monoclonal antibody was purchased from Upstate (Millipore, Newtownabbey, Northern Ireland).

Inductions and drug treatments. Physiological hypoxia was achieved by incubating cells in 1% oxygen, 5% carbon dioxide and 94% nitrogen in a LEEC (Nottingham, UK) dual gas incubator (GA-156). The hypoxic mimetic agent, deferoxamine mesylate (DFX), was used at a final concentration of 500 μM. RITA was obtained from the National Cancer Centre, Drug Therapeutic Program, Frederick, MD, USA (NSC-652287) and dissolved in dimethyl sulfoxide. Wortmannin (Sigma, Gillingham, UK) was used at a final concentration of 10 μM. The caspase-3 inhibitor Z-DEVD-FMK (Calbiochem) was used at 50 μM.

Western blot analysis. After treatment, cells were washed in ice-cold phosphate-buffered saline and lysed in 2 $\times$ sample buffer (125 mM Tris (pH 6.8), 4% SDS, 0.01% bromophenol blue, 10% β-mercaptoethanol, 10% glycerol). Alternatively, cells were harvested in NP-40 lysis buffer (100 mM Tris (pH 8.0), 100 mM $NaCl_2$, 1% NP-40) containing an EDTA-free protease inhibitor cocktail (Boehringer Mannheim-Roche Diagnostics Ltd, Burgess Hill, UK) to determine total protein concentration using a standard protein assay (Biorad, Hemel Hempstead, UK).

Flow cytometric analysis. Cell death was analysed by FACS using a Beckman Coulter Diagnostics machine (High Wycombe, UK). Briefly, total populations of cells, including floating and adherent cells, were fixed in 70% ethanol and stained with propidium iodide (50 μg/ml). Ribonuclease was added at 100 μg/ml. The percentage of cells with sub-G1 DNA content was taken as a measurement of apoptosis.

Comet assay. The comet assay was performed using reagents from Trevigen (Gaithersburg, MD, USA) and according to the manufacturer's instructions. Cells were dosed for 24 h with RITA (at the concentrations indicated) in complete media. Following dosing, cells were harvested, mixed with low-melting agarose at 2×10^5 cells/ml ($\sim$1000 cells/slide) and spread onto preprepared comet slides. Cells were lysed and incubated in alkaline buffer to select for single-strand DNA breaks. Slides were then electrophoresed using alkaline electrophoresis buffer at 4 °C, 18 v and 300 mA for 40 min. Following electrophoresis, slides were fixed, dried and stained using Sybr green (Trevigen). Comets were viewed using a Zeiss (Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) fluorescent microscope at $\times$ 20 magnification, and images were captured over 20 fields of view for each slide using ImagePro software (Media Cybernetics Inc., Bethesda, MD, USA). The relative length and intensity of Sybr green-stained nuclei (comets) were proportional to DNA damage in individual nuclei. This was quantified using an algorithm for Olive tail moment on the CometScore software (TriTek Corporation, Sumerduck, VA, USA). At least 100 comets were analysed for each treatment. A 20-min dose of 100 μM hydrogen peroxide at 4 °C was used as a positive control for DNA damage.

DNA fibre assay. Replication tracks were labelled in culture medium containing 25 μM BrdU. RITA (500 nM) was added 16 h before each experiment. DNA fibre spreads were prepared, as previously described.[24] BrdU-labelled tracks were detected with BrdU anti-sheep antibody (Biodesign, Lewisville, TX, USA; M20105S; 1:1000 dilution; 1 h at 20 °C) using either Cy3- or AlexaFluor-488-conjugated donkey anti-sheep secondary antibody (Invitrogen Ltd, Paisley, UK). Fibres were examined using a Zeiss LSM 510 confocal microscope using a $\times$ 100 (1.4NA) lens, labelled tracks measured using the LSM software (Zeiss Ltd) (white bars on individual images show examples of measurements recorded) and converted to kbp using a conversion factor of 1 μm = 2.59 kbp. Measurements were recorded in randomly selected fields (selected at low power) from dispersed, untangled areas of the DNA spread. As the analysis of single, unbroken fibres is a key, routine quality control for spreading of different cell types under different experimental conditions was performed using direct DNA labelling with YOYO.[23] For the S-phase analysis, cells were grown on microscope coverslips and pulsed labelled for 20 min with 25 μM BrdU. RITA (500 nM) was added 16 h before each experiment. The cells were fixed using 4% PF, and BrdU detected as described above and previously.[25]

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Anna EO Fisher, Nadia Lovegrove and Keith W Caldecott (Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton, UK) for their initial input with comet assays. We thank David Gillespie (The Beatson Institute for Cancer Research, Glasgow, UK) for helpful discussions. We also thank Bert Vogelstein (Johns Hopkins School of Medicine, Baltimore, MD, USA) for the $p53^{-/-}$HCT116 and $p53^{+/+}$HCT116 cells.[38] Finally, we thank all other members of the Ashcroft lab for their input. AA and JY were funded by the Cancer Research UK studentships C7358/A8020 and C7358/A4420, respectively. AM-M and DAJ were funded by a BBSRC grant (reference BBS/B/06091).

1. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010; **40**: 294–309.
2. Poon E, Harris AL, Ashcroft M. Targeting the hypoxia-inducible factor (HIF) pathway in cancer. *Expert Rev Mol Med* 2009; **11**: e26.
3. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW *et al.* Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996; **379**: 88–91.
4. Stempien-Otero A, Karsan A, Cornejo CJ, Xiang H, Eunson T, Morrison RS *et al.* Mechanisms of hypoxia-induced endothelial cell death. Role of p53 in apoptosis. *J Biol Chem* 1999; **274**: 8039–8045.
5. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q *et al.* Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev* 2000; **14**: 34–44.
6. Kaluzova M, Kaluz S, Lerman MI, Stanbridge EJ. DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1alpha in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. *Mol Cell Biol* 2004; **24**: 5757–5766.
7. Salnikow K, Costa M, Figg WD, Blagosklonny MV. Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer. *Cancer Res* 2000; **60**: 5630–5634.
8. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Pharmacological rescue of mutant p53 conformation and function. *Science* 1999; **286**: 2507–2510.
9. Bykov VJ, Wiman KG. Novel cancer therapy by reactivation of the p53 apoptosis pathway. *Ann Med* 2003; **35**: 458–465.
10. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z *et al. In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; **303**: 844–848.
11. Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H *et al.* Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci USA* 2006; **103**: 1888–1893.
12. Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M *et al.* 2004 Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* **10**: 1321–1328.
13. Rivera MI, Stinson SF, Vistica DT, Jorden JL, Kenney S, Sausville EA. Selective toxicity of the tricyclic thiophene NSC 652287 in renal carcinoma cell lines: differential accumulation and metabolism. *Biochem Pharmacol* 1999; **57**: 1283–1295.
14. Nieves-Neira W, Rivera MI, Kohlhagen G, Hursey ML, Pourquier P, Sausville EA *et al.* DNA protein cross-links produced by NSC 652287, a novel thiophene derivative active against human renal cancer cells. *Mol Pharmacol* 1999; **56**: 478–484.
15. Yang J, Ahmed A, Poon E, Perusinghe N, de Haven Brandon A, Box G *et al.* Small-molecule activation of p53 blocks hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression *in vivo* and leads to tumor cell apoptosis in normoxia and hypoxia. *Mol Cell Biol* 2009; **29**: 2243–2253.
16. Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A, Selivanova G. MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer Cell* 2009; **15**: 171–183.
17. Yang J, Ahmed A, Ashcroft M. Activation of a unique p53-dependent DNA damage response. *Cell Cycle* 2009; **8**: 1630–1632.
18. Kao J, Milano MT, Javaheri A, Garofalo MC, Chmura SJ, Weichselbaum RR *et al.* gamma-H2AX as a therapeutic target for improving the efficacy of radiation therapy. *Curr Cancer Drug Targets* 2006; **6**: 197–205.

19. Ashcroft M, Kubbutat MH, Vousden KH. Regulation of p53 function and stability by phosphorylation. *Mol Cell Biol* 1999; **19**: 1751–1758.
20. Bradbury JM, Jackson SP. ATM and ATR. *Curr Biol* 2003; **13**: R468.
21. Chehab NH, Malikzay A, Stavridi ES, Halazonetis TD. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. *Proc Natl Acad Sci USA* 1999; **96**: 13777–13782.
22. Marti TM, Hefner E, Feeney L, Natale V, Cleaver JE. H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks. *Proc Natl Acad Sci USA* 2006; **103**: 9891–9896.
23. Merrick CJ, Jackson D, Diffley JF. Visualization of altered replication dynamics after DNA damage in human cells. *J Biol Chem* 2004; **279**: 20067–20075.
24. Jackson DA, Pombo A. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J Cell Biol* 1998; **140**: 1285–1295.
25. Maya-Mendoza A, Petermann E, Gillespie DA, Caldecott KW, Jackson DA. Chk1 regulates the density of active replication origins during the vertebrate S phase. *EMBO J* 2007; **26**: 2719–2731.
26. Niida H, Murata K, Shimada M, Ogawa K, Ohta K, Suzuki K *et al.* Cooperative functions of Chk1 and Chk2 reduce tumour susceptibility *in vivo*. *Embo J* 2010; **29**: 3558–3570.
27. Paulsen RD, Cimprich KA. The ATR pathway: fine-tuning the fork. *DNA Repair (Amst)* 2007; **6**: 953–966.
28. Robinson HM, Jones R, Walker M, Zachos G, Brown R, Cassidy J *et al.* Chk1-dependent slowing of S-phase progression protects DT40 B-lymphoma cells against killing by the nucleoside analogue 5-fluorouracil. *Oncogene* 2006; **25**: 5359–5369.
29. Petermann E, Maya-Mendoza A, Zachos G, Gillespie DA, Jackson DA, Caldecott KW. Chk1 requirement for high global rates of replication fork progression during normal vertebrate S phase. *Mol Cell Biol* 2006; **26**: 3319–3326.
30. Petermann E, Woodcock M, Helleday T. Chk1 promotes replication fork progression by controlling replication initiation. *Proc Natl Acad Sci USA* 2010; **107**: 16090–16095.
31. Collis SJ, Barber LJ, Clark AJ, Martin JS, Ward JD, Boulton SJ. HCLK2 is essential for the mammalian S-phase checkpoint and impacts on Chk1 stability. *Nat Cell Biol* 2007; **9**: 391–401.
32. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 2009; **29**: 625–634.
33. Shimura T, Toyoshima M, Adiga SK, Kunoh T, Nagai H, Shimizu N *et al.* Suppression of replication fork progression in low-dose-specific p53-dependent S-phase DNA damage checkpoint. *Oncogene* 2006; **25**: 5921–5932.
34. Agarwal ML, Agarwal A, Taylor WR, Chernova O, Sharma Y, Stark GR. A p53-dependent S-phase checkpoint helps to protect cells from DNA damage in response to starvation for pyrimidine nucleotides. *Proc Natl Acad Sci USA* 1998; **95**: 14775–14780.
35. Vaziri C, Saxena S, Jeon Y, Lee C, Murata K, Machida Y *et al.* A p53-dependent checkpoint pathway prevents rereplication. *Mol Cell* 2003; **11**: 997–1008.
36. Sorensen CS, Hansen LT, Dziegielewski J, Syljuasen RG, Lundin C, Bartek J *et al.* The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol* 2005; **7**: 195–201.
37. Sengupta S, Linke SP, Pedeux R, Yang Q, Farnsworth J, Garfield SH *et al.* BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. *EMBO J* 2003; **22**: 1210–1222.
38. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998; **282**: 1497–1501.
39. Carroll VA, Ashcroft M. Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. *Cancer Res* 2006; **66**: 6264–6270.

are already in clinical testing to interfere with TGF-β signaling, PD-1, Tregs, and IDO, and it is not difficult to envision combination studies to block two or more pathways in concert, in the near future. Finally, signal transduction inhibitors that block the activity of specific mutated kinases in melanoma, including B-Raf and c-kit, have shown high rates of clinical activity in patients bearing melanomas with the relevant mutations. Agents that target other pathways are also in clinical development. As many of the clinical responses to these agents appear to be of short duration, integrating an immunotherapeutic strategy in combination is logical to consider, perhaps to capitalize on the rapid tumor cell death that occurs that could deliver tumor-derived antigens to the host immune system.

It is indeed exciting to have a spectrum of active agents in clinical development for melanoma. A thoughtful path forward is likely to improve the outcome of patients with this disease, while catering therapeutic choices to specific biologic properties of the tumors in individual patients.

References

Camacho, L.H., Antonia, S., Sosman, J. et al. (2009). Phase I/II trial of tremelimumab in patients with metastatic melanoma. J. Clin. Oncol. *27*, 1075–1081.

Gajewski, T.F., Meng, Y., Blank, C. et al. (2006). Immune resistance orchestrated by the tumor microenvironment. Immunol. Rev. *213*, 131–145.

Harlin, H., Meng, Y., Peterson, A.C. et al. (2009). Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res. *69*, 3077–3085.

O'Day, S.J., Maio, M., Chiarion-Sileni, V. et al. (2010). Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Ann. Oncol. doi: 10.1093/annonc/mdq013.

TRP-ing off the p53 apoptotic switch

Afshan Ahmed and Margaret Ashcroft

e-mail: m.ashcroft@ucl.ac.uk

Sustained angiogenesis is a common hallmark of many tumours and contributes significantly to the invasion and metastatic potential of tumour cells. The hypoxia inducible factor (HIF) is a key transcription factor responsible for maintaining oxygen homeostasis and regulating angiogenesis (Bertout et al., 2008; Kaelin and Ratcliffe, 2008). HIF is deregulated across a broad range of cancers and is associated with increased mortality and treatment failure (Semenza, 2010). Previous studies have demonstrated that HIF-α, the regulatory subunit of HIF, is not only involved in cellular adaptation to oxidative stress, but also has an important function in regulating p53-dependent cell death responses (Carmeliet et al., 1998). Tumour cells that adapt to evade cell death and induce angiogenesis in response to changes in the tumour microenvironment are generally more aggressive and metastatic. However, precisely how HIF and p53 co-operate to maintain cellular integrity is unclear and many avenues of HIF-p53 regulation and interaction are yet to be explored.

A recent study by Sendoel and colleagues has utilized the nematode *Caenorhabditis elegans* to provide novel insights into mechanisms linking HIF and apoptosis (Sendoel et al., 2010). Both HIF and the apoptotic machinery are evolutionarily conserved and well characterised in *C. elegans*. Irradiation induced DNA damage and cell death in the germ line of *C. elegans* is regulated by Cep-1 (the *C. elegans* homologue of p53). Intriguingly, Sendoel and colleagues identify a mechanism by which Cep-1 is transcriptionally repressed by HIF-1 (Sendoel et al., 2010). Sendoel and colleagues begin their investigation by demonstrating that in wildtype *C. elegans*, DNA damage induced by ionizing radiation (IR) increases germ cell apoptosis. However, in *C. elegans* that overexpress HIF-1 due to *vhl-1* (von-Hippel Lindau) mutation [*vhl-1(ok161)*], apoptosis is significantly impaired. HIF-1 is subsequently shown to protect *C. elegans* against apoptosis by targeting Cep-1 specifically (Sendoel et al., 2010). In contrast to these findings, a previous report has shown that HIF-1α binds and stabilizes p53, thus promoting p53-mediated activation (An et al., 1998). Other studies have shown that p53 activation inhibits HIF-1α-mediated responses in tumour cells (Yang et al., 2009).

Sendoel and colleagues now present a novel mechanism for Cep-1 inhibition by HIF-1 that involves transcriptional upregulation of the tyrosinase family member tyrosinase-related protein 2 (TYR-2). Tyrosinases are specific enzymes involved in catalysing production of the pigment melanin in melanocytes. TYR-2 expression is induced by stabilised HIF-1α in *vhl* mutant *C. elegans* and this occurs specifically in ASJ neurons present in the head of *C. elegans*. TYR-2 is secreted by the ASJ neurons and is taken up by endocytosis to inhibit apoptosis specifically in germ cells, in a non-autonomous fashion. The dependency of this effect on TYR-2 was confirmed by Sendoel and colleagues using RNAi to knock down TYR-2 which restored sensitivity to IR-induced apoptosis in the germ cells of *vhl-1* mutant *C. elegans* (Sendoel et al., 2010). The fascinating model proposed by Sendoel and colleagues in which neurons are able to regulate HIF transcription and transmit a long range signal to protect distant tissues from DNA damage and stress is summarised in Figure 1A.

The anti-apoptotic functions of TYR-2 in *C. elegans* appear to translate to human cells: Sendoel and colleagues found that shRNA inhibition of human

Coverage on: Sendoel, A., Kohler, I., Fellmann, C., Lowe, S.W., and Hengartner, M.O. (2010). HIF-1 antagonizes p53-mediated apoptosis through a secreted neuronal tyrosinase. Nature *465*, 577–583.

doi: 10.1111/j.1755-148X.2010.00746.x

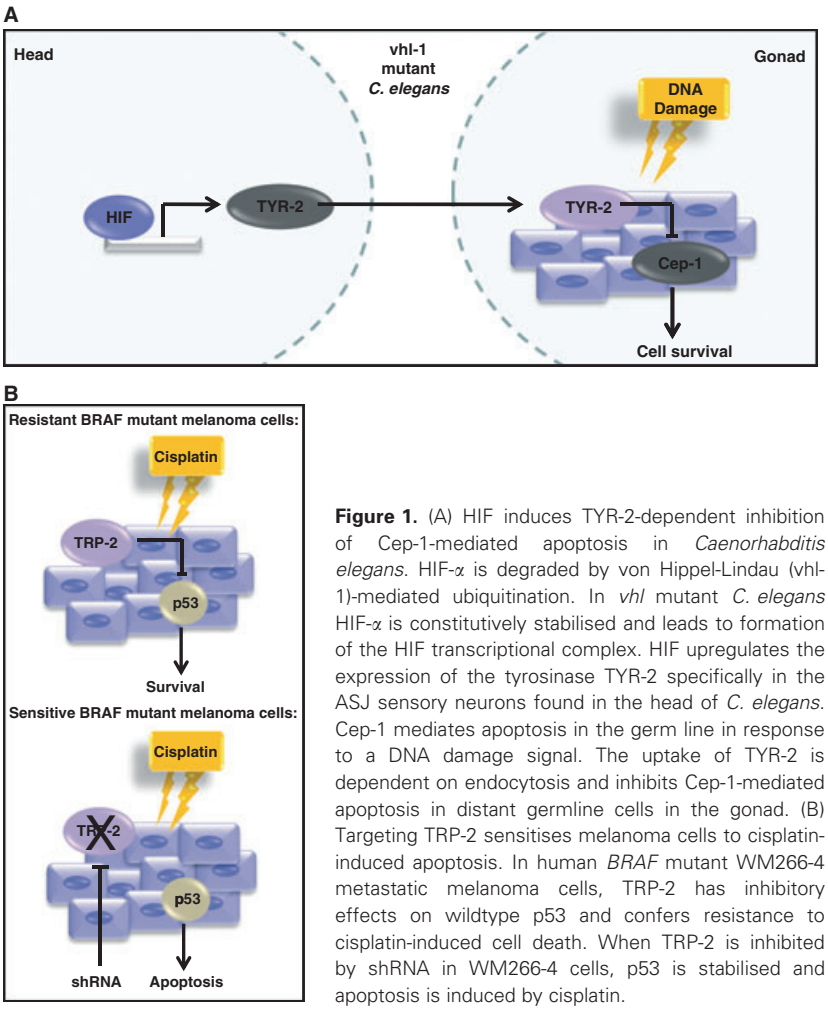


Figure 1. (A) HIF induces TYR-2-dependent inhibition of Cep-1-mediated apoptosis in *Caenorhabditis elegans*. HIF-α is degraded by von Hippel-Lindau (vhl-1)-mediated ubiquitination. In *vhl* mutant *C. elegans* HIF-α is constitutively stabilised and leads to formation of the HIF transcriptional complex. HIF upregulates the expression of the tyrosinase TYR-2 specifically in the ASJ sensory neurons found in the head of *C. elegans*. Cep-1 mediates apoptosis in the germ line in response to a DNA damage signal. The uptake of TYR-2 is dependent on endocytosis and inhibits Cep-1-mediated apoptosis in distant germline cells in the gonad. (B) Targeting TRP-2 sensitises melanoma cells to cisplatin-induced apoptosis. In human *BRAF* mutant WM266-4 metastatic melanoma cells, TRP-2 has inhibitory effects on wildtype p53 and confers resistance to cisplatin-induced cell death. When TRP-2 is inhibited by shRNA in WM266-4 cells, p53 is stabilised and apoptosis is induced by cisplatin.

tyrosinase-related protein 2 (TRP-2), the homologue of TYR-2 in the WM266-4 metastatic melanoma cell line sensitises these cells to cisplatin-induced apoptosis (Sendoel et al., 2010) (Figure 1B). As an enzyme that functions in synthesising melanin, TRP-2-mediated inhibition of p53 allows for an effective cell survival mechanism in melanocytes that are often exposed to damage induced by UV radiation. Increased expression of TRP-2 has been shown to correlate with decreased apoptosis in several human melanoma cell lines, and hence confers resistance to radiation therapy and chemotherapy (Pak et al., 2004). Interestingly, despite this, the frequency of p53 mutations in metastatic melanoma is only present at 25%, much lower compared to other cancers. Instead, deregulated p53 signalling often co-operates with other pathways, such as the Rb pathway and BRAF signalling to contribute to melanocyte transformation.

Indeed, Sendoel and colleagues propose that TRP-2 may act downstream of *BRAF* through its transcriptional regulation by microphthalmia-associated transcription factor (MITF) to affect tumour progression. Activating mutations in *BRAF* occur with a frequency of 50–70% in melanoma. Therefore targeting TRP-2 may offer an attractive strategy to the treatment of aggressive melanomas that have transcriptionally repressed wildtype p53. Increased expression of TRP-2 has also been observed in other cancer lines. Thus it would be of particular interest to extend the study by Sendoel et al. to other aggressive cancers such as renal cell carcinoma where HIF signalling is deregulated by loss of VHL tumour suppressor function.

One obvious drawback of the study by Sendoel and colleagues is that it concentrates on phenotypes where HIF-1 is overexpressed in the context of *vhl-1* mutation. Therefore, the contribution that HIF-1 specifically makes to the anti-apoptotic effect of TRP-2 in response to hypoxia remains to be clarified. Despite this, the novelty of Sendoel et al.'s study lies in the characterisation of TYR-2 in *C. elegans* as an important pro-survival signalling molecule that has not been previously implicated in regulating apoptosis in response to deregulated HIF-1. Furthermore, the non-autonomous regulation of apoptosis by this mechanism may potentially provide another level of complexity within the tumour microenvironment whereby HIF is able to transcriptionally regulate p53-mediated apoptosis in other, distant regions of the tumour. Finally, the study by Sendoel and colleagues highlights a mechanism by which HIF co-operates with p53 to mediate resistance in highly metastatic and aggressive cancers such as melanoma and provides important information for designing novel targeted therapies.

References

An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., and Neckers, L.M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature *392*, 405–408.

Bertout, J.A., Patel, S.A., and Simon, M.C. (2008). The impact of O_2 availability on human cancer. Nat. Rev. Cancer *8*, 967–975.

Carmeliet, P., Dor, Y., Herbert, J.M. et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485–490.

Kaelin, W.G. Jr, and Ratcliffe, P.J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol. Cell *30*, 393–402.

Pak, B.J., Lee, J., Thai, B.L., Fuchs, S.Y., Shaked, Y., Ronai, Z., Kerbel, R.S., and Ben-David, Y. (2004). Radiation resistance of human melanoma analysed by retroviral insertional mutagenesis reveals a possible role for dopachrome tautomerase. Oncogene *23*, 30–38.

Semenza, G.L. (2010). Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene *29*, 625–634.

Yang, J., Ahmed, A., Poon, E. et al. (2009). Small-molecule activation of p53 blocks hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression *in vivo* and leads to tumor cell apoptosis in normoxia and hypoxia. Mol. Cell. Biol. *29*, 2243–2253.