The role of p53 in hypoxia-induced apoptosis

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List of abbreviations

ADR: Adriamycin

APAF1: Apoptotic peptidase activating factor 1

APH: Aphidicolin

ASPP: Apoptosis-stimulating protein of p53

ATM: Ataxia-telangiectasia mutated

ATR: ATM and Rad3-related

ATRIP: ATR interacting protein

Bak: BCL2-antagonist/killer

Bax: BCL2-associated X protein

BCL-2: B-cell lymphoma-2

BH3: Bcl2 homology domain 3

BRCA: Breast cancer associated

BTG2: B-cell translocation gene 2

CBP: CREB binding protein

Cdk: Cyclin dependent kinase

ChIP: Chromatin immunoprecipitation

Chk1: Checkpoint kinase 1

Chk2: Checkpoint kinase 2

$CoCl_2$: Cobalt chloride

DBD: DNA binding domain

DFO: Desferrioxamine

DNA-PKcs: DNA-dependent protein kinase catalytic subunit

GADD45: Growth arrest and DNA-damage-inducible 45

HDAC: Histone deacetylase

HIF: Hypoxia-inducible factor

HIPK2: Homeodomain interacting protein kinase

HR: Homologous recombination

HU: Hydroxyurea

IgG: Immunoglobulin G

INPP5D: Inositol polyphosphate-5-phosphatase

IP: Immunoprecipitation

Kap-1: Krüppel-associated box (KRAB) domain-associated protein 1

MCM: Mini-chromosome maintenance deficient

MDM2: Mouse double minute 2

MDMX: Mouse double minute X

miRNA: microRNA

MLH1: mutL homolog 1

MOMP: Mitochondrial outer membrane potential

NHEJ: Non-homologous end joining

NOXA: Phorbol-12-myristate-13-acetate-induced protein 1 (also known as PMAIP1)

p53AIP1: p53-regulated apoptosis inducing protein-1

PAGE: Polyacrylamide gel electrophoresis

PCAF: p300/CBP associated factor

PH: Plekstrin homology

PHLDA3: Pleckstrin homology-like domain, family A, member 3

PIG3: p53-induced gene-3

Pin-1: Peptidylprolyl cis/trans isomerase, NIMA-interacting 1

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PIP_3: Phosphatidylinositol 3,4,5-trisphosphate

PTEN: Phosphatase and tensin homologue

PUMA: Bcl2 binding component 3 (also called BBC3)

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction

ROS: Reactive oxygen species

RPA: Replication protein A

rRNA: Ribosomal RNA

SDS: Sodium dodecyl sulphate

SH3: Src homology 3

SIRT1: Sirtuin-1

SNP: Single nucleotide polymorphism

ssDNA: Single stranded DNA

SULF2: Sulfatase 2

TAD: Transactivation domain

TSA: Trichostatin A

UTR: Untranslated region

Wip1: Wild-type p53-induced phosphatase 1

XIAP: X-linked inhibitor of apoptosis

WT: Wild type

Abstract

Tumour hypoxia is associated with resistance to therapy, increased aggressiveness and a poor patient prognosis. The tumour suppressor p53 causes apoptosis in response to hypoxia and therefore hypoxic tumours select for the loss of p53.

In this work was we have investigated the post-translational modifications and binding partners of p53 in hypoxia. Furthermore, we investigated the p53-dependent gene expression changes in hypoxia.

We have shown that p53 is stabilised and phosphorylated in hypoxia, and that there are some differences between p53 modifications in hypoxia and those in response to DNA damage. We have also shown that N-terminal phosphorylations of p53 at serines 20 and 46 are important, but not essential for induction of apoptosis in hypoxia. Strikingly we observed that DNA binding of p53 is essential for hypoxia-induced apoptosis. We have seen, for the first time, that p53 induces a subset of its targets in hypoxia and that these proteins do have previously reported roles in apoptosis. In addition, it is possible that p53 can induce miRNA expression in hypoxia. Moreover, preliminary work suggests that p53 is part of a complex in hypoxia. Elucidating the proteins that make up this complex could prove important for understanding the mechanism of p53-induced apoptosis in hypoxia.

1. Introduction

The p53 protein was discovered through its association with the simian virus (SV40) large T-antigen in 1979 (Lane and Crawford, 1979, Linzer and Levine, 1979, Melero et al., 1979) and has since attracted much attention due to its important and varied roles. p53 has been found to play a major role in the response to cellular stress including DNA damage, hypoxia, hyperthermia and oncogene activation (Shieh et al., 1997, Graeber et al., 1996, Guan et al., 2002, Sherr and Weber, 2000, Graeber et al., 1994). Following its activation, p53 brings about one of a range of cellular responses appropriate for tissue type and severity of damage. These responses include cell cycle arrest, senescence, autophagy or apoptosis and prevent the replication of damaged cells that could lead to tumour formation (Yee and Vousden, 2005, Riley et al., 2008, Green and Kroemer, 2009). p53 has been called "the guardian of the genome" (Lane, 1992) and the "cellular gatekeeper" (Levine, 1997) due to its central role in coordinating the cellular response to stress. It is considered to be perhaps the most important tumour suppressor protein and is mutated in over 50% of human cancers (Levine, 1997).

1.1 Domain structure

p53 brings about many of its downstream responses through its role as a transcriptional transactivator. It binds DNA as a tetramer (Friedman et al., 1993) and is split into four functional domains (Joerger and Fersht, 2008) (Figure 1). The N-terminal domain consists of a transactivation domain (TAD) and a proline rich region. The TAD is further divided into two subdomains, TAD1 (amino acids 1-40) (Chang et al., 1995) and TAD2 (amino acids 41-61) (Walker and Levine, 1996) and provides the binding site for a number of proteins. These include components of the transcriptional machinery, transcriptional coactivators and negative regulators (Teufel et al., 2007, Popowicz et al., 2008). The transactivation domain is

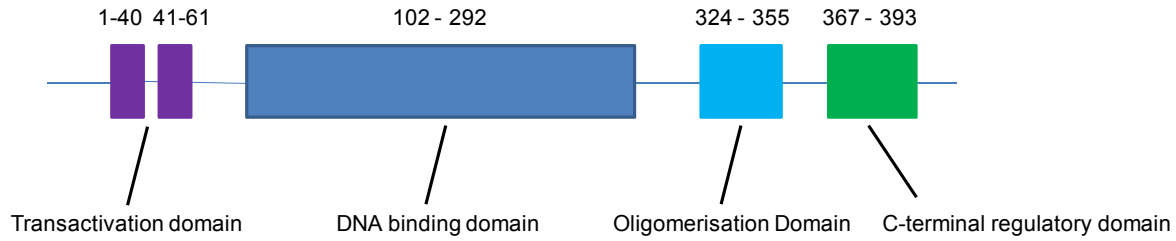


Figure 1: The domain structure of p53

p53 can be split into four domains. The N-terminal transactivation domains consist of two transactivation subdomains and a proline rich region. The central domain is responsible for sequence specific DNA binding. The C-terminal domain is split into an oligomerisation domain and a C-terminal regulatory domain.

required for transcriptional activation of genes by p53. p53 mutated at residues 22 and 23 (Q22, S23) is impaired for transactivation and induction of apoptosis (Roemer and Mueller-Lantzsch, 1996). Additional mutations of p53 at residues 53 and 54 (Q22, S23, Q53, Q54), which affect TAD2, results in a protein that is more completely impaired in its ability to transactivate and is also incapable of transcriptional repression (Venot et al., 1999). The histone acetyltransferase paralogues p300 and CREB binding protein (CBP) bind to the N-terminal transactivation domain of p53 (Teufel et al., 2007). p53-dependent recruitment of p300/CBP to promoters facilitates local chromatin unwinding brought about through histone modifications (Barlev et al., 2001, Liu et al., 2003).

The proline rich domain contains five repeats of the PXXP motif where P represents proline and X is any amino acid. This motif plays a role in signal transduction due to its Src homology 3 (SH3) domain-binding activity (Walker and Levine, 1996). The proline rich region is dispensable for transactivation; deletion of all five PXXP motifs leaves a p53 protein with significant structural alterations but ability to drive a number of p53 reporter genes with activity greater than or equal to the wild type protein (Walker and Levine, 1996). However, p53 protein lacking the proline rich domain (Δpro p53) is impaired in its ability to suppress growth in response to DNA damage, attributed largely to a defect in induction of apoptosis (Venot et al., 1998). Δpro p53 is unable to transactivate a subset of genes, including the pro-apoptotic p53-induced gene 3 (PIG3) and is also impaired in its ability to repress genes (Venot et al., 1998).

The central DNA binding domain (DBD) is responsible for sequence specific DNA binding and allows binding to the major and minor groove of DNA (Bargonetti et al., 1993, Cho et al., 1994). The C-terminal domain of p53 is split into the C-terminal regulatory domain and the oligomerisation domain (Joerger and Fersht, 2008).

1.2 p53 regulation

p53 is tightly regulated by a number of different processes to ensure that its response is appropriate for the stress and tissue type. There are multiple levels of p53 regulation which can broadly be split into two groups: regulation of p53 levels and regulation of the downstream response. In normal, unstressed cells p53 is present at low levels. Upon DNA damage and other cellular stresses protein levels rapidly increase. This occurs because p53 is usually degraded by the proteasome following ubiquitylation by the E3 ubiquitin ligase Mouse double minute 2 (MDM2). p53 is stabilised by phosphorylation at the N-terminus, preventing the binding of MDM2 (Shieh et al., 1997). In response to DNA damage the ataxia telangiectasia mutated (ATM) kinase is active and phosphorylates a number of substrates, including p53. Immunoprecipitated ATM phosphorylates p53 at serine 15 *in vitro* (Khanna et al., 1998) and serine 15 phosphorylation following DNA damage is reduced in Ataxia telangiectasia (A-T) cells (Nakagawa et al., 1999). ATM is a member of the PI-3K (phosphatidyl inositol-3- kinase) like kinases (PIKK) family along with Rad3-related kinase (ATR) and the two proteins have similar functions and overlapping substrates. However ATM and ATR do differ in the stimuli that they are induced by; ATM primarily responds to double strand breaks and is active following γ-irradiation (Canman et al., 1998), while ATR is induced by replication stresses such as UV, hydroxyurea and hypoxia (Heffernan et al., 2002, Hammond et al., 2003a). The situation is more complex however as many stresses that cause a replication arrest also lead to DNA damage and therefore activate the ATM pathway. There is evidence that ATR, in response to UV treatment, is able to phosphorylate and activate ATM (Stiff et al., 2006). An additional mechanism for p53 stabilisation occurs following oncogene activation. p14ARF induced by oncogene activation can inhibit the E3 ligase activity of MDM2 and sequester it in the nucleoli (Sherr and Weber, 2000).

p53 is post-translationally modified on a number of residues. Phosphorylation of p53 on serines 15 and 20 occurs rapidly following DNA damage. Serine 20 is in the MDM2 binding site (Kussie et al., 1996); phosphorylation here reduces the binding of p53 to MDM2 and is thought to be necessary for p53 stabilisation, particularly in response to DNA damage (Chehab et al., 1999). Mutation of serine 20 to alanine makes p53 very sensitive to turnover by MDM2 (Dumaz et al., 2001). Phosphorylation of serines 15 and 37 may also contribute to p53 stabilisation due to their proximity to the MDM2 binding site and the observation that phosphorylation here stops MDM2 binding p53 (Kapoor et al., 2000). It is also possible for p53 stabilisation to occur in the absence of N-terminal phosphorylations. This is the case in response to heat shock, oncogene activation and actinomycin D treatment, where p53 stabilisation is seen in the absence of serine 15 phosphorylation (Ashcroft et al., 2000, Sherr and Weber, 2000). Phosphorylation-independent mechanisms to stabilise p53 can occur through inhibition of *MDM2* transcription or through p53 and MDM2 occupying different areas of the nucleus, as in the case of actinomycin D treatment (Ashcroft et al., 2000).

As well as DNA-damage induced p53 modifications, the DNA damage response kinases can also modify p53 regulators, including MDM2 as a means of increasing p53 stability. ATM-dependent phosphorylation of MDM2 on a number of residues occurs in response to DNA damage. Phosphorylation occurs on serines 386 and 429 following DNA damage and this inhibits the poly-ubiquitination of p53 by preventing MDM2 RING finger oligomerisation (Cheng et al., 2009). Furthermore, it has been shown that ATM mediates phosphorylation of MDM2 between residues 154 and 167 in response to X-rays and that this has a role in p53 stabilisation (de Toledo et al., 2000). ATM-dependent phosphorylation of MDM2 at serine 395 is thought to stabilise p53 by reducing the ability of MDM2 to shuttle it to the cytoplasm (Maya et al., 2001). ATM is not the only stress-activated kinase that has been shown to play a role in MDM2 regulation. ATR can phosphorylate MDM2 on serine 407 following

camptothecin or hydroxyurea treatment and this promotes nuclear localisation of p53 possibly as a consequence of defective shuttling of p53 into the cytoplasm (Shinozaki et al., 2003).

There are a number of other proteins involved in the stabilisation of p53. The protein MDMX, is, along with MDM2 one of the main regulators of p53 stability. MDMX is an MDM2 homologue that inhibits p53 function by hindering its transactivation capacity. In addition it appears to alter the substrate preference of MDM2, promoting degradation of p53 (Okamoto et al., 2009). MDMX can also bind to MDM2 and increase its stability by preventing autoubiquitination of MDM2. Deletion of either MDM2 or MDMX in mice is embryonic lethal, and this can be rescued by deletion of p53, showing the vital role that these proteins play in regulating p53 activity (Parant et al., 2001). In addition, both of these proteins are oncogenes and are overexpressed in a number of human tumours (Riemenschneider et al., 1999, Danovi et al., 2004). Studies have shown that controlling MDMX levels is crucial for p53 stabilisation following DNA damage. Phosphorylation of MDMX on a number of serine residues seems to be important for regulation of its stability (Pereg et al., 2005, Chen et al., 2005). The phosphorylations are ATM and Chk2 dependent and so we have a situation whereby ATM signalling increases p53 stability in response to DNA damage through MDM2 and MDMX. Furthermore, Chk1-mediated phosphorylation of serine 367 on MDMX occurs in response to UV and mediates its interaction with 14-3-3γ. This suppresses MDMX-activated ubiquitination of p53 (Jin et al., 2006). The stress activated kinase c-Abl is also able to phosphorylate MDMX and disrupt its interaction with p53 (Zuckerman et al., 2009). C-Abl has also been shown to phosphorylate MDM2, and this is necessary for its interaction with and ubiquitination of MDMX (Waning et al., 2010). MDM2 and MDMX both bind the N-terminus of p53 and prevent its transactivation ability.

Both proteins have been shown to prevent ASPP1 and ASPP2 from stimulating the apoptotic functions of p53 by inhibiting its transcriptional activity (Bergamaschi et al., 2005).

It is clear that regulation of p53 stability is a very complex process involving a number of proteins and can be fine-tuned depending upon the type of stress encountered by the cell. p53 is able to activate transcription of its negative regulator *MDM2*, and this acts as a negative feedback loop to control p53 accumulation. However, the discovery that stress activated kinases also modify MDM2 and MDMX provides a means of extending p53 stabilisation despite the increase in MDM2 protein. Nonetheless, it is necessary for p53 degradation to take place once the cellular stress is no longer present. The wild-type p53-induced phosphatase 1 (Wip1) plays a role in curbing the p53 response by dephosphorylating both MDM2 and MDMX resulting in their stabilisation and increased affinity for p53 (Lu et al., 2007). Wip1 phosphatase can also dephosphorylate p53, at serine 15, as well as ATM kinase (Shreeram et al., 2006), Chk1 (Lu et al., 2005) and Chk2 (Oliva-Trastoy et al., 2007) and so acts to reverse the p53 response.

The post-translational modifications of p53 mediate the downstream responses it brings about. p53 can be modified in a number of ways including phosphorylation, acetylation, NEDDylation, SUMOylation and ubiquitination (Xirodimas et al., 2004, Kwek et al., 2001, Chuikov et al., 2004, Hollstein and Hainaut, 2010). Modifications of p53 are thought to influence its ability to bind to other transcription factors, its ability to bind promoters and the recruitment of histone modifying enzymes. Of particular importance for p53 function are phosphorylation and acetylation of p53. Phosphorylation at serines 15 and 20 is important in mediating the p53 apoptotic response (Unger et al., 1999). Mutation of these residues does not affect expression, DNA-binding or transactivation capacity of p53 but it is impaired in its ability to mediate apoptosis, possibly due to a defect in stabilisation (Unger et al., 1999). Serine 46 is phosphorylated in response to γ-irradiation and UV and p53 mutated at this

residue is impaired in its ability to cause apoptosis (Oda et al., 2000b, D'Orazi et al., 2002). Phospho-serine 46 appears to be essential for p53 binding to the promoter of pro-apoptotic target gene p53-regulated apoptosis inducing protein-1 p53AIP1 (Oda et al., 2000b) and there are also data suggesting that it is involved in the transcription of PUMA (Bcl2 binding component 3) (Li et al., 2005). DNA damage induces phosphorylation of p53 at ser/thr-pro motifs which include serines 33, 46 and 315 and threonine 81. These phosphorylations enable the binding of prolyl isomerase Pin-1 to p53 which in turn leads to a conformational change which increases the transactivation capacity of p53 (Zheng et al., 2002, Zacchi et al., 2002). Pin-1 deficient cells are defective in p53 activation and have impaired checkpoint activation following DNA damage (Zheng et al., 2002).

p53 is acetylated at various C-terminal residues, and this alters its capacity to transactivate genes. N-terminal phosphorylation of p53 at serine 15 increases its association with acetyltransferases p300/CBP and also prevents binding of MDM2, and so p53 is able to 'switch' into an active state upon cellular stress (Lambert et al., 1998, Dumaz and Meek, 1999). However, p53 acetylation is seen in response to most stresses, including those which do not induce N-terminal phosphorylation (Ashcroft et al., 2000, Ito et al., 2001). Acetylation of p53 has been shown to allow recruitment of coactivators and acetylation of histones (Barlev et al., 2001). The co-activator p300/CBP acetylates p53 on lysines 373 and 380 *in vivo* and this acetylation is suppressed by MDM2 (Ito et al., 2001). p300/CBP associated factor (PCAF) acetylates p53 on lysine 320 (Liu et al., 1999). Acetylation of p53 has been shown to abrogate MDM2-mediated repression by blocking the recruitment of MDM2 to p53-responsive promoters (Tang et al., 2008). Loss of acetylation abolishes p53-dependent growth arrest and apoptosis (Tang et al., 2008) and p53 acetylation at lysine residues 320, 373 and 382 is required for the transcription-independent apoptotic functions of p53 including reactive oxygen species (ROS) generation (Yamaguchi et al., 2009). The

importance of p53 acetylation by p300/CBP is highlighted by the findings that p300 mutations are found in a number of tumour types (Goodman and Smolik, 2000). It has remained uncertain whether post-translational modifications of p53 are essential for its function, or simply act to fine-tune the p53 response. Recent data has shown that unacetylated p53 is still able to induce *MDM2*, but cannot induce cell cycle arrest or apoptosis (Tang et al., 2008). p53 simultaneously mutated at all acetylation sites abolishes activation of the p21 promoter. Acetylation of p53 blocks the recruitment of MDM2 and MDMX to the p21 promoter, and the defects of unacetylated p53 can be rescued by removing MDM2 and MDMX from the cell (Tang et al., 2008).

1.3 Classical role of p53 in apoptosis

One role of p53 that has been well characterised is the apoptotic response. The intrinsic apoptosis pathway involves mitochondrial outer membrane permeabilisation (MOMP) leading to the release of pro-apoptotic factors and caspase cleavage (Tait and Green, 2010). p53 brings about apoptosis in part by activating transcription of a variety of pro-apoptotic B-cell lymphoma-2 (BCL-2) family members (Miyashita and Reed, 1995, Sax et al., 2002, Nakano and Vousden, 2001, Oda et al., 2000a). The BCL-2 family is divided into anti-apoptotic, pro-apoptotic and BH3 only proteins. Anti-apoptotic BCL-2 family members, including BCL-2 and BCL-XL act to inhibit pro-apoptotic family members such as Bax and Bak. Upon stimulation, Bax and Bak are activated and form a channel in the mitochondrial outer membrane leading to MOMP. The BH3 only proteins which include PUMA and NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) are also pro-apoptotic. They bind and inhibit anti-apoptotic BCL-2 family members and may also cause direct activation of Bax and Bak.

Another pro-apoptotic p53 target gene is p53AIP1. Phosphorylation of p53 at serine 46 is essential for induction of p53AIP1 which causes apoptosis by dissipating mitochondrial membrane potential (Oda et al., 2000b).

Transactivation deficient p53 has also been shown to cause apoptosis (Haupt et al., 1995). Indeed generation of knock-in mice expressing a chimeric p53 capable of transactivation but mutant in regions required for other functions has shown that roles of p53 other than transactivation are required for induction of apoptosis (Johnson et al., 2008). Some apoptosis-inducing stresses cause localisation of p53 at the mitochondria where it induces MOMP (Mihara et al., 2003). This leads to release of pro-apoptotic cytochrome C which binds apoptosis peptidase activating factor 1 (APAF1) causing its conformational change and oligomerisation to form the apoptosome. The apoptosome then recruits and activates procaspase 9 which in turn cleaves pro-caspases 3 and 7 leading to apoptosis. Furthermore, release of SMAC and OMI from the mitochondria blocks X-linked inhibitor of apoptosis (XIAP) mediated inhibition of caspase activity.

1.4 Hypoxia and the normal cellular response

Hypoxia is a broad term encompassing a wide range of oxygen concentrations and is a normal feature in developing embryos as well as being present in certain disease states and during wound healing. Tumours rapidly outgrow their blood supply, and new blood vessels formed in the tumour are leaky and inefficient. Areas of acute, perfusion limited hypoxia arise due to blockages in the tumour vasculature. In some cases the vessels reopen leading to reoxygenation-induced DNA damage. Furthermore, tumours contain areas of chronic, diffusion limited hypoxia in those areas furthest away from blood vessels. Hypoxia can be

measured using an oxygen electrode and in tumours oxygen concentrations can range between anoxia (0%) and 8% oxygen (Brown, 2007).

Hypoxia is a unique stress that activates components of the DNA damage response in the absence of detectable DNA damage (Bencokova et al., 2009) (Figure 2). In hypoxia, cells arrest in S-phase resulting in the accumulation of regions of single stranded DNA (ssDNA) (Pires et al., 2010). To further establish hypoxia as a unique physiological stress, the DDR is altered when compared to the classical response triggered by either double strand breaks or replication-induced stress. In hypoxia, as in DNA damage situations, autophosphorylation of ATM occurs at serine 1981 and the kinase is active. However, unlike the situation following double strand breaks, it is not chromatin bound (Bencokova et al., 2009). ATM phosphorylates a number of its downstream targets in hypoxia, including Krüppel-associated box (KRAB) domain-associated protein 1 (Kap-1) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Bencokova et al., 2009), though it is not required for H2AX phosphorylation (γH2AX) or focus formation in hypoxia. Furthermore, 53BP1 foci do not form and while γH2AX is present in hypoxic cells, it occurs in the absence of DNA damage and the staining pattern is atypical (Bencokova et al., 2009). ATR is known to mediate the response to stalled replication, and indeed in hypoxia it is known to phosphorylate p53, Chk1 and γ-H2AX (Hammond et al., 2004, Hammond et al., 2003a, Hammond et al., 2002). Studies have shown that ATR is the dominant kinase in hypoxia, while ATM is required to maintain these phosphorylations following reoxygenation, when ROS-dependent DNA damage occurs (Hammond et al., 2003a). S-phase cells are the most sensitive to hypoxia/reoxygenation as they accumulate more damage than cells in other phases of the cell cycle, leading to higher levels of apoptosis. Knockdown of ATR, as well as its target Chk1 further sensitises these cells to hypoxia/reoxygenation suggesting that Chk1 and ATR have a role in stabilising stalled replication forks (Hammond et al., 2004).

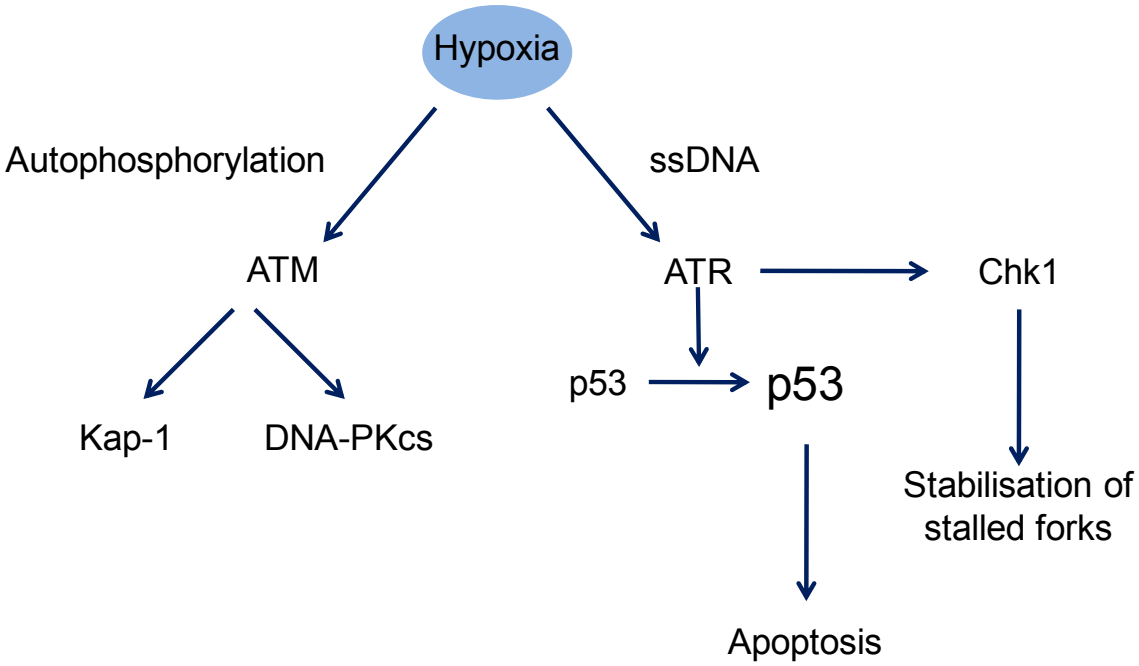


Figure 2: The DNA-damage response in hypoxia

The DNA damage response is active in hypoxia in the absence of detectable DNA damage. ATM and ATR are active and phosphorylate downstream targets including p53, Chk1, Kap-1 and DNA-PKcs.

Tumour hypoxia is an independent prognostic factor and can predict poor patient survival in a variety of cancers including head and neck and cervix (Hockel et al., 1996, Nordsmark et al., 1996, Brizel et al., 1996). Moreover hypoxic tumours are more aggressive and resistant to therapy (Teicher, 1994). This is in part due to the difficulty in delivering chemotherapeutic drugs and to the reduction of radiation-induced damage in low oxygen conditions. Additionally, hypoxic cells undergo S-phase arrest and many chemotherapeutic drugs are designed to target rapidly proliferating cells. Some chemotherapeutic drugs are not taken into the cell effectively in hypoxia due to the low pH conditions outside the cell. p53 induces apoptosis in severely hypoxic cells and therefore hypoxia can act as a selective pressure for the loss of p53 (Graeber et al., 1996).

Hypoxia has been shown to contribute to genomic instability through the downregulation of a number of DNA-repair genes. Hypoxic cells have a diminished repair capacity and a higher rate of mutagenesis (Yuan et al., 2000). Functional suppression of homologous recombination (HR) and mismatch repair has been shown in hypoxia, due to the downregulation of genes in these pathways, including RAD51, RAD52, BRCA1, BRCA2 and Mlh1. Despite the repression of certain non-homologous end joining (NHEJ) genes in hypoxia, no functional deficiency in this pathway has been demonstrated (Bindra et al., 2005) leading to the hypothesis that hypoxia may force cells to repair DNA damage by the more error prone NHEJ pathway, contributing to genetic instability. Repression of RAD51 and BRCA1 is due to repressive E2F family members at the promoters of these genes (Bindra et al., 2005, Bindra and Glazer, 2007). It has been suggested that the downregulation of HR sensitises cells to hypoxia and that this may provide a mechanism to offset hypoxia-induced radio- and chemo-resistance (Chan et al., 2008). Recently it has been shown that two hypoxia-induced microRNAs, miR-210 and miR-373 may play a role in the downregulation

of RAD52 and the nucleotide excision repair protein RAD23B providing another mechanism for hypoxia-induced suppression of DNA repair (Crosby et al., 2009).

1.5 p53 stabilisation in hypoxia

Hypoxia, along with hyperthermia is an example of a non-genotoxic stress that causes p53 stabilisation. p53 is also induced in response to hydroxyurea- and aphidicolin-induced S-phase arrest, though here the situation is slightly different due to the DNA damage that they cause. While p53 is required for a G_1 and G_2 arrest following gamma irradiation, replication blocks such as hypoxia and hydroxyurea treatment cause a p53-independent S-phase arrest (Gottifredi et al., 2001, Pires et al., 2010). In response to hydroxyurea- and hypoxia-induced replication block, p53 accumulates in the nucleus in an ATM-independent manner and is phosphorylated at various residues including serines 15, 20, and 37 (Hammond et al., 2003b, Gottifredi et al., 2001). However, despite high levels of nuclear p53, many of its targets including p21, GADD45 and MDM2 are not induced, or are induced more weakly than with γ-irradiation. In response to hydroxyurea treatment, levels of c-fos and cyclinE mRNA were increased which rules out global inhibition of RNA synthesis (Gottifredi et al., 2001). The same is true in hypoxia where, for example, transcription of many Hypoxia-inducible factor (HIF) target genes is increased. Studies on the p21 gene after hydroxyurea treatment suggest that the defect in transcription is due to impaired elongation and reduced occupancy of RNA polymerase II downstream of the start site (Mattia et al., 2007). p53 binding to the promoter, recruitment of TATA-binding protein (TBP), TFIID and RNA polymerase II were all unchanged, as was histone acetylation (Mattia et al., 2007). Exposure to γ-irradiation following hydroxyurea treatment fails to restore p53 transcriptional activity suggesting that in response to hydroxyurea p53 is actively repressed rather than simply lacking a specific

cofactor or modification needed for transactivation. Strikingly, despite this, PIG3 is strongly induced in a p53-dependent following hydroxyurea treamtment (Gottifredi et al., 2001). Thus is appears that a blockage in DNA synthesis actively represses p53 activity in a selective manner, allowing some genes to be expressed but preventing the transcription of others. In contrast, treatment of hypoxic cells with the DNA damaging agent adriamycin does lead to induction of known pro-apoptotic target genes and allows binding of p300/CBP to p53 responsive promoters (Koumenis et al., 2001).

Hypoxia is known to inhibit certain enzymes in the deoxyribonucleotide biosynthetic pathway. Ribonucleotide reductase castalyses the conversion of ribonucleotides to deoxyribonucleotides and is dependent upon molecular oxygen to regenerate a free radical at its active site (Probst et al., 1989, Reichard and Ehrenberg, 1983). Another enzyme, dihydroorotate dehydrogenase, catalyses a step in the formation of UMP and is coupled with the respiratory chain, rendering it oxygen dependent (Loffler et al., 1997). It has been suggested that hypoxia-induced S-phase arrest could be due to a shortage of deoxyribonucleotides preventing DNA replication. Nucleotide levels in cell culture fall in severe hypoxia (below 0.1% oxygen) concomitant with an S-phase arrest, while in mild hypoxia where no arrest is seen, there is no nucleotide depletion (Pires et al., 2010). However, it has also been observed that growing cells in media complemented with all 8 ribonucleotides and deoxyribonucleotides does not affect the kinetics of the S-phase arrest (Hammond et al., 2003b), though these experiments are technically challenging. The hypoxia-induced S-phase arrest has been well characterised and is due to a decrease in origin firing, stalled replication forks and slower fork rates (Pires et al., 2010). Upon reoxygenation, G_1 cells are able to progress through the cell cycle. However, S-phase cells can only restart replication if the hypoxic exposure was short. Cells that are exposed to hypoxia for more than 16 hours are unable to restart and this is at least in part due to the

dissociation of the replisome machinery. Expression levels of MCM3, MCM4, MCM5 and MCM6 are decreased and the proteins are no longer associated with chromatin. The E2F family of transcription factors, also responsible for the hypoxic repression of DNA repair, seem to mediate this effect (Pires et al., 2010).

It has been shown that ATR is responsible for p53 phosphorylation at serine 15 in hypoxia. ATR forms nuclear foci in hypoxia and phosphorylation of serine 15 is reduced when a dominant negative ATR protein is used to inhibit ATR function (Hammond et al., 2002). Moreover, in this system the total levels of p53 are reduced supporting the hypothesis that ATR activity leads to p53 accumulation in response to hypoxia (Hammond et al., 2002). Stabilisation of p53 in hypoxia can also be partly attributed to the levels of MDM2. MDM2 is itself a p53 target, and is induced upon DNA damage to act in a negative feedback loop by degrading p53 following its stabilisation. Hypoxia-induced p53 fails to activate transcription of MDM2 and in fact protein levels have been shown to fall in hypoxia leading to reduced p53 degradation (Koumenis et al., 2001, Alarcon et al., 1999). As stalled replication has been shown to lead to the accumulation of ssDNA, it is thought that this is the mechanism through which ATR is activated. Replication protein A (RPA) binds to regions of single stranded DNA and is known to recruit ATR via binding of ATRIP to RPA (Zou and Elledge, 2003).

1.6 p53 and hypoxia-induced apoptosis

p53 is known to cause apoptosis in response to severe hypoxia. However this apoptosis occurs in the absence of induction of many of its known pro-apoptotic target genes. ChIP analysis shows that while p53 is bound at the promoters of these target genes in hypoxia it fails to recruit the coactivator CBP (Hammond et al., 2006). Furthermore, p53 is not acetylated at lysine 382 and therefore is unable to bind p300 in hypoxia (Koumenis et al.,

2001). Instead hypoxic p53 has been shown to be bound to a corepressor mSin3a. The corepressor mSin3a acts to recruit histone deacetylases (HDACs) to the promoters of genes resulting in histone deacetylation and subsequent transcriptional repression. The deacetylase inhibitor TSA is able to reduce hypoxia-induced apoptosis and p53-dependent transrepression suggesting that p53-dependent repression is at least in part responsible for apoptosis in hypoxia (Koumenis et al., 2001). Little is understood about how p53 causes apoptosis in hypoxia. What is clear is that hypoxic p53 is behaving in a very different manner to that activated in response to DNA damage. A study in mice showed that the p53 mutant $p53^{QS}$ (L25Q, W26S) was selectively compromised in transactivation despite retaining DNA binding activity and being more stable due to an inability to bind MDM2. The $p53^{QS}$ mutant is refractory to apoptosis caused by doxorubicin or UV but retains almost wild type apoptosis levels following hypoxia (Johnson et al., 2005). These results support other literature which suggests that hypoxia-induced apoptosis either does not require transactivation, or requires transactivation of a different set of genes to that seen in response to other stresses. This highlights again how the way in which p53 responds to various stresses is complex and specific. Expression profiling of the mouse genome showed that p53-induced genes in hypoxia were different to those induced in response to adriamycin treatment (Hammond et al., 2006). Strikingly, known p53 targets *Noxa, Perp, Bax and Puma* were not induced. Furthermore p53 was seen to repress a number of genes in hypoxia including *Survivin* and *Runx2*. A p53 construct mutated at residues 25, 26, 53 and 54 was unable to cause repression or apoptosis, and a DNA binding mutant was also defective in these responses.

1.7 The role of microRNAs in p53-induced apoptosis

MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs that play an important role in regulating gene expression (Zamore and Haley, 2005, Pillai, 2005). The first step in the production of a mature miRNA is the transcription of primary-miRNAs (pri-miRNAs). These transcripts are processed by the RNAse III enzyme Drosha in the nucleus, resulting in the production of a stem-loop precursor molecule (pre-miRNA). These are transported to the cytoplasm and further processed by another RNAse III enzyme, Dicer, into a short-lived dsDNA molecule of 20-25 nucleotides. This molecule is unwound and one strand becomes the mature miRNA. miRNAs are incorporated into protein complexes and guided to partially complementary sites in the 3' untranslated regions (UTRs) of genes where they either inhibit transcription, or reduce the stability of the mRNA thereby causing gene repression. In recent years miRNAs have been widely studied. In particular they have been linked to different forms of cancer and can function both as oncogenes, as in the case of miR-21 and tumour suppressors (Raver-Shapira et al., 2007, Frankel et al., 2008, Lodygin et al., 2008, Luan et al., 2010).

miR-21 functions as an oncogene and is the most often over-expressed miRNA in solid tumours (Volinia et al., 2006). Inhibition of this miRNA leads to growth suppression and apoptosis. One of its targets is the tumour suppressor PTEN (Meng et al., 2007) and several of its other known targets are p53 target genes including BTG2, APAF1 and FAS (Frankel et al., 2008). As well as the p53 pathway, miR-21 targets are also involved in the TGF-β and mitochondrial apoptosis pathways (Papagiannakopoulos et al., 2008). In addition, the miRNA downregulates some p53 coactivators and generally acts to antagonise the p53 pathway.

p53 has been implicated in the regulation of a number of miRNAs. miR-34a has been well established as a p53 target and p53 activates its transcription through direct binding to the promoter (Raver-Shapira et al., 2007). miR-34a has been shown to play a role in cell cycle regulation, senescence and apoptosis (Fujita et al., 2008, Yamakuchi et al., 2008, Tarasov et al., 2007). It is silenced in a number of cancers, most notably prostate cancer, by methylation of CpG islands in the promoter (Lodygin et al., 2008, Chang et al., 2007). miR-34a has been shown to downregulate cell cycle genes such as Cdk4, Cdk6, CycE2, E2F5 Aurora Kinase B and CDC25a (Bommer et al., 2007). Furthermore it has also been shown to repress expression of the anti-apoptotic Bcl2 protein (Bommer et al., 2007). Reintroduction of miR-34a has been shown to increase chemosensitivity and inhibit tumoursphere formation in a gastric cancer cell line. The mechanism by which miR-34a induces apoptosis has been studied, and evidence suggests that miR-34a downregulates Sirtuin 1 (SIRT1) which leads to an increase in acetylated p53. This in turn results in increased expression of the p53 target gene PUMA and increased apoptosis. This apoptosis is p53-dependent as presumably it relies upon transcription of p53 targets. As miR-34a is itself a target of p53, a potential positive feedback loop is formed.

As p53-mediated gene repression seems to be dependent upon DNA binding activity, miRNAs offer a possible mechanism for this and indeed for apoptosis in hypoxia. p53-dependent miRNA regulation in hypoxia has recently been demonstrated by a study that saw p53-dependent repression of the miR-17-92 cluster in hypoxia (Yan et al., 2009). This miRNA cluster is induced by the oncogene C-myc and is a common signature in several human tumours. p53-dependent repression of miR-17-92 occurs in severe hypoxia (0.1% O_2). Repression is not due to p53-mediated repression of C-Myc and occurs through repression of the promoter. Repression is dependent upon a p53 binding site which overlaps with the TATA box. Overexpresion of the miRNA cluster has been shown to inhibit

hypoxia-induced apoptosis and knockdown of specific miRNAs in the cluster increases the levels of apoptosis.

Other miRNAs have been implicated in modulating the apoptotic response including miR-630, miR-181a and miR-449a/b (Galluzzi et al., 2010, Lize et al., 2010). Over recent years the amount of data linking miRNAs to p53 and apoptosis has increased rapidly and so a role for these molecules in hypoxia-induced apoptosis seems likely.

1.8 Aims of the project

This project aims to further elucidate the mechanism of hypoxia-induced apoptosis by investigating the post-translational modifications and binding partners of p53 in hypoxia. Another aim is to determine the gene expression changes brought about by p53 in hypoxia, and investigate how these lead to apoptosis:

- It is known that p53 has the potential to be modified on a number of residues in response to stress. In this project I will investigate the phosphorylation status of hypoxic p53
- The binding partners of p53 can modulate its downstream function. I will investigate whether p53 has any binding partners in hypoxia, what these binding partners are, and if possible, how they might fit into the mechanism of apoptosis
- p53 is a transcription factor and works by bringing about a number of gene expression changes. I aim to use microarray technology to look at the p53-dependent gene expression changes in hypoxia

2. Materials and Methods

2.1 Tissue culture and hypoxia treatment

Cell lines used were as follows: RKO (colorectal carcinoma), HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$ (colorectal carcinoma, courtesy of B. Vogelstein), mouse embryonic fibroblast (MEF) $p53^{+/+}$, MEF $p53^{-/-}$ and H1299 (non-small cell lung carcinoma). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% Foetal Bovine Serum and 10% penicillin/streptomycin or in the case of the HCT116 cell lines, McCoys 5A medium (Gibco) with 10% FBS and penicillin/streptomycin.

Before exposure to hypoxia, cells were plated onto glass dishes and allowed to grow until they reached 80% confluency. Cells were harvested into equilibrated PBS in hypoxia and were spun down and the supernatant removed.

Hypoxic exposures were carried out in a Bactron anoxic chamber (Shel Lab) (0.02% oxygen) or an Invivo$_2$ hypoxic workstation (Ruskinn) (0.1%-2% oxygen). Oxygen concentrations were verified using an Oxylab pO_2 tissue oxygenation monitor (Oxford Optronix).

2.2 SDS-polyacrylamide gel electrophoresis and western blotting

Cell pellets were resuspended in UTB lysis buffer (9M urea, 75mM Tris-HCl pH 7.5, 0.15M β-mercaptoethanol) and frozen at -20^{o}C overnight. The extracts were then thawed, sonicated briefly and spun to remove any insoluble material.

50 µg of whole cell extract (WCE) was heated in sample buffer (6 M urea, 17 mM Tris-HCl pH 7.5, 0.07 M β-mercaptoethanol, 3.3% SDS, 0.01% bromophenol blue) and run out on a polyacrylamide gel at 100 V for 90 minutes in a Tris-Glycine-SDS buffer. Transfer onto a

nitrocellulose membrane was carried out for 1 hour at 100 V in Tris-Glycine buffer with 20% methanol. Membranes were washed in phosphate buffered saline (PBS) and blocked in 50% Licor blocking buffer in PBS for 1 hour. Membranes were left in primary antibody solution (in 50% Licor blocking buffer/PBS-Tween (0.1%)) overnight at 4°C. Membranes were washed in PBS-Tween and then incubated with fluorescent secondary antibody (Alexa Fluor 680 (Invitrogen)) in 50% Licor/PBS-Tween at room temperature for 1 hour. They were then washed in PBS-Tween and finally in PBS before imaging on the Odyssey (Licor).

2.3 Size Exclusion Chromatography

4 x 10^7 cells were lysed in 5 ml of NETN buffer (150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% N-P40) and spun (17,000 x g, 15 minutes) to remove insoluble material. The lysate was then concentrated to a final volume of 1 ml using a centrifugal filter unit (Millipore). The size exclusion column was prepared by running size exclusion buffer (10 mM Tris, 150 mM NaCl pH 7) through the column. The concentrated lysate was passed through a sterile filter and 200 µl loaded onto the column. Fractions of 80 µl were collected at 4°C every 5 seconds. This process was repeated until all of the concentrated lysate had been used up with equivalent fractions being collected in the same well of a 96 well plate. Protein containing fractions were subjected to SDS-PAGE and western blotting performed to identify proteins of interest.

2.4 Antibody-Bead Crosslinking

Protein A beads were washed with PBS then equilibrated to 0.2 M boric acid pH 9.0. Antibody and protein A beads (3 µg mAB/1 µl packed beads, volume made up to 1 ml with

boric acid) were mixed for 1 hour at room temperature. The beads were washed with 0.2 M boric acid and mixed in 1.5 ml 0.2 M boric acid with 20 mM dimethyl pimelimidate for 30 minutes at room temperature. The reaction was stopped by washing with 0.2 M ethanolamine pH 8.0 for 1 hour. The beads were then washed sequentially with PBS, water and 100 mM glycine pH 3.0 and resuspended in PBS with 0.02% azide.

2.5 Immunoprecipitation

Cells were washed with PBS and lysed at 4°C in 1 ml NETN buffer (150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% N-P40) then spun (17,000 x g, 15 minutes) to remove insoluble material. 10 µl (bead volume) of cross linked beads equilibrated to NETN were added to each extract and rotated at 4°C overnight. The beads were washed six times with NETN and all traces of the last wash removed. Protein was eluted from the antibody by incubation with 5 volumes of 100 mM glycine pH 2.5 for 5 minutes at room temperature. The eluate was neutralised with 0.1 volumes of 1 M Tris-HCl pH 7.5 and made up with sample buffer for SDS-PAGE.

2.6 Coomassie Staining

Coomassie staining was carried out using the EZBlue reagent (Sigma). Following electrophoresis the gel was rinsed in an excess of deionised water (3 x 5 minutes) to remove the SDS. Staining reagent was then added to cover the gel and the container was shaken gently for 45 minutes – 1 hour until staining reached a maximum. The gel was then washed with water until the background was clear and visualised using the Odyssey imaging system (Licor).

2.7 Silver Staining

Following polyacrylamide gel electrophoresis of immunoprecipitates, the gel was shaken in fixing buffer (50% ethanol, 10% acetic acid in water) for 15 minutes. The gel was then washed in deionised water (3 x 5 minutes) and in 1.26 mM sodium thiosulphate (2 minutes). The gel was then washed in deionised water and stained for 25 minutes in the dark with 11.7 mM silver nitrate. The gel was briefly washed in water before the developing solution (0.56 M disodium carbonate, 0.1% formaldehyde) was added. Stop solution (39 mM disodium EDTA) was added as soon as bands started to appear. The gel was then photographed.

2.8 Mass Spectrometry

Coomassie stained protein bands were digested using an in gel digestion protocol (Benedikt Kessler) and were then run on an LC-MS/MS tandem mass spectrometer (HCTplus, Bruker).

2.9 RNA extraction

Approximately 400,000 cells were washed with PBS then lysed in 1 ml of TriZol reagent (Invitrogen) by pipetting up and down. Lysates were then typically stored at -20°C overnight or the preparation of RNA was continued. To extract RNA, 0.2 ml of chloroform was added to the lysates, vortexed and incubated at room temperature for 3 mins. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new tube and 0.5 ml of isopropanol was added. Samples were left to stand at room temperature for 10 minutes and then centrifuged at 12000 x g for 10 minutes at 4°C. The RNA pellet was then washed in 1 ml 75 % ethanol and then air dried before being

resuspended in 50-100 µl DEPC treated water. RNA concentrations were determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific).

2.10 Microarray Analysis

RNA extracted from cells was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and the quality was checked; the 260/280 and 260/230 ratios were required to be <1.8. Gene expression and miRNA array analysis was carried out using Agilent Sure Print HD arrays. The microarrays and initial data analysis were carried out by Oxford Gene Technology (www.ogt.co.uk).

2.11 cDNA preparation

cDNA was prepared from total RNA using the Superscript VILO kit (Invitrogen). The components of the reaction are: 4 µl 5 X VILO reaction mix, 2 µl 10 X SuperScriptR enzyme mix and 2.5 µg RNA. This was made up to a total volume of 20 µl with DEPC-treated water.

2.12 miRNA-specific cDNA preparation

cDNA for TaqMan microRNA (miRNA) assays was prepared using a miRNA reverse transcription kit (Applied Biosystems). The reverse transcription was carried out according to manufacturers instructions using the GeneAmp PCR system 9700 (Applied Biosystems).

2.13 miRNA Assays

miRNA levels were quantified using the TaqMan miRNA assay (Applied Biosystems) used according to manufacturers instructions. The PCR reaction was carried out using the 7500 Fast Real Time PCR System (Applied Biosystems).

2.14 qRT-PCR

Quantitative reverse transcription PCR (q-RT PCR) was performed using the Verso SYBR 2-step kit (Thermo Scientific). Primers are listed in Table 2. The PCR reaction was carried out using the 7500 Fast Real Time PCR system (Applied Biosystems) according to manufacturer's instructions.

2.15 Bacterial transformation

One Shot Max Efficiency chemically competent DH5α *E. coli* (Invitrogen) were mixed with 100 ng of plasmid DNA (Table 3) and incubated on ice for 30 minutes. This was followed by heat shock at 42°C for 30 seconds and a 2 minute incubation on ice. The *E. coli* were shaken in super optimal broth with catabolite repression (S.O.C.) medium (Invitrogen) for 1 hour at 37°C and 20 µl were spread on a pre-warmed selective plate. The plates were incubated at 37°C overnight.

2.16 Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Stratagene Quick Change mutagenesis kit. This is a PCR based method, using primers to introduce point mutations. Mutagenic primers were designed to introduce single amino acid changes in the p53 sequence (Table 2).

2.17 DNA sequencing

DNA was sequenced on both strands to verify the mutagenesis was successful and to ensure no random mutations were introduced. DNA sequencing was performed by Geneservice using 100 ng/µl DNA and 3.2 pmol/µl custom designed primers (Tm 55-60°C) (Table 2). Approximately 900 bp of reliable sequence was generated per reaction.

2.18 Small scale DNA preparation

Single bacterial colonies were picked and grown for 4-6 hours in 5 ml of Terrific Broth (Sigma) at 37°C. Bacteria was pelleted by spinning (900 x g, 5 minutes) and resuspended in

100 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1mM EDTA). 200 µl of Solution II (0.2 M NaOH, 1% SDS) and 150 µl of cold solution III (3 M KOAc, pH 4.8) were added followed by brief vortexing and a 5-minute incubation on ice. Tubes were spun (13,000 x g, 5 minutes) and 500 µl of phenol/chloroform/isoamyl alcohol was added to the supernatant. Tubes were vortexed and spun (13,800 x g, 5 minutes). The aqueous phase was taken to a new tube containing 2 volumes of ethanol, mixed and spun (13,000 x g, 15 minutes). The DNA pellet was washed with 75% ethanol air dried and resuspended in 50 µl water with 0.1 µg/ml RNase A.

2.19 Large scale DNA preparation

Single bacterial colonies were picked and grown in 2 ml of Terrific Broth (Sigma) for 4-6 hours. 100 µl of this culture were then used to inoculate 100 ml of Terrific Broth and a culture grown overnight. The DNA preparation procedure was carried out using a Qiagen Maxi Prep kit according to manufacturers' instructions.

2.20 Transient transfection

Transfections were carried out using Lipofectamine (Invitrogen) according to manufacturers' instructions. Media was changed 4 hours after the transfection and cells were left overnight to recover before hypoxia treatment.

2.21 siRNA transfection

siRNA transfection was carried out using Dharmafect (Dharmacon) according to manufacterers instructions. Transfection reagent was removed if cell toxicity was observed and media was changed before cells were treated with hypoxia. Knockdown of SIRT1 was performed using Ambion siRNA ID s223591 (50 nM final concentration) and siRNA treatment was performed for 48 hours.

2.22 Apoptosis assays

Cells were fixed in 4% paraformaldehyde (PFA) in PBS and mounted on slides with DAPI. Apoptotic cells were counted by morphology (total cells counted: 100-300). Western blotting for cleaved PARP and cleaved-caspase 3 was also carried out. Additionally, the Caspase-Glo

3/7 luminescence assay (Promega) was performed in triplicate in 96 well plate format. Luminescence was read using the Envision multilabel reader (PerkinElmer).

2.23 Immunofluorescence

Cells were fixed in 4% PFA in PBS for 15 minutes then washed with PBS. Cells were then lysed for 10 minutes in PBS-Triton (1%) and blocked for 1 hour in blocking buffer (PBS-Triton (0.1%), 2% bovine serum albumin (BSA)). Cells were washed with PBS-Triton (0.25%) and incubated with primary antibody (diluted in blocking buffer) for 1-1.5 hours at 37°C in a humidified chamber. The slides were then washed with PBS-Triton (0.25%) and incubated with secondary antibody (1:250 in blocking buffer) for 1 hour (37°C in a humidified chamber). Slides were washed with PBS-Triton (0.25%) and PBS before being mounted with DAPI. Immunofluorescence was visualised using a fluorescence microscope (Nikon 90i).

Luciferase Reporter Assay

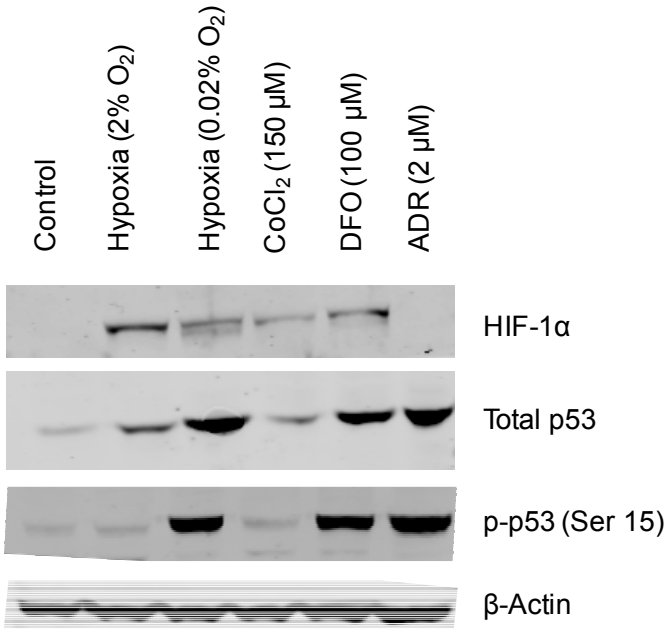
H1299 cells were transfected with 250 ng of 5 x HRE p53 construct and 250 ng of pGI3-luc p53 reporter construct in a 96 well plate. In addition cells were co-transfected with 1 ng of pCMV renilla luciferase construct. The transfection reagent was Lipofectamine LTX (Invitrogen) used according to manufacturers instructions. Cells were exposed to hypoxia (0.02% O_2) for 16 hours, then 100 µl Dual-Glo luciferase reagent (Promega) was added to each well. After 10 minutes luminescence was measured using a plate reader. To stop the reaction and measure Renilla luciferase background reading, 100 µl Dual-Glo Stop and Glo reagent was added to each well. After 10 minutes a further luminescence reading was taken. Cells expressing HRE driven luciferase were used as a positive control. Cells expressing either p53 alone or pGI3-luc alone were used as negative controls.

3. Results

3.1 p53 stabilisation and modification in hypoxia

3.1.1 p53 is stabilised in hypoxia concurrent with S-phase arrest

It has been established that p53 is phosphorylated at serine 15 in hypoxia and that this is predominantly ATR-dependent. Phosphorylation at serines 15 and 20 is thought to be responsible for p53 stabilisation due to disruption of the binding of E3 ligase MDM2. In addition in hypoxia it has been shown that levels of MDM2 fall, presumably in part due to the failure of p53 to activate its transcription (Alarcon et al., 1999). We sought to confirm that p53 stabilisation occurs in severe hypoxia (<0.02% O_2). RKO cells were exposed to 0.02% oxygen, 2% oxygen, HIF stabilising agents cobalt chloride ($CoCl_2$) and desferrioxamine (DFO), and the double strand break inducing agent adriamycin (ADR) (Figure 3). The drug treatments were all carried out in normoxia. We found that p53 was indeed stabilised in severe hypoxia, but not at 2% oxygen. With the exception of ADR treatment, p53 was only stabilised in response to those stresses known to cause S-phase arrest, namely severe hypoxia and DFO (Hammond et al., 2003b). Hypoxia-induced p53 stabilisation was comparable to that seen following induction of double strand breaks by adriamycin. Furthermore, we observed that p53 stabilisation was accompanied by its phosphorylation at serine 15 in all cases. It has been suggested that p53 stabilisation in hypoxia is mediated by the hypoxia inducible transcription factor (HIF) (An et al., 1998). Our results suggest that p53 stabilisation occurs independently of HIF as despite HIF-1α stabilisation in response to 2% oxygen and $CoCl_2$ treatment, p53 stabilisation did not occur. It is however possible that stabilisation of p53 could require HIF-1α along with another factor, or factors, specific to severe hypoxia.

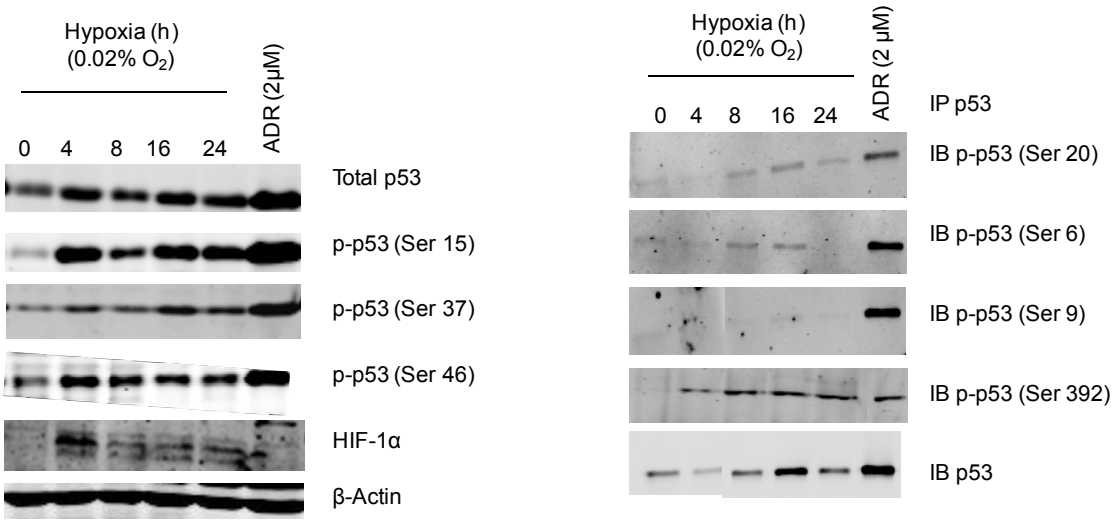


<u>Figure 3:</u> p53 was stabilised and phosphorylated in severe hypoxia and in response to other stimuli that cause an S-phase arrest

Cells were exposed to severe (0.02% O_2) or mild (2% O_2) hypoxia (16 hours), hypoxia mimetic drugs $CoCl_2$ (150 µM, 6 hours) and DFO (100 µM, 6 hours) or the double strand break inducing agent ADR (2 µM, 6 hours). Western blotting for total p53 and phospho-p53 (serine 15) was carried with the protein lysates. p53 was stabilised in response to severe hypoxia and DFO to a level similar to that observed with the positive control ADR. Little p53 stabilisation was seen in response to mild hypoxia or $CoCl_2$ despite stabilisation of the HIF-1α protein.

3.1.2 Hypoxia-induced p53 modifications differ to those observed following DNA damage

p53 functions in a variety of ways in response to a range of different stresses. Work to date has shown that hypoxia is a unique physiological stress, being able to initiate S-phase arrest and a DNA damage response, in the absence of DNA damage detectable by comet assay (Bencokova et al., 2009). Hypoxia-induced p53 behaves in a way that differs from its response to genotoxic stress. The post-translational modifications of p53 are believed to play a large role in determining the downstream effects of p53 stabilisation and because of this we hypothesised that hypoxia-induced p53 might be modified differently to p53 induced by DNA damage. RKO cells were exposed to severe hypoxia for 4, 8, 16 and 24 hours or treated with ADR to induce double strand breaks (Figure 4). We carried out western blotting with antibodies against specific phosphorylated residues of p53. Serine residues 15, 20, 37, 46 and 392 were phosphorylated strongly following ADR treatment. As shown previously, we observed phosphorylation at serine 15 in response to hypoxia (Figure 3). Some of the phospho-specific antibodies are weak and so to look at these, we first immunoprecipitated p53 from hypoxic or ADR-treated cells and then blotted for the specific phosphorylations. We observed phosphorylation at serine 392 and weak phosphorylation at serine 20 in response to hypoxia. Two sites, serines 6 and 9 showed differing phosphorylation between DNA damage and hypoxia. Serine 6 was only very weakly phosphorylated in hypoxia and serine 9 was not phosphorylated at all. Both showed strong phosphorylation in response to ADR. These results suggest that there are differences in the way p53 is modified following hypoxia and that these could mediate its hypoxia-specific downstream effects. However, it should be noted that the differences in p53 phosphorylation could be due to slower kinetics of phosphorylation rather than a complete absence of a particular modification in hypoxia. Nonetheless these kinetic differences could still affect the downstream response to p53.



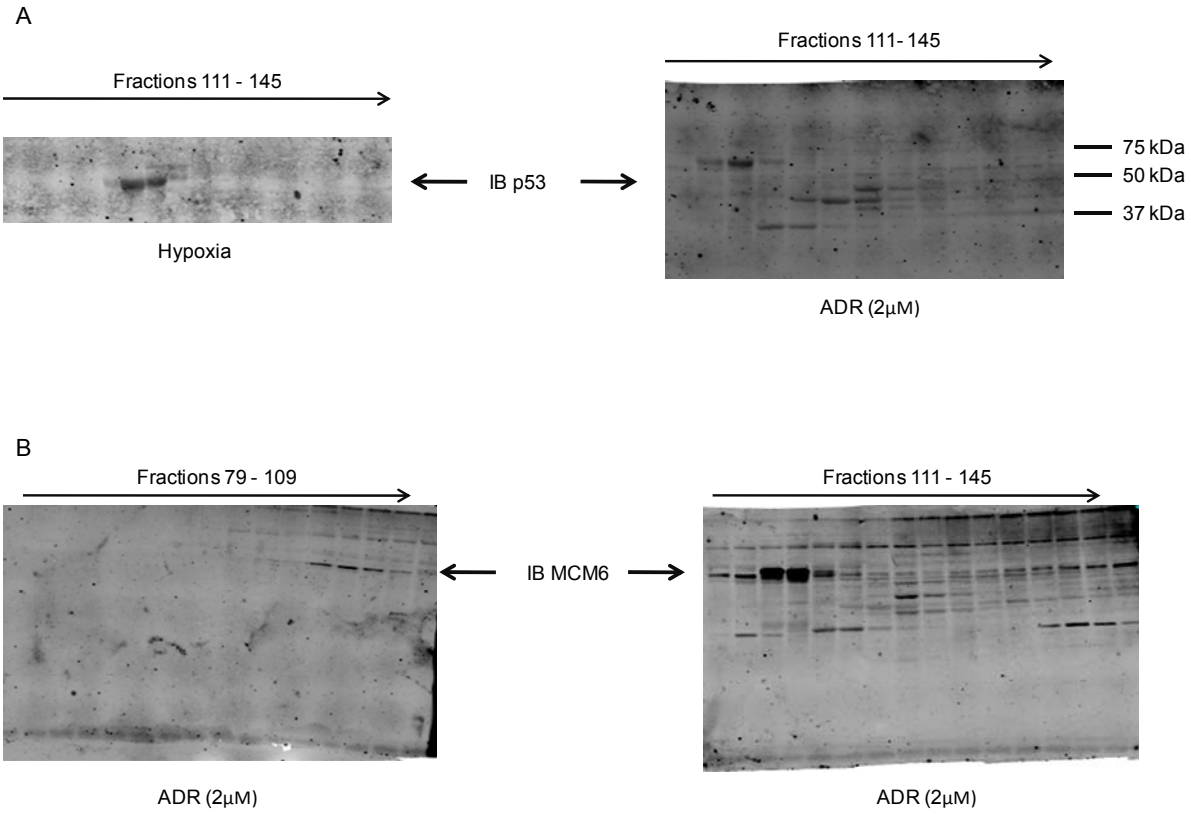
<u>Figure 4:</u> p53 was phosphorylated on a number of serine residues following exposure to severe hypoxia

Antibodies specific for phosphorylated residues of p53 were used to determine the post-translational modifications of p53 in severe hypoxia (0.02% O_2) by western blotting. We saw that p53 is phosphorylated on serines 15, 37, 46 and 392. Weak phosphorylation is also seen at serine 20 and serine 6. The phosphorylation pattern differs slightly to that seen in response to the DNA damaging agent ADR (2 μM, 6 hours).

Serines 6 and 9 are known to be phosphorylated by casein kinase-1δ (CK1) *in vitro* (Knippschild et al., 1997, Milne et al., 1992, Higashimoto et al., 2000). The alpha isoform of this kinase is thought to be active in hypoxia (Mou et al., 2006), and recent data has shown that the delta isoform is able to phosphorylate HIF-1α *in vitro* (Kalousi et al., 2010).

3.1.3 p53 is part of a complex in hypoxia

One way in which p53 modifications can influence its downstream response is through altering its binding partners. For example, phosphorylation at serines 33, 46 and 315 allow the binding of a prolyl isomerase Pin-1 to p53 (Zacchi et al., 2002). We decided to carry out size exclusion chromatography to see whether p53 formed a complex in hypoxia, and, if so, to estimate the size of the complex. Cells were exposed to hypoxia for 16 hours or ADR for 6 hours. These were then lysed and the concentrated lysate was filtered and passed through a size exclusion column. Eluate was collected in fractions and those containing high levels of protein were prepared for western blotting. The blots were probed with the monoclonal p53 antibody DO-1 and the sizes of complexes containing p53 were calculated from their elution time, when compared to the elution of known protein standards. Size exclusion chromatography separates proteins on the basis of their size, with large proteins coming off earlier than smaller ones. We observed that in hypoxia, p53 was eluted earlier than the predicted point for the 212 kDa tetramer alone, at a molecular weight of about 400 kDa, suggesting that p53 is bound to another protein or protein complex in hypoxia (Figure 5A). Following treatment with ADR, p53 also came off the column in a number of places earlier than for the tetramer alone. This included one at around the same size as the hypoxic p53 complex confirming that in response to DNA damage it is bound to a number of other proteins. Compared to the elution profile of hypoxic p53 there were more p53 complexes of



<u>Figure 5:</u> Size exclusion chromatography suggests that p53 is present in a complex in hypoxia

(A). Cells were exposed to hypoxia (0.02%, 16 hours) or ADR (2 μM, 6 hours) and protein lysates were separated by size exclusion chromatography. Protein fractions were run on a polyacrylamide gel and western blotting for p53 was carried out. Those fractions containing p53 are shown. Some of the bands at lower molecular weights may represent p53 splice variants. The time that p53 is eluted from the column correlates to the size of the complex that it is present in. p53 was eluted earlier than predicted for the tetramer alone, suggesting that it is part of a complex in both hypoxia and in response to DNA damage. Complex sixes were estimated by comparison to the elution times of known protein standards.

(B). Cells were treated with ADR (2 μM, 6 hours) and separated by size exclusion chromatography. Fractions were run on a polyacrylamide gel and subjected to western blotting for MCM6. MCM6 is known to be present in complexes of various sizes and this was used as a control for the separation abilities of the column. MCM6 was eluted from the column at a number of different times, confirming the validity of the approach.

differing sizes in response to ADR. In order to validate the technique we also blotted for MCM6 which should be present as part of an MCM2-7 hexamer and also in complexes of various other sizes (Remus et al., 2009, Komata et al., 2009). We observed that MCM6 came off the column at a position corresponding with approximately 600 kDa, as expected for the hexamer. It also came off the column in a number of places corresponding to lower molecular weights and so we could be confident that the column had effectively separated complexes of differing sizes (Figure 5B).

Having found that p53 is bound in a protein complex in hypoxia, we next sought to work out what its binding partners were. In order to optimise the immunoprecipitation technique, we immunoprecipitated p53 from RKO cells that had been exposed to the proteasome inhibitor MG 132. Initially, many non-specific protein bands were present on the western blot and our controls showed that even in controls with antibody alone (no protein) there was a band at the same molecular weight as p53. As the IgG heavy chain runs at the same molecular weight as p53, and mouse IgGs are sometimes recognised by rabbit secondary antibodies, we could not be sure that the bands we saw were p53. To optimise the procedure we reduced the amount of antibody used to pull down p53 and blotted for total p53 protein with the goat polyclonal antibody C-19 to avoid antibody cross-reactivity. Western blotting confirmed that we were able to bring down p53 and the blots were slightly cleaner. However, the best results were obtained when we used C-19 to immunoprecipitate p53 and blotted with DO-1 (Figure 6), presumably because the monoclonal antibody does not recognise non-specific proteins pulled down by the goat antibody. Beads alone, and antibody alone controls were carried out to ensure that we were detecting a band that corresponded to p53, and not a non-specific protein. Coomassie and silver staining were carried out to detect protein bands (Figure 6). In both cases we were able to detect p53 in the MG 132- treated samples but no other bands were seen. It is likely that the amount of p53 protein pulled down was not sufficient to be

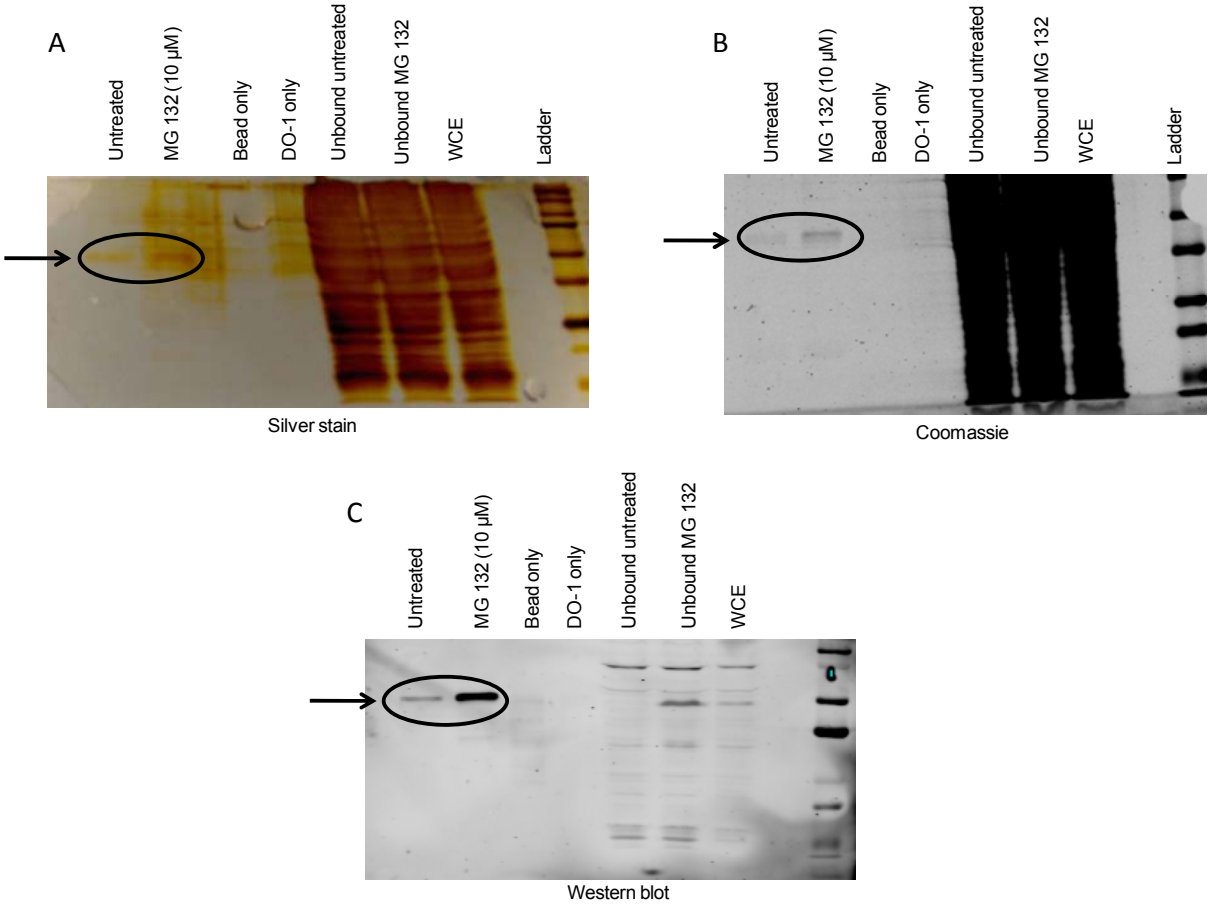


Figure 6: Optimising immunoprecipitation of p53

The goat polyclonal p53 antibody C-19 was used to immunoprecipitate p53 from untreated cells and cells treated with the proteasome inhibitor MG 132 (10 µM, 6 hours). p53 was stabilised to a greater extent in the MG 132 treated cells. Protein was detected by silver staining (A), coomassie staining (B) and western blotting (C) to confirm the presence of p53. As the IgG heavy chain runs at the same molecular weight as p53 so to confirm that the band we observe is in fact p53 we ran several controls. The bead only control shows any proteins that bind non-specifically to the beads and antibody alone control was a negative control without any protein. Whole cell extract shows that p53 was initially present but then disappears from the unbound fraction following immunoprecipitation.

able to detect its binding partners. As our ultimate aim was to be able to look at p53 modifications and detect binding partners by mass spectrometry, we cut out the visible p53 band from the coomassie-stained gel and carried out a gel extraction. LC-MS/MS tandem mass spectrometry was carried out, resulting in the successful identification of p53. However p53 levels were not sufficient to allow identification of post-translational modifications.

In order to immunoprecipitate more p53 from the cell lysates we tried exogenous overexpression of p53. A hypoxia inducible p53 construct was used (discussed in detail in section 3.2.1). In addition to requiring high protein concentrations, mass spectrometry is also very sensitive to contamination. In order to reduce the number of contaminating proteins in the sample it was necessary to change the immunoprecipitation procedure. The monoclonal antibody DO-1 was used to pull down p53 in this case. To avoid the problem of contaminating IgG protein, we crosslinked the antibody to protein A beads to enable elution of the protein whilst leaving the antibody on the beads (Figure 7A). This led to a visible p53 band on the gel following coomassie staining (Figure 7B). Furthermore there were some extra bands that may have represented binding partners. All the protein bands were cut out and subjected to mass spectrometry analysis. p53 was successfully identified, however there was not a high enough protein concentration in the other samples to identify any possible p53 binding partners.

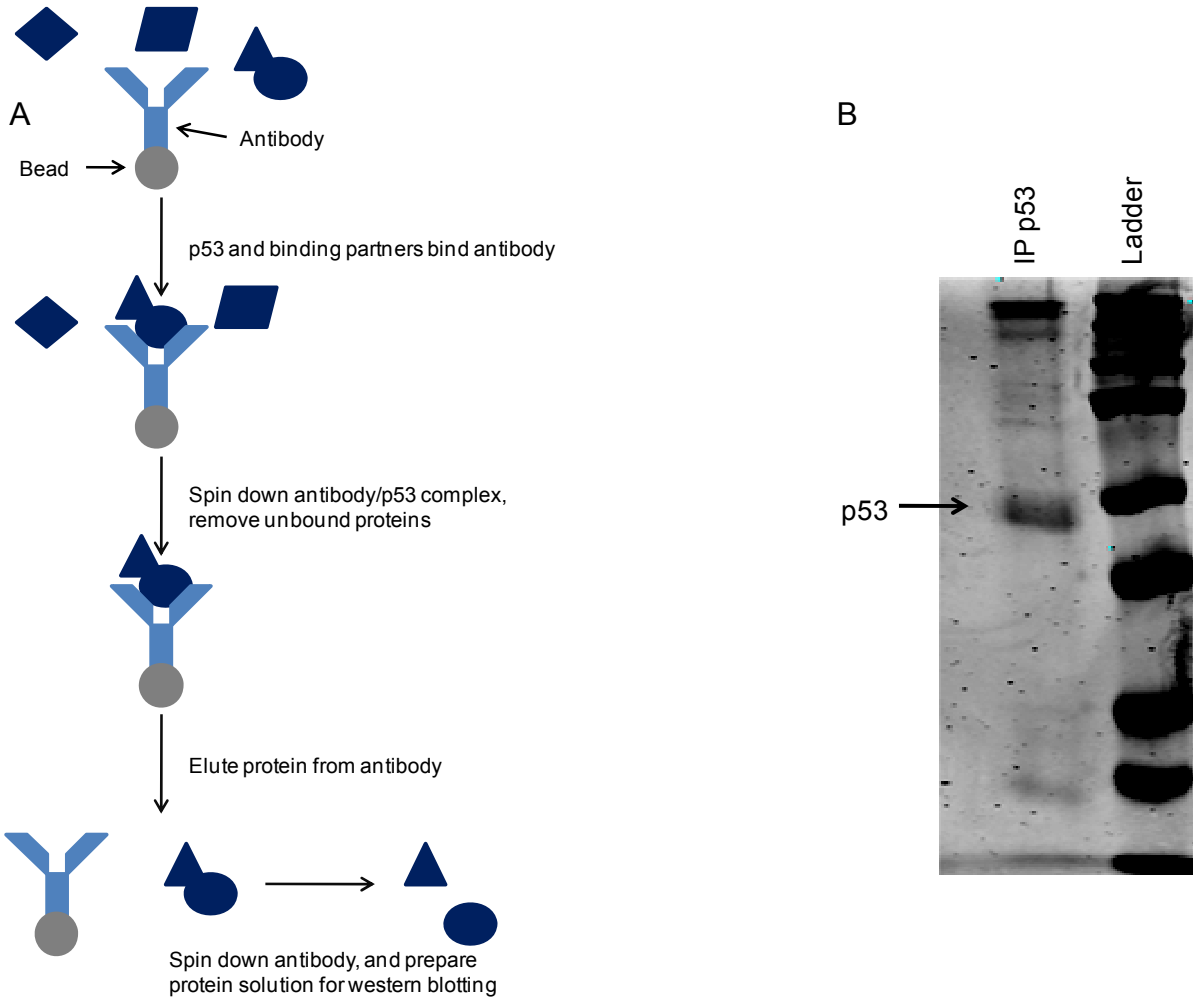


Figure 7: Immunoprecipitation of exogenous p53

A hypoxia-inducible p53 construct was expressed in H1299 cells and they were exposed to hypoxia for 16 hours. p53 was immunoprecipitated from the cell lysate and SDS-PAGE was carried out (Procedure shown in (A)). (B). The gel was stained with coomassie and a band corresponding to 53 kDa was cut out. Other visible bands were also cut out and a gel digestion protocol carried out to isolate the protein. All protein samples were analysed using mass spectrometry. p53 was successfully identified using mass spectrometry, however it was not possible to identify the proteins present in the additional bands.

3.2 p53-induced apoptosis in hypoxia

3.2.1 Characterising the hypoxia-inducible p53 construct

In order to look specifically at hypoxia-induced p53 we used a hypoxia-inducible p53 construct transfected into the p53 null lung carcinoma cell line H1299. We optimised the technique by changing the amount of reagent and transfection time, using GFP as a quick readout for transfection (Figure 8). We were able to achieve 60% transfection efficiency. H1299 cells have lost p53, but still undergo apoptosis if p53 is put back in. This makes them a good model system in which to study hypoxia-induced apoptosis. Although p53 expressed from this construct is induced by a different mechanism, it will presumably be modified according to the hypoxic environment and therefore behave in a similar fashion to endogenous hypoxia-induced p53. In order to validate this we looked at the expression and apoptotic capabilities of the p53 construct. H1299 cells were transfected with the construct for 24 hours and exposed to hypoxia (Figure 9A). Western blotting and immunofluorescence confirmed that the construct is expressed only in hypoxic cells, and there are only very low levels of p53 expression in normoxia (Figures 9B and 9C). Furthermore, the p53 was shown to be entirely nuclear by immunofluorescence (Figure 9D). Expression of the construct in severe hypoxia causes H1299 cells to undergo apoptosis as seen by western blotting for cleaved PARP (Figure 9B), cell morphology, and caspase cleavage (Figure 10). One important difference between the exogenous p53 construct and endogenous hypoxic-p53 is that while endogenous p53 is expressed only in S-phase cells in severe hypoxia, the exogenous construct is expressed in all hypoxic cells, whenever HIF-1α is present. Nonetheless, when examined for their apoptotic capability, H1299 cells expressing the p53 construct only underwent apoptosis in severe hypoxia providing good evidence that despite its different mechanism of induction, the hypoxic p53 still behaves in a manner similar to endogenous p53.

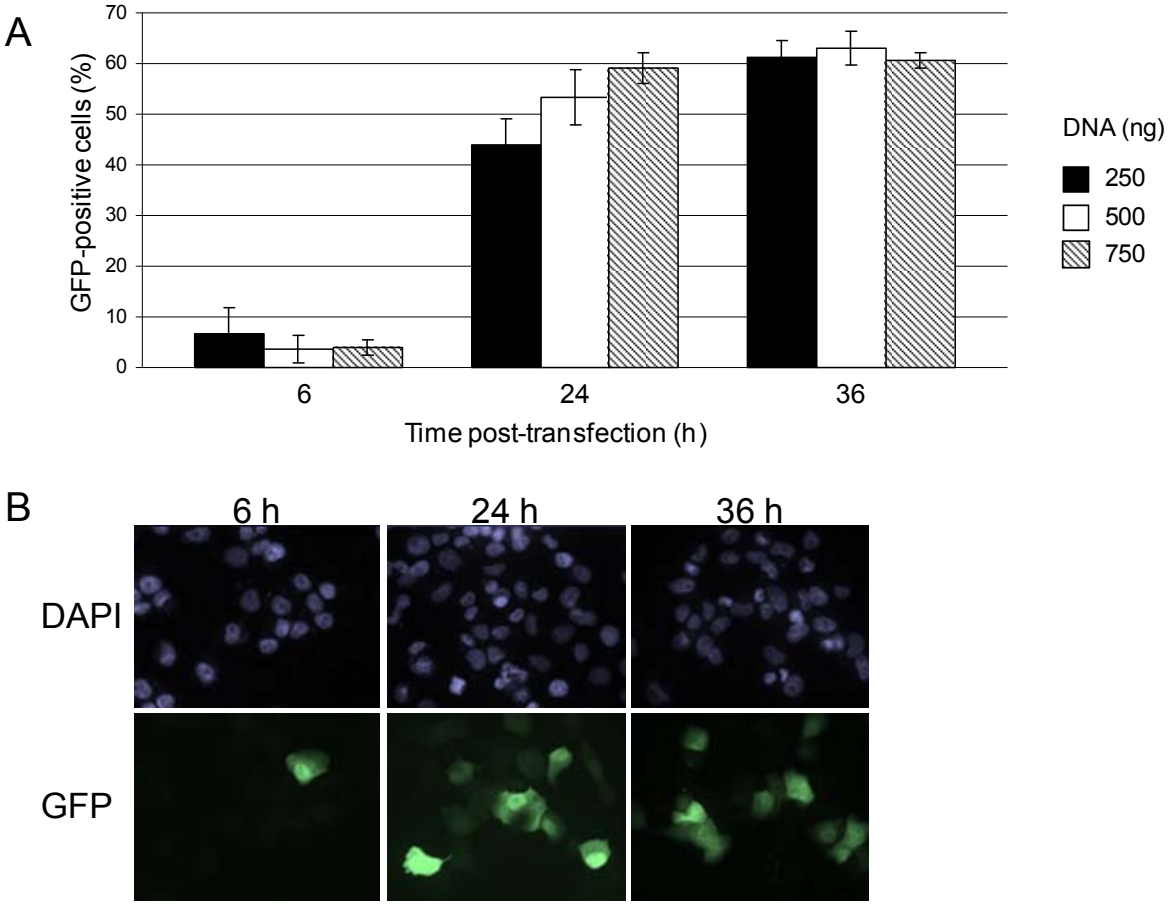


Figure 8: Optimising transfection efficiency in H1299 cells

(A and B). 250 ng, 500 ng or 750 ng of DNA containing the GFP gene was transfected into H1299 cells using lipofectamine (LTX). The number of GFP positive cells was counted after 6, 24 and 36 hours. The amount of DNA seemed to only have a minimal effect on transfection efficiency, and approximately 60% efficiency was seen following 24 hours. In light of these results we chose to carry out transfections with 500 ng of DNA for 24 hours for future experiments.

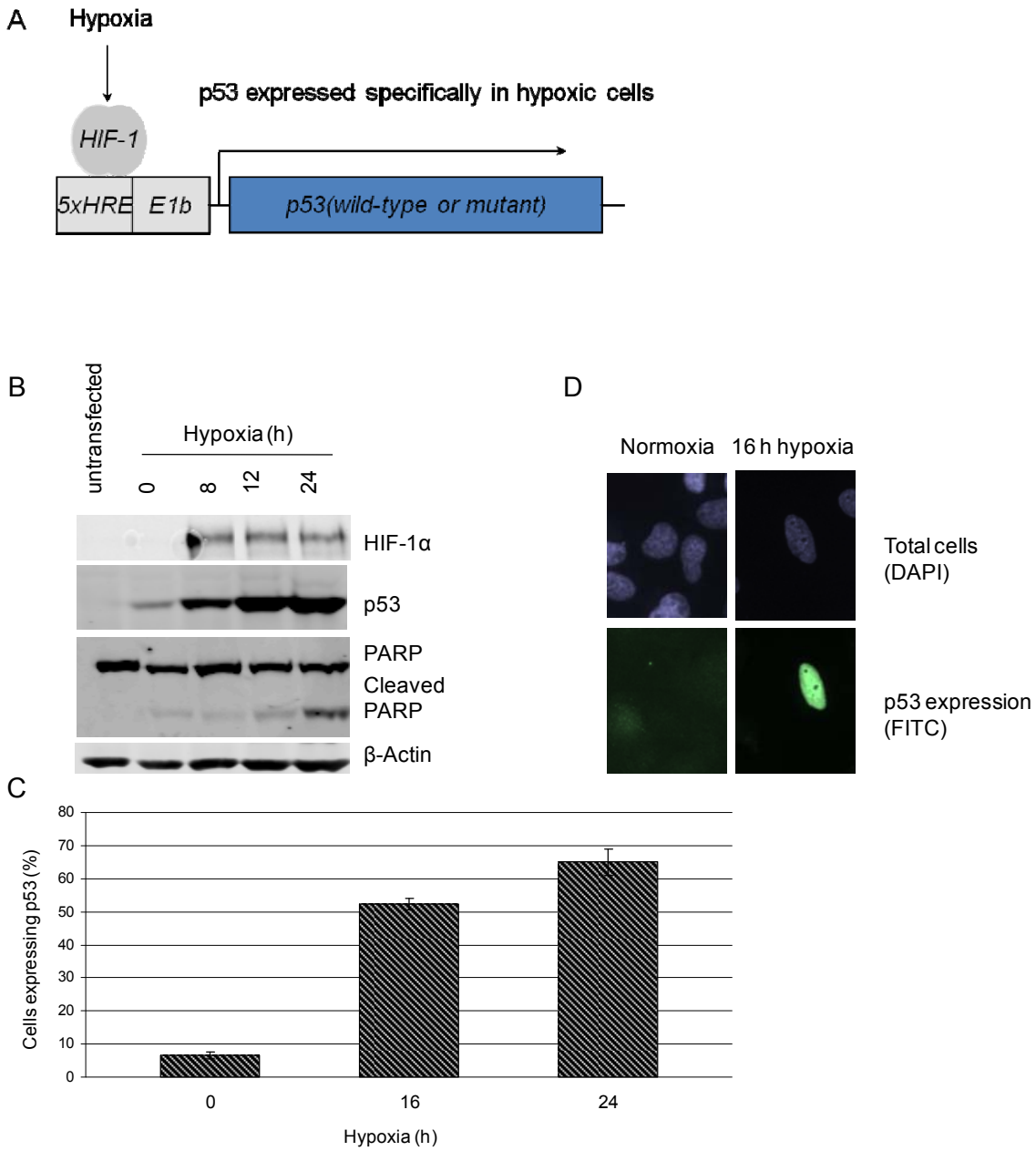


Figure 9: Characterisation of the hypoxia-inducible p53 construct

(A). p53 that has been cloned downstream of the HRE from the VEGF gene and the E1b promoter from adenovirus. It is activated in hypoxia by the binding of a HIF-1α/HIF-1β heterodimer.
(B). The hypoxia-inducible p53 construct was transfected into the p53 null cell line H1299 and, 24 hours later, cells were exposed to hypoxia (0.02% O_2) for 8, 16 and 24 hours. Western blotting for p53 shown that there is minimal p53 expression in normoxia and good hypoxia-inducibility. Western blotting for cleaved PARP shows that this p53 construct can induce apoptosis in severe hypoxia.
(C). The number of cells expressing p53 were counted and expressed as a percentage of the total cells stained with DAPI. In each case at least 100 cells were counted in each of three fields of view. (D). Approximately 60% of cells express p53 following 24 hours of hypoxia (0.02%). Immunofluorescence with a p53 antibody and DAPI counterstain shows that p53 expression is entirely nuclear in hypoxia.

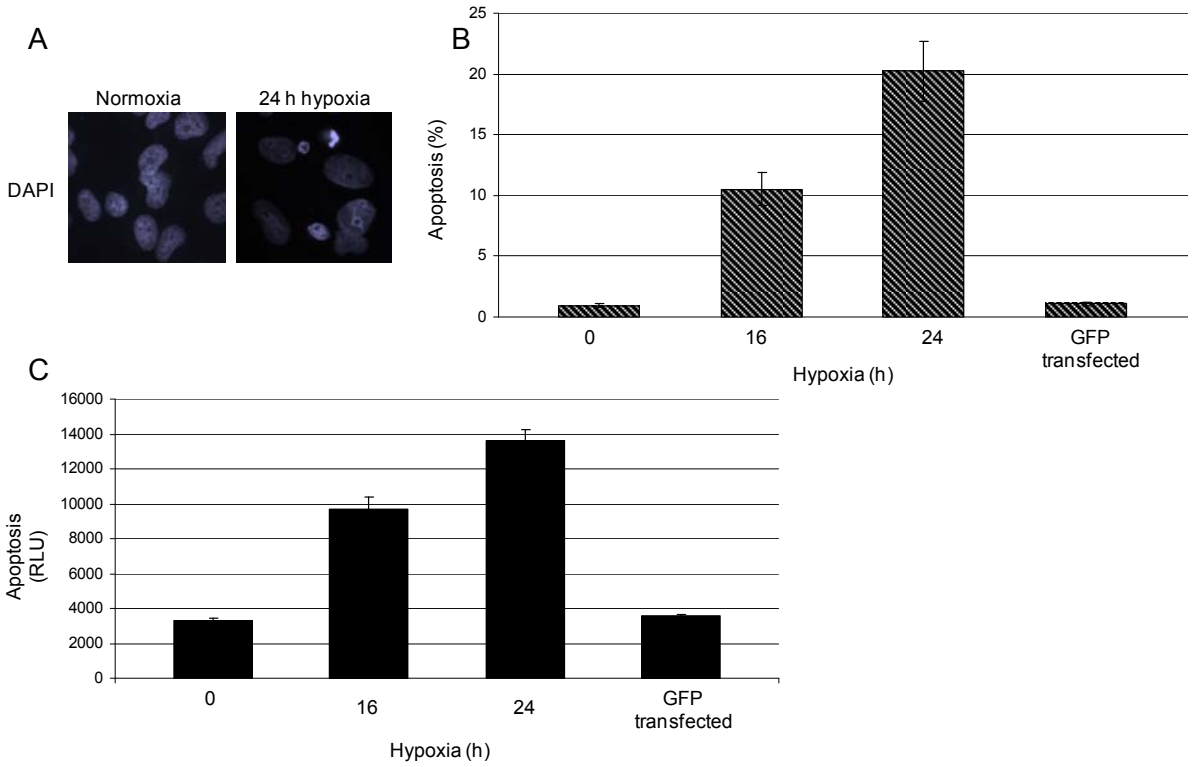


Figure 10: Hypoxia-inducible p53 causes apoptosis in severe hypoxia

H1299 cells transfected with the hypoxia-inducible p53 construct were exposed to 16 and 24 hours of hypoxia (0.02% O_2). As a control, cells transfected with GFP were also exposed to 24 hours hypoxia. Apoptosis was assessed by cell morphology following DAPI staining (A and B). Or using the Promega caspase-glo 3/7 assay (C). For quantification of apoptosis by morphology, at least 100 cells were counted in each of three fields of view. Due to the fact that we achieved 60% transfection efficiency we assumed that cells undergoing apoptosis were expressing p53.

3.2.2 Post-translational modifications of p53 play a role in hypoxia-induced apoptosis

We have hypothesised that the post-translational modifications of p53 would mediate the apoptotic response in hypoxia. Serine 46 is robustly phosphorylated in hypoxia and has been previously shown to play a role in apoptosis by induction of the p53 target gene p53AIP1 (Oda et al., 2000b). We hypothesised that this residue may be important for hypoxia-induced apoptosis and so carried out site-directed mutagenesis of the hypoxia-inducible construct to change the serine to an alanine. In addition, we also carried out mutagenesis of serine 20 as this is a Chk2 target known to be involved in transactivation of genes as well as being important for stabilisation of p53 (Unger et al., 1999, Chehab et al., 1999, Amano et al., 2009). The mutant constructs were sequenced to confirm presence of the mutations and were tested for their ability to drive a p53 reporter gene (Figure 11) We observed that despite being expressed at similar levels the mutant p53 proteins were both able to drive a reporter construct better than wild type. This may be attributed to the mutant proteins being more stable than wild type. In contrast, the DNA binding mutant R175H was completely impaired in its ability to drive a p53 reporter. The wild type (WT) and mutant constructs along with the tumour derived DNA binding mutant p53 R175H were transfected into H1299 cells and exposed to severe hypoxia for 16 and 24 hours (Figure 12). Levels of apoptosis were measured using the Caspase-glo 3/7 assay. We saw that both mutant constructs were slightly impaired in their ability to cause apoptosis, though neither mutation resulted in complete abrogation. More striking was the result seen with the DNA binding mutant p53. This construct was completely unable to cause apoptosis in hypoxia, suggesting that the DNA binding ability of p53 is essential for apoptosis in hypoxia (Figure 12).

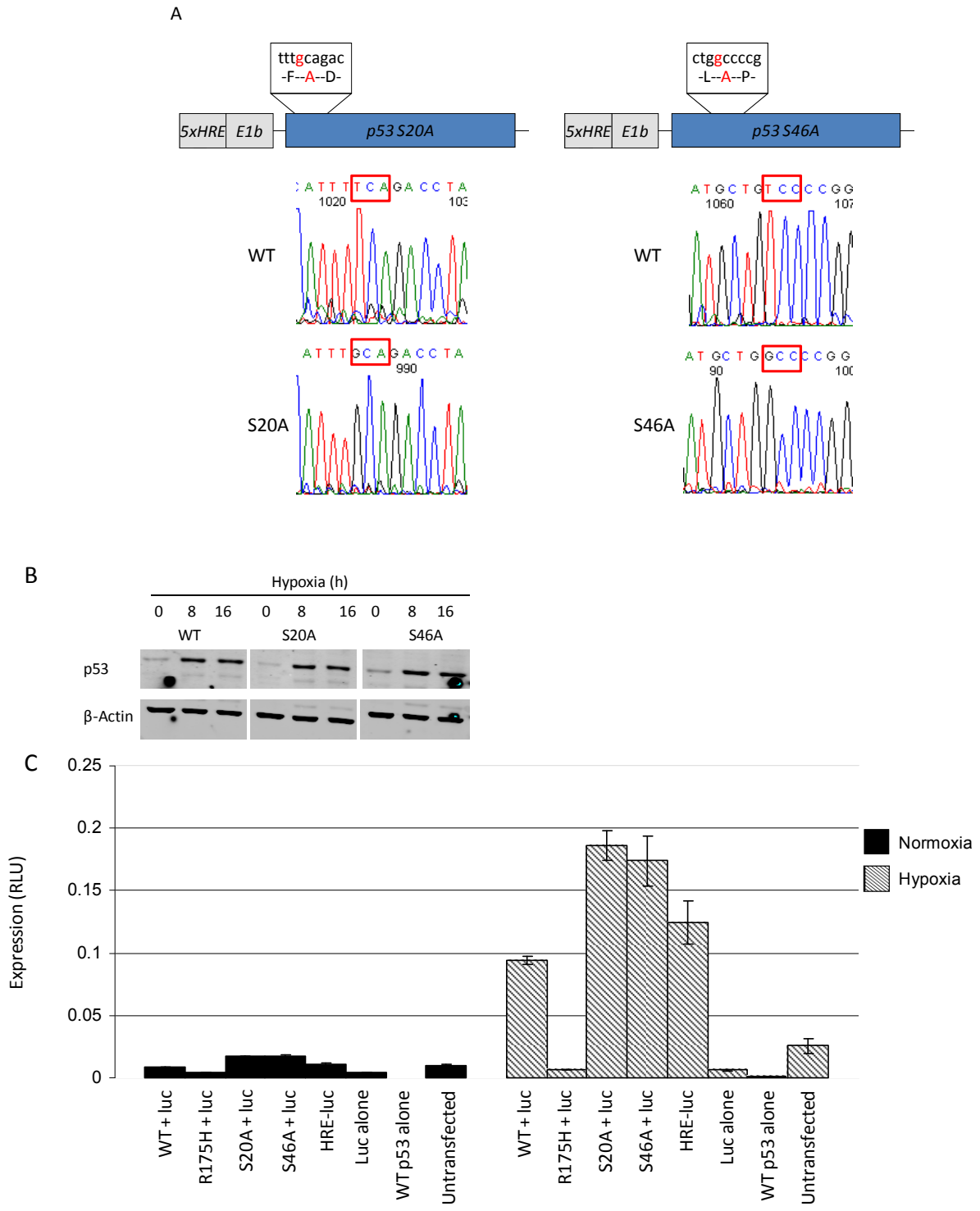
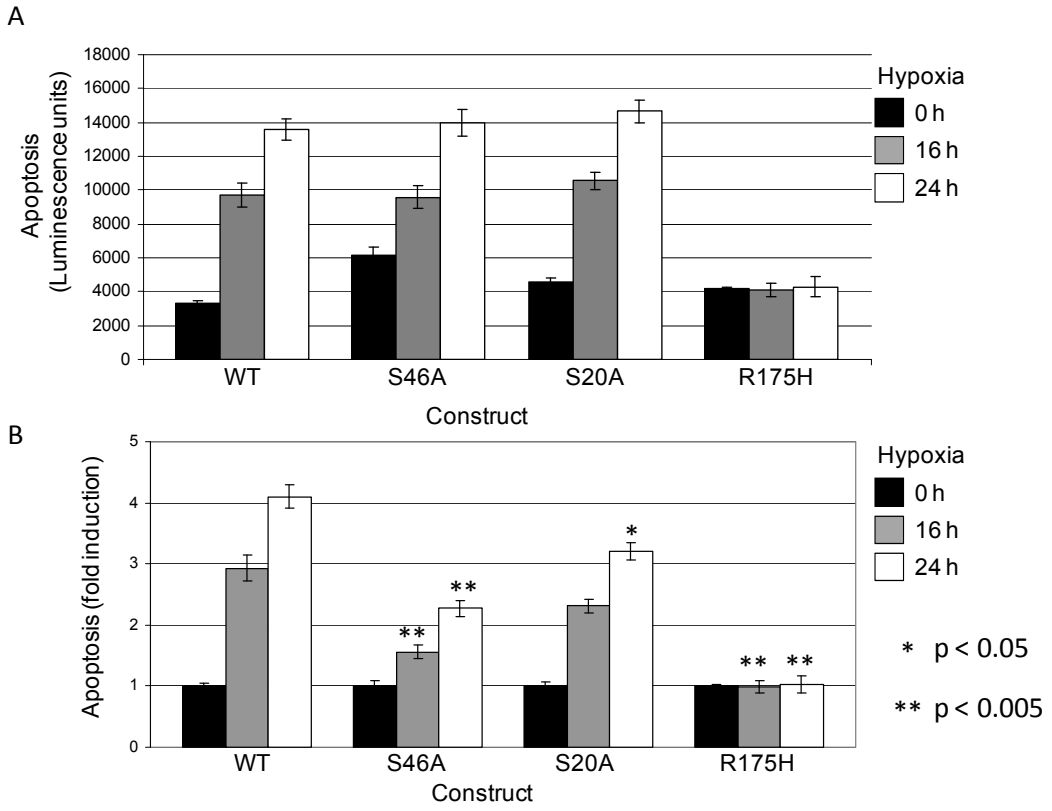


Figure 11: Site-directed mutagenesis of the hypoxia-inducible p53 construct

(A). Site directed mutagenesis was carried out on the hypoxia-inducible p53 construct. Serines 20 and 46 were mutated to alanine. The presence of the mutations was confirmed by sequencing. (B). Expression of the mutant constructs was checked by western blotting and seen to be equal to that of the WT construct. (C). The mutant constructs were able to drive a p53 reporter gene more effectively than the WT construct in hypoxia (0.02% O_2). All cells were transfected with renilla luciferase.



<u>Figure 12:</u> Post-translational modifications of p53 are important for induction of apoptosis in severe hypoxia

H1299 cells were transfected with mutant or WT p53 constructs for 24 hours and subjected to 16 or 24 hours of severe hypoxia (0.02% O_2). Apoptosis was quantified with the caspase-glo 3/7 assay, the results are based upon three replicates.

(A) Raw data showing apoptosis levels in cells expressing either mutant or WT p53 reveals that the basal level of apoptosis is higher in the S46A and S20A constructs. Despite this, the levels of hypoxia-induced apoptosis are approximately equal for WT, S46A and S20A expressing cells. This suggests that cells expressing the mutant constructs may be impaired in hypoxia-induced apoptosis.

(B) Data showing the fold induction of apoptosis in hypoxia compared to normoxia show that p53 mutated at serine 20 or serine 46 is impaired in its ability to cause apoptosis. In cells containing the DNA binding mutant R175H, apoptosis is completely abrogated suggesting that DNA binding of p53 is essential for hypoxia-induced apoptosis.

3.2.3 Microarray analysis of p53-dependent gene expression changes in hypoxia

The striking defect in apoptosis observed with the DNA binding mutant supported work previously done in myc/ras transformed mouse embryonic fibroblasts (MEFs) (Hammond et al., 2006). Microarray analysis of p53-dependent gene expression in hypoxia was carried out in this mouse system, and we decided to repeat the analysis in the human system using the H1299 cells as we felt it would be more relevant to human disease. We performed microarray analysis on RNA extracted from cells expressing either WT or mutant p53 following 16 hours of hypoxia. Our aim was to compare gene expression levels in hypoxia (0.02% O_2) in cells expressing either WT or R175H mutant p53. Before sending the RNA for analysis we checked that HIF target gene vascular endothelial growth factor (VEGF) was expressed (Figure 13) and that the cells were expressing p53. We saw a 5-7 fold induction of VEGF with both wild-type and mutant p53 suggesting that in our system p53 status does not affect expression of HIF target genes. We also performed an apoptosis assay in parallel to check that the WT cells underwent apoptosis and the mutant expressing cells did not. The RNA samples were sent for gene expression array analysis and miRNA array analysis to elucidate p53-dependent expression changes in hypoxia. We chose the five most significantly activated, and the five most significantly repressed genes for validation by qRT-PCR (Appendix I). While DNA-binding capacity is necessary for p53 to cause apoptosis in hypoxia, it has previously been shown that hypoxic p53 fails to activate known pro-apoptotic target genes (Koumenis et al., 2001). Indeed it has been suggested that p53 is primarily behaving as a trans-repressor in hypoxia, and that it brings about apoptosis through repression of genes needed for survival. Despite the requirement for DNA binding, few p53-repressed genes have been discovered to date. In a genome wide study of p53 under hypoxic conditions, ChIP was carried out with at the promoters of genes known to be repressed by p53 and was only seen at the promoters of ankyrin-like repeat protein and Cdc25C

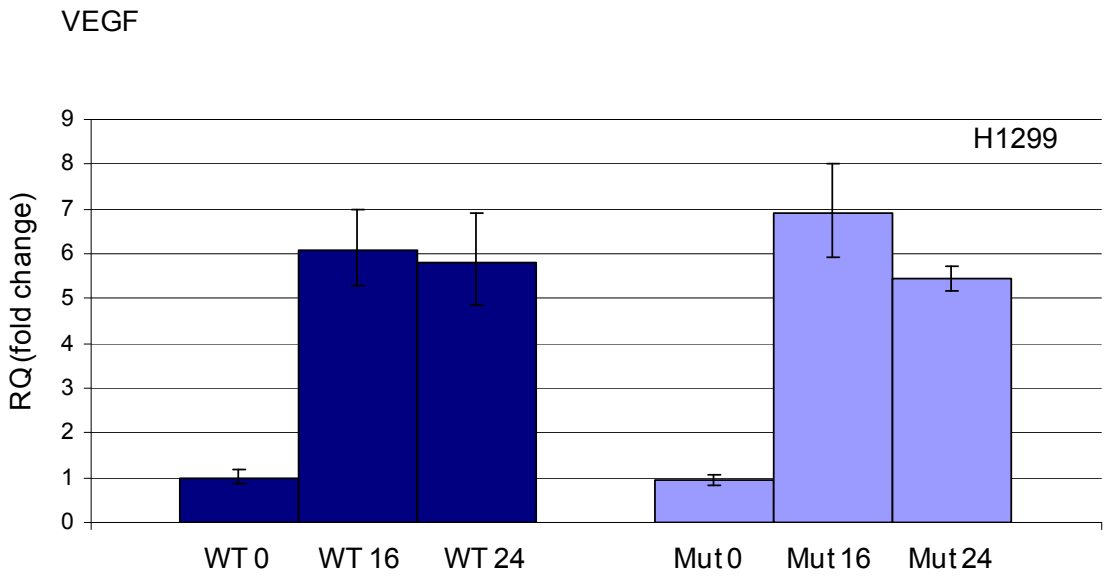


Figure 13: VEGF expression in hypoxia does was not dependent upon p53 status

Before sending the RNA for microarray analysis we carried out qRT-PCR to check for the induction of HIF target gene VEGF. VEGF was induced 5-7 fold by hypoxia in both cell lines confirming that the hypoxic chamber had worked correctly. Furthermore, our results suggest that the expression of HIF target genes is not affected by p53 status, though this would need to be repeated with other HIF targets to confirm the observation.

(Hammond et al., 2006). While this does not mean that p53 is absent from the promoters of the other repressed genes, it does leave open the possibility that p53 may be utilising a number of mechanisms to repress genes in hypoxia. Another possibility is that p53 is repressing genes through activation of miRNA transcription. Furthermore it is still possible that p53 can activate as yet unknown targets in hypoxia, either directly, or through repression of a miRNA (Figure 14). From previous data, we predicted that we would see p53-repressed genes in higher numbers than p53-activated genes (Hammond et al., 2006) and so we were surprised to observe that there were relatively equal numbers in each group. It is important to note that due to the experimental design, those genes that are lower in WT than in mutant could represent genes that are repressed by wild type, or activated by a gain of function in mutant p53. Similarly, genes that are higher in WT than mutant could be activated by wild type or repressed by mutant p53. Perhaps the most striking feature of the data was that we did not see any activation of the well-known p53 target genes such as Puma, Noxa or Bax, confirming that hypoxia-induced p53 brings about apoptosis via a different mechanism. Nonetheless four out of the five WT p53-activated genes we chose for validation were known to be p53 targets and so we were reassured that our data was valid. We began by choosing some of the most significantly altered genes for validation by qRT-PCR. Validation was carried out in the H1299 system and with a pair of HCT116 cell lines that are either WT or null for p53 (Bunz et al., 1999). The chosen genes are highlighted in Appendix I. Of the genes we chose to validate, those activated by WT p53 gave the most convincing results (Figure 15). B-cell translocation gene 2 (BTG2), Inositol polyphosphate-5-phosphatase (INPP5D), Sulfatase 2 (SULF2) and Plekstrin homology-like domain protein A family member 3 (PHLDA3) were all upregulated by hypoxia in a p53-dependent manner. These genes are known p53 targets and have been implicated in apoptosis or reduced proliferation

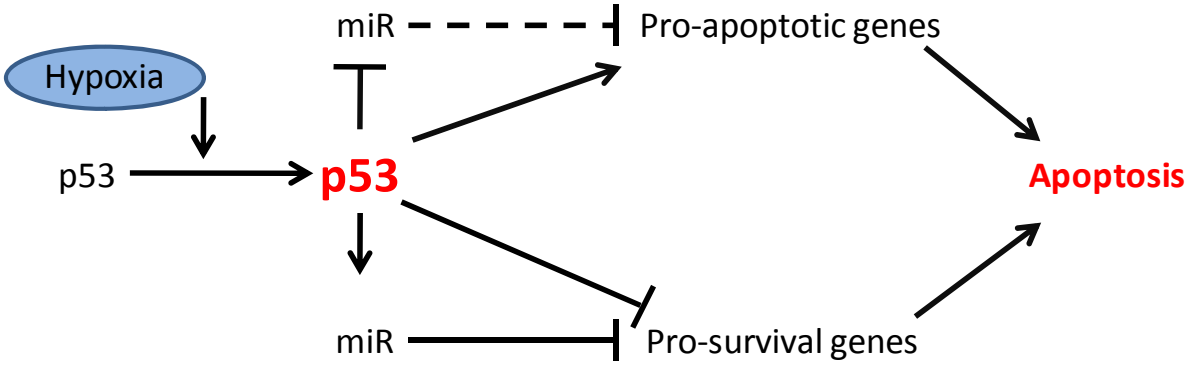
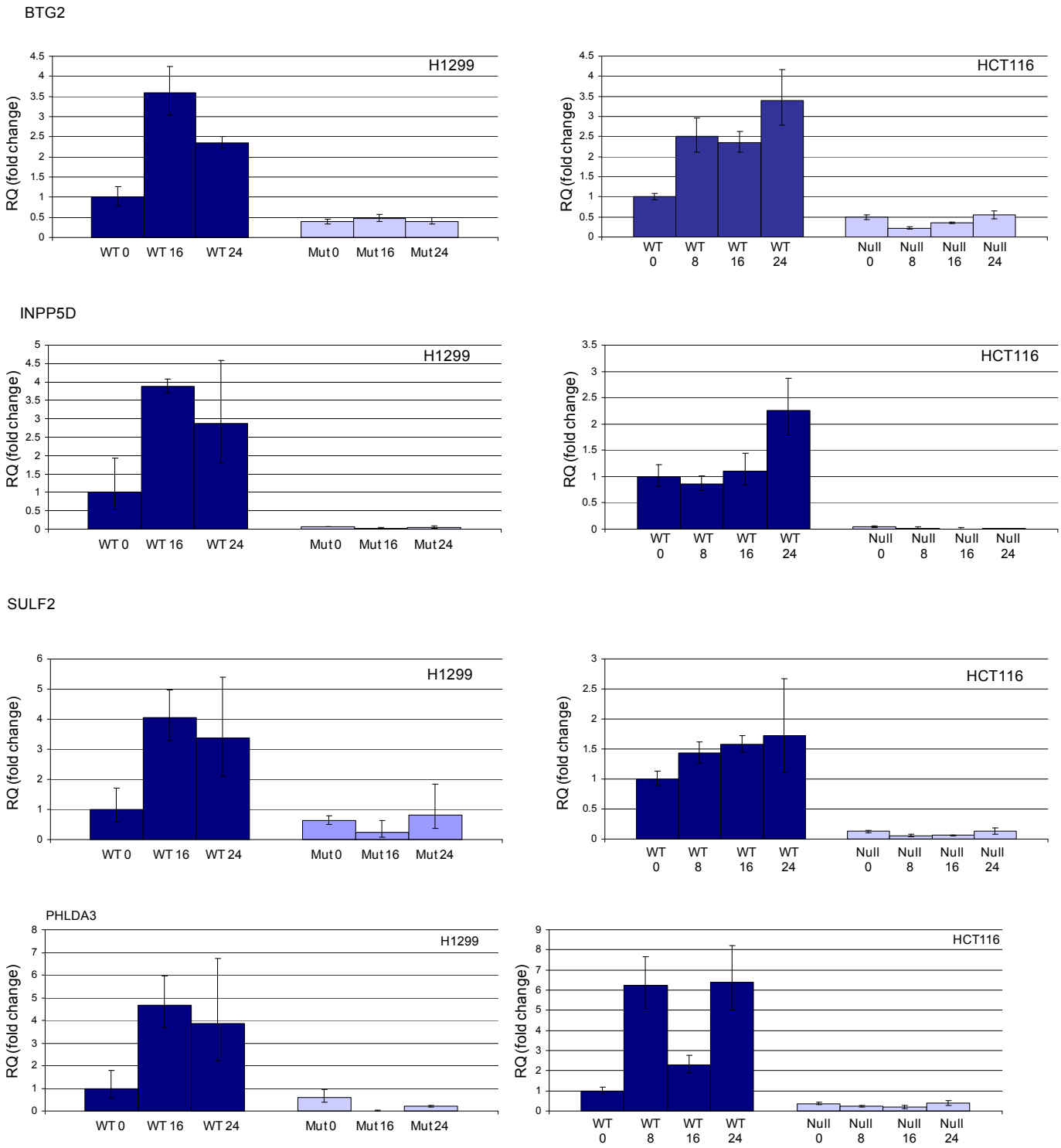


Figure 14: A Model for p53-induced apoptosis in hypoxia

p53 stabilised in hypoxia could mediate the apoptotic response in a number of ways. p53 may activate apoptotic target genes or repress survival genes. Furthermore p53 could do this either directly or by altering the expression of a miRNA.



<u>Figure 15:</u> Validation of p53-induced genes in hypoxia

qRT-PCR was carried out in H1299 cells transfected with WT or mutant p53 (R175H), and in HCT116 cells WT or null for p53. BTG2, INPP5D and SULF2 were all induced by hypoxia (0.02% O_2) in a p53-dependent manner. The results are based on three replicates. 18S rRNA was used as an endogenous control.

and therefore make good candidates for further investigation into the mechanism of hypoxia-induced apoptosis.

In addition to the gene expression changes, three miRNAs, miR-34a, miR-630 and miR-1290 were seen to be upregulated by p53 in hypoxia. miR-34a is a known p53 target and has been shown to have a role in apoptosis (Raver-Shapira et al., 2007). Of the three, this miRNA looked to be the most likely to play a role in hypoxia-induced apoptosis and so we validated the microarray data by qRT-PCR in H1299 cells (Figure 16). Our results confirm that miR-34a is upregulated in cells expressing WT p53, as expected of a p53 induced gene. However, there was no statistically significant hypoxia inducibility despite an apparent increase in miR-34a expression following 16 hours of hypoxia in the p53 WT cells. There was a large amount of variation between replicates and a repeat of this experiment may reveal that miR-34a is indeed induced by p53 in hypoxia. A known target of miR-34a is the histone deacetylase SIRT1. SIRT1 is also able to deacetylate p53, and so repression of this protein leads to an increase in active, acetylated p53. This in turn has been shown to lead to an increase in transcription of pro-apoptotic p53 targets, and a concurrent increase in apoptosis. If p53 is upregulating genes in hypoxia to lead to apoptosis then a miR-34a-mediated repression of SIRT1 could be an important factor in this. SIRT1 has been shown to be repressed in hypoxia (Lim et al., 2010) and so we looked in H1299 cells and the HCT116 pair to see if SIRT1 was downregulated in a p53-dependent manner. Preliminary data did not show a significant p53-dependent downregultation, though the results are preliminary and needs to be repeated to reduce the error (Figure 17).

Taking into account the current data it is clear that p53 can still activate gene expression in hypoxia, though this is only true for a specific subset of genes. In addition, p53 may also able to activate the transcription of a number of miRNAs and that these may act to repress genes and bring about apoptosis (Figure 14).

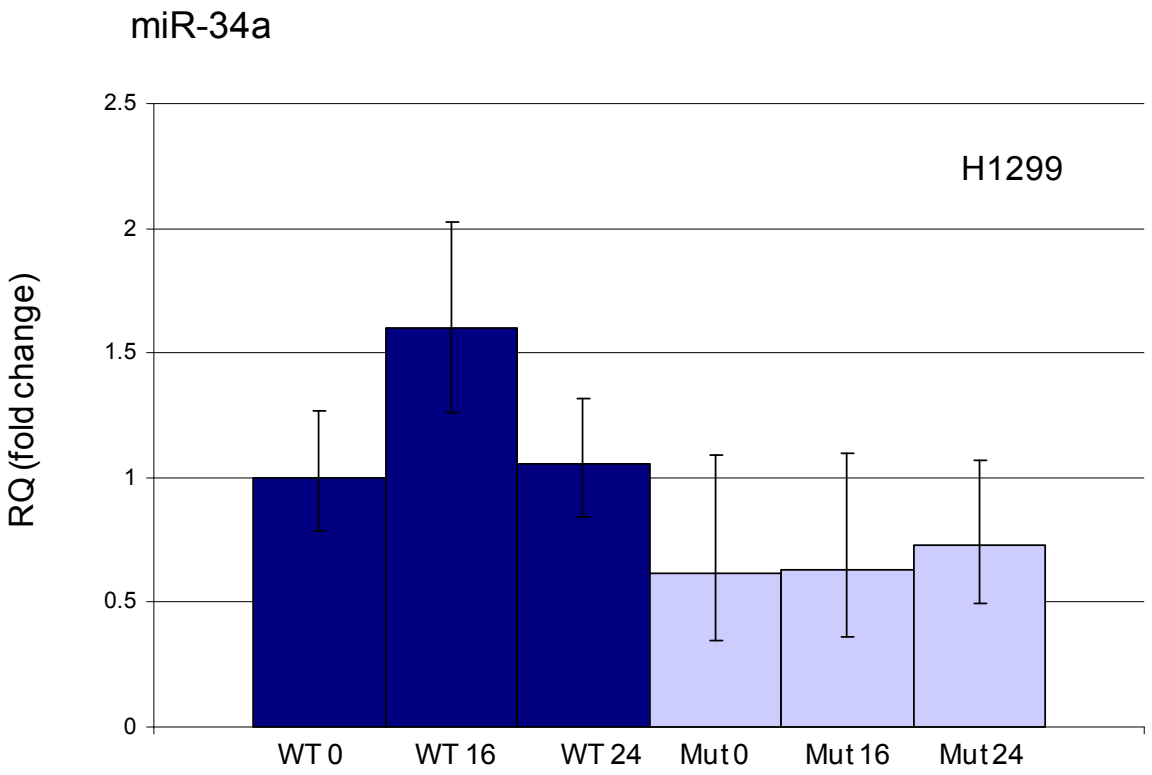
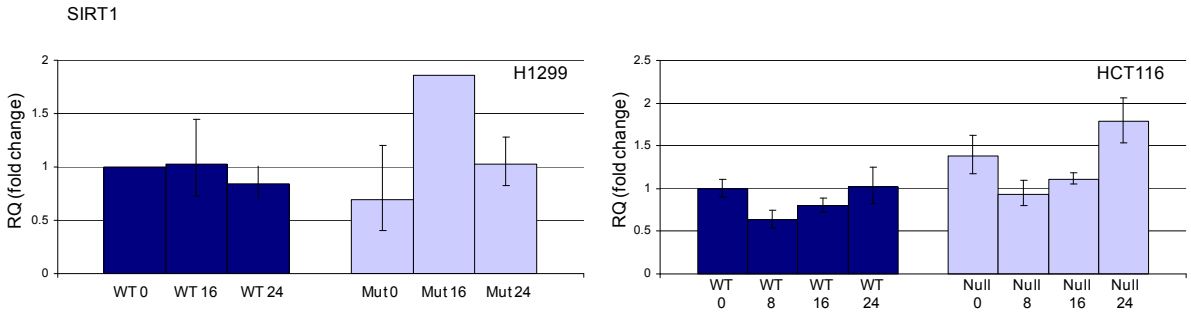


Figure 16: miR-34a is induced in a p53-dependent manner, but is not hypoxia-inducible

qRT-PCR for miR-34a was carried out in H1299 cells expressing either WT or mutant p53 (R175H) in hypoxia (0.02% O_2). miR-34a is higher in those cells containing WT p53, but there was no statistically significant hypoxia inducibility based upon 4 replicates.



<u>Figure 17:</u> SIRT1 does not appear to be repressed by p53 in hypoxia

SIRT1 is a possible target of miR-34a. qRT-PCR for SIRT1 was carried out in H1299 cells and in the HCT116 pair in hypoxia (0.02% O_2). There is some evidence of p53-dependent repression in hypoxia, although the results were not statistically significant based upon 4 replicates.

4. Discussion

It is already known that p53 is stabilised predominantly by ATR in hypoxia (Hammond et al., 2002). ATR usually responds to replication arrest, for example in response to UV, hydroxyurea or aphidicolin (Wright et al., 1998, Heffernan et al., 2002). Severe hypoxia is known to cause an S-phase arrest due to decreased origin firing, stalled forks and slower fork rates (Pires et al., 2010) and it has been shown that p53 stabilisation only occurs when oxygen concentrations are low enough to see this (Hammond et al., 2002). As it is known that the hypoxia-induced S-phase arrest results in regions of ssDNA it seems likely that this is responsible for activating ATR through the binding of ATRIP to RPA (Zou and Elledge, 2003). Recent data has shown that the hypoxia-induced S-phase arrest is concurrent with a fall in dNTP levels. Furthermore, chronic exposure to hypoxia causes replisome disassembly which means that cells fail to restart replication upon reoxygenation (Pires et al., 2010). A topic debated in the literature is the involvement of HIF in the hypoxic stabilisation of p53 (An et al., 1998, Wenger et al., 1998). While we have done little in this work to directly study the involvement of HIF, we have shown that the presence of HIF1-α is not, on its own, enough to give p53 stabilisation and we have seen no evidence to suggest that induction of HIF target genes such as VEGF is affected by p53 mutation.

p53 is a heavily modified protein and the post-translational modifications are known to play a role in determining the downstream effects of p53. The downstream response to p53 in hypoxia differs to that following DNA damage and we hypothesised that the post-translational modifications of p53 in hypoxia may be responsible for this. We did indeed observe some subtle differences between phosphorylation status following DNA damage and hypoxia. Serine 9 was not phosphorylated in hypoxia and serine 6 was only weakly phosphorylated while in response to DNA damage these residues are both strongly phosphorylated. These differences may be enough to mean that p53 transactivates a different

subset of genes. p53 exerts its transcriptional effects through the binding and recruitment of other factors to DNA and this is, in part, mediated by its post-translational modifications.

4.1 Serine 20 and serine 46 phosphorylation of p53 are involved in hypoxia-induced apoptosis

We have demonstrated for the first time that serine 46 is robustly phosphorylated in hypoxia. p53 phosphorylated at this residue is involved in transactivation of the pro-apoptotic gene p53AIP1, and mutation of serine 46 to alanine specifically abrogates the transactivation of p53AIP1 due to reduced binding of p53 to the response element (Oda et al., 2000b). p53 mutated at this residue is also impaired in its ability to cause apoptosis in response to UV irradiation (Oda et al., 2000b, D'Orazi et al., 2002). p53AIP1 protein is located at the mitochondria and causes apoptosis by dissipation of mitochondrial membrane potential (Oda et al., 2000b). It is expressed in response to UV and γ-irradiation and correlates with the induction of apoptosis. Serine 46 is phosphorylated by Homeodomain-interacting protein kinase (HIPK2) and p38 in response to UV irradiation and ATM following γ-irradiation (Bulavin et al., 1999, Saito et al., 2002, D'Orazi et al., 2002). Furthermore, HIPK2 is thought to be important for phosphorylation of serine 46 following cisplatin treatment (Di Stefano et al., 2004). HIPK2 binds to CBP, and in response to UV, phosphorylation of p53 at serine 46 by this kinase facilitates acetylation of p53 at lysines 373 and 382 (Hofmann et al., 2002). Acetylation at these residues increases the transcriptional activity of p53. Serine 46 phosphorylation can also alter the promoter selection of p53 to activate the tumour suppressor PTEN in preference to MDM2. It was postulated that this creates an amplification cycle where p53 is protected from MDM2-mediated degradation allowing it to cooperate with PTEN to mediate apoptosis (Mayo et al., 2005). Further evidence of the involvement of serine 46 in apoptosis is that a p53 variant with single nucleotide polymorphism (SNP) at residue 47 is impaired in its apoptotic ability. The SNP results in a proline to serine change,

preventing the proline-directed phosphorylation of serine 46 by p38. This variant is specifically impaired in its ability to transactivate p53AIP1 and PUMA (Li et al., 2005). HIPK2 has recently been shown to be degraded in hypoxia by proteasomal degradation involving the ubiquitin ligase Siah1 (Moehlenbrink et al., 2010). This results in a reduction in serine 46 phosphorylation and apoptosis induction. This is at odds with our results, where we see an increase in serine 46 phosphorylation in hypoxia. This could suggest that the kinase responsible for serine 46 phosphorylation in hypoxia is not HIPK2, or that the situation is different in severe hypoxia, as the experiments in this study were carried out at 0.2% O_2 and above. Indeed, lack of serine 46 phosphorylation at oxygen levels above 0.2% O_2 could in part be responsible for the failure to induce apoptosis under these conditions, even when p53 is exogenously expressed. Furthermore, HIPK2 is targeted for degradation by MDM2, which is known to be downregulated under severe hypoxia providing another mechanism for the potential activation of this protein under these conditions. Our results support the findings of others and suggest a role for phosphorylated serine 46 in hypoxia-induced apoptosis. It is not known what the mechanism is in hypoxia, and investigations into the activation of p53AIP1 proved inconclusive. However, it is possible that phosphorylation at serine 46 and the subsequent acetylations it facilitates are required for the induction of a specific set of pro-apoptotic genes in hypoxia.

Our results also support a role for serine 20 phosphorylation in apoptosis. Serine 20 is rapidly phosphorylated by Chk2 following DNA damage (Shieh et al., 1999, Hirao et al., 2000). Indeed, mutation at this residue has been shown to reduce the apoptotic capabilities of p53, despite not affecting its expression, DNA-binding ability or its ability to transactivate certain promoters (Unger et al., 1999). Simultaneous phosphorylation of serines 15 and 20 have recently been implicated in the apoptotic response in glioma cells (Amano et al., 2009). Serine 20 resides in the MDM2 binding site and its mutation to alanine can render p53 more

susceptible to inhibition by MDM2 following γ-irradiation and UV (Chehab et al., 1999). Phosphorylation of serine 20 interferes with MDM2 binding, leading to increased p53 stabilisation and this may contribute to its importance for apoptotic activity. Despite this, there is still confusion in the literature and there are specific cases where phosphorylation at the N-terminus of p53 is not required for stabilisation (Ashcroft et al., 1999, Ashcroft et al., 2000). Nonetheless, it is possible that in our system, phosphorylation at serine 20 could regulate protein stability and/or activity so as to increase apoptosis. It would be interesting to look at the half life of p53 mutated at serine 20 compared with that of WT p53. Western blotting suggests that the mutant p53 proteins are equally as stable as the WT p53. However, in this exogenous system, transcription of p53 is being constantly driven by the HIF response element and this could mask subtle changes in the final stability of the p53 protein.

4.2 DNA binding of p53 is required for hypoxia-induced apoptosis

p53 is able to induce apoptosis by a number of mechanisms both transcription dependent and independent. Traditionally, due to lack of activation of the classical pro-apoptotic target genes in hypoxia, it has been hypothesised that p53 is primarily repressive in hypoxia due to an interaction with the co-repressor mSin3a and a failure to recruit co-activators (Koumenis et al., 2001, Hammond et al., 2006). Indeed data has shown that a number of genes are repressed in a p53-dependent manner in hypoxia. p53 in hypoxia is entirely nuclear and so it has always been thought that it induces apoptosis in a transcription-dependent manner under these conditions. However the mechanism of repression has not been demonstrated for all p53-repressed genes (Hammond et al., 2006) leaving open the possibility that apoptosis induction in hypoxia may not always require DNA binding. Our data strongly suggest that DNA binding is required for p53-dependent apoptosis in hypoxia, though it is still unclear

whether it is transcriptional repression or activation that leads to apoptosis. p53 has recently been linked to the transactivation of a number of miRNAs and so it is possible that repression or activation of genes could be mediated via a miRNA intermediate. Our work has shown that p53 is in fact able to transactivate some genes in hypoxia, though it is as yet unclear how, if at all, they mediate apoptosis.

Of the five p53-activated genes in hypoxia that we selected for validation, 4 were known p53 target genes. This is reassuring as this suggests that the microarray results are valid. There is the possibility that some p53 target genes are shown as being induced in hypoxia simply due to the increased levels of p53 and leakiness of the HIF-inducible system. However, the absence of many well known p53 target genes from our list is reassuring and suggests that we are seeing real hypoxia-induced genes. The five most significant p53-activated and p53-repressed genes from our microarray list were chosen for validation by qRT-PCR. Expression was examined in H1299 cells expressing either WT or DNA binding mutant p53 (R175H). Furthermore, qRT-PCR was carried out in HCT116 cell lines that were either WT or null for p53.

B-cell translocation gene-2 (BTG2) was seen to be upregulated in hypoxia in a p53 dependent manner. BTG2 is a known p53 target gene involved in cell cycle control, and preferentially expressed in quiescent cells (Rouault et al., 1996, Cortes et al., 2000). It is induced in a p53-dependent manner in response to DNA damage and causes a G_1 growth arrest. Overexpression of BTG2 causes a decrease in growth rate and in cells where BTG2 is deficient, increased apoptosis is seen following DNA damage (Rouault et al., 1996). BTG2 is known to be a tumour suppressor and its levels are reduced in prostate cancer (Ficazzola et al., 2001). Forced expression of BTG2 in PC-3 cells decreases tumourogenicity *in vivo*. Data also suggest that BTG2 plays an important role in the suppression of Ras-induced transformation (Boiko et al., 2006) and high levels of BTG2 correlate with increased survival

in breast cancer (Mollerstrom et al., 2010). BTG2 has been shown to enhance the apoptotic response to doxorubicin by upregulating generation of H_2O_2 (Lim et al., 2008). As an antiproliferative protein with roles in cell cycle arrest and apoptosis, BTG2 may be a good candidate for further investigation.

Sulfatase 2 (SULF2) was upregulated by p53 in hypoxia. It encodes an extracellular heparin sulphate 6-O-endosulphatase that modulates binding of growth factors to their receptors by attenuating growth factor-mediated signalling. It has been shown to function as a tumour suppressor and has recently been identified as a p53 target gene (Adamsen et al., 2007, Chau et al., 2009). Sulphatase enzymes including SULF2 have been shown to inhibit myeloid tumour growth (Dai et al., 2005) though it is thought to have an oncogenic effect in a variety of other cancers (Lai et al., 2010a, Lai et al., 2010b, Lemjabbar-Alaoui et al., 2010). Due to its tumour suppressive effects, SULF2 may contribute to hypoxia-induced apoptosis.

Plekstrin homology-like domain family A member 3 (PHLDA3) is a p53 target gene involved in repression of AKT (Kawase et al., 2009). Our microarray data suggested that it was upregulated by p53 in hypoxia. PHLDA3 induces apoptosis through inhibiting translocation of AKT to the plasma membrane and therefore preventing its activation. AKT activation occurs through binding of phospholipids PIP_2 and PIP_3 at the plasma membrane through its plekstrin homology (PH) domain. PHLDA3 also possesses a PH domain and is therefore able to competitively bind to PIP_2 and PIP_3 to inhibit AKT activation (Kawase et al., 2009). This gene is a candidate for further investigation as it has a previously known role in apoptosis induction.

Inositol polyphosphate-5-phosphatase (INPP5D, also known as SHIP1) is also a negative regulator of AKT signalling and is known to be induced by p53 in response to cisplatin (Metzner et al., 2009, Kerley-Hamilton et al., 2005). INPP5D was shown to reduce

proliferation of CD34(+) cells from patients with acute myeloid leukaemia (Metzner et al., 2009). This gene was found to be induced by p53 in hypoxia and could be mediating hypoxia-induced apoptosis.

p53 is seen to upregulate a subset of genes in hypoxia which is in contrast with the literature suggesting that p53 is unable to bind coactivators and is not transcriptionally active under these conditions. However, regulation of transcription by p53 is complex and it is plausible that it could activate a specific set of genes in hypoxia. Bioinformatic analysis of the promoters of genes activated by p53 in hypoxia may reveal common features which allow them to be activated. Additionally p53 may bind to another coactivator at the promoters of certain genes allowing it to activate their transcription. siRNA knockdown of these genes in hypoxia followed by apoptosis and colony survival assays could reveal if they play a role in apoptosis. It may be the case that a number of genes work together to bring about apoptosis which may make the effects of individual genes more difficult to elucidate. Although the activated genes include known p53 targets, it may be that p53 is in fact repressing a miRNA in hypoxia which is then leading to increased expression of these genes. Bioinformatic analysis of miRNAs targeting these genes could prove to be informative.

4.3 p53 may activate transcription of miR-34a in hypoxia

Preliminary work has shown that miR-34a may be upregulated in hypoxia in a p53-dependent manner. In other systems, p53 transactivates miR-34a by direct binding to a response element in its promoter. miR-34a, itself a tumour suppressor that is frequently inactivated in human tumours, is able to inhibit cell growth and induce apoptosis when ectopically expressed in a p53 mutant glioma cell line (Luan et al., 2010) or prostate cancer cells (Fujita et al., 2008). Some of the tumour-suppressive effects of miR-34a are due to downregulation

of the histone deacetylase SIRT1. Repression of SIRT1 has been shown to occur in hypoxia (Lim et al., 2010) and so we investigated whether this occurred in our system and whether repression was p53-dependent. miR-34a has been shown to bind directly to SIRT1 through a binding site in the 3'UTR (Yamakuchi et al., 2008) which serves to downregulate the gene. This downregulation leads to an increase in acetylated p53 and therefore an increase in transcription of cell cycle arrest and pro-apoptotic genes (Yamakuchi et al., 2008). Inhibition of SIRT1 by miR-34a also inhibits endothelial progenitor cell angiogenesis and increases senescence at least in part through increasing levels of acetylated FoxO1 (Zhao et al., 2010). In our system, preliminary results do not suggest that SIRT1 is repressed in severe hypoxia in a p53 dependent manner. This is in accordance with previous data showing that p53 is not acetylated at lysine 382 in hypoxia, and does not recruit co-activators to the promoters of its target genes. Furthermore acetylation of p53 has been shown to be reduced at lysine 320 in hypoxia (Xenaki et al., 2008). Inhibition of histone deacetylases in hypoxia reduces transrepression and hypoxia-induced apoptosis (Koumenis et al., 2001) suggesting that the acetylation status of p53 in hypoxia is important for induction of apoptosis in hypoxia. If SIRT1 repression was increasing p53 acetylation in hypoxia we might expect p53 to play a more classical role, and thus we would see activation of known pro-apoptotic genes including Bax and PUMA.

4.4 Future work

Having established a possible link between serine 46 phosphorylation and hypoxia-induced apoptosis, possible future work would involve identifying the kinase that phosphorylates this residue in hypoxia. In addition it would be interesting to know the hypoxia-induced activating stimulus for the kinase. siRNA-mediated knockdown and/or pharmacological

inhibition of candidate kinases, followed by western blotting for phospho-serine 46 would be a good starting point, although problems with the available phospho-specific antibodies may hinder this approach. Once the kinase has been identified, the next step would be to look into the mechanisms by which it is regulated. Following on from this, as serine 46 phosphorylation is involved in transactivation of genes, it would be interesting to compare transcriptional responses to WT p53 versus S46A mutated p53 in severe hypoxia. This may give insight into the transcriptional programme brought about by this phosphorylation in hypoxia, and therefore the mechanism of apoptosis. Indeed, several p53-activated genes in hypoxia were identified by microarray analysis in this work. A good starting point would be to compare induction of these genes by WT p53 to that by S46A p53. A pro-apoptotic gene known to require serine 46 phosphorylation for activation is p53AIP1. This gene was not one of the p53-activated genes in the microarray data but it would be important to find out if expression of this gene is activated in hypoxia and if so, whether it is dependent upon phosphorylation at serine 46.

This work has identified a number of p53-activated genes in hypoxia. Future work could focus on elucidating the mechanism of their activation. ChIP experiments could be carried out to determine whether p53 is bound at the promoters, and if so whether it brings with it any coactivators. As p53 is also seen at the promoters of genes that it does not activate in hypoxia, it may also be necessary to make reporter constructs for these genes to see if they can be driven by hypoxic p53. siRNA-mediated knockdown and overexpression of these genes could be carried out followed by apoptosis and survival assays to determine their role, if any, in apoptosis. Perhaps the biggest question arising from this work is how p53 is able to activate some genes and not others in hypoxia. Bioinformatic analysis of the promoter could be informative here as well as a detailed analysis of p53 post-translational modifications and binding partners by mass spectrometry.

The data on miR-34a and SIRT1 is inconclusive and would need further validation. However, if it was the case that p53 activated miR-34a in hypoxia it is possible that this could also have a role to play in p53-induced apoptosis. The levels of known miR-34a targets could be investigated in hypoxia, to see if there are any obvious candidates for a role in apoptosis. We have looked at one known target, SIRT1 and preliminary work has suggested that it is not downregulated in hypoxia, though the results are unclear. If indeed SIRT1 proved to be downregulated in hypoxia it would be necessary to show that the downregulation of SIRT1 in hypoxia is mediated by miR-34a. This could be done by using anti-miR-34a to knockdown levels of this miRNA in hypoxia, followed by qRT-PCR and western blotting to see if the levels of SIRT1 change as a result. In addition, it would be necessary to show that inhibition of SIRT1 in hypoxia leads to apoptosis. As it is already known that SIRT1 knockdown leads to increased levels of acetylated p53, and therefore increased apoptosis, knocking down SIRT1 in normoxia and monitoring apoptosis would not be a valid approach. Additionally, as stress specific modifications of p53 are important for transactivation, knocking down SIRT1 without another cellular stress may not be sufficient to lead to apoptosis. Another approach may be to overexpress SIRT1 to reduce levels of apoptosis in hypoxia. However, it is possible that this may have a dominant negative effect, masking the real mechanism of hypoxia-induced apoptosis. One approach may be to create a stable knockdown of miR-34a, or to find a cell line that expresses low levels, and knockdown SIRT1 in an attempt to augment the apoptotic response.

5. Conclusions

We have shown that p53 is stabilised and phosphorylated in hypoxia, and that there are some differences between p53 modification in hypoxia and its modification following DNA damage. We have also shown that N-terminal phosphorylation at serines 20 and 46 are important, but not essential for induction of apoptosis by p53 in hypoxia. Strikingly we see that DNA binding of p53 is essential for hypoxia-induced apoptosis. We have seen, for the first time that p53 induces a subset of its targets in hypoxia, and these proteins do have previously reported roles in apoptosis. There is also the possibility that p53 could activate transcription of miRNAs in hypoxia, and that these could mediate some of its downstream effects through repression of genes. Additionally we have preliminary work to suggest that p53 is part of a complex in hypoxia. Elucidating the proteins present in this complex may prove important for understanding the mechanism of p53-induced apoptosis.

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Tables and Appendices

Table 1: Antibodies used in this study

Protein	Company	Species	Dilution
p53 (DO-1)	Santa Cruz Biotechnology	Mouse monoclonal	WB: 1:1000, IF: 1:500
p53 (C-19)	Santa Cruz Biotechnology	Goat polyclonal	WB: 1:1000
p-p53 (ser 6)	Cell Signalling	Rabbit polyclonal	WB: 1:1000 (IP first)
p-p53 (ser 9)	Cell Signalling	Rabbit polyclonal	WB: 1:1000 (IP first)
p-p53 (ser 15)	Cell Signalling	Rabbit polyclonal	WB: 1:1000
p-p53 (ser 20)	Cell Signalling	Rabbit polyclonal	WB: 1:1000 (IP first)
p-p53 (ser37)	Cell Signalling	Rabbit polyclonal	WB: 1:1000
p-p53 (ser 46)	Cell Signalling	Rabbit polyclonal	WB: 1:1000
p-p53 (ser 392)	Cell Signalling	Rabbit polyclonal	WB: 1:1000 (IP first)
HIF-1α	Becton Dickinson Biosciences	Mouse monoclonal	WB: 1:500
PARP1	Cell Signalling	Rabbit polyclonal	WB: 1:1000
B-Actin	Santa Cruz Biotechnology	Mouse monoclonal	WB 1:10,000
MCM6	Santa Cruz Biotechnology	Rabbit polyclonal	WB 1:1000
Alexa Fluor 680 anti-mouse IgG	Invitrogen	Goat	WB: 1:10000
Alexa Fluor 680 anti-rabbit IgG	Invitrogen	Goat	WB: 1:10000
Alexa Fluor 680 anti-rat IgG	Invitrogen	Goat	WB: 1:10000
Alexa Fluor 680 anti-goat IgG	Invitrogen	Rabbit	WB: 1:10000
Alexa Fluor 488 anti-mouse IgG	Invitrogen	Goat	IF: 1:250

Table 2: Primers used in this study

Primer Name	Sequence 5'-3'	Details
p53 F1	GTCAGATCCTAGCGTCGAG	Sequencing primer
p53 R1	CAAGTCACAGACTTGGCTG	Sequencing primer
p53 F2	CAGCTACGGTTTCCGTCTG	Sequencing primer
p53 R2	GTAGTGGATGGTGGTACAG	Sequencing primer
p53 F3	CGACATAGTGTGGTGGTGC	Sequencing primer
p53 R3	GCTCTCGGAACATCTCGAAG	Sequencing primer
p53 S46A sense	ATGGATGATTTGATGCTGGCCCCGGACGATATTGA	Mutagenesis primer
p53 S46A antisense	TCAATATCGTCCGGGGCCAGCATCAAATCATCCAT	Mutagenesis primer
p53 S20A sense	CCCTCTGAGTCAGGAAACATTTGCAGACCTATGGAAAC	Mutagenesis primer
p53 S20A antisense	GTTTCCATAGGTCTGCAAATGTTTCCTGACTCAGAGGG	Mutagenesis primer
BTG2 F1	TGGGCTTAGGGAACCATCTCT	qRT-PCR primer
BTG2 R1	TTCAGCCAAGGAATACATGCAA	qRT-PCR primer
INPP5D F1	GAGAGGAGGGAGCAGAAGGT	qRT-PCR primer
INPP5D R1	GCTTTCTGCTTGGTGTAGGC	qRT-PCR primer
PHLDA3 F1	GCGCCACATCTACTTCACG	qRT-PCR primer
PHLDA3 R1	GACCAGGCCTAGGGTGATCT	qRT-PCR primer
SULF2 F1	GACCCCTACCAGCTGATGAA	qRT-PCR primer
SULF2 R1	GCTTGTAACCCTTGCAGCTC	qRT-PCR primer

Table 3: Plasmids used in this study

Plasmid Name	Resistance Marker	Details
5xHRE hp53 neo	Amp	Hypoxia-inducible human p53
5xHRE hp53 S20A neo	Amp	Mutant hypoxia-inducible p53
5xHRE hp53 S46A neo	Amp	Mutant hypoxia-inducible p53
5xHRE hp53 R175H neo	Amp	DNA binding mutant hypoxia-inducible p53