An Investigation of Mutant p53 Function

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B. Science

B. Health Science (Hons)

Thesis submitted for the Degree of Doctor of Philosophy

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July 2011

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ABSTRACT

The *TP53* tumour suppressor gene is mutated in approximately 50% of all human cancers. The majority of these mutations are missense mutations resulting in the expression of a mutated form of the full-length p53 protein. This mutant protein exhibits a loss of tumour suppressive activity, dominant-negative activity to inactivate functional p53 and gain-of-function properties to drive tumour progression and metastasis. Investigation into mutant p53-mediated oncogenic pathways and the mechanisms through which they are controlled plays an integral role in identifying new therapeutic targets for a range of mutant p53-expressing tumours.

To model the initial events that occur in cancer following sporadic p53 mutation, an isogenic panel of cell lines was established in the p53 null, H1299 lung cancer cell line, expressing wild-type or various p53 hotspot mutants under the control of an inducible promoter. These cell lines were harnessed to investigate a range of wild-type and mutant p53 functions. The induced wild-type p53 protein is demonstrated to be transcriptionally and biologically active, and its function can be further mediated by DNA damaging agents or expression of regulatory proteins. Conversely, induced mutant p53 exhibits a loss of the majority of the normal wild-type transcriptional activity while mediating gain-of-function, oncogenic phenotypes in H1299 cells. This system is demonstrated to provide an important platform with which to investigate both wild-type and mutant p53 function.

Mutant p53 is reported to function as an aberrant transcription factor, re-programming the cellular transcriptome to enhance oncogenic pathways. The mechanisms

underlying this were specifically examined through expression microarray analysis, which identified a number of mutant p53-regulated targets. Surprisingly, these targets were predominately also direct targets of wild-type p53. A novel mechanism for mutant p53 activity is subsequently suggested, whereby mutant p53 is recruited to the DNA through its interaction with p63.

A key function of mutant p53 is its ability to drive tumourigenesis through the initiation of a range of oncogenic pathways. Through utilising the inducible system, mutant p53 is demonstrated to influence mitotic pathways, resulting in multinucleation, and enhance the invasive and migratory properties of cancer cells. Importantly, an endogenous protein, ANKRD11, is identified with the capacity to suppress the oncogenic properties of mutant p53 and provide a potential target for the development of new cancer therapeutics.

The role of mutant p53 in driving the invasive and metastatic potential of breast cancer cells was further explored and a relationship between mutant p53 and a micro-RNA (miR-155) established. Mutant p53 expression is shown to correlate with miR-155 expression, with miR-155 target genes involved in invasive pathways. ZNF652 is specifically identified as a target of miR-155 and loss of ZNF-652 is correlated with increased invasion and poor prognosis in breast cancer.

Collectively, these studies identify key mechanisms through which mutant p53 functions to enhance tumourigenesis and importantly identify novel targets, ANKRD11, miR-155 and ZNF652, for the development of cancer therapies.

DECLARATION

I, Jacqueline Elise Noll, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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LIST OF PUBLICATIONS

Noll JE, Jeffery J, Al-Ejeh F, Kumar R, Khanna KK, Callen DF and Neilsen PM. (2011) Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11. *Oncogene.* Submitted. Second Revision.

Muller PAJ, Trinidad AG, Timpson P, Morton J, Nixon C, Karim S, Caswell P, **Noll JE**, Coffill CR, Lane DP, Sansom O, Neilsen PM, Norman JC and Vousden KH. (2011) Mutant p53 induces c-Met signalling to drive cell scattering and invasion by inhibiting TAp63 and Dicer. *Nature Cell Biology.* Submitted, Second Revision

Chee JLY, Saidin S, Lane DP, Leong SM, Phua YT, **Noll JE**, Neilsen PM, Gabra H and Lim TM. (2011) Wild type and mutant p53 mediate cisplatin resistance through interaction and inhibition of caspase-9. *Carcinogenesis.* Submitted

Neilsen PM*, **Noll JE***, Tay BS, Bracken C, Schulz R, Lim S, Gregory P, Kumar R, Goodall G and Callen DF. (2011) Mutant p53 drives invasion in breast tumors through a pathway involving miR-155 and ZNF652. *Text in Manuscript*

* These authors contributed equally to this work

ABBREVIATIONS

ANK – Ankyrin

ANKRD11 – Ankyrin repeat domain 11

BCA – Bicinchoninic acid

CBP – CREB binding protein

CIP – Calf intestinal phosphatase

cDNA – Complimentary DNA

ChIP – Chromatin immunoprecipitation

DBD – DNA binding domain

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DN – Dominant negative

DNA – Deoxyribonucleic acid

ECL – Enhanced Chemiluminescence

EDTA – Ethylenediaminetetraacetic acid

EI – Ecdysone inducible

EMT – Epithelial to mesenchymal transition

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

GFP – Green fluorescent protein

GOF – Gain of function

HRP – Horseradish peroxidase

LOF – Loss of function

MDM2 – Murine double minute 2

mRNA – Messenger RNA

ORF – Open reading frame

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PonA – Ponasterone A

P/CAF – p300/CBP associated factor

RE – Response element

RNA – Ribonucleic acid

RT – Room temperature

RT-PCR – Reverse transcription PCR

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

shRNA – Short hairpin RNA

SNP – Single nucleotide polymorphism

ssDNA – Salmon sperm DNA

SV40 – Simian virus 40

UTR – Untranslated region

ACKNOWLEDGEMENTS

To my supervisors – Prof. David Callen, Dr. Paul Neilsen and Dr. Raman Sharma. Thank you for providing me with such a wonderful opportunity and for guiding me along the way: to study, to learn and to develop my skills and understanding.

To the members of the Cancer Therapeutics Laboratory/Breast Cancer Genetics Group – Thank you for providing such a fun and supportive atmosphere to work in. I would particularly like to thank Renee; no matter where I was at you were there – to lift my spirits or to celebrate with me. Your support and friendship over the past few years has been immensely important to me and I will never forget it.

To my friends and family – Thank you for always showing an interest in what I was doing, even when you didn't understand it. And most importantly thank you for believing in me and my abilities, even when I doubted myself.

To my amazing husband, Ben – Thank you for your constant love and support. I know I can achieve anything while I have you by my side. Your continuing and never-ending positive outlook on life helped me focus my attention to where it mattered most. Whether I needed a shoulder to cry on or someone to celebrate with you were there, and knowing that made all the difference. I couldn't have made it through without you. Love you, babe.

CHAPTER 1 – Introduction

1.1 Tumour suppressor protein: p53

The p53 protein is encoded by the *TP53* gene, located on the short arm of chromosome 17. In 1979, p53 was identified as a proto-oncogene due to its association with the SV40 large T-antigen, which is involved in cellular transformation (Lane and Crawford 1979, Linzer and Levine 1979). Furthermore, high levels of p53 mRNA were detected in transformed, but not normal cells, indicating its expression may promote cellular transformation (DeLeo et al 1979, Linzer and Levine 1979). However, after a decade of research, p53 was re-classified as a potential tumour suppressor protein as this aberrantly expressed p53 was shown to contain specific mutations within highly conserved regions of the protein (Baker et al 1989, Nigro et al 1989). Loss-of-heterozygosity of 17p, encompassing the *TP53* gene, is found in tumours, and provides additional evidence for p53 as a tumour suppressor (Kaelbling et al 1992, Shimizu et al 1993). These findings initiated intensive research centred on p53, its cancer-associated mutations, and their role in tumour development. The p53 tumour suppressor is now described as the "guardian of the genome" (Lane 1992) due to the critical importance of its function as a DNA sequence specific transcription factor which regulates the transcription of genes involved in a variety of tumour suppressor pathways, specifically growth arrest, DNA repair and apoptosis (see Figure 1.1). The normal function of p53 is as a central monitoring point for DNA damage and cellular insults to prevent the onset and progression of tumorigenesis.

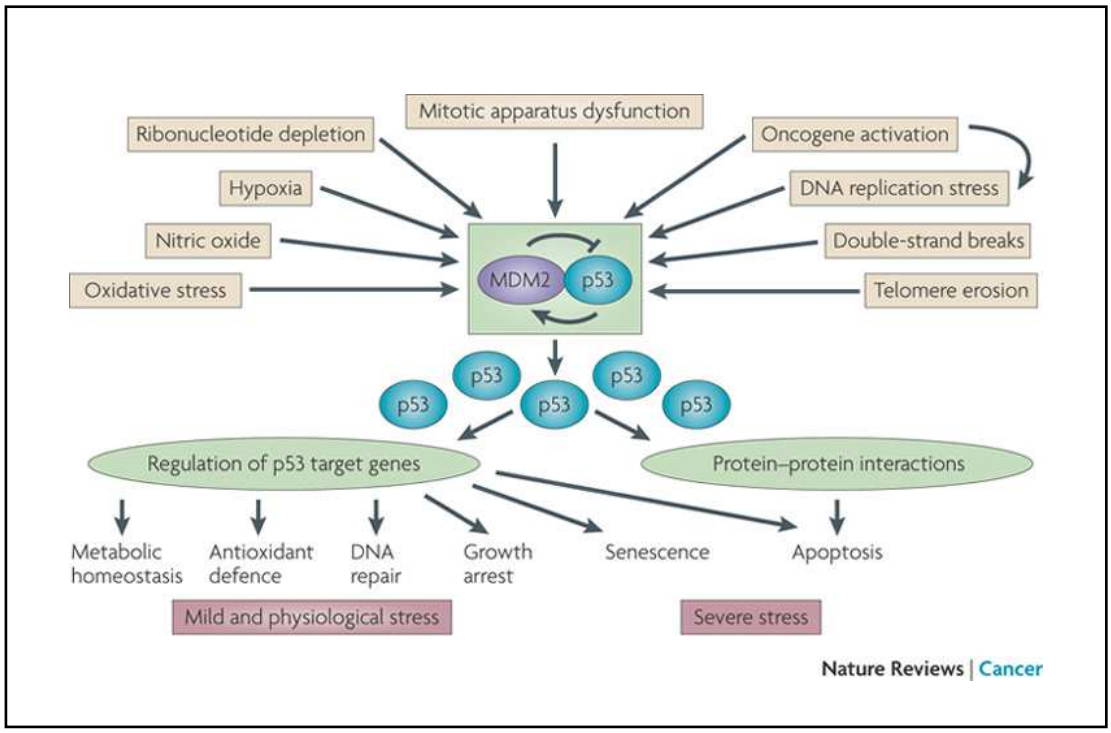


Figure 1.1 – Activation of p53 regulated pathways.

Schematic of the activation signals of p53 and subsequent pathways regulated by activated p53 (Levine and Oren 2009).

1.1.1 Regulation of p53

The regulation of p53 involves cross-talk between a complex network of signalling pathways and regulatory proteins. To protect against cellular damage and prevent the onset of tumorigenesis it is vitally important that the p53 protein is tightly regulated. Below are some descriptions of various regulatory mechanisms in place that control p53 expression and activity.

1.1.1.1 Negative regulation

1.1.1.1.1 MDM2

The p53 protein has a short half-life, with low protein levels maintained in unstressed cells through modulation by negative regulators, thus preventing the onset of apoptosis or cell-cycle arrest in healthy cells (Harris and Levine 2005). The most prominent negative regulator of p53 is MDM2. MDM2 was reported to interact with the N-terminal, transactivation domain of p53 (Haupt et al 1997), although a recent study has found that the C-terminal domain of p53 is also involved in the interaction with MDM2 (Poyurovsky et al 2010). MDM2 is an E3 ubiquitin ligase, which covalently links multiple ubiquitin moietes to the C-terminus of the p53 protein, leading to its subsequent proteasome-mediated degradation (Oren 2003). *MDM2* itself is also a p53 target gene, therefore p53-mediated regulation of *MDM2* expression results in a negative feedback loop that maintains relatively low levels of p53 protein in the cell (Wu et al 1993).

While poly-ubiquitination at the C-terminus leads to degradation of the p53 protein, mono-ubiquitination has been shown to result in nuclear export of p53 (Brooks et al 2004). Furthermore, MDM2 has been shown to associate with p53 at the promoter of p53 target genes and inhibit its transcriptional activity (Arva et al 2005). It is therefore clear that MDM2 can function at multiple levels to negatively regulate p53 function and activity.

Importantly, MDM2 is shown to be amplified in >10% of human cancers (as reviewed by Toledo and Wahl 2006). This amplification generally occurs in tumours in which

wild-type p53 is expressed, presenting an interesting challenge for the development of cancer therapies (as discussed in Section 1.3.1).

1.1.1.1.2 MDM4

MDM4 (also called MDMX) was identified as a binding partner of p53 which had significant sequence homology to MDM2 (Shvarts et al 1996). MDM4 also negatively regulates p53 (Finch 2002, Parant 2001), but in a manner quite different from MDM2. While MDM2 functions as a ubiquitin ligase to target the p53 protein for degradation, MDM4 enhances the stability of p53 and prevents p53 dependent transcription through the inhibition of the p300/CBP-mediated acetylation of the p53 C-terminus (Migliorini et al 2002, Stad et al 2001, Sabbatini and McCormick 2002). Similarly to MDM2, MDM4 amplification is demonstrated to contribute to the onset of tumorigenesis and has been shown to be amplified in tumours with wild-type p53 (Danovi et al 2004, Toledo and Wahl 2006).

1.1.1.2 Activation of p53

Stabilisation and activation of p53 occurs in response to various cellular stresses including DNA damage, abnormal growth signals from oncogenic proteins, low extracellular pH, hypoxia and chemical oxidants (Liu and Chen 2006, Vogelstein et al 2000). For example, in response to oncogene-induced stress, $p14^{ARF}$ binds MDM2, resulting in direct inhibition of the p53-MDM2 interaction and subsequent p53 stabilisation (Ryan et al 2001) (see Figure 1.2). However, modulation of p53 activity occurs primarily through post-translational modifications of the p53 protein (Harris and Levine 2005, Liu and Chen 2006). These involve many sites of the protein and include phosphorylation, acetylation, ubiquitylation, sumoylation, methylation and

neddylation (Bode and Dong 2004, Feng et al 2005a). Acetyl transferase proteins such as p300/CBP and P/CAF specifically function to enhance the DNA binding and transcriptional activity of p53 through increased acetylation of the p53 protein, which in turn leads to an increased efficacy of p53-mediated transactivation of target genes (Avantaggiati et al 1997, Liu et al 1999). The specific pattern of post-translational modifications, as well as cell type and the nature of the cellular stress, is believed to be critical in determining activation of downstream p53 pathways (Appella and Anderson 2001).

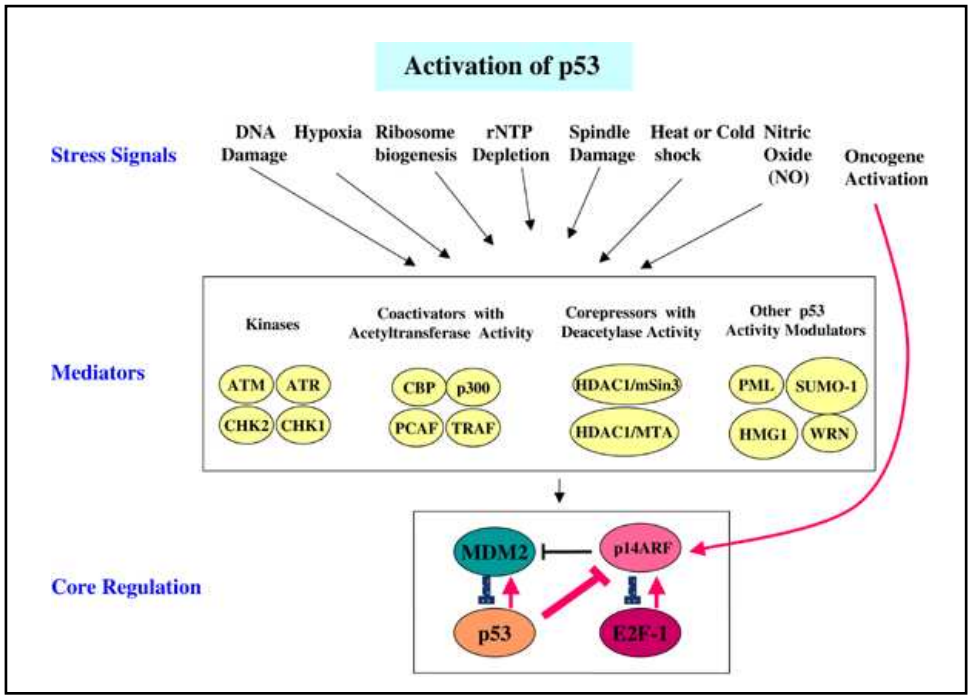


Figure 1.2 – Activation of p53.

Schematic of activation signals and mediator proteins that are involved in regulating p53 activity (Harris and Levine 2005).

1.1.2 Regulation of p53 target genes

The three major biological outcomes of p53 activation are cell cycle arrest, apoptosis and senescence. However, p53 also activates a range of genes involved in other pathways including: the regulation of the extracellular matrix and cellular cytoskeleton (Riley et al 2008), DNA repair mechanisms (Smith et al 1994, Tanaka et al 2000), metabolism (Vousden and Ryan 2009), cellular migration and invasion (Moskovits et al 2006, Muller et al 2011, Roger et al 2006, Wang et al 2009) and autophagy (Feng et al 2005b, Tasdemir et al 2008).

Common p53 regulated genes include: *CDKN1A*, encoding the p21 protein – involved in regulating cell cycle progression through G1 (el-Deiry et al 1993), *GADD45* – involved in cell cycle arrest after DNA damage (Kastan et al 1992), *BAX* – which inhibits the apoptosis-suppressing gene *BCL2* resulting in a pro-apoptotic response (Miyashita et al 1994) and *MDM2* – a negative regulator of p53 (Wu et al 1993).

1.1.2.1 Consensus p53 response elements

The wild-type p53 protein binds directly to DNA as a tetramer at a well-defined consensus motif (5' RRRCWWGYYY 3' where R = purine, Y = pyrimidine and W = A or T) known as the p53 response element (p53-RE). The p53-RE invariably consists of two copies of this motif separated by a linker of 0-13 base pairs, and it has been demonstrated that a single copy of the motif is insufficient for p53 binding (el-Deiry et al 1992, Funk et al 1992). A recent study by Riley *et al.* has compiled a list of 129 target genes of wild-type p53. The genes on this list meet at least 3 of 4 selection criteria: (1) a putative p53-RE is located close to, or within, the gene; (2) the gene (at mRNA or protein level) is up- or down-regulated by activated wild-type p53; (3) the

p53-RE cloned into a reporter assay shows direct control of activity by p53; and (4) p53 protein binds directly to the reported p53-RE (Riley et al 2008). Of these identified p53 target genes, approximately 50% contain a p53-RE within the 5' promoter region of the gene upstream of the transcription initiation site, making this classical promoter region the most common location of p53-REs. However, 25% of genes contain a validated p53-RE within the first intron, while the remainder of genes have a p53-RE located elsewhere within the gene, though most frequently within the 5' and 3' UTR regions (Riley et al 2008).

1.1.2.2 Identifying p53 regulated genes

Due to the importance of wild-type p53 in tumour suppression, the identification of novel targets directly regulated by p53 provide a means of defining important pathways that may be de-regulated in tumour growth and development. Recently there have been significant advances in identifying novel p53 regulated genes, using methods including chromatin immunoprecipitation (ChIP) and expression microarray analysis. ChIP assays involve the immunoprecipitation of chromatin, with its bound and associated proteins immobilised by cross-linking. Through the use of specific antibodies that detect the protein of interest, such as a transcription factor, the regions of DNA that can bind the protein can be identified. A p53 ChIP followed by PET (paired-end ditag) sequencing in the HCT116 colon carcinoma cell line (expressing wild-type p53), identified 542 p53 binding sites, 98 of which were previously unidentified (Wei et al 2006). It has however become clear in a number of studies that binding of p53 to a p53-RE is not necessarily sufficient for its activation (Riley et al 2008). Furthermore, the binding of p53 to the promoters of target genes may also be tissue or cell line specific and dependent on the precise mechanism of p53 activation.

Therefore, this study is incomplete in its goal of identifying all functional p53-REs and the corresponding target genes. It is necessary to confirm the functionality of the p53-REs identified through ChIP-PET sequencing using direct methods, including direct ChIP, promoter reporter assays and investigation of specific gene regulation by p53.

Another method used to identify p53 targets is by expression microarray analysis to determine changes in gene expression using RNA extracted from cells where p53 is induced following exposure to stress or by synthetic induction of p53 (Zhao et al 2000). The use of various stress signals to activate p53 is flawed in that the induction of DNA damage, in addition to activating p53, will also activate related pathways and genes that are independent of p53. For this reason, the use of a p53 inducible system would be an ideal system to identify genes whose expression is directly regulated by p53. However, through the use of an inducible system, the expressed p53 protein may not be modified, therefore to comprehensively confirm target genes of p53 multiple methods of p53 activation and expression are required. Furthermore, these targets should be confirmed by direct ChIP analyses and investigation in a variety of cell lines to discount cell line specific results.

1.2. Mutations of p53

Approximately 50% of all human cancers contain a mutation within the *TP53* gene, while in cancers retaining a wild-type p53 the protein is frequently inactivated (Hollstein et al 1991, Vogelstein et al 2000). This high rate of mutation and inactivation emphasises the importance of p53 in tumour suppression.

Approximately 95% of p53 mutations occur within the core DNA binding domain (DBD) of the protein (Bullock and Fersht 2001), highlighting the importance of the DNA binding function of p53 in its tumour suppressor role. Interestingly, 75% of these mutations are missense mutations that result in a single amino acid substitution within the full-length protein. This is in contrast to the majority of tumour suppressor protein mutations that typically exhibit deletions, frame-shifts or truncations (Olivier et al 2010, Petitjean et al 2007). In recent years the International Agency for Research on Cancer (IARC) have released recommendations for the detection of *TP53* mutations, advising direct sequencing of exons 4 to 10 of the *TP53* gene, which includes the entire coding region of p53 (Petitjean et al 2007). The reported extremely high incidence of mutations detected within the DNA binding domain of p53 may be biased due to the previous methods of sequencing which did not always incorporate the entire p53 coding region but rather focussed around the DNA binding domain.

1.2.1 p53 "Hotspot" Mutants

Within the DNA binding domain, six specific residues have been identified as mutational "hotspots" (see Figure 1.3). Eight different mutations at these six sites account for approximately 25% of all p53 cancer mutations (Petitjean et al 2007). These mutations can be classified as either DNA contact or structural mutants (Cho et al 1994).

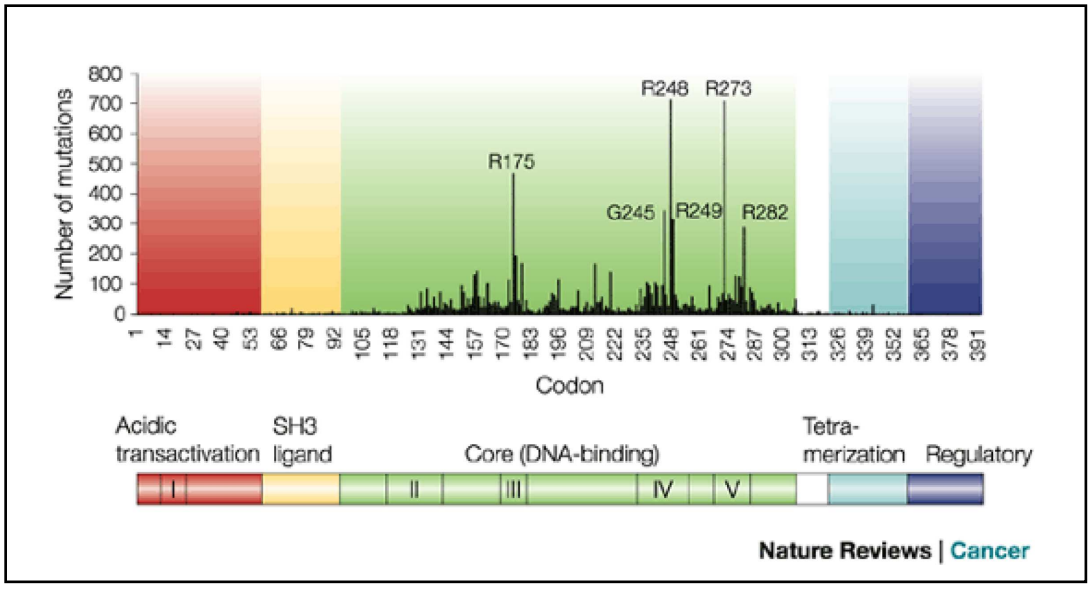


Figure 1.3 – p53 mutational hotspots

Schematic representing the number of missense mutations found in p53. Enrichment for mutations is seen within the core DNA binding domain, with six "hotspot" sites over-represented (Bullock and Fersht 2001).

The DNA contact mutants (R248Q, R248W, R273H and R273C) involve residues that directly contact the DNA, adversely affecting the proteins ability to bind DNA. Alteration of these residues however does not notably change the physical structure or stability of the DBD *in vitro* (Bullock et al 2000, Joerger et al 2006). The wild-type p53 DBD has a relatively low melting temperature, therefore any destabilising mutation can have a profound effect on the overall structure and folding of the DBD. The structural mutants (R175H, G245S, R249S and R282W) have alterations to residues that are crucial in maintaining the structural integrity of the DBD, resulting in an unstable protein (Bullock and Fersht 2001, Cho et al 1994). The degree of protein stability, however, does differ between the mutant p53 proteins. The R175H and R282W mutants are globally distorted (Brosh and Rotter 2009) in comparison to G245S and R249S which exhibit only localised changes in core domain structure in *in*

vitro assays (Wong et al 1999). Interestingly, the DNA contact mutants R248Q and R273H were shown to be distorted to a similar degree to that observed for the structural mutants G245S and R249S (Wong et al 1999), which is further confirmed as the R273H protein endogenously expressed in cell lines can be immunoprecipitated with the mutant specific antibody, PAb240 (Milner et al 1987, Muller et al 2008). Therefore, the historical grouping of p53 mutants into "DNA contact" and "structural" classes may not be a valid way of identifying and studying p53 mutant activity.

1.2.2 Mutant p53 loss-of-function and dominant-negative function

Mutant p53 proteins are unable to directly bind canonical wild-type p53 binding sites (Bullock and Fersht 2001, Joerger et al 2006). For example, the R273H mutant exhibits ~1000-fold decrease in binding to a specific p53 response element compared to wild-type p53 (Ang et al 2006). This loss of DNA binding results in a subsequent loss of transactivation activity on common p53 target genes. This phenomenon is commonly referred to as a "loss-of-function" (LOF) as the wild-type protein loses the ability to function as a tumour suppressor through its transactivation activity. Furthermore, mutant p53 has been shown to function in a dominant-negative manner with wild-type p53, as a non-functional mutant p53 monomer within the active p53 tetramer reduces its ability to bind the p53-RE within its target promoters and subsequently induce cell cycle arrest (Willis et al 2004).

It is interesting to note that one of the most common mechanisms to investigate mutant p53 function is through the re-expression of mutant p53 in a p53 null background. This can bias results towards identifying gain-of-function (GOF) properties of mutant p53 (discussed in detail below). Studies are yet to determine

whether the LOF, dominant-negative or GOF properties of mutant p53 impact the most on its ability to drive tumour formation and progression, or whether it is in fact a combination of these three phenomenon that provide mutant p53 with its power to enhance tumorigenesis.

1.2.3 Mutant p53 gain-of-function

A number of studies have shown both *in vitro* and *in vivo* that mutant p53 expression can lead to the enhanced growth of tumour cells, both in the presence and absence of wild-type p53. Specifically, it has been demonstrated through xenograft studies in mice that the re-expression of a p53 hotspot mutant in either a p53 null or wild-type p53 cell line results in increased tumour growth (Dittmer et al 1993, Khromova et al 2009). A transgenic mouse model co-expressing the R270H mutant (equivalent to human R273H) and the SV40 T-antigen, to inactivate wild-type p53, also results in increased invasion and a higher frequency of metastases (Heinlein et al 2008). Furthermore, knockdown of endogenous mutant p53 in cell lines leads to decreased cellular proliferation and decreased tumour formation in mouse models (Bossi et al 2006, Bossi et al 2008). These findings indicate that tumorigenesis is driven not just by a loss of normal p53 function, but also by a GOF associated with the presence of mutant p53 (see Figure 1.4). The high levels of mutant p53 expression in tumour cells has been associated with the acquisition of new oncogenic properties such as increased proliferation, drug resistance, angiogenesis, inhibition of apoptosis, genetic instability, invasion and metastasis (Brosh and Rotter 2009, Oren and Rotter 2010).

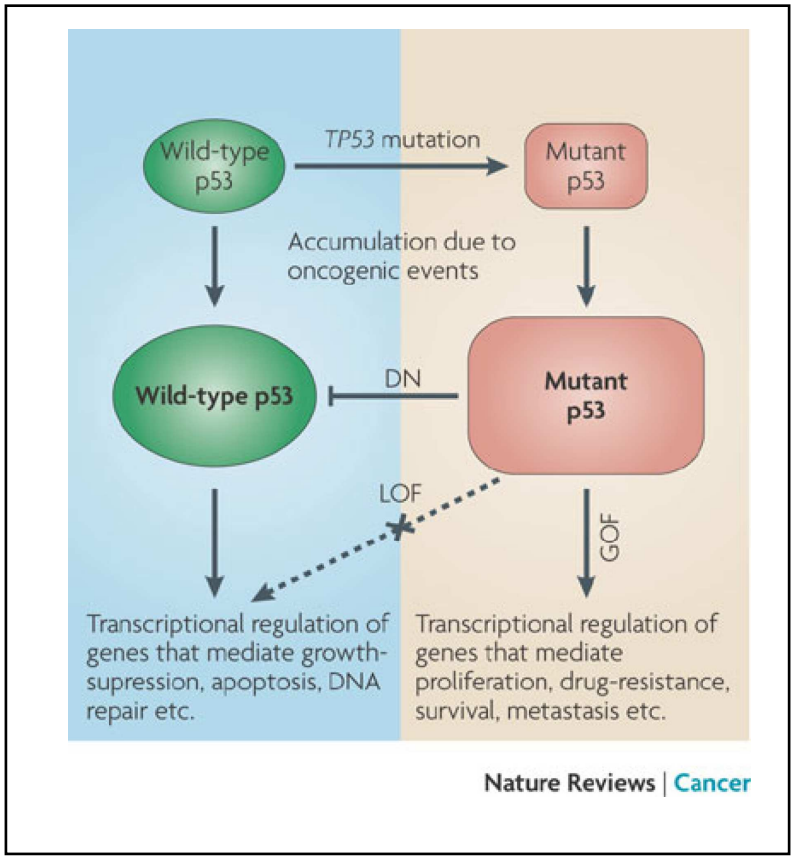


Figure 1.4 – Mutant p53 mode of action with WT p53

Schematic of the mechanisms through which mutant p53 may function with or independently of wild-type p53 in tumorigenesis (Brosh and Rotter 2009)

1.2.3.1 Mutant p53 transcriptional activity

To-date, no consensus binding site for mutant p53 has been established, however mutant p53 has been demonstrated to selectively bind to particular DNA structures in oligonucleotide binding assays (Gohler et al 2005, Kim and Deppert 2004) indicating that the loss of binding to canonical p53 binding sites does not necessarily indicate a complete loss of DNA binding ability. Furthermore, mutations within the N-terminal transactivation domain of mutant p53 prevent mutant p53 from interfering with chemotherapy induced apoptosis and other GOF pathways (Matas et al 2001, Yan and Chen 2010), indicating that the transactivation activity of mutant p53 is likely to be

important in modulating its GOF pathways. This however does not discount the importance of other regions of the p53 protein in driving oncogenic pathways, as the C-terminus of p53 has been shown to be required for the invasive GOF of p53 null H1299 cells with stable expression of p53 mutants (Muller et al 2009).

Various mechanisms have been harnessed to identify genes up-regulated by mutant p53. These have largely involved microarray analysis from cell lines stably over-expressing different p53 mutants. For example, microarray analysis of three common p53 mutants (R175H, R273H and D281G) over-expressed in the p53 null H1299 cell line show that mutant p53 expression leads to an increase in the expression of genes commonly involved in growth promoting pathways, and this expression is specifically reliant on mutant p53 transactivation function (Scian et al 2004a, Scian et al 2004b). Similar expression microarray studies on p53 null cell lines and their mutant p53 expressing derivatives have identified *EGR1* (Weisz et al 2004) and *NFκB2* (Scian et al 2005) as potential mutant p53 target genes. *NFκB2* expression was subsequently shown to be constitutively activated by mutant p53 in circumstances of chronic inflammation (Weisz et al 2007). While the precise mechanism through which mutant p53 activates these genes is unknown, mutant p53 has been shown to associate with the *EGR1* promoter (Weisz et al 2004), though it has not yet been determined if this represents a direct interaction with DNA.

A microarray analysis on the wild-type p53 expressing LNCaP prostate cancer cells stably expressing G245S, R248W, R273C or R273H p53 mutants also identified a list of 95 genes that were differentially regulated (compared to a vector control) following androgen ablation in these cells (Tepper et al 2005). The information gathered from

this study provides an insight into how wild-type p53 activity can be influenced by mutant p53 expression. The genes identified in this study may represent both true GOF targets of mutant p53, as well as de-regulated targets of wild-type p53.

1.2.3.2 Mutant p53 and transcription factors

A number of studies have shown transcriptional regulation by mutant p53 requires interaction with other transcription factors. It is suggested that mutant p53 does not bind directly to DNA, but rather "piggy-backs" these transcription factors on their specific response elements, resulting in increased activation of target genes. The Ets-1 transcription factor has been shown to synergise with mutant p53 to transactivate the multiple drug resistance gene, *MDR1*. This activation was shown to require an Ets binding site in the promoter region of the gene (Sampath et al 2001). Furthermore, a mutant p53/E2F1 complex has been identified on the promoter of *ID4*, resulting in up-regulation of this gene involved in cell proliferation and tumorigenesis (Fontemaggi et al 2009). A number of NF-Y target genes are also up-regulated by mutant p53 in response to DNA damage due to the binding of a mutant p53/NF-Y complex within the promoter (Di Agostino et al 2006). These studies indicate that the interaction between mutant p53 and particular transcription factors is likely to contribute significantly to the aberrant transcriptional regulation observed in mutant p53 expressing cells. Targeting these interactions may be a novel method of inhibiting the induction of growth promoting genes by mutant p53.

1.2.3.3 Mutant p53 and the p53 family members, p63 and p73

Family members of the p53 protein, p73 and p63, were identified in 1997 and 1998 respectively (Kaghad et al 1997, Osada et al 1998, Yang et al 1998). The p53, p63 and

p73 proteins have very similar domain structures and share a high sequence homology within their DNA binding domains (Harms and Chen 2006). Both p63 and p73 also function as transcription factors and are able to transactivate a number of p53 target genes, as well as an independent group of target genes (Harms et al 2004). Furthermore, despite mutations in p63 and p73 being relatively rare, both proteins have been implicated in tumour suppression pathways. Mice heterozygous for *p63* and *p73* are predisposed to spontaneous tumour development. However, mice heterozygous for *p63* or *p73*, in combination with *p53* heterozygosity, exhibit a greatly increased tumour burden and rate of metastases compared to that seen in the presence of p53 heterozygosity alone (Flores et al 2005).

Specifically, p63 is published to play a role in the suppression of invasion and metastasis (Su et al 2010, Urist et al 2002) and p73 expression is required to maintain chemo-sensitivity in cell lines (Bergamaschi et al 2003, Irwin et al 2003, Lunghi et al 2009). Mechanistically, mutant, but not wild-type, p53 is reported to interact with both p63 and p73, subsequently inhibiting their transactivation functions and contributing to tumorigenesis and resistance to chemotherapies (Di Como et al 1999, Strano et al 2000, Strano et al 2002).

1.2.3.3.1 Targeting the interaction between mutant p53 and its family members

The mutant p53•p73 interaction is specific to cancer cells and therefore provides an ideal therapeutic target for the development of agents to prevent chemo-resistance. Peptides, referred to as SIMPs (**S**hort **I**nterfering **M**utant p53 **P**eptides) have been described which inhibit the mutant p53•p73 interaction and subsequently increase the cellular response to chemotherapeutic agents such as adriamycin and cisplatin (Di

Agostino et al 2008). Similarly a small molecule called RETRA (**R**eactivation of **T**ranscriptional **R**eporter **A**ctivity) has been demonstrated to increase p53 transcriptional activity in mutant p53 expressing cells in a p73-dependent manner, resulting in growth suppression of these cells (Kravchenko et al 2008). To-date no agent has been described to target the mutant p53•p63 interaction. However the above examples provide proof-of-principle for the future identification of peptides or small molecules that may function by targeting the GOF interactions of mutant p53 with transcription factors.

1.2.3.4 Mutant p53 in invasion and metastasis

Transgenic mouse models have shown that although p53 knockout mice exhibit an increased tumour burden, these p53 null tumours are infrequently invasive and metastatic (Attardi and Jacks 1999), while the expression of mutant p53 results in an increased frequency of tumour metastases (Duan et al 2002, Heinlein et al 2008, Morton et al 2010). These studies indicate that the presence of mutant p53 drives an increasingly aggressive tumour phenotype. Indeed, mutant p53 expression in non-small cell lung carcinoma (NSCLC) cases is seen to be associated with poor metastasis-free survival (Wang et al 2009). Wild-type p53 suppresses cellular migration and invasion (Moskovits et al 2006), therefore the mutation of p53 may result in a LOF or dominant-negative effect to drive cancer cell invasion and subsequent metastasis. However, the re-expression of mutant p53 in a p53 null background also drives invasion (Adorno et al 2009, Muller et al 2009), suggesting that the GOF properties of mutant p53 are sufficient to drive the invasive phenotype.

Metastasis requires the physical translocation of a cancer cell from the primary tumour, followed by colonisation of this tumour cell at a secondary site or organ (Chaffer and Weinberg 2011). Epithelial-mesenchymal transition (EMT) is suggested to be the process through which cancer cells are able to spread from their primary sites, through the loss of tight cell-cell junctions and cell polarity and the acquisition of migratory and invasive properties (Thiery et al 2009). Wild-type p53 has been shown to function to suppress EMT and associated invasion through enhancing E-cadherin expression, required for cell-cell adhesion (Wang et al 2009), and opposing the pro-migratory function of transcription factors such as Twist (Shiota et al 2008). Furthermore, a recent study has identified miR-200c as a direct transcriptional target of p53, which functions to suppress the expression of genes that mediate EMT (Chang, 2011). Loss of regulation by wild-type p53 therefore results in an inability to suppress EMT and subsequent cancer cell invasion. In addition to the loss of wild-type p53-mediated regulation, mutant p53 can also function as a transcription factor that enhances the invasive nature of cancer cells through the aberrant regulation of genes involved in cell growth, migration and invasion (Muller et al 2011). Furthermore, loss of p63 expression in cell lines leads to enhanced invasion, phenocopying mutant p53 expression (Muller et al 2009), and it has been demonstrated that the mutant p53-mediated inhibition of p63 function promotes cancer cell invasion (Adorno et al 2009, Muller et al 2009). Specifically, p63 has been shown to suppress tumorigenesis by inhibiting metastatic pathways through the activation of genes *Sharp-1* and *Cyclin G2* (Adorno et al 2009) as well as the regulation of Dicer and downstream microRNAs (Su et al 2010). The mutant p53•p63 interaction therefore represents an attractive therapeutic target to specifically inhibit

cancer cell metastasis through the restoration of p63 activity, although no molecules to target this interaction have yet been described.

1.3. Targeting p53 in Cancer Therapies

The high incidence of p53 mutations in cancer which are associated with increasingly aggressive tumours, accompanied by the observation that mutant p53 proteins are selectively expressed at high levels in tumours (Goh et al 2011, Terzian et al 2008) provides an attractive target for the design of new therapies specific to mutant p53 expressing tumours, aiming to restore a normal function to the mutant p53 protein. Since the *TP53* gene is mutated in only 50% of cancers, those tumours expressing wild-type p53 will require alternative strategies. Such p53 targeted strategies will also require that the *TP53* mutational status be determined for each tumour.

1.3.1 Reactivation of wild-type p53 in tumours

Wild-type p53 can be inactivated in tumours via a number of mechanisms including the amplification of negative regulators (i.e. MDM2) and deletion of positive regulators (i.e. $p14^{ARF}$) (Brown et al 2011, Vogelstein and Kinzler 2001). A summary of small molecule activators of wild-type p53, their mechanism of action and the stage of action in the clinic is presented in Table 1.1 below.

1.3.2 Mutant p53 rescue

Over the past decade there has been a major focus on developing cancer treatments to reactivate mutant p53. The restoration of normal p53 functions would have drastic consequences in tumour cells expressing mutant p53, resulting in the induction of apoptosis and regression of the tumour. There have been a number of attempts to

achieve the restoration of tumour suppressive activity of p53 mutants, including the introduction of second site mutations in p53, binding of specific p53 mutant antibodies and delivery of short peptides or chemical compounds (reviewed by (Brown et al 2011, Selivanova and Wiman 2007, Wiman 2006)). The peptides and small molecules that have been identified to have some function in restoring activity to p53 mutants are listed in Table 1.2 and are discussed below.

Table 1.1 – Chemical structures and proposed mechanisms of small molecule p53 activators (Neilsen et al 2011)

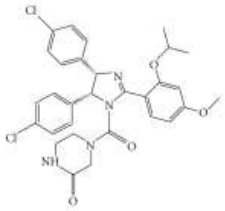
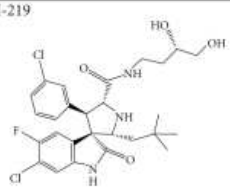
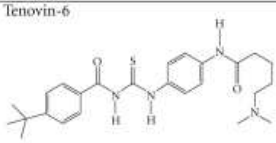
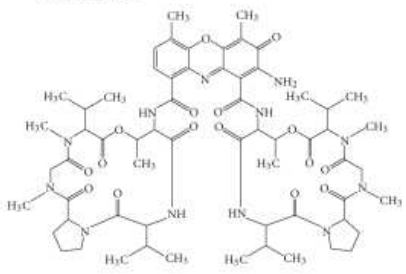
Compound	Molecular formula	MWT	Mechanism	Stage in clinical testing
Nutlin-3a 	$C_{30}H_{30}Cl_2N_4O_4$	581.5	Binds to MDM2 and inhibits p53-MDM2 interaction	Phase I
MI-219 	$C_{27}H_{32}Cl_2FN_3O_4$	552.5	Binds to MDM2 and inhibits p53-MDM2 interaction	Phase I
RITA 	$C_{14}H_{12}O_3S_2$	292.4	Binds to p53 and inhibits p53-MDM2 interaction	Preclinical
Tenovin-6 	$C_{25}H_{34}N_4O_2S$	454.6	SIRT1 and SIRT2 inhibition	Preclinical
Actinomycin D 	$C_{62}H_{86}N_{12}O_{16}$	1255.5	RPL11 and RPL5 release	Clinically approved

Table 1.2 – Restoration of mutant p53 activity by small molecules and peptides

Molecule	Ligand Type	p53 Mutants Effective Against	Method of Idenitification	Reference
CBD3	Peptide (REDEDEIEW-NH2)	R249, G245, I195	Rational design	Friedler (2002)
CP31398	Small molecule	R173, R249	*in vitro* assay of mutant p53 unfolding	Foster (1999)
PRIMA-1	Small molecule	R273, R175	Cell based	Bykov (2002)
PhiKan083	Small molecule	Y220	Virtual Screening	Boeckler (2008)
SCH529074	Small molecule	R273, R249, R175, R248, S241	DNA-binding assay	Demma (2010)

1.3.2.1 PRIMA-1/APR-246

PRIMA-1 (**p**53 **R**eactivation and **I**nduction of **M**assive **A**poptosis) was identified in 2002 as a chemical compound that suppresses human tumour cell growth in mutant p53 expressing cells, both *in vitro* and in mouse xenograft models (Bykov et al 2002, Bykov et al 2003, Li et al 2005). Treatment of cells with PRIMA-1 stimulated the specific DNA binding of multiple p53 mutants and resulted in the activation of the endogenous p53 target genes *MDM2*, *CDKN1A* and *PUMA* (Bykov et al 2002, Bykov et al 2003), thereby providing a plausible mechanism for its observed tumour suppressive function. This effect is possibly due to the partial restoration of the native conformation to the DBD of the mutant p53 proteins. This was specifically demonstrated for the R273H and R175H mutants (Bykov et al 2002). Furthermore, it has been shown that the degradation of the PRIMA-1 molecule results in covalent modifications of the mutant p53 protein that are sufficient to induce an apoptotic response in p53 null cells stably expressing mutant p53 (Lambert et al 2009). Although no toxic effects have been observed in p53 wild-type or p53 null cell lines following administration of PRIMA-1 at the doses required to kill mutant p53 expressing cells, higher concentrations of PRIMA-1 have been shown to adversely effect wild-type p53 expressing cells (Li et al 2005). Despite this, current observations

indicate that the use of PRIMA-1 may be beneficial in the treatment of human cancers containing mutant p53 and remain non-toxic to normal cells.

PRIMA-1^{MET} (also called APR-246), a structural analogue of PRIMA-1, has been demonstrated to work synergistically with chemotherapeutic drugs such as cisplatin to enhance tumour cell apoptosis *in vitro* and inhibit tumour xenograft growth in mouse models (Bykov et al 2005), suggesting it may have potential in combination therapies in the clinic. Furthermore, administration of PRIMA-1^{MET} has been shown to target mutant p53 expressing tumours in mice leading to growth suppression (Zache et al 2008) and this preclinical data has lead to the commencement of clinical trials (Lane et al 2010). Further to these findings, a recent study has demonstrated that PRIMA-1^{MET} can activate wild-type p53 in melanoma cells, resulting in the induction of pro-apoptotic target genes and subsequent p53-dependent apoptosis. These findings were also extended to xenograft studies in mice, where PRIMA-1^{MET} was shown to suppress the growth of melanoma xenograft tumours *in vivo* (Bao et al 2011). Together, these data indicate that the efficacy of PRIMA-1^{MET} in the treatment of human tumours may be extended to both mutant and wild-type p53 expressing tumours.

1.3.2.2 CDB3

CDB3 is a short peptide derived from the p53 interacting protein 53BP2 (also known as ASPP2), which has been shown to bind the DBD of p53 and stabilise the structure of p53 mutant proteins (Friedler et al 2002, Issaeva et al 2003). It is hypothesised that CDB3 acts as a chaperone, binding mutant p53 during protein biosynthesis, allowing it to maintain a native, wild-type conformation. The p53 protein in its native state may

then enter the nucleus and bind regulatory proteins or DNA elements that act to further stabilise the protein and reactivate its ability to regulate transcription (Issaeva et al 2003). However, peptide-based therapeutics generally exhibit low bioavailability which severely impacts on the usefulness of CDB3 as an anti-cancer treatment, although there are mechanisms more recently established that may be utilised to improve the prospects of peptide based therapies (discussed in Section 1.3.2.6 below)

1.3.2.3 CP-31398

CP-31398 was identified in a chemical library screen as a compound that could stabilise the DBD of wild-type p53 and the V173A p53 mutant *in vitro* (Foster et al 1999). Similarly to PRIMA-1, administration of CP-31398 inhibits the growth of tumours containing various p53 mutations in nude mice (Foster et al 1999). Similarly to CDB3, CP-31398 was believed to act as a chaperone, stabilising the p53 protein in its active conformation, resulting in p53-mediated transcription of target genes. However, no interaction between CP-31398 and the core domain of p53 was detected (Rippin et al 2002). CP-31398 treatment has also been shown to alter the expression of non-p53 target genes (Takimoto et al 2002) and result in non-specific, p53-independent side effects (Rippin et al 2002), suggesting that CP-31398 is not an ideal molecule for treatment of mutant p53-expressing tumours.

1.3.2.4 PhiKan083

PhiKan083 was identified through a rational drug design approach as a small molecule that could specifically target the Y220C p53 mutant (Boeckler et al 2008). The Y220C mutation creates a destabilising cavity on the surface of the p53 mutant protein, which has been referred to as a "druggable surface" (Basse et al 2010). The

Y220C mutant is therefore commonly used to identify molecules that may function to stabilise the mutant conformation through direct binding at this cavity. The binding of PhiKan083 is shown to increase the melting temperature and stabilise the p53 protein, which when coupled with its small size indicates that it may be a potential lead molecule for the design of specific mutant p53 stabilising molecules (Boeckler et al 2008). The clinical use of PhiKan083 may however be limited by its specificity to the Y220C mutant, as it is yet to be demonstrated if it has any effects on other common cancer mutations of p53.

1.3.2.5 SCH529074

The small molecule SCH529074 was identified in a DNA binding assay as a molecule that could bind specifically to the DNA binding domain of p53 (Demma et al 2010). It is believed to function to restore wild-type function to a range of p53 mutants (see Table 1.2) by acting as a chaperone molecule, as previously discussed for CDB3 and CP-31398. Specifically, SCH529074 has been shown to restore DNA binding and transcriptional activity to p53 mutants endogenously expressed in cancer cell lines and subsequently induce an apoptotic response (Demma et al 2010). SCH529074 is also shown to inhibit the MDM2-dependent ubiquitination of wild-type p53 (Demma et al 2010) indicating that it may also have a potential function in the treatment of wild-type p53 cancers. Due to the recent identification of this molecule, there is no further data currently available relating to its efficacy *in vivo* or its potential in clinical trials.

1.3.2.6 Peptide aptamers

Peptide aptamers (PAs) are peptides of up to 20 amino acids in length that are held by a scaffold protein in a unique conformation and can be specifically targeted to various

proteins and affect their function (Borghouts et al 2005, Colas 2008). PAs have previously been described that can inhibit cell cycle regulators, such as the cyclin dependent kinase, Cdk2 (Colas et al 1996) and inhibit signalling cascades (Buerger et al 2003). More recently a random peptide library screen has identified PAs that interact with structural p53 mutants and specifically induce apoptosis in mutant p53 expressing cells (Guida et al 2008).

The use of peptides in the clinic is impaired due to the necessity of high binding affinity, increased stability and means of cellular uptake. However, the binding affinity and stability of PAs can be improved and they can provide the basis for rational drug design. For example, the binding affinity of a peptide to its target protein can be increased by replacement of a single amino acid with an analogue based on the crystal structure of the interaction (Borghouts et al 2005). Importantly, studies have shown that the delivery of peptides to treat tumours *in vivo* is a feasible option (Harada et al 2002, Mai et al 2001, Snyder et al 2004). These findings open the door for the discovery of new proteins and peptides and provide the springboard for rationally designed compounds that can target p53 and suppress tumour formation and growth.

1.4. ANKRD11 (Ankyrin Repeat Domain 11)

Breast cancer commonly exhibits LOH (loss of heterozygosity) on the long arm of chromosome 16, specifically a region identified as 16q24.3 (Miller et al 2003) and this region has been shown to harbour a number of putative tumour suppressor genes (Powell et al 2002). The *ANKRD11* gene is located within this common LOH region, indicating it may play a role as a breast cancer tumour suppressor. ANKRD11 is a

300kDa protein containing an ankyrin (ANK) domain consisting of five tandem ankyrin repeats. The ANK domain is found in many proteins and consists of a variable number of highly conserved repeats, the major function of which is to facilitate protein-protein interactions (Michaely and Bennett 1992, Mosavi et al 2002, Mosavi et al 2004).

1.4.1 ANKRD11 in human disease

ANKRD11 has been implicated in a number of human diseases. The *ANKRD11* gene was identified as one of the most frequently deleted and down-regulated genes in Ewing sarcoma family of tumours (Savola et al 2009) and as a candidate gene for autism and cognitive impairment (Willemsen et al 2010). More recently, a small deletion was identified within the *ANKRD11* gene in two patients exhibiting with cognitive impairment and related dysmorphic features (Isrie et al 2011), indicating that *ANKRD11* does in fact play a role in this syndrome. A missense mutation in the *Ankrd11* gene was shown to be homozygous lethal in mice, while mice heterozygous for the mutation presented with the 'Yoda' phenotype, including short snouts and deformed nasal bones typified by reduced bone mineral density (Barbaric et al 2008). These studies indicate that ANKRD11 may play an integral role in development and human disease.

1.4.2 Role of ANKRD11 in transcriptional repression and activation

ANKRD11 was shown to localise in discrete nuclear foci and colocalize with histone deacetylases. Specifically, ANKRD11 was thought to repress transcriptional activity through the recruitment of HDAC's to nuclear receptor complexes, resulting in inhibition of steroid receptor transactivation (Zhang et al 2004). Subsequently, the

ANKRD11 protein was shown to contain both repressor and activation domains (Zhang et al 2007) indicating it may have the potential to act both as a repressor and activator of transcription.

1.4.3 ANKRD11 and p53

Our laboratory has identified a link between ANKRD11 and the p53 pathway. ANKRD11 was identified as a novel p53 interacting protein, directly interacting with p53 through its ANK domain (Neilsen et al 2008). Furthermore, ANKRD11 has been reported to interact with the co-activator ADA3 and associate with the histone acetyltransferase protein, P/CAF (Li et al 2008, Neilsen et al 2008). This association was shown to be related to an increase in acetylation of p53 by P/CAF at lysine-320. This increased acetylation resulted in increased transactivation function and was accompanied by an increase in DNA binding ability, indicating ANKRD11 can function as a p53 coactivator (Neilsen et al 2008). Stable ANKRD11 expression in the wild-type p53 expressing MCF-7 cells also resulted in a decrease in proliferation rates and a reduced capacity to form colonies on plastic (Neilsen et al 2008). In addition, *ANKRD11* was demonstrated to be a p53 target gene, suggesting that a positive-feedback loop exists between ANKRD11 and p53 during the activation of the p53 pathway. When coupled with the location of the *ANKRD11* gene in a common breast cancer LOH region, these observations suggest ANKRD11 may have a role as a tumour suppressor protein, and this function may be dependent on p53.

1.4.3.1. ANKRD11 restores activity to the R273H mutant

Interestingly, it was demonstrated that stable expression of ANKRD11 in the breast cancer cell-line MDA-MB-468, which harbours the endogenous R273H mutant, also

resulted in decreased colony growth and reduced proliferation rates, similarly to that observed for the MCF-7 cells expressing wild-type p53 (Neilsen et al 2008). Mechanistically, ANKRD11 was shown to restore some DNA binding capacity to the R273H mutant, resulting in the activation of the common p53 target gene, *p21* (Neilsen et al 2008). This data indicates that ANKRD11 can function with mutant p53 to restore its transcriptional activity and tumour suppressive properties and therefore may provide a novel therapeutic target.

1.5 Thesis themes and structure

There are several challenges facing researchers that study mutant p53:

(i) the vast number of mutant variants of p53 in cancer.

(ii) the broad genetic background of cancer cell lines that endogenously express these p53 mutants.

(iii) partitioning loss-of-function, dominant-negative and gain-of-function properties of a p53 mutation.

This thesis overcomes these challenges through describing the generation and applications of an isogenic panel of cell lines in the p53 null H1299 lung cancer cell line which express various p53 mutants under the control of an inducible promoter. An inducible wild-type p53 derivative was also generated. Although inducible wild-type (Teodoro et al 2006, Yu et al 1999, Zhao et al 2000) and mutant (Goldstein et al 2011) p53 cell lines have been generated previously, this panel of inducible cell lines is the most comprehensive panel reported thus far in the literature, encompassing the eight most frequently observed p53 mutants in cancer and a wild-type p53 control. The work presented throughout this thesis includes the generation and application of this system to uncover the mechanisms of mutant p53 GOF in cancer.

- Chapter 3 contains an in depth description of the initial characterisation of the EI H1299 cell lines. Further to this, the inducible system was used to follow up previous studies on ANKRD11 and its regulation of p53 performed in our laboratory and published in *The Journal of Cell Science* (Neilsen et al 2008). Although I am included as a co-author on this original article it is not included in this thesis, as the data contributing to this publication was generated in my honours year. This chapter includes some initial observations of ANKRD11 function with both wild-type p53 and a p53 mutant.

- Chapter 4 describes the investigation of mutant p53 regulated genes using the inducible system and expression microarrays. The work described in this chapter identifies a putative mechanism through which mutant p53 may aberrantly regulate a number of wild-type p53 target genes.

- Chapter 5 is a manuscript under the final stages of review in *Oncogene*, which investigates the ability of ANKRD11 to suppress a range of mutant p53 GOF pathways, including centrosomal abnormalities, multinucleation, EMT and invasion.

- Chapter 6 is a manuscript in preparation which further investigates the mechanisms through which mutant p53 can drive EMT and invasion through the regulation of a microRNA (miR-155).

Each chapter presented in this thesis represents various means in which the inducible system can be utilised to study p53 function and the regulatory mechanisms affecting its function *in vitro*. The advantages of this inducible system are realised through the international collaborations that have arisen, including with Prof. Karen Vousden at the Beatson Institute for Cancer Research in Glasgow and Sir David Lane at the A*STAR Institute for Biomedical Research, Singapore. Prof. Vousden has utilised this isogenic system to investigate how mutant p53 function in cancer is influenced by growth factor signalling. This work is in the final stages of review in *Nature Cell Biology*. Sir David Lane has utilised the isogenic system to investigate the mechanism of cisplatin resistance mediated by wild-type and mutant p53, and this work is currently in preparation for submission to *Oncogene*. Although I am included as a co-author on these manuscripts, these findings will not be presented in this thesis.

CHAPTER 2 – General Materials and Methods

2.1 Cloning and expression vectors

In general, inserts for cloning were generated by PCR amplification of the ORF of interest using Platinum High Fidelity Taq Polymerase (Invitrogen, Carlsbad, CA; Cat. #11304-029) or digested with specific restriction enzymes (NEB, Ipswich, MA, USA). Vector DNA was prepared by restriction digest with specific restriction enzymes followed by the addition of cold CIP (Finnzymes, Espoo, Finland; Cat. # F-201S) and incubation at 37ºC for 30 minutes. Insert and vector DNA was gel purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany; Cat. #28704). Ligation reactions contained 1 × ligation buffer, 1 U T4 DNA ligase (NEB; Cat. #M0202S) and purified vector and insert DNA in a 1:5 molar ratio. Reactions were made up to a total volume of 20 μL with sterile water and incubated at 4ºC overnight. Ligated DNA was ethanol precipitated and resuspended in 8 μL sterile water. 4 μL was added to 40 μL JM109 competent cells and electroporated, followed by addition of SOC media. Cells were incubated at 37ºC in water bath for 1 hour prior to plating $1/5^{th}$ of total volume on an agar plate containing appropriate antibiotic selection. Plates were incubated at 37ºC overnight.

Graphical representation of expression vectors used throughout the course of this study is shown in Figure 2.1.

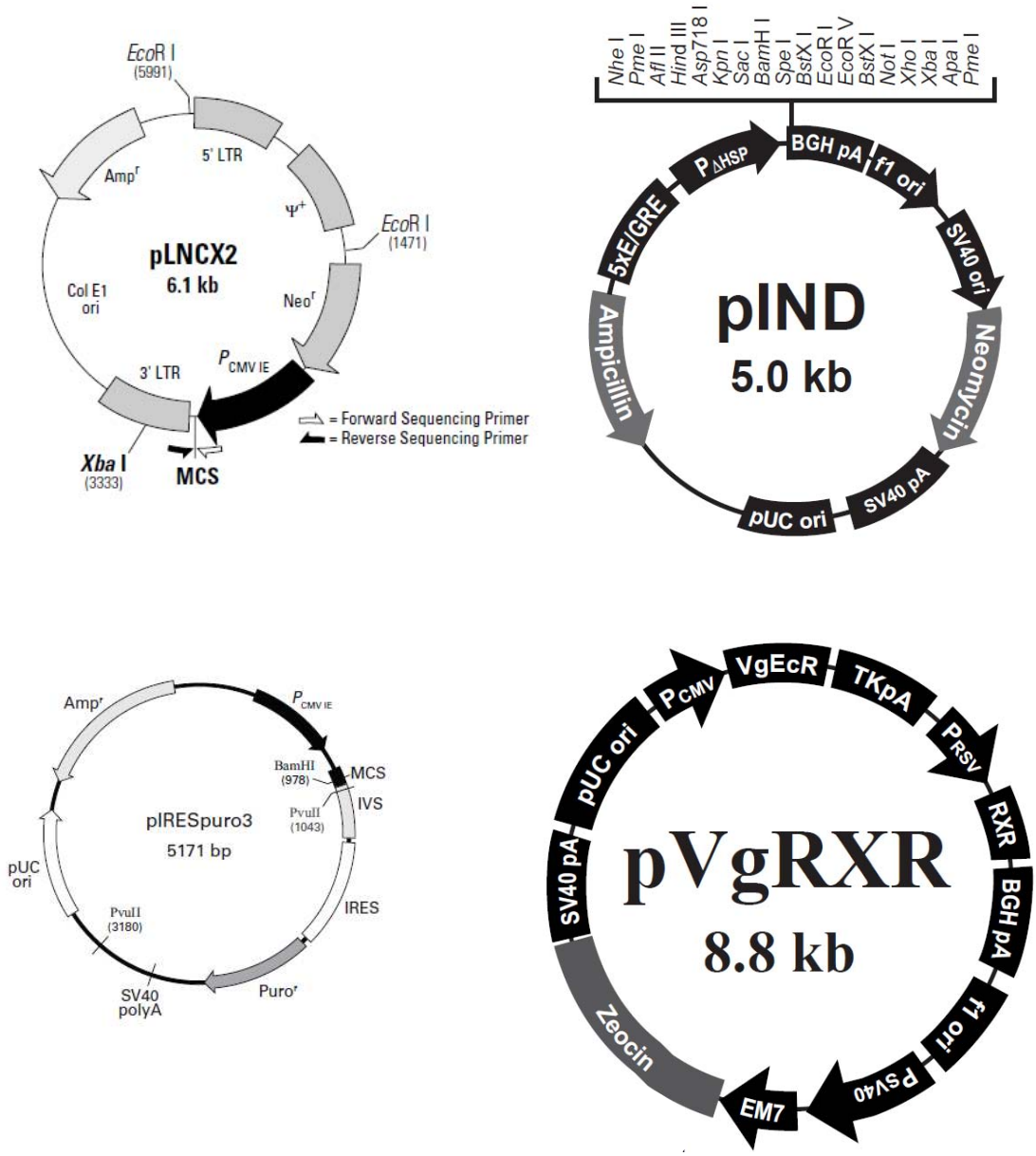


Figure 2.1 – Vector maps for cloning and expression vectors

pLNCX2, pIND and pIRESpuro3 were used as base vectors for cloning and subsequent expression of ORF's of interest. pVgRXR is an expression vector with dual expression of the VgEcR and RXR nuclear receptor proteins for generation of the inducible cell lines.

2.1.1 Generation of expression constructs

The pIRES-luciferase construct was generated by subcloning the luciferase ORF from the pGL3-basic plasmid into the pIRES-puro3 vector (Clontech Laboratories, Mountain View, CA, USA) at *EcoR*V-*Nhe*I sites. The wild-type (WT) and mutant p53 expression constructs were obtained from various sources (WT, R175H and G245S from Chikashi Ishioka; R248Q from Maria Lung; R282W from Sumitra Deb, R249S and R248W from Bert Vogelstein). The R273H ORF was PCR amplified from MDA-MB-468 cDNA. The WT and hot spot mutant p53 ORFs were subsequently PCR amplified using specific primers (Forward: 5' – ATG GAG GAG CCG CAG TCA GAT CCT AG - 3' and reverse: 5' – AGT CTG AGT CAG GCC CTT CTG TCT TG - 3') and sub-cloned at *BamH*I-*Xho*I of pI-TK-Hygro. pI-TK-Hygro is an expression plasmid containing hygromycin resistance and the multiple cloning site (MCS) from the pIND vector, downstream of an ecdysone-responsive promoter (Stolarov et al 2001). All p53 constructs (WT and mutant) in pI-TK-Hygro have the p53 Pro72 SNP. To generate pIND-GFP, the EGFP ORF was digested from pEGFP-N1 (Clontech Laboratories) with *Bam*HI-*Not*I and sub-cloned into same sites in pIND. To generate pLNCX2-ANKRD11-myc, the ANKRD11 ORF was PCR amplified and sub-cloned at *Bgl*II-*Cla*I of pLNCX2 (Clontech Laboratories) with a C-terminal MYC-tag. To generate pLNCX2-GFP-ANKRD11-myc the EGFP ORF was PCR amplified from pEGFP-C1 (Clontech Laboratories) and sub-cloned at the *Bgl*II site in pLNCX2-ANKRD11-myc. The pIND-GFP and pLNCX2-GFP-ANKRD11-myc constructs were generated prior to the commencement of this work by Dr. Raman Sharma.

2.2 Cell lines

H1299 (p53 null, non small cell lung carcinoma cells). HEK-293T and Phoenix cells were maintained in DMEM (Quantum Scientific, Brisbane, Aus; Cat. #E15-009) supplemented with 10% FBS (Sigma Aldrich, St Louis, MO, USA; Cat. #12003) under 5% CO_2. MCF-10A cells (an immortalized non-malignant mammary epithelial cell line expressing wild-type p53) were maintained in DME/F12 (Sigma Aldrich; Cat. #51445) supplemented with 5% horse serum (Invitrogen; Cat. #26050-070). MCF-7 cells (a breast cancer cell line expressing wild-type p53) were maintained in RPMI (Quantum Scientific; Cat. #E15-039) with 10% FBS and 10 μg/mL insulin. Cell lines were sourced from the ATCC.

2.2.1 Routine passaging of cell lines

In general, cells were passaged twice weekly. Briefly, growth media was removed, followed by 2 × washes with sterile PBS. Trypsin-EDTA (Sigma Aldrich; Cat. #T4049) was added to cells and incubated until all cells detached. Cells were subsequently resuspended in growth medium containing serum (to inactivate the trypsin) and a proportion of cells returned to flask.

2.2.2 Maintaining frozen cell stocks

Frozen cell stocks were maintained for all cell lines generated throughout the course of this work. Cell stocks were prepared for each cell line between passage 2 and passage 6. Cells were grown to confluence in a T75 flask, trypsinised and collected in growth medium (as described in Section 2.2.1). The cell suspension was centrifuged for 5 minutes at 300 × *g* to pellet cells, followed by removal of supernatant. The cell pellet was resuspended in FBS with 10% DMSO (Sigma Aldrich; Cat. #D2650) and 1

ml transferred to 3 × cryovials. The cryovials were placed in a Nalgene freezing container containing isoproponol and stored at -80ºC overnight. Cells were subsequently transferred to liquid nitrogen for long term storage.

2.2.3 Generation of cell lines

The generation of cell lines used throughout this thesis is a multi-step process, which is summarized in Figure 2.2. The processes employed for this purpose are outlined below.

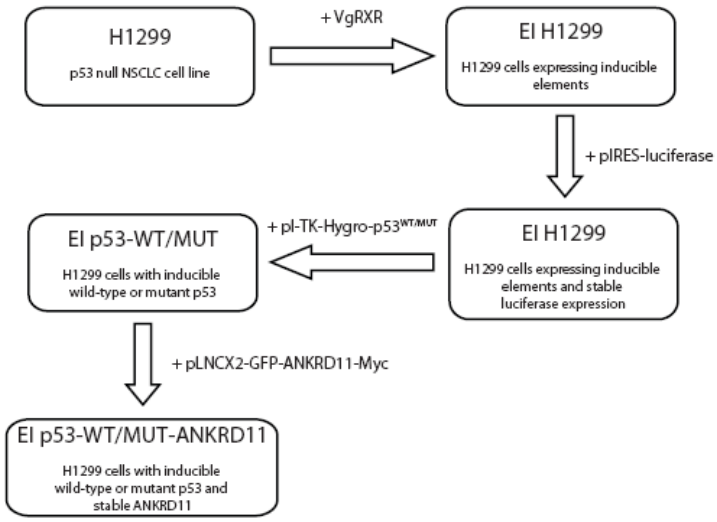


Figure 2.2 – Schematic of cell line methodology for generation of ecdysone-inducible (EI) H1299 cell lines

2.2.3.1 Generation of the base EI H1299 cell line

To generate the base ecdysone-inducible H1299 cell line (referred to as EI H1299) the inducible elements VgEcR and RXR were first constitutively expressed in H1299 cells. To achieve this, the pVgRXR vector (5 μg) was linearised using *Mlu*I followed by purification using the QIAquick PCR purification kit (Qiagen; Cat. #28104).

Briefly, DNA was mixed with binding buffer and bound to supplied column. Column was subsequently washed and DNA eluted with sterile water. Purified DNA was transfected into H1299 cells in a 6-well format using Lipofectamine 2000 transfection reagent (Invitrogen; Cat. #11668-019) as per manufacturer's protocol. Following transfection, cells were plated at limiting dilutions in 10 cm dishes and single clones incorporating the inducible elements selected in 100 μg/mL zeocin (Invitrogen; Cat. #R250-05). Approximately 20 single colonies were collected from the 10cm dish and grown in individual wells of a 96-well plate in media containing 100 μg/mL zeocin. Cells were expanded followed by screening for presence of the inducible elements as detailed in Section 2.2.3.1.1 below.

2.2.3.1.1 Screening EI H1299 cell lines

Cells were screened for the incorporation and expression of the VgEcR and RXR inducible elements by transient transfection with pIND-GFP (inducible GFP reporter) expression construct followed by treatment with or without 2.5 μg/mL Ponasterone A (PonA; Invitrogen; Cat. #H101-01). The inducible nature of cells was determined by fluorescence microscopy (Figure 2.3A). Clones expressing GFP in the presence, but not the absence, of PonA were subsequently subjected to a second round of screening. Second round clones were further screened through transient transfection with pI-TK-Hygro-$p53^{WT}$ followed by treatment with or without PonA, as described above. Western blot analysis determined that p53 protein was expressed in the presence, but not in the absence, of PonA (Figure 2.3B).

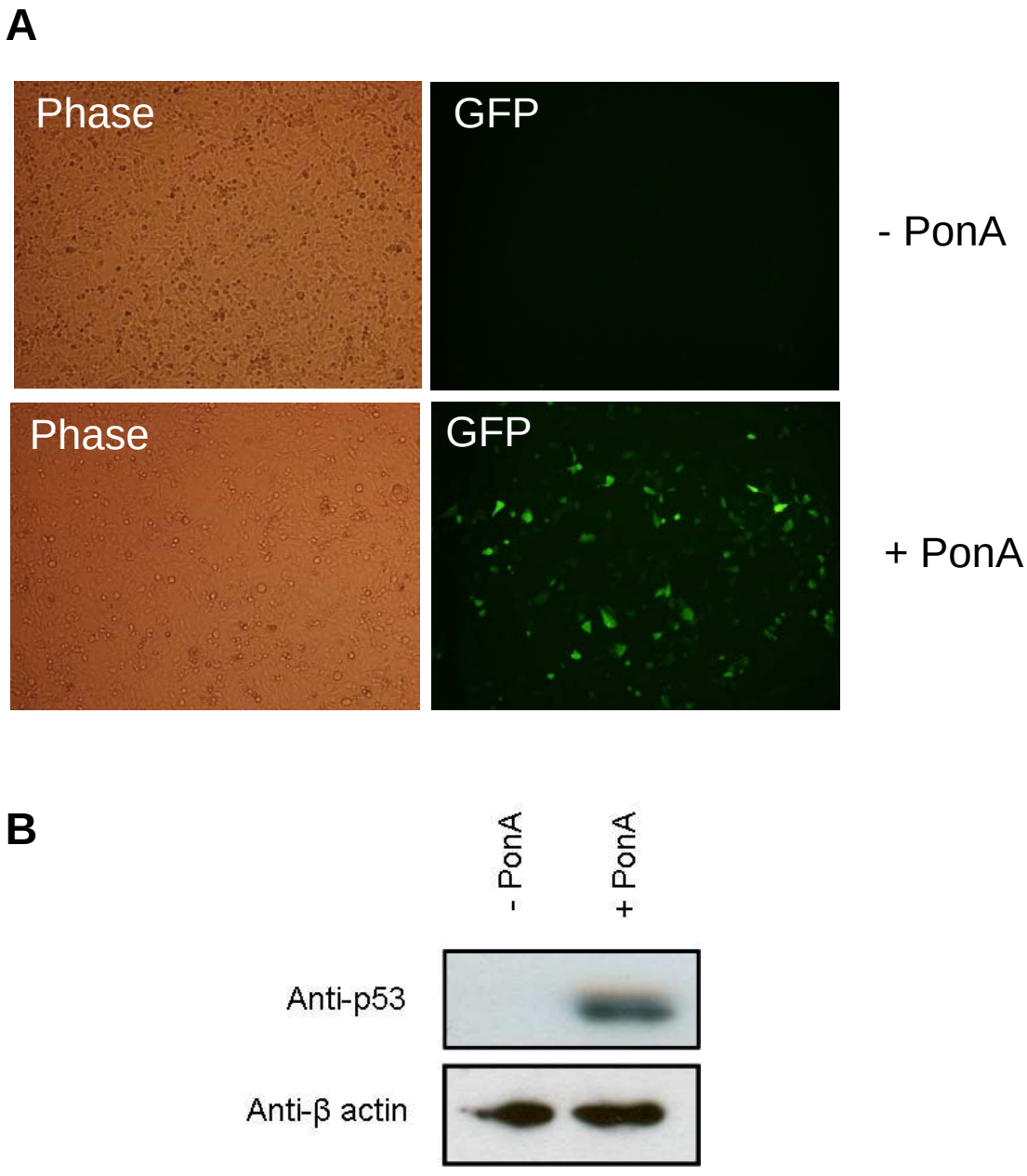


Figure 2.3 – Screening EI H1299 cell lines for expression of inducible elements.

A. The expression of GFP under the control of an inducible promoter was determined by fluorescent microscopy in EI H1299 cells following treatment with PonA or vehicle control.

B. EI H1299 cells were transfected with pI-TK-Hygro-p53^{WT}, treated with PonA or vehicle control and p53 protein expression subsequently determined by wesern blot analysis

2.2.3.2 Generation of EI H1299 cells stably expressing luciferase

As these inducible H1299 cells may in the future be used in mouse xenograft studies, stable luciferase expression was incorporated to allow for easy and regulated bio-imaging. To incorporate stable luciferase expression, the pIRES-luciferase construct linearised with *Nru*I was transfected into EI H1299 cells, followed by selection in 500 ng/mL puromycin (Sigma Aldrich; Cat. #P8833). This is the EI H1299 base cell line used for subsequent generation of the inducible wild-type and mutant p53 cell lines.

2.2.3.3 Generation and screening of EI H1299 cells with inducible wild-type or mutant p53 expression

Inducible p53-expression constructs (pI-TK-Hygro-p53$^{WT/MUT}$) were linearised with *Xba*I and transfected into the EI H1299 base cell line (with stable luciferase expression, as described in Section 2.2.3.2) using Lipofectamine 2000. Following transfection, cells were plated at limiting dilutions and selected in 600 μg/mL Hygromycin B (Sigma Aldrich; Cat. #10687-010) for incorporation of the inducible p53 constructs. To screen, individual clones were plated in the presence or absence of 2.5 μg/mL PonA. Cells were harvested, lysed and subjected to western blot analysis to determine p53 protein expression using a specific p53 antibody. A panel of ecdysone-inducible cell lines with the prefix EI-H1299 were generated: p53-WT, p53-R175H, p53-G245S, p53-R248Q, p53-R248W, p53-R249S, p53-R273C, p53-R273H and p53-R282W. A minimum of 2-3 positive clones were selected per cell line generated and frozen stocks made as described in Section 2.2.2.

2.2.3.4 Generation of EI p53 cells with stable ANKRD11 expression

To investigate the effects of ANKRD11 expression on wild-type or mutant p53, the EI p53-WT/MUT cell lines were stably integrated with an ANKRD11 expression vector via retroviral transduction. To generate recombinant retroviruses, Phoenix cells (a derivative of 293T cells with integrated constructs to express gag-pol envelope proteins for amphotropic virus production) were transfected with pLNCX2-GFP-ANKRD11-Myc or pLNCX2 vector (negative control) with Lipofectamine 2000. Forty-eight hours post-transfection, culture medium containing retrovirus particles was collected and filtered. Polybrene (8 ng/mL; Sigma Aldrich; Cat. # H9268) was added to enhance transduction efficiency, followed by addition of the retrovirus-containing media to the recipient cell line (plated at ~50% confluence). Forty-eight hours post-transduction, the transduced recipient cells were plated at limiting dilutions and selected in 500 μg/mL Geneticin (Invitrogen; Cat. #10131-035). Successful integration of GFP-ANKRD11-Myc was assessed using fluorescent microscopy to screen for GFP positive colonies. Colonies were then transferred to 96-well plates and expanded prior to use in cellular assays.

2.2.3.5 Generation of knockdown cell lines

Cell lines with silenced expression of p53 or p63 were generated using a GIPZ lentiviral shRNAmir system. HEK-293T cells were seeded at 50% confluence and transfected using the Translentiviral GIPZ packaging system (Open BioSystems, Huntsville, AL, USA; Cat. #TLP4614). Briefly (for 1× 6-well), 5 μL Expression Arrest™ Trans-Lentiviral Packaging Mix was added to 250 μL OPTI-MEM reduced serum medium (Invitrogen; Cat. #31985070) with 1.5 μg DNA and 10 μL Arrest-In Transfection Reagent was added to 250 μL OPTI-MEM. DNA and Arrest-In solutions

were mixed and incubated at room temperature for 20 minutes. 1 mL OPTI-MEM was added prior to addition of transfection mix to 293T cells. Growth medium was changed after 4-6 hours and cells incubated at 37°C with 5% CO_2 for 48 hours. Recipient cells were seeded at 50% confluence, filtered media containing lentivirus added and incubated for 48 hours. Growth medium was subsequently changed and cells were selected in puromycin (1 μg/mL for MCF-10A; 500 ng/mL for MDA-MB-468 and 200 ng/mL for MDA-MB-231). Cells were subsequently expanded and screened for successful knockdown by western blot or real-time RT-PCR analysis.

2.3 Western blot analysis

Cells were lysed in 50mM Tris-HCl (pH 7.5), 250mM NaCl, 1mM EDTA, 50mM NaF, 0.5% Triton-X 100, 0.1mM Na_3VO_4 with 1× protease inhibitor cocktail (Roche, Indianapolis, IN, USA; Cat. #1697498). Lysates were sonicated 1 × 15 seconds at 25% amplitude (Sonics Vibra Cell Sonicator) and clarified (13,200 rpm, 4ºC) for 15 min. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA; Cat. #23227). Equal amounts of protein were resolved on SDS-PAGE and transferred on to Hybond-C Extra (GE Healthcare, Uppsala, Sweden; Cat. #RPN203E). Transfers were performed at 35V for 2 hours at 4ºC in transfer buffer (25mM Biscine, 25mM Bis-Tris, 1.025mM EDTA, 20% methanol). Protein transfer was confirmed by staining membranes with Ponceau stain. Membrane was subsequently washed with TBST (20mM Tris-HCl pH7.5, 150mM NaCl, 0.1% Tween-20) followed by blocking for 30 to 60 minutes in 10% skim milk solution (in TBST). Membranes were incubated with the indicated primary antibodies (diluted in 2% skim milk solution) for 2 hours at RT followed by incubation with the appropriate horseradish peroxidise-conjugated secondary antibodies (diluted in 2%

skim milk solution) for 2 hours at RT. Between each incubation, membranes were washed 3 × with TBST. Proteins were visualised using an ECL detection system (GE Healthcare; Cat. #RPN2109) on chemiluminescence film (GE Healthcare; Cat. #28-9068-37).

2.3.1 Antibodies

Antibodies used were: mouse α-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA; Cat. #sc-126), mouse α-β actin (Sigma Aldrich; Cat, #A5441), mouse α-p21 (Thermo Scientific; Cat. #MS-230), mouse α-MDM2 SMP14 (Santa Cruz; Cat, #sc-965), α-mouse IgG HRP linked (GE Healthcare; Cat. #NA931).

2.4 RNA isolation

Total RNA was extracted from cultured cell lines using the RNAeasy Plant Mini Kit (Qiagen; Cat. #74904) as per manufacturer's protocol. Briefly, cells were trypsinised and pelleted and resuspended in lysis buffer. Cell lysates was mixed with ethanol, applied to supplied column and centrifuged to bind RNA. Column was washed and DNase (Qiagen; Cat. #79254) was applied to the coumn and incubated at RT for 15 minutes. Column was washed twice followed by elution of RNA in 40 μL RNase-free water. Purified RNA was stored at -80ºC until required for use.

2.5 Complimentary DNA synthesis

Isolated RNA (as described in Section 2.4) was used to synthesis cDNA. Briefly, 1 μg RNA wass added to 200 ng random primers (Promega, Madison, WI, USA; Cat. #C1181) and sterile, RNase-free water to give a volume of 18.25 μL. Samples wre incubated at 72ºC for 4 minutes followed by 4ºC for 4 minutes. To each tube was

added: 1 × RT buffer, 1mM dNTPs and 1 U MMLV H- reverse transcriptase (Promega; Cat, #M3682). Tubes were incubated for 1.5 hours at 42ºC followed by 15 minutes at 70ºC.

2.6 Real-time PCR analysis

The mRNA expression levels of specific genes of interest were determined by real time RT-PCR analysis using specific forward and reverse primers (see Table 2.1). Real-time PCR reactions were performed on a BioRad iCycler (BioRad, Hercules, CA, USA) using IQ SYBR Green Supermix (BioRad, Cat, #170-8884) with the following parameters: denaturation at 95ºC for 15 seconds, annealing at 57ºC for 15 seconds and elongation at 72ºC for 20 seconds for a total of 45 cycles. Changes in the mRNA expression of specific genes was subsequently determined by the ΔΔCT method (Livak and Schmittgen 2001), with the levels of gene expression normalised to the average Ct value of the peptidylpolyl isomerise G (PPIG) or β-actin housekeeping genes. Expression data was normalised to the uninduced control (where applicable).

2.7 Expression microarray analysis

The EI p53-WT, R175H, R248Q, R248W, R249S, R273H and R282W cell lines were treated in the presence of 2.5 μg/mL PonA (or vehicle control) for 24 hours. Cells were collected and total RNA extracted as described in Section 2.4. Expression profiling was performed at the Adelaide Microarray Centre using Affymetrix Human Gene 1.0 ST array as per manufacturer's protocol. Briefly, the arrays are scanned using a GeneChip 3000 7G scanner and Affymetrix GeneChip Command Console software (AGCC). The scan information is saved as a CEL file using AGCC. The

CEL files are then imported into Partek Genomics Suite to normalise the data using a quantile normalisation method. The probesets are summarised into a single intensity value for each gene (in log2 format). Two independent biological replicates were performed per treatment and cell line. Subsequent analysis was performed by Prof. David Callen.

2.8 Chromatin Immunoprecipitation (ChIP)

EI-H1299-p53-WT or –MUT cell lines were treated with or without 2.5μg/mL PonA for 24 hours to induce WT or mutant p53 expression. Cells were collected and DNA and proteins were cross-linked by addition of 1% formaldehyde for 9 min with rotation at RT. 625mM cold glycine was added to stop cross-linking, mixed and centrifuged for 5 minutes at 300 *g*. Cells were subsequently washed twice with 50 mL cold PBS. Cell pellets were lysed in 400 μL SDS Lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) with protease inhibitors, followed by sonication (6 × 15 sec; 30% amplitude). Following clarification, lysates were diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) and inputs taken. Lysates were precleared with Protein A sepharose beads (GE Healthcare; Cat. #17-1279-01) with BSA and sonicated salmon sperm DNA (ssDNA) at 4ºC with rotation for 2 hours. Lysates were subsequently incubated with 4 μg mouse α-p53 DO-1, rabbit α-p63 H-129 (Santa Cruz; Cat. #sc-8344), mouse IgG (Sigma Aldrich; Cat. #15381) or rabbit IgG (Sigma Aldrich; Cat. #15006) at 4ºC with rotation overnight. Immune complexes were precipitated with Protein A sepharose with ssDNA at 4ºC with rotation for 2 hours. Beads were washed once each with low salt immune complex wash buffer (20mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), high salt immune complex wash

buffer (20mM Tris-HCl pH 8, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), LiCl immune complex wash buffer (10mM Tris-HCl pH 8, 1mM EDTA, 0.25M LiCl, 1% NP-40, 1% sodium deoxycholate) and twice with TE buffer (10mM Tris-HCl pH 8, 1mM EDTA). Specific immune complexes were eluted in 250μL SDS Elution Buffer (1% SDS, 0.1M $NaHCO_3$). Cross-links were reversed by addition of 10μL 5M NaCl and heating at 65ºC for 16 hours, followed by addition 10μL 0.5M EDTA, 20μL 1M Tris-HCl pH 6.5 and 4μL 10mg/mL Proteinase K (Roche; Cat, #3115836001) and heating at 45ºC for 1 hour. DNA was extracted using standard PCR purification kit. Levels of specific promoter DNAs were determined by real-time RT-PCR using primers spanning the p53 response elements (see Table 2.2).

2.9 Cell Proliferation Assays

EI p53-WT or –MUT cell lines were seeded in quadruplicate at 1×10^4 cells in a 96-well plate. Cells were left untreated (0 hour) or treated with 2.5 μg/mL PonA for 24, 48 or 72 hours. Cell growth was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega; Cat. #G3580). Briefly, media was removed from wells, followed by addition of 25 μL fresh media (at room temperature) and 25 μL CellTiter-Glo reagent. Plates were placed on an orbital shaker at ~200 rpm for 2 min followed by equilibration at room temperature for 10 minutes. Plates were read on a luminometer (Lumistar Galaxy). Alternatively, the EI p53-R175H cell line was induced for 24 hours prior to exposure to the PLK inhibitor (BI2536) at indicated concentrations for 48 hours. Cells were then seeded at low density (1:30 split) and their growth rate monitored over the following 5 days. Proliferation was determined in real-time through the collection of phase contrast images at 30 minute intervals using Incucyte (Essen).

2.10 Cell Cycle Analysis

Cells were grown to <80% confluence and treated as indicated. Growth media, including any detached cells, was collected together with trypsinized cells and pelleted at 300 *g* for 5 minutes. Cell pellets were subsequently washed twice with cold PBS and fixed in ice-cold ethanol (70%) overnight at 4°C. Fixed cells were stained with propidium iodide solution (50 μg/mL) (Sigma Aldrich; Cat. #P4170) and 100 μg/mL RNase A (Sigma Aldrich; Cat. #R5503) for 45 minutes at 37°C. DNA content was examined with the use of a FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with cell cycle profiles analysed using WinMDI v2.8 software (Scripps Research Institute).

2.11 B-galactosidase senescence assays

EI-H1299-p53-WT cells were seeded at 2×10^5 cells in a 60mm plate and treated with 2.5 μg/mL PonA (or vehicle control) for 72 hours. Senescence was determined using the Senescence β-galactosidase Staining Kit (Cell Signaling, Danvers, MA, USA; Cat. #9680) as per the manufacturer's protocol. Briefly, cells were washed once with 5 mL PBS, fixed with 2.5 mL fixative solution followed by 2×PBS washes. Cells were incubated in 2.5 mL staining solution (2.325 mL staining solution, 25 μL staining supplement A, 25 μL staining supplement B, 125 μL 20 mg/ml Xgal in DMSO) at 37ºC for 4-6 hours. Senescence staining was visualised as a blue stain using a light microscope and images taken at 10× or 40× objective.

Table 2.1 Real-time PCR primers

Primer Name	Sequence (5'→3')	Direction
ANKRD11-F	AGCCAGGGTGACGAGAACAAGTC	Forward
ANKRD11-R	CACACACAGGATCCTCAGTCGTCGTTGACGT	Reverse
BCL2L1-F	GGGGTAAACTGGGGTCGCATT	Forward
BCL2L1-R	ACCAGCGGTTGAAGCGTTC	Reverse
CDO1-F	ACCGAGTGGGCAATGTACGC	Forward
CDO1-R	GAGCCCGAAGTTGCATTTGGA	Reverse
Cyclin G1-F	GTCCCATTGGCAACTGACTT	Forward
Cyclin G1-R	TGACATGCCTTCAGTTGAGC	Reverse
DDIT4-F	TTTGGGACCGCTTCTCGTCG	Forward
DDIT4-R	GTAAGCCGTGTCTTCCTCCGG	Reverse
DKK1-F	AACGCTGCATGCGTCACGCTAT	Forward
DKK1-R	TGAGGCACAGTCTGATGACCGGA	Reverse
FAS-F	ATGCTGGGCATCTGGACCCT	Forward
FAS-R	GCCATGTCCTTCATCACACAA	Reverse
Gadd45a-F	GGAGGAAGTGCTCAGCAAAG	Forward
Gadd45a-R	ATCTCTGTCGTCGTCCTCGT	Reverse
GPR17-F	GTCCTAGGGAAAGTGCCAGCCG	Forward
GPR17-R	GCCCACCAACTCCGTTTGGACA	Reverse
HDM2-F	TCTACAGGGACGCCATCGA	Forward
HDM2-R	CTGATCCAACCAATCACCTGAA	Reverse
LAMC2-F	TGAACAGGAGATTGGGAGTCTGAA	Forward
LAMC2-R	GACATCATGGGCCGCAGTTG	Reverse
METTL7B-F	GAGCGGTTTGTGGTGGCTCCTG	Forward
METTL7B-R	GCCACATGAAGGCCCAGCTTCC	Reverse
NOXA-F	AGAGCTGGAAGTCGAGTGT	Forward
NOXA-R	GCACCTTCACATTCCTCTC	Reverse
NR2F2-F	TGCGGAGACAAGTCGAGCGG	Forward
NR2F2-R	GGCATCCTGCCCCTCTGCAC	Reverse
OCEL1-F	CCCCACCCAGTGCCCGACTA	Forward
OCEL1-R	GCAGCGAGCCTGCTTGTCCA	Reverse
p21-F	TGGACCTGGAGACTCTCAGGGTCG	Forward
p21-R	TTAGGGCTTCCTCTTGGAGAAGATC	Reverse
p53-R2-F	GCCAGGACTCACTTTTTCCA	Forward
p53-R2-R	TCAGGCAAGCAAAGTCACAG	Reverse
PLK2-F	ACCAGCTCTCAGACCACACCGT	Forward
PLK2-R	CCAGACATCAGCAGAGTTGTCAGCC	Reverse
SERPINA1-F	GTAGGCGGGCGACTCAGATC	Forward
SERPINA1-R	GACGGCATTGTCGATTCACTGT	Reverse
STX11-F	TCGCACTCTCGCTCCCAGTCC	Forward
STX11-R	TGTCCTCGTGGGGCGAGTCAA	Reverse
TFPI2-F	TGGGCTTCTGCGCACCAAAGA	Forward
TFPI2-R	GCACGTTTGCAATCCTCCCTGC	Reverse
TMEM205-F	CAGGTGCCTGGGGCATGCAA	Forward
TMEM205-R	GCCCACATGGCAGCTGTGGT	Reverse

Table 2.2 ChIP Primers

Primer Name	Sequence (5'→3')	Direction
GPR17-BS1-F	CCCCCACCTCGCTGGCATGC	Forward
GPR17-BS1-R	GGACACGCTGCTGCTGGGGC	Reverse
METTL7B-BS1-F	CATATCTGTACAATGCAATGCTACTTGGAC	Forward
METTL7B-BS1-R	TCCCCATCCCAACCCCAGTACCA	Reverse
OCEL1-BS2-F	TGGCAGCTGAGCTAGACCTCG	Forward
OCEL1-BS2-R	GTCCCCACACCTGGTTGTCCTTC	Reverse
PLK2-BS1-F	CACCCCTAAGCCCCACCCCTTA	Forward
PLK2-BS1-R	AGGCTTTGCCATTAGCGAGGAGAA	Reverse
STX11-BS1-F	CATGAACCATCATGCCTGGCCCAAT	Forward
STX11-BS1-R	TCACAGACCACAGCCTGCTAGCAA	Reverse
TFPI2-BS1-F	AAAGGACAGTTGCTTTGGGTGTCA	Forward
TFPI2-BS1-R	TGGGAAGAGGAAGAACAGGAAGTGC	Reverse
TFPI2-BS2-F	TGGCAGCTGTTGGAGGCTAGCT	Forward
TFPI2-BS2-R	TTGTCCCTACCTCCACTCGCACC	Reverse
TMEM205-BS1-F	CAGCCCCTCCCAGTGTCCCC	Forward
TMEM205-BS1-R	ACAGCGACCCTCTTTTGGCCG	Reverse

CHAPTER 3 – Generation and characterisation of a panel of inducible p53 cell lines

3.1 PREFACE

This chapter addresses the generation and characterisation of an isogenic system, designed to express wild-type or mutant p53 under the control of an ecdysone-inducible promoter. These cell-lines provide the basis for the work discussed in Chapters 3 to 5 of this thesis. This chapter also contains data that further supports and contributes to research performed in our laboratory and previously published in *The Journal of Cell Science* (Neilsen et al 2008) and on which I am a co-author, investigating a role for ANKRD11 in the regulation of the p53 pathway.

Some figures presented in this chapter are also replicated in Chapter 5 as they form part of a manuscript in the final stages of review at *Oncogene*. For simplicity and to maintain a holistic view of the research presented within this chapter, the data is included within this chapter. Specifically, Figure 3.1 is also presented in Chapter 5, Figure S2 and Figure 3.4C is presented in part in Chapter 5, Figure S11. Furthermore, although the work presented here represents that achieved throughout the course of my PhD, I have not directly generated all the data presented. I would therefore like to acknowledge Kira Height for data presented in Figures 3.1B and 3.8; Fares Al-Ejeh for data presented in Figures 3.4B & C; Kristen Ho and Dr. Paul Neilsen for data presented in Figure 3.5.

3.2 INTRODUCTION

3.2.1 Wild-type p53 activity in cells

The tumour suppressor protein p53 is widely known to function in many cellular pathways including cell cycle arrest, DNA repair and apoptosis (see Chapter 1; Figure 1.1). As expression of p53 can lead to either cell cycle arrest or apoptotic cell death, it is of interest to investigate the processes required for the choice of this cellular fate. Deregulation of these pathways may lead to cancer development, for example through increased survival of DNA damaged cells. Earlier studies suggested that the level of p53 protein is sufficient to dictate a specific cellular response (Chen et al 1996), however more recent observations suggest that the choice between cell cycle arrest and apoptosis is more complex, and may in fact be based on specific interactions of proteins with p53 (Hsieh et al 2002, Samuels-Lev et al 2001, Shikama et al 1999) and/or post-translational modifications of the p53 protein (Knights et al 2006, Oda et al 2000, Sykes et al 2006, Tang et al 2006, Yoshida et al 2006).

Examples of specific proteins that interact with p53 include the apoptosis stimulating proteins of p53 (ASPP) which directly interact with p53 and stimulate its binding to the promoters of pro-apoptotic target genes (Samuels-Lev et al 2001). Similarly, Junction-mediating and regulatory protein, JMY, when complexed with p53 and the histone acetyl-transferase protein p300, specifically enhances the expression of the pro-apoptotic target gene *BAX*, while having no effect on genes regulating cell cycle arrest, such as *CDKN1A* (Shikama et al 1999).

Critical modifications of p53 that influence its cellular responses include the phosphorylation of Ser46 in response to DNA damage which has been demonstrated to direct p53 towards an apoptotic response (Oda et al 2000, Yoshida et al 2006). Specific acetylation patterns of p53 at commonly acetylated lysine residues have also been demonstrated to effect the target gene selection of activated p53 (Knights et al 2006, Sykes et al 2006, Tang et al 2006). However, these biological responses do not occur in cells expressing a mutated p53 protein. Therefore, to comprehensively investigate p53 activity and how it is disrupted by mutations of the p53 protein, it is necessary to establish a tightly regulated system that allows for direct analysis of the consequences of p53 expression and allow the comparison between the expression of wild-type and mutant p53 in a common genetic background.

3.2.2 Systems to investigate p53 function

There are three key issues that must be addressed in the investigation of mutant p53 function:

(i) The range of mutant p53 proteins expressed in cancer.

(ii) The different genetic backgrounds of cell lines used to investigate mutant p53 function.

(iii) The ability to identify the initial events caused by mutant p53 expression.

To address these issues, this chapter describes the implementation of an inducible system expressing wild-type or mutant p53s.

A major shortfall of previous studies is the limited range of p53 mutants that have been investigated (Adorno et al 2009, Scian et al 2004a, Scian et al 2004b). There is a

significant bias in the literature towards studies involving the p53 mutants R175H and R273H. While these represent DNA contact (R273H), and structural (R175H), classes of mutations, it may be ill-advised to extrapolate these findings to all p53 mutants. The inducible system described in this thesis includes the expression of the eight most common p53 hotspot mutants and a wild-type control and therefore allows discovery of properties that are common across a range of p53 mutants.

Published studies of wild-type and mutant p53 have used a range of cell lines or tumours with multiple genetic backgrounds that make the analysis of p53 specific effects on cellular function difficult. Furthermore, previous studies have utilised stable clones with constitutive expression of mutant p53 in p53 null cell lines to identify mutant p53 regulated genes (Scian et al 2004a, Scian et al 2004b). Through the use of stable clones, it is not possible to elucidate the precise role of p53 in initial events relating to the pathways under investigation. In comparison, an inducible system allows for tightly regulated control of mutant p53 expression in a single genetic background and thereby provides a means through which to identify the primary events driven by p53 to regulate oncogenic pathways.

There are previous reports where mutant p53 function was investigated using inducible systems, including inducible knockdown of endogenously expressed mutant p53 in cancer cell lines (Yan and Chen 2009), and induction of p53-R175H in a p53 null cell line using the ecdysone-inducible system (Fontemaggi et al 2009) to identify mutant p53 responsive genes. Furthermore, dual-inducible systems have been used to regulate both mutant and wild-type p53 expression in a single system to investigate the dominant-negative activity of mutant p53 (Willis et al 2004). However, to-date no

single system has incorporated such a broad range of p53 mutants, while also incorporating a wild-type p53 control cell line, as that described in this chapter.

A number of studies have also used inducible systems to investigate wild-type p53 function. Tetracycline-regulated (Yu et al 1999), ecdysone-inducible (Teodoro et al 2006) and zinc-inducible (Zhao et al 2000) systems have been reported that have identified a range of p53-regulated genes. These systems have also been useful in the past to examine the biological effects of wild-type p53 expression and activation in cells (Shaw et al 1992, Teodoro et al 2006, Wang et al 1998). This validates the use of an inducible system to comprehensively investigate protein function.

3.2.3 The Ecdysone-Inducible System

The ecdysone-inducible system is a stringent system, offering high levels of control of gene expression and has previously been used both for inducible protein expression (Stolarov et al 2001) and inducible expression of short hairpin RNA (shRNA) molecules to silence gene expression (Gupta et al 2004, Rangasamy et al 2008). Importantly, the ecdysone system exhibits greater sensitivity to the inducing agent and lower basal activity than the other commonly used inducible systems, such as tetracycline (No et al 1996). The critical elements of the ecdysone inducible system were originally derived from *Drosophila melanogaster* and have no counterparts in human cells, providing the basis for the negligible background observed. The system requires expression and heterodimerisation of the modified nuclear receptor proteins VgEcR and RXR, which in turn recognise, and tightly repress transcription of, a unique synthetic response element upstream of the gene of interest. In the presence of the inducing agent (Ponasterone A; an analogue of the insect hormone ecdysone), co-

repressors are released from the complex and activation of the gene of interest is initiated (No et al 1996). Using this system, an isogenic panel of cell lines was generated in the p53 null H1299 non small cell lung carcinoma background. This panel expresses various p53 mutants (and wild-type p53) under the control of an inducible promoter for the study of wild-type and mutant p53 function and their subsequent biological effects.

3.3 RESULTS

3.3.1 Generation and characterisation of p53 inducible H1299 cells

3.3.1.1 Inducible Expression of wild-type p53 in EI p53-WT cells is tightly controlled

Previous studies have indicated that the level of p53 expression may be critical in determining subsequent cellular function (Chen et al 1996), therefore it was of interest to examine the degree to which p53 protein expression could be controlled in this system. Treatment of the ecdysone-inducible H1299 cell lines expressing WT-p53 (called EI p53-WT) with increasing concentrations of the inducing agent Ponasterone A (PonA) for 8 or 24 hours showed a time- and dose-dependent increase in the expression of p53 (Figure 3.1A). This ecdysone inducible system can therefore be utilised to investigate p53 function while tightly regulating the protein expression levels.

3.3.1.2 Wild-type and mutant p53 are induced at physiologically relevant levels

To ascertain the physiological relevance of this induced p53, we compared the level of p53 protein induction in this system to the levels of endogenous wild-type p53 induced by DNA damage in the breast cancer cell line, MCF-7. The basal level of p53 in MCF-7 cells is seen to be comparable to the levels in low-dose PonA (0.75 μg/mL) induction in H1299 cells. The DNA damage induced p53 levels in MCF-7 are comparable to EI p53-WT cells induced with 2.5 μg/mL PonA (Figure 3.1B). For subsequent experiments, 2.5 μg/mL PonA was chosen as the maximum dose to induce a high level of p53 protein, as it is comparable to physiologically-relevant levels of

biologically-active wild-type p53 seen in a cancer cell lines following DNA damage. Similarly, levels of mutant p53 are also induced in these H1299 cells at a level comparable to endogenous p53 mutants expressed in cancer cell lines (Figure 3.1C). Subsequently we examined the dynamics of PonA in the H1299 inducible system and determined that a single treatment with PonA added to the growth medium was sufficient to maintain stable protein expression for up to 72 hours. The expression of the induced protein was also maintained by changing the growth media with fresh PonA every three days.

Figure 3.1

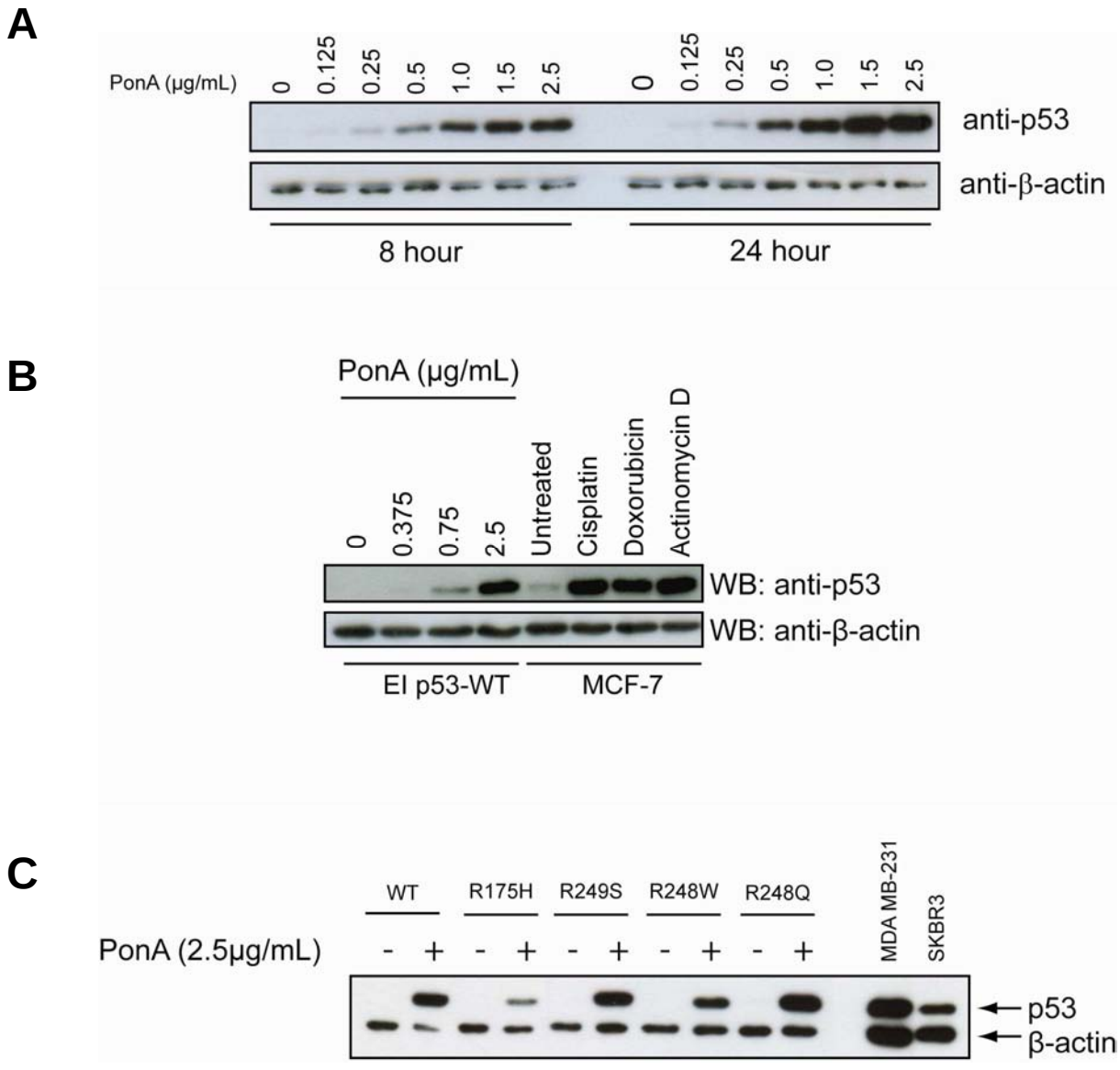

A
PonA (µg/mL)
0
0.125
0.25
0.5
1.0
1.5
2.5
0
0.125
0.25
0.5
1.0
1.5
2.5
anti-p53
anti-β-actin
8 hour
24 hour
B
PonA (µg/mL)
0
0.375
0.75
2.5
Untreated
Cisplatin
Doxorubicin
Actinomycin D
WB: anti-p53
WB: anti-β-actin
EI p53-WT
MCF-7
C
WT
R175H
R249S
R248W
R248Q
MDA MB-231
SKBR3
PonA (2.5µg/mL)
- + - + - + - + - +
p53
β-actin

Figure 3.1 - Induction of p53 in the EI p53-WT cell line is tightly regulated

A. EI p53-WT cells were treated with 0, 0.125, 0.25, 0.5, 1, 1.5 or 2.5 µg/mL PonA for 8 or 24 hours as indicated. Induction of p53 protein was determined by western blot analysis using the specific α-p53 (DO-1) antibody; β-actin is a loading control.

B. The level of wild-type p53 induced in EI p53-WT cells was compared to p53 protein levels following activation of the p53 pathway in MCF-7 cells by DNA damage. EI p53-WT cells were treated with 0, 0.375, 0.75 or 2.5 µg/mL PonA, while MCF-7 cells were treated with 20 µg/mL cisplatin, 20 µg/mL doxorubicin or 100 ng/mL actinomycin D for 16 hours.

C. The level of wild-type and mutant p53 induced in EI H1299 cells was compared to the basal level of endogenously expressed mutant p53 in cancer cell lines. MDA-MB-231 express p53-R280K, SKBR3 express p53-R175H.

3.3.1.3 Wild-type and mutant p53 protein stability

Activated wild-type p53 is degraded in the cell and it has been reported that mutant p53 is maintained at high levels in cancer cells (Brooks and Gu 2006). However, it has also been observed that accumulation of the mutant p53 protein occurs only in tumour tissues and not in adjacent normal tissues that also express mutant p53 (Terzian et al 2008). Therefore, we aimed to determine the stability of the wild-type and mutant p53 proteins in the inducible H1299 system.

To examine this, the EI p53-WT cells were induced for 24 hours followed by withdrawal of PonA (by replacing media without PonA). Within 6 hours of PonA withdrawal, the level of wild-type p53 protein had decreased marginally and by 24 hours post-withdrawal, p53 protein was only faintly detectable. Forty-eight hours post-withdrawal, no visible p53 protein remained (Figure 3.2A). To examine mutant p53 protein stability, the EI p53-R273H cell line was similarly induced followed by PonA withdrawal. Within 24 hours of withdrawing PonA, the level of mutant p53 expression had significantly decreased and was back to basal, uninduced levels within 48 hours (Figure 3.2B). These data indicate rapid turnover of induced wild-type and mutant p53 protein in H1299 cells.

Figure 3.2

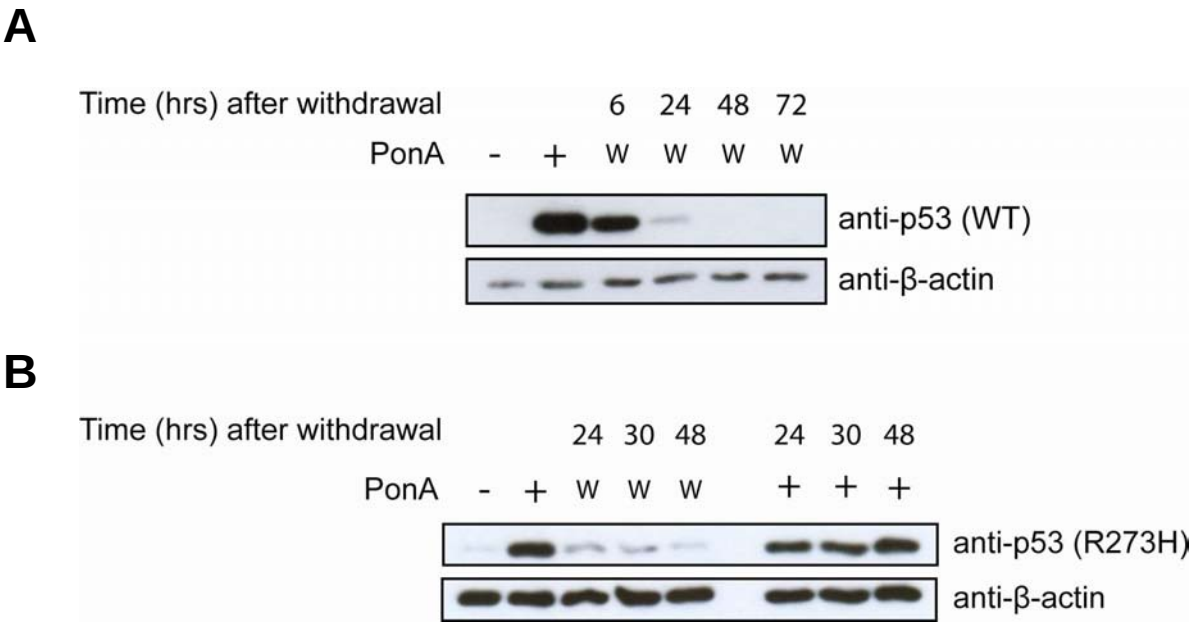


Figure 3.2 – Stability of induced wild-type and mutant p53

A. EI p53-WT cells were treated in the presence of 2.5 µg/mL PonA (or vehicle control) for 24 hours, followed by withdrawal (W) of PonA for 6, 24, 48 or 72 hours. Protein expression of p53 was determined by western blot analysis; β-actin is a loading control.

B. EI p53-R273H cells were treated with 2.5 µg/mL PonA for 24 hours, followed by withdrawal (W) of PonA for 24, 30 or 48 hours. Induction of p53-R273H is consistent at 24, 30 and 48 hours, while withdrawal of PonA leads to a decrease in p53-R273H expression.

3.3.1.4 Transcriptional activity of induced wild-type and mutant p53

The function of wild-type p53 as a transcription factor represents one of its most important roles in the regulation of cellular growth and the suppression of tumorigenesis. Cellular stresses such as DNA damage result in post-translational modifications of the p53 protein, such as acetylation, which can enhance the activation of target genes (reviewed by (Beckerman and Prives 2010)). However, p53 target genes have also been shown to be activated through the use of inducible systems in the absence of any stress signals (Zhao et al 2000). Similarly, the inducible system described here demostrates induction of a range of p53 target genes (as assessed by real-time RT-PCR) encompassing apoptosis, cell cycle, negative feedback and DNA repair pathways (Figure 3.3A). In addition to the 'basal' activity of the induced p53 protein, the transcriptional activity of p53 was assessed following treatment with the DNA damaging agent, cisplatin. The EI p53-WT cells were treated with 10 μg/mL cisplatin for 24 hours followed by an 8 hour induction of p53. The expression of a range of p53 target genes was then assessed by real-time PCR. Interestingly, the transcriptional activity on all genes tested was enhanced when p53 protein induction was coupled with DNA damage, with a significant increase ($p<0.05$) observed for 5 of the 6 genes examined (Figure 3.3B). This provides preliminary data which suggests the inducible system can be useful to investigate the p53 response following activation of the p53 pathway by new or existing chemotherapeutic drugs.

It is also of importance to note that the induced mutant p53 proteins in this system are largely unable to activate transcription of these wild-type p53 target genes (Figure 3.3A), which is expected as mutant p53 is widely reported to be non-functional as a transcription factor on normal wild-type p53 target genes (Bullock and Fersht 2001,

Joerger et al 2006). It is of interest to note that *CDKN1A* (p21) and *GADD45A* mRNA are increased in the presence of the induced R248W and R249S mutants, indicating these mutants may have some capacity to activate transcription, although this is less than observed in the wild-type system.

Figure 3.3

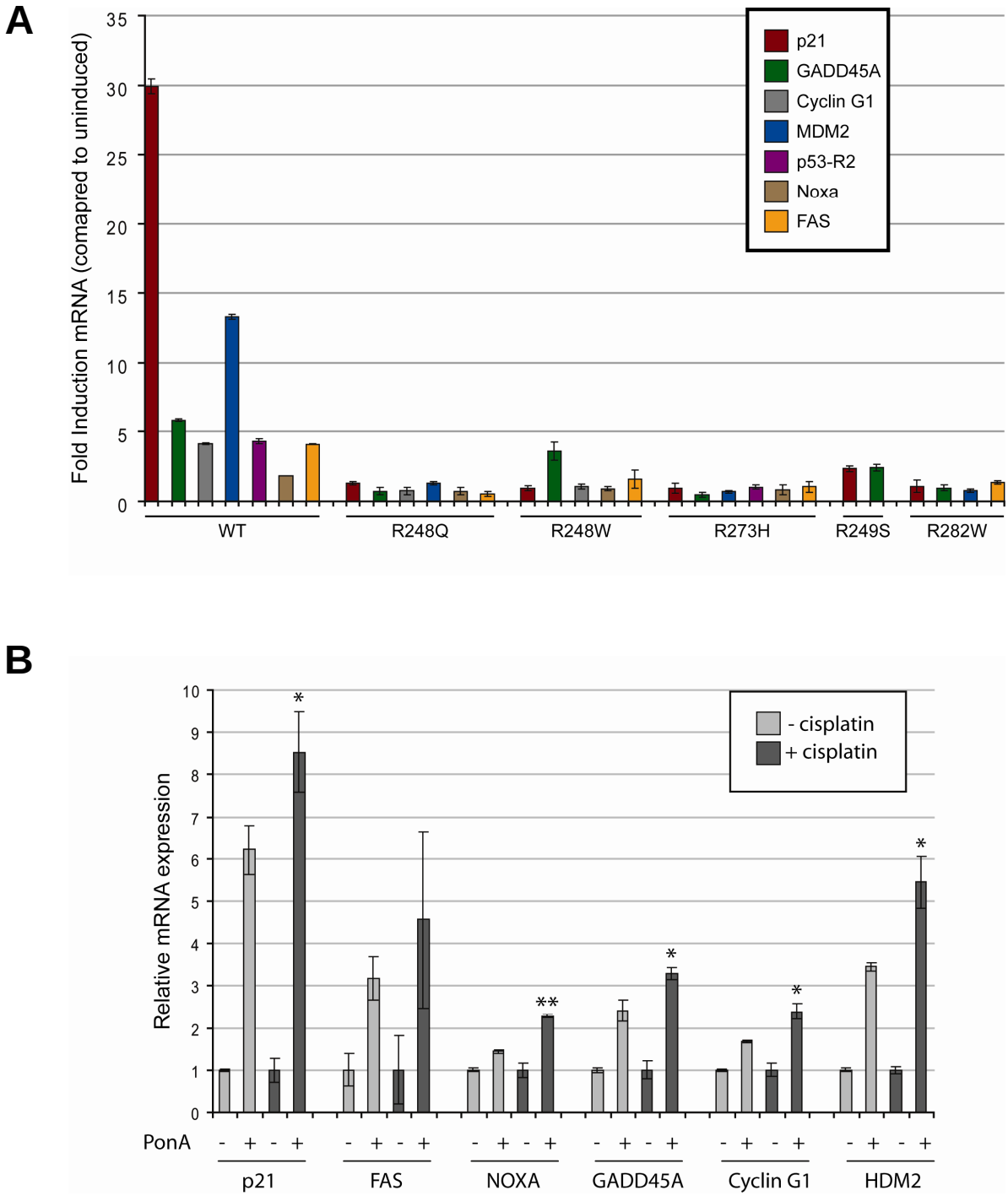

A
Fold Induction mRNA (comapred to uninduced)
35
30
25
20
15
10
5
0
p21
GADD45A
Cyclin G1
MDM2
p53-R2
Noxa
FAS
WT
R248Q
R248W
R273H
R249S
R282W
B
Relative mRNA expression
10
9
8
7
6
5
4
3
2
1
0
- cisplatin
+ cisplatin
*
**
PonA
- + - +
p21
FAS
NOXA
GADD45A
Cyclin G1
HDM2

Figure 3.3 – Transcriptional activity of induced p53 in H1299 cells

A. WT or mutant p53 expressing cell lines (as indicated) were treated with 2.5 µg/mL PonA (or vehicle control) for 24 hours. Following induction, RNA was extracted from cells and cDNA generated. Real-time PCR was performed to determine the mRNA expression levels of a range of p53 target genes (see Materials and Methods for specific primers). Expression levels are relative to the uninduced control.

B. The EI p53-WT cell line was treated with 10 µg/mL cisplatin for 24 hours, followed by induction of p53 for 8 hrs. Real-time PCR was performed as above to determine target gene expression. (* = $p<0.05$; ** = $p<0.0001$; n=3)

3.3.1.5 Wild-type p53 induction leads to growth suppression

To investigate the biological outcome of induced wild-type or mutant p53 expression in H1299 cells, we initially performed cell proliferation assays. The induction of a variety of different p53 mutants had no effect on the proliferation and growth of the cells, however cell proliferation was significantly inhibited within 24 hours of wild-type p53 induction (Figure 3.4A). This was subsequently demonstrated to represent a G_1 arrest in the EI p53-WT cell line (Figure 3.4B), which was accompanied by a decrease in the number of cells undergoing mitosis, as seen by a decrease in MPM2 staining (Figure 3.4C). In contrast, the mutant induced cells did not exhibit any changes in their cellular profile or mitotic entry (Figure 3.4B & C). Therefore, the primary cellular response observed following wild-type p53 induction in this H1299 system is a cell-cycle arrest in G_1.

Figure 3.4

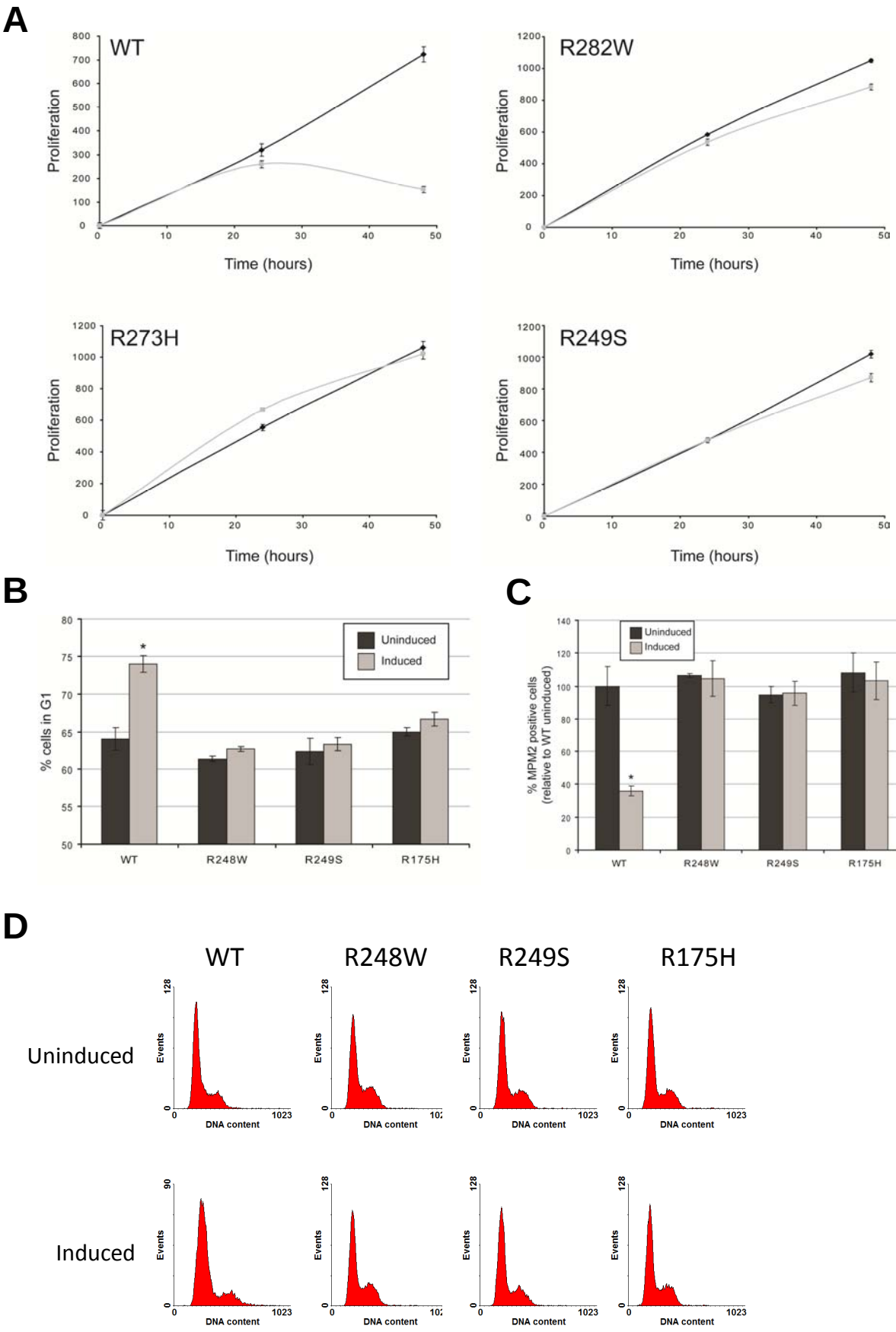

A
WT
R282W
R273H
R249S
Proliferation
Time (hours)
B
% cells in G1
Uninduced
Induced
WT
R248W
R249S
R175H
C
% MPM2 positive cells (relative to WT uninduced)
Uninduced
Induced
WT
R248W
R249S
R175H
D
WT
R248W
R249S
R175H
Uninduced
Induced
Events
DNA content

Figure 3.4 – Wild-type p53 induction leads to a G_1 arrest

A. EI p53-WT or –MUT cells (as indicated) were treated with 2.5 µg/mL PonA (or vehicle control) for 0, 24 or 48 hours and their proliferation measured using the CellTiter-Glo Luminescent Cell Viability assay (Promega).

B. EI p53-WT or –MUT cells were treated with 2.5 µg/mL PonA (or vehicle control) for 72 hours. Cells were collected, fixed and stained with propidium iodide followed by FACS analysis. The percentage of cells present in G_1 is presented here. Data is presented as the mean with standard error (* = p<0.01, n=3).

C. EI p53-WT or –MUT cells were treated as above and stained for MPM2, followed by FACS analysis. The percentage of cells staining positive for MPM2 (i.e. cells undergoing mitosis) is presented here. Data is presented as the mean with standard error, relative to the EI p53-WT uninduced control (* = p<0.01, n=3).

D. Representive histograms (raw data) showing cell cycle profiles, as graphically represented in (B). Cell cycle profiles were analysed using WinMDI v2.8 software (Scripps Research Institute). Data supplied by Fares Al-Ejeh.

3.3.1.6 Wild-type p53 induction results in a senescent-like phenotype

To determine if the growth arrest phenotype observed following wild-type p53 induction (Figure 3.4) was associated with a senescent phenotype, EI p53-WT cells were seeded at low density and treated with 2.5 μg/mL PonA for 72 hours. Following induction of the wild-type p53 protein, the EI p53-WT cells stained positive for β-galactosidase, a common marker of senescence (Figure 3.5). This indicates that wild-type p53 expression can result in a senescent-like phenotype, although we have not yet determined if this is true senescence or merely a reversible senescent-like phenotype as seen in previous studies following activation of wild-type p53 (Huang et al 2009; as discussed in Section 3.4.3.2).

Figure 3.5

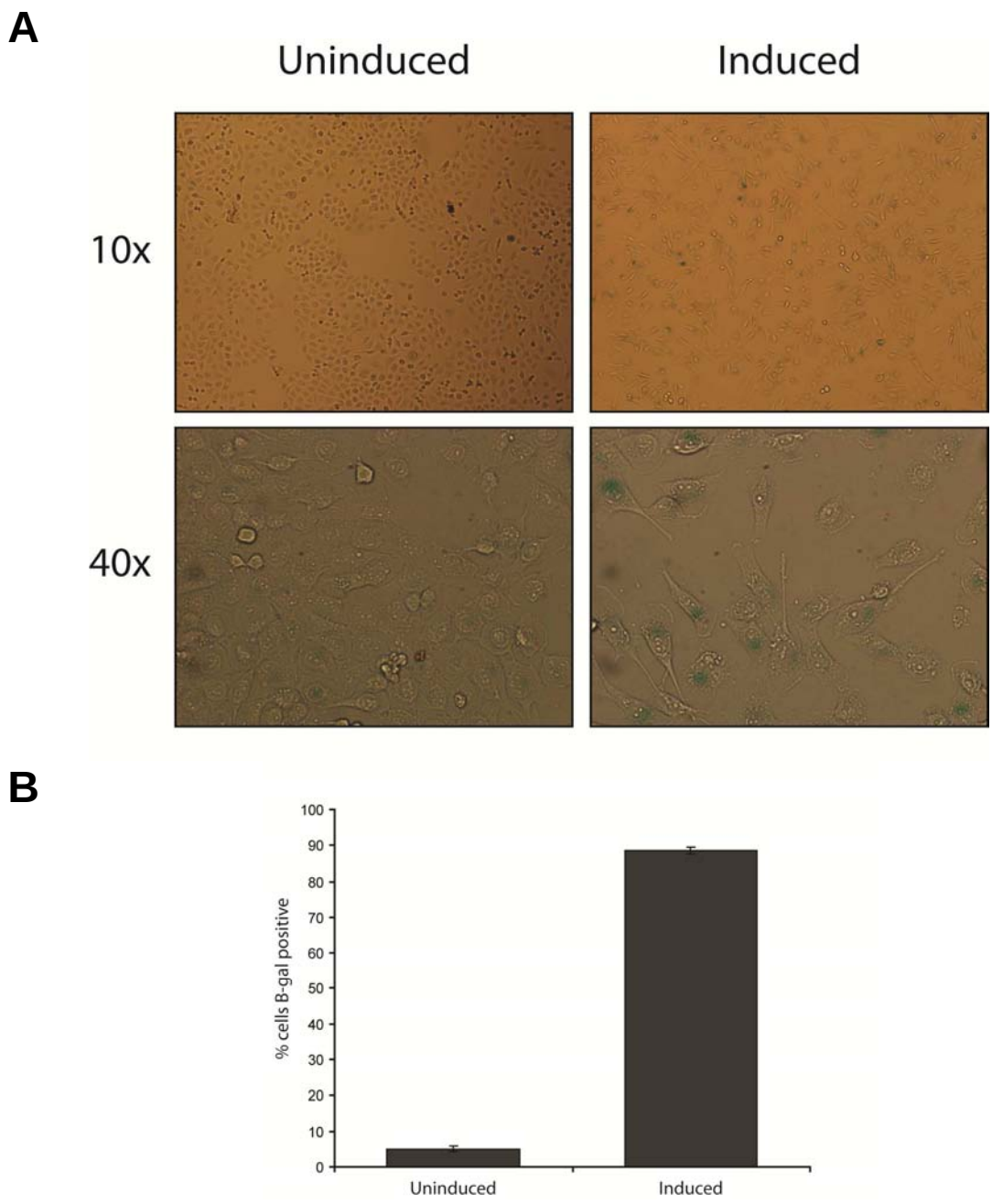


Figure 3.5 – Wild-type p53 induction results in a senescent-like phenotype

A. The EI p53-WT cell line was plated at low density ad treated with 2.5 µ/mL PonA (or vehicle control) for 72 hours followed by detection of senescence-associated β-galactosidase.

B. Graphical representation of cells staining positive for β-galactosidase. Cells were counted from 5 independent views with a minimum of 500 cells counted per view.

3.3.1.7 Mutant p53 induction enhances chemo-resistance in H1299 cells

The wild-type p53 protein induced in H1299 cells is transcriptionally (Figure 3.3) and biologically (Figure 3.4 & Figure 3.5) active and mutant p53 proteins do not transactivate common p53 target genes (Figure 3.3). It was next of interest to determine if the induced mutant p53 proteins mediate a gain-of-function in H1299 cells. Mutations in p53 are predictive of decreased sensitivity to chemotherapeutic agents *in vivo* (Buttitta et al 1997) and silencing of endogenous mutant p53 has been demonstrated to decrease chemo-resistance in cancer cell lines (Bossi et al 2006). Therefore, the sensitivity of EI p53-R175H cells to the chemotherapeutic agent cisplatin in the presence or absence of mutant p53 expression was investigated. EI p53-R175H cells were treated in the presence or absence of PonA for 24 hours prior to treatment with various concentrations of cisplatin for 48 hours. Cell viability was subsequently determined. The uninduced (p53 null) cells exhibited a 90% decrease in cell viability with the lowest concentration of cisplatin, while the p53-R175H expressing cells had little change in viability at this concentration (Figure 3.6). It is evident that expression of mutant p53 in this system can confer resistance to chemotherapy and therefore does exhibit gain-of-function properties.

Figure 3.6

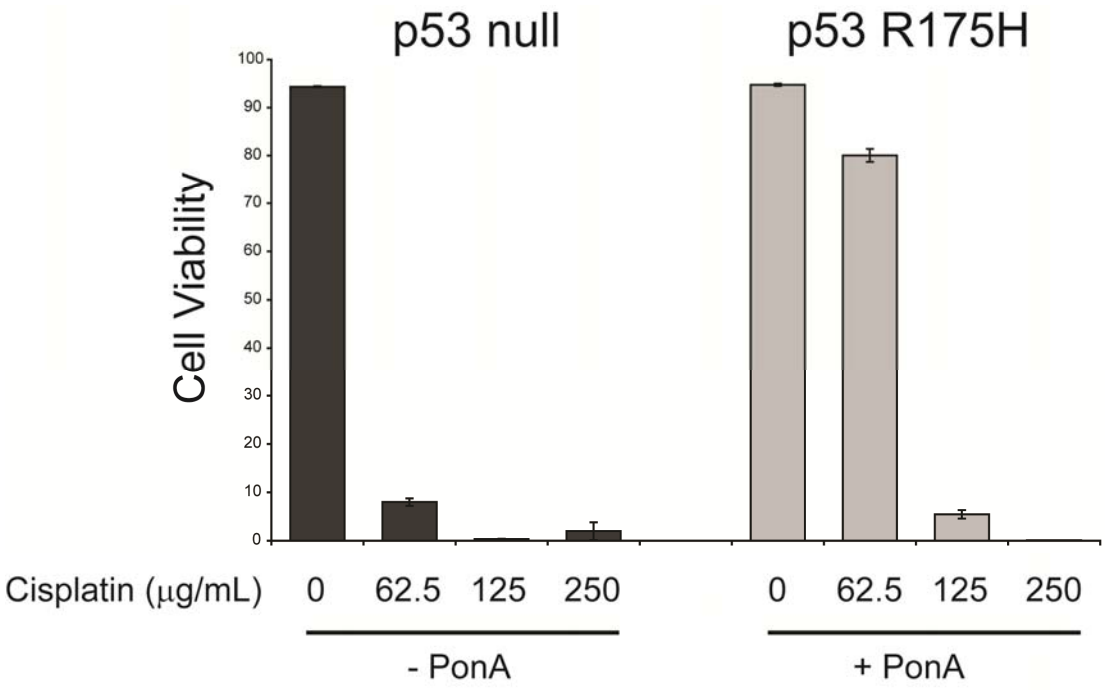


Figure 3.6 – Induction of mutant p53 in H1299 enhances resistance to cisplatin treatment

EI p53-R175H cells were induced with PonA (or vehicle control) for 24 hours prior to treatment with the indicated concentrations of cisplatin for 48 hours. Cell viability was subsequently determined through 7-AAD staining and flow cytometry, and analysed using FlowJo software.

3.3.1.8 Promoter demethylation may synergise with p53 to activate target genes

Wild-type p53 in the inducible system described here is able to enhance the expression of a variety of target genes (Figure 3.3). *ANKRD11* has previously been described as a p53 target gene that can in turn function to enhance p53 activity (Neilsen et al 2008), however *ANKRD11* expression is not induced by the addition of PonA in EI p53-WT cells i.e. induction of p53 expression alone does not result in increased expression levels of *ANKRD11* (Figure 3.7). It is hypothesised that this may be due to aberrant promoter methylation of *ANKRD11* in H1299 cells resulting in a p53-RE which is inaccessible to the induced p53 protein. To investigate this, EI p53-WT cells were treated with 100 μM zebularine (a common methylation inhibitor) for 72 hours, resulting in a 3-fold increase in *ANKRD11* expression. Furthermore, zebularine treatment coupled with induction of p53 for 16 hours resulted in a 10-fold increase in *ANKRD11* expression above the basal level expressed in these H1299 cells (Figure 3.7). This data infers that promoter demethylation, through an agent such as zebularine, can function synergistically with p53 to increase target gene expression. It has since been confirmed that CpG islands in the *ANKRD11* gene promoter are indeed methylated in breast tumours (Sue Lim, personal communication, unpublished data).

Figure 3.7

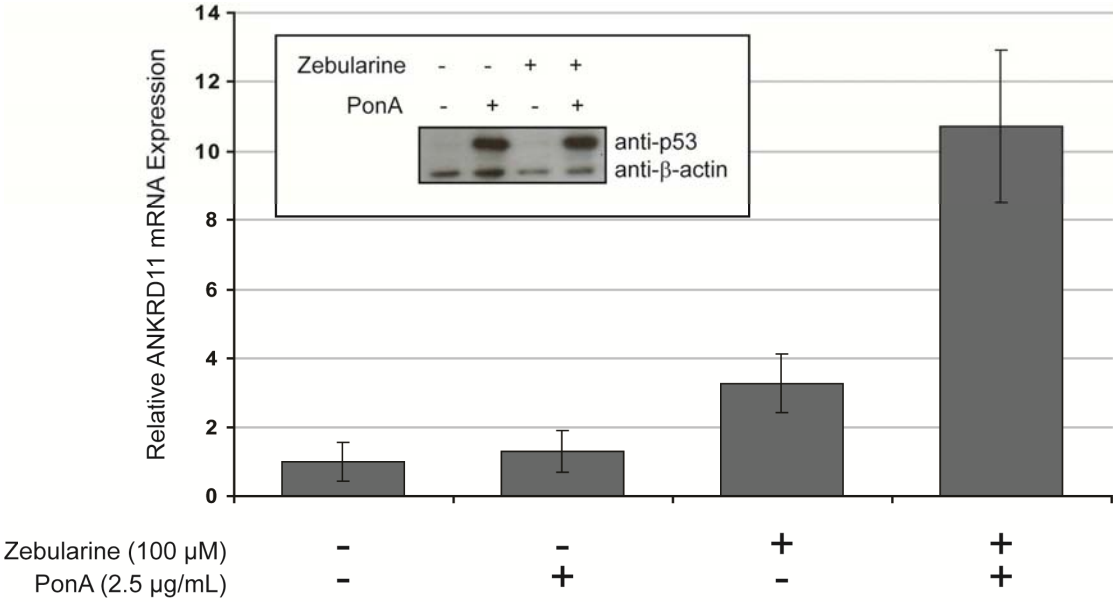


Figure 3.7 – ANKRD11 promoter demethylation results in synergistic activation by p53

EI p53-WT cells were plated at 10% confluence and treated with 100 μM zebularine or vehicle control for 72 hours, refreshing growth medium and zebularine daily, followed by induction with 2.5 μg/mL PonA (or vehicle control) for 16 hours. The level of *ANKRD11* mRNA expression was determined by real-time PCR. **Inset:** Western blot analysis shows p53 levels are unaffected by zebularine treatment.

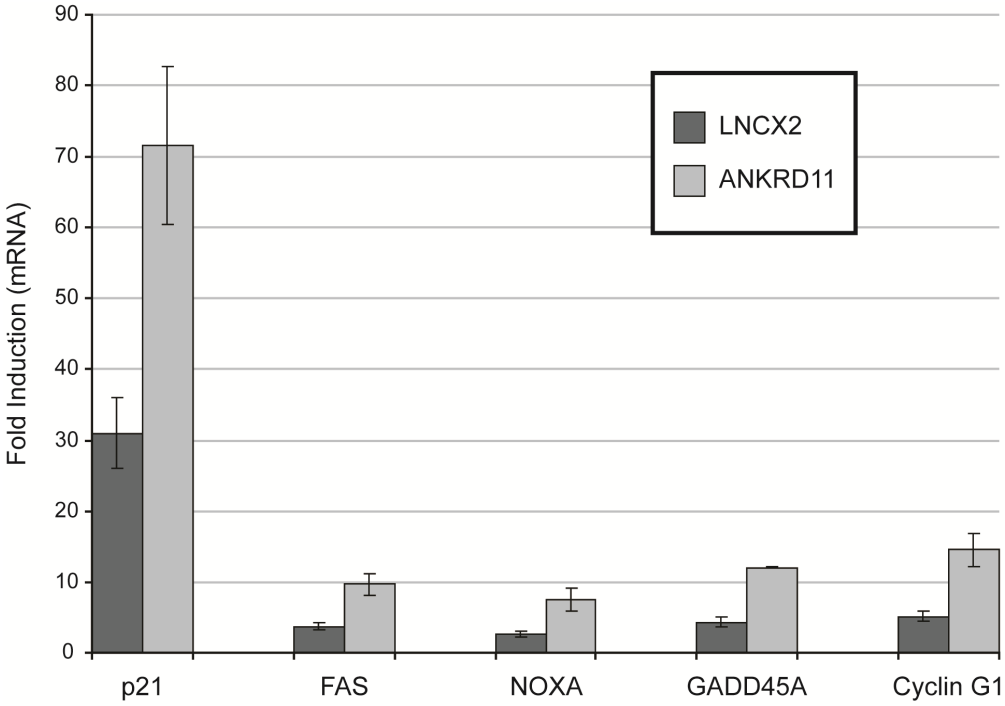
3.3.2 Investigation of the role of ANKRD11 in p53 function

3.3.2.1 ANKRD11 enhances wild-type p53 transcriptional activity

Our laboratory has previously described ANKRD11 as an endogenous protein that can interact with wild-type p53 and function as a p53-coactivator (Neilsen et al 2008). To further these findings, the EI p53-WT cell line was utilised in the presence or absence of stable ANKRD11 expression (see Section 2.1.6) to investigate the p53-dependent effect of ANKRD11 on the expression of a range of p53 target genes. Following induction of p53, the expression of the common p53 target genes *CDKN1A* (p21), *FAS*, *NOXA*, *GADD45A* and *CYCLIN G1* was increased. Interestingly, this expression was further enhanced by greater than 2-fold in the presence of ANKRD11 (Figure 3.8A). It was also established that p53 target gene expression was not enhanced by stable expression of ANKRD11 alone (Figure 3.8B). In fact, ANKRD11 expression resulted in a slight repression of target genes. This data supports previous findings that ANKRD11 can function as a co-activator of wild-type p53 transcriptional activity.

Figure 3.8

A



	LNCX2	ANKRD11	Fold change
p21	31	71.6	2.3
FAS	3.7	9.6	2.6
NOXA	2.6	7.5	2.9
GADD45A	4.3	12	2.8
Cyclin G1	5.1	14.5	2.8

B

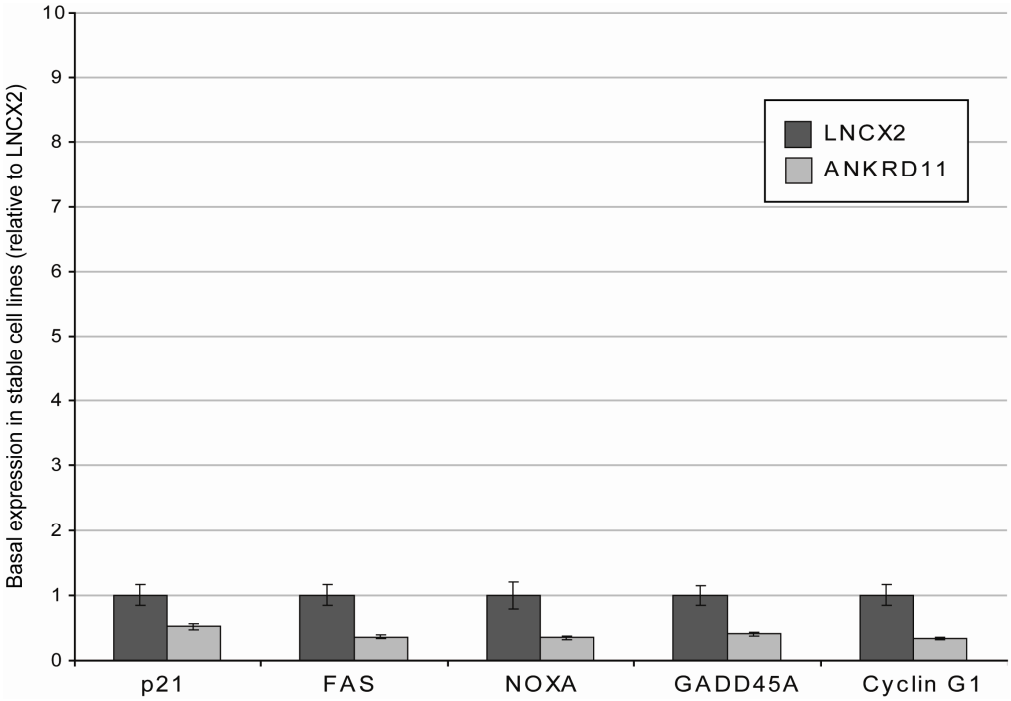


Figure 3.8 – Induction of target genes by WT-p53 is enhanced by ANKRD11

A. EI p53-WT-LNCX2 or EI p53-WT-ANKRD11 cells were treated with 2.5 µg/mL PonA for 24 hours. Real-time PCR using specific primers (see Materials and Methods) was used to determine the mRNA expression of a range of p53 target genes. Induction of target genes in the presence of ANKRD11 (light bars) was greater than that observed in the LNCX2 control cells (dark bars). Expression was normalised to β-actin and data is represented as a fold induction of indicated gene expression relative to the uninduced control (n=3).

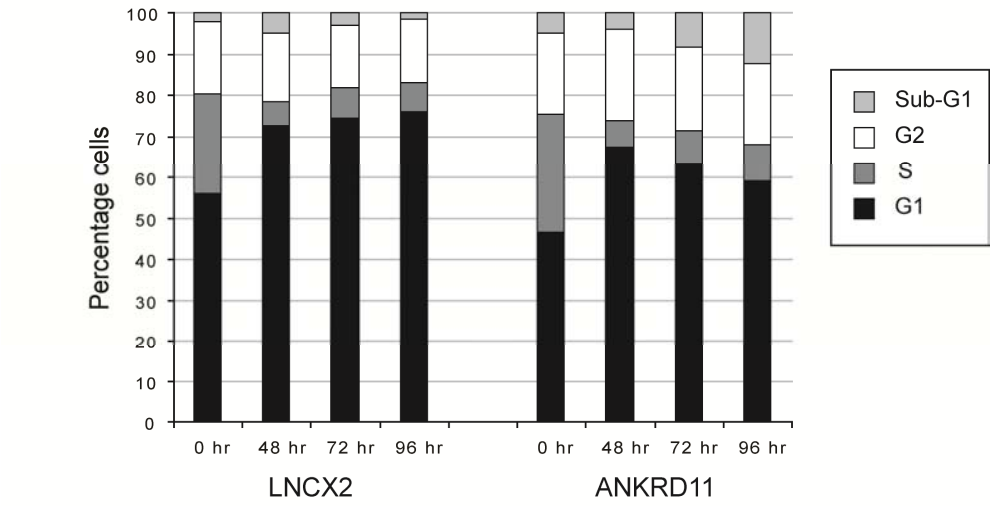
B. EI p53-WT-LNCX2 or EI p53-WT-ANKRD11 cells were treated with a vehicle control for 24 hours, and expression of p53 target genes analysed as in **(A)**.

3.3.2.2 ANKRD11 expression results in apoptosis of H1299 cells with induced wild-type p53

The expression of wild-type p53 at high levels in H1299 cells results in a G_1 arrest (Figure 3.4). Previous studies have identified p53-interacting proteins that can modulate the choice between cell cycle arrest and apoptosis in response to p53 (Hsieh et al 2002, Samuels-Lev et al 2001, Shikama et al 1999). As ANKRD11 expression in H1299 cells is able to increase the capacity of wild-type p53 to activate target gene expression (Figure 3.8), it was hypothesised that ANKRD11 could also alter the cellular outcome of H1299 cells expressing wild-type p53. To investigate this, the EI p53-WT cell line (stably expressing ANKRD11 or a vector control) was induced with 2.5 μg/mL PonA for 0, 48, 72 or 96 hours. Induction of wild-type p53 in the absence of ANKRD11 resulted in an increased number of cells in G_1 phase from 57.1 to 76.4% in 48 hours, with no increase in the sub-G_1 (or apoptotic) population, indicating a cell cycle arrest (Figure 3.9A), which is consistent with previous data (Figure 3.4). In contrast, induction of wild-type p53 in cells expressing ANKRD11 gave an increase in the G_1 population (from 48.8 to 70.2% in 48 hours) accompanied by an increase in apoptosis, with 9.2% of cells in the sub-G_1 population 72 hours post-induction, reaching a maximum of 14.1% at 96 hours (Figure 3.9B). Therefore it is probable that ANKRD11 not only alters the transcriptional activity of wild-type p53 but can affect the cellular outcome of p53 expression in H1299 cells, however further experiments are required to demonstrate this conclusively.

Figure 3.9

A



		0 hr	48 hr	72 hr	96 hr
LNCX2	**G1**	57.1	76.4	76.7	77
	S	25.1	6.1	7.8	7.3
	G2	17.7	17.5	15.5	15.7
	Sub-G1	2.3	5.2	3.1	1.5
ANKRD11	**G1**	48.8	70.2	69.1	67.4
	S	30.5	6.7	8.9	10.1
	G2	20.7	23.1	22	22.4
	Sub-G1	5.1	4.3	9.2	14.1

B

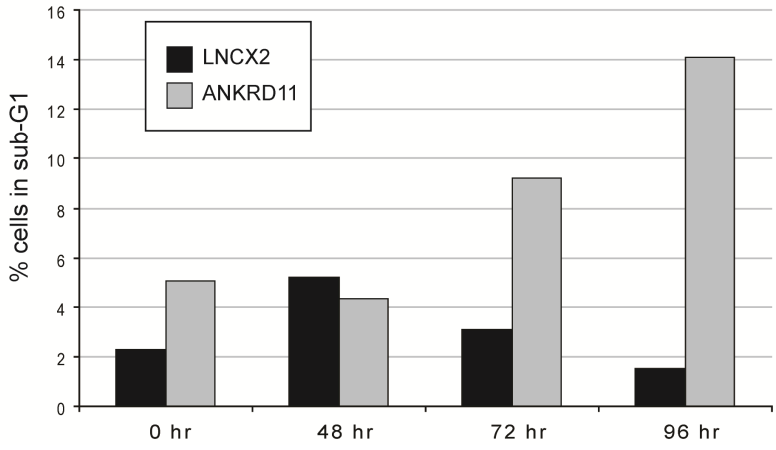


Figure 3.9 – ANKRD11 expression enhances the p53-mediated apoptotic response of H1299 cells

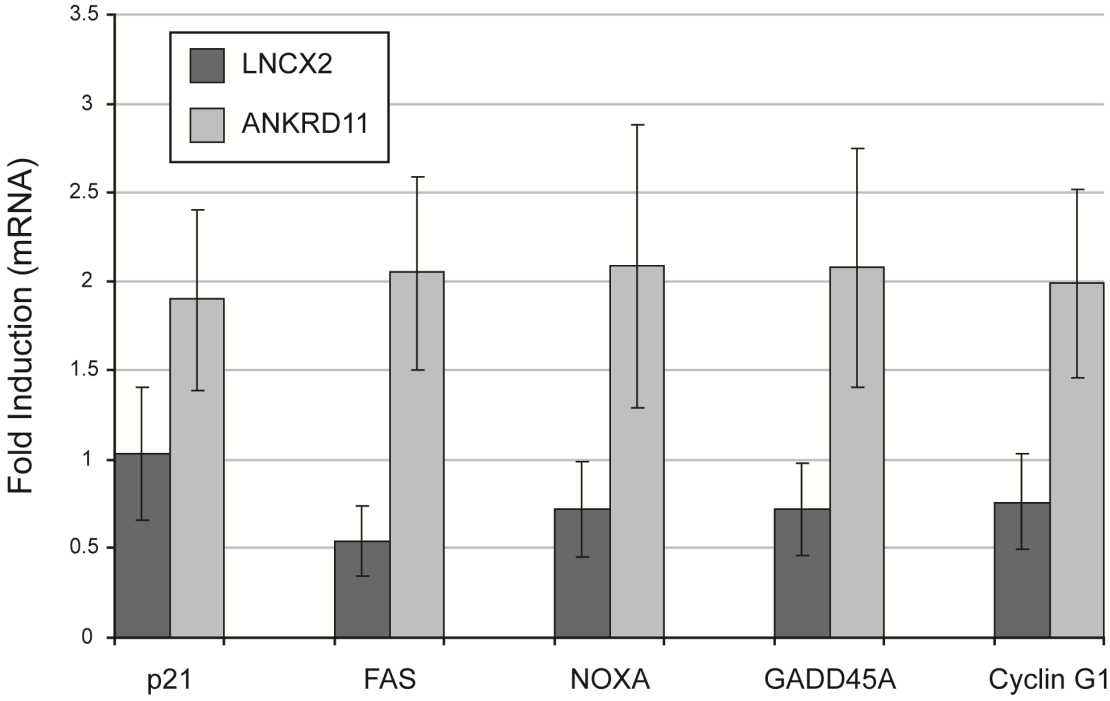
A. EI p53-WT-LNCX2 or EI p53-WT-ANKRD11 cells were treated with 2.5 µg/mL PonA (or vehicle control) for 0, 48, 72 or 96 hours. Cells were collected, fixed and stained with propidium iodide followed by FACS analysis.

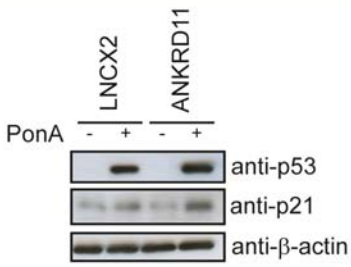
B. The percentage of cells in the sub-G_1 population, representing apoptotic cells, is presented here. An increase in the apoptotic population is evident at 72 and 96 hours post-induction in the presence of ANKRD11 (light bars) compared to a vector control (dark bars). NB. This is initial data from a single experiment. More biological replicates will be required.

3.3.2.3 ANKRD11 can restore transcriptional activity to a p53 mutant

Stable expression of ANKRD11 in the MDA-MB-468 cell line (endogenously expressing the R273H mutant) exhibits an increase in *p21*, *FAS* and *NOXA* expression (Neilsen et al 2008). It was of interest to determine if a similar increase in p53 target gene expression could be observed for any other common p53 cancer mutants. In the absence of ANKRD11, induction of the R248Q mutant failed to activate transcription of p53 target genes. In fact, a slight repression was seen in most cases, a finding consistent with previous studies (Zalcenstein et al 2003). However, in the presence of stable ANKRD11 expression the expression of these targets was enhanced, ranging from a 1.9-fold increase in *p21* mRNA to a 4-fold increase in *FAS* expression levels (Figure 3.10). These observations were also consistent with an increase in p21 protein levels (Figure 3.10). Although not significant, these data, coupled with a previously published report (Neilsen et al 2008), suggest that ANKRD11 may have a role in the rescue of a variety of p53 mutants. Therefore, potential ANKRD11-based therapies may be applicable to a broad range of mutant p53-expressing tumours.

Figure 3.10





	LNCX2	ANKRD11	Fold change
p21	1	1.9	1.9
FAS	0.5	2	4.0
NOXA	0.7	2.1	3.0
GADD45A	0.7	2.1	3.0
Cyclin G1	0.8	2	2.5

Figure 3.10 – ANKRD11 restores transcriptional activity to the R248Q p53 mutant

The expression of a range of p53 target genes was determined by real-time PCR following 24 hours of mutant p53 induction in the EI p53-R248Q-LNCX2 control or EI p53-R248Q-ANKRD11 cell lines. Stable ANKRD11 expression resulted in an increase in target gene expression (light bars) relative to the empty vector control (dark bars) (n=3). A representative western blot indicates an increase in p21 protein expression following R248Q induction in the presence of stable ANKRD11 expression.

3.4 DISCUSSION

This chapter describes the generation and initial characterisation of an isogenic panel of cell lines expressing wild-type or mutant p53 under the tightly regulated control of an ecdysone-inducible (EI) promoter. These cell lines provide an important resource that are currently being harnessed at national (Peter MacCallum Cancer Centre, Melbourne) and international (A*STAR Institute for Biomedical Research, Singapore and The Beatson Institute for Cancer Research, Glasgow) research institutes to investigate the functional consequences of both wild-type and mutant p53 expression and delve into the mechanistic basis behind these functions. Importantly, these cell lines fill a void in the current systems available, as previous systems (using either stable or inducible expression) invariably focus on the R175H and R273H mutants, while this system provides a means to investigate and compare the functional consequences of the eight most frequent cancer-associated p53 hotspot mutants.

3.4.1 Wild-type and mutant p53 protein stability

The initial observations using this inducible system relate to the stability of the wild-type and mutant p53 proteins. The isogenic inducible system provides a useful tool to investigate protein stability as the induced proteins are expressed in an identical genetic background, therefore the mechanisms available to degrade or stabilise a protein are consistent across inducible cell lines. Preliminary experiments in this system indicate that both the wild-type and mutant (specifically R273H) protein is turned over 24-48 hours after the withdrawal of the inducing agent (Figure 3.2). This is despite the inability of the mutant p53 protein to up-regulate *MDM2* expression (see Figure 3.3A).

There are a number of reported mechanisms through which p53 can be degraded. Mdm2 is the most commonly reported E3 ubiquitin ligase that targets p53 for degradation through poly-ubiquitination of specific lysine residues within the C-terminus of the protein (Haupt et al 1997, Honda et al 1997). Throughout the past decade Pirh2 (Leng et al 2003), Cop1 (Dornan et al 2004), TRIM24 (Allton et al 2009, Jain and Barton 2009), ARF-BP1 (Chen et al 2005), CARP1/2 (Yang et al 2007), TOPORS (Rajendra et al 2004), Synoviolin (Yamasaki et al 2007), CHIP (Esser et al 2005) and MKRN1 (Lee et al 2009) have all been reported as E3 ubiquitin ligase proteins that can directly target the p53 protein for degradation. Furthermore, a micro-RNA has recently been reported to repress p53 expression via a conserved sequence in the 3' untranslated region (Swarbrick et al 2010). It has been observed that mutation of the lysine residues required for ubiquitination of p53 by Mdm2 does not prevent the normal degradation of the p53 protein (Feng et al 2005a, Krummel et al 2005). This is not unexpected since it is now evident that p53 degradation is much more complex than originally thought, with a number of proteins and mechanisms demonstrated to target p53 for degradation.

Mutant p53 protein has previously been shown to accumulate in tumours (Lang et al 2004, Olive et al 2004) and this is reportedly due to an inability to up-regulate *MDM2* expression. Interestingly, the mutant p53 present in non-malignant tissues (adjacent to tumours) is not stabilised to the high levels evident in tumours and this is shown to be dependent on Mdm2 expression (Terzian et al 2008). This indicates that although mutant p53 can be degraded in some circumstances, tumour-specific stabilisation of mutant p53 is likely to occur through other compounding signals. Indeed, mutant p53

has been demonstrated to be ubiquitinated independently of Mdm2, although this ubiquitination does not necessarily correlate to increased protein degradation (Lukashchuk and Vousden 2007, Muller et al 2008). The induced mutant p53 protein in the system described in this thesis is seen to be unstable due to its rapid turnover following the withdrawal of PonA, therefore it is likely that the mutant protein can be actively degraded in this system.

To comprehensively determine similarities between wild-type and mutant p53 protein stability in the inducible H1299 system, p53 protein should be induced followed by treatment with cyclohexamide to block further protein synthesis. Subsequent analysis will determine the half-life of the wild-type and mutant proteins. Silencing of Mdm2 expression, or other ubiquitin ligases of interest, would provide further insight into the mechanisms of p53 protein turnover. These conditions would also be optimal to investigate the half-life of endogenous, activated wild-type p53 in comparison to the wild-type p53 induced in this system, which would be required to further determine the biological relevance of the artificial induction system for investigating wild-type p53 function. This inducible system provides an ideal model to establish the mechanisms through which mutant p53 may be degraded.

3.4.2 Wild-type p53 transcriptional activity

Induction of wild-type p53 in the EI p53-WT cell line results in increased expression of established p53 transcriptional targets (Figure 3.3A). These results are consistent with a previous study in which a colon tumour-derived cell line with inducible wild-type p53 was subjected to expression microarray analysis to determine p53-regulated genes (Zhao et al 2000). Therefore, it is evident that induction alone is sufficient for

p53 activity. However, preliminary data presented here suggests that treatment with the DNA damaging agent cisplatin further increases the transcriptional activity of wild-type p53 on a selection of p53 target genes (Figure 3.3B), however further studies are required to ascertain the functional significance of this increase in target gene expression.

Post-translational modifications of p53 are important in the regulation of p53 activity and it is suggested that acetylation of key lysine residues of the p53 C-terminus results in increased transcriptional activity of p53 (Avantaggiati et al 1997, Liu et al 1999). The post-translational modifications of p53 in this inducible scenario have not as yet been assessed and it may be of interest to define the acetylation and phosphorylation status of p53, in the presence and absence of extraneous stresses, to further identify the modifications required for activation of particular classes of target genes. This inducible system provides an ideal model to investigate the functional activation of p53, including its response to various DNA damaging agents and the resultant post-translational modifications and protein interactions that may occur.

Induction of the various mutant p53 proteins in the EI p53-MUT cell lines results in very low or no induction of the p53 target genes investigated (Figure 3.3A). This is indicative of a loss-of-function phenotype most likely due to an inability of the mutant p53 proteins to effectively bind to the consensus p53 response element (Bullock and Fersht 2001). In the presence of induced p53-R248Q, *GADD45A*, *CYCLIN G1*, *NOXA* and *FAS* expression is repressed below the basal levels seen in p53 null H1299 cells. This is consistent with a previous study showing direct repression of the *CD95* (*FAS*) gene by mutant p53. This study showed repression of *FAS* through mutant p53

association with the promoter at a region distinct from the consensus wild-type p53 response element, although it was not determined if this was a direct association with DNA (Zalcenstein et al 2003).

3.4.3 Cellular fate in response to p53 induction

3.4.3.1 G_1 arrest

Induction of the wild type p53 in the EI p53-WT cell line resulted in a reduction in cell growth and proliferation correlating with a G_1 arrest. This p53-dependent effect on cell growth was not observed for the mutant p53 expressing H1299 cells (Figure 3.4). This cell cycle arrest is most likely due to the preferential up-regulation of the master cell cycle regulator, p21 (as seen in Figure 3.3A), which is known to inhibit the G_1-S phase transition when expressed at high levels (Weinberg and Denning 2002). A homozygous deletion of *p21* in wild-type p53 expressing HCT116 cells results in a complete inhibition of the G_1 arrest phenotype observed following activation of p53, indicating p21 expression is required for p53-dependent G_1 arrest (Waldman et al 1995). However, the data reported here contradicts a previous study in which high levels of p53 expression induced through the tetracycline-regulated system in the p53 null cell lines Saos2 and H1299 was shown to result in apoptosis (Chen et al 1996). This data suggests that the choice of cellular fate by p53 is likely to require much more complex signalling than p53 levels alone. It has been suggested that the cellular response to p53 expression is also dependent on the activity and stability of the p53 protein, which in turn is reliant on post-translational modifications or protein-protein interactions (Braithwaite et al 2006, Zuckerman et al 2009).

A common single nucleotide polymorphism (SNP) was identified at codon 72 of p53 (resulting in either Pro72 or Arg72) (Matlashewski et al 1987). This SNP is maintained at various allelic frequencies around the world, varying with both race and physical location (Sjalander et al 1995). The codon 72 SNP has been reported to impact on cellular fate in response to p53 activation. A number of studies have demonstrated that a greater induction of G_1 arrest is seen in response to p53 containing Pro72, while Arg72 is more efficient at driving apoptosis (Dumont et al 2003, Pim and Banks 2004, Sullivan et al 2004, Thomas et al 1999). The EI p53-WT cells used in this study express p53 with Pro72, which may account for the G_1 arrest phenotype observed following p53 induction. However it is not stated which polymorphic variant is expressed in the Chen study, therefore it is difficult to draw any conclusions from the differences seen in these two studies in relation to this SNP. Another difference between this system and that employed by Chen *et al.* is the mechanism of induction. The system described in this thesis adopts an ecdysone-inducible promoter while the Chen study uses a tetracycline based system. To-date it is unclear if the method of induction may play a role in the subsequent activity of the induced protein.

3.4.3.2 Cellular senescence

Cellular senescence relates to an irreversible state of growth arrest which mammalian cells will reach after a defined number of cellular divisions *in vitro* (Hayflick 1965). Senescent cells can be identified through their increased size and changed morphology, as well as by the expression of senescence-associated β-galactosidase (SA-β-Gal) (Dimri et al 1995). Previously, p53 has been shown to play a role in inducing senescence pathways (Ben-Porath and Weinberg 2005) and indeed, upon

induction of wild-type p53 for 72 hours, the EI p53-WT cells stained positive for SA-β-Gal (Figure 3.5). A previous study has shown that activation of wild-type p53 in epithelial cells by the MDM2 antagonist nutlin results in a senescent-like phenotype. Following nutlin treatment, cells stained positive for SA-β-Gal, however the senescent phenotype was reversed upon withdrawal of nutlin (Huang et al 2009). The nature of the senescent-like phenotype induced in the EI p53-WT cell lines has not yet been determined. This may be achieved through withdrawal of the inducing agent and investigation of a possible reversion of the cells to a proliferating culture. The induction of senescence in tumour cells can lead to tumour regression *in vivo* due to the removal of senescent cells by the immune system (Xue et al 2007). Therefore, the ability to investigate the mechanisms through which p53 can drive true cellular senescence will play a critical role in the development of future cancer therapies.

3.4.4 Mutant p53 and chemo-resistance

The resistance of tumours to treatment with chemotherapeutic agents has been linked to mutant p53 expression in mice (Lowe et al 1994) and in human ovarian carcinomas (Buttitta et al 1997). Current hypotheses for mechanisms through which mutant p53 can confer this chemo-resistance include its inhibition of p73, which has been linked to the response to chemotherapeutic agents (Bergamaschi et al 2003, Irwin et al 2003) as well as specific up-regulation of genes involved in drug resistance pathways, such as *MDR1* (multiple drug resistance 1) (Chin et al 1992) and *MRP1* (multidrug resistance-associated protein 1) (Tsang et al 2003).

Chemo-resistance has previously been investigated in the p53 null H1299 cells expressing mutant p53 proteins. The expression of the R175H mutant was shown to

result in a decreased apoptotic response of cells to treatment with etoposide, while both R175H and R273H expression resulted in resistance to low, but not high, concentrations of cisplatin (Blandino et al 1999). The inducible system described in this thesis responds similarly to systems previously reported, with expression of the R175H mutant resulting in resistance to low, but not high, concentrations of cisplatin (Figure 3.6). The resistance conferred by induction of mutant p53 in these H1299 cells is likely to be a true phenomenon as there is no observed difference in the growth rate of p53 null cells compared to cells expressing mutant p53 (see Figure 3.4A), which may otherwise explain increased resistance.

Further investigation of the cisplatin resistance conferred by wild-type or mutant p53 in this inducible system is currently being undertaken at the A*STAR Institute for Biomedical Research, Singapore and a manuscript pertaining to this area has recently been submitted to *Carcinogenesis*.

3.4.5 Demethylating agents and p53

Zebularine is a DNA methylation inhibitor that has previously been demonstrated to specifically target cancer cells and induce and maintain demethylation of silenced genes in bladder cancer cells (Cheng et al 2004a, Cheng et al 2004b). Recently acquired data indicates that the reduced expression of *ANKRD11* in tumours and cancer cell lines can arise from methylation of its promoter (Sue Lim, personal communication; unpublished data). It is speculated that the *ANKRD11* gene promoter is methylated in H1299 cells, as although it has previously been identified as a p53 target gene (Neilsen et al 2008), no increase in *ANKRD11* expression is observed following p53 induction in EI p53-WT cells (Figure 3.7). This is supported by the

observation that treatment with zebularine results in an increase in *ANKRD11* expression, which is further enhanced by p53 expression (Figure 3.7). These data suggest that methylation of p53 target gene promoters, or of genes involved in p53 regulation and activation, may inhibit the ability of p53 to regulate the expression of its target genes. If down-regulation of p53 interacting proteins or critical p53 target genes by promoter methylation is a common occurrence in cancer, then combination therapies of demethylating agents with p53 activators, such as nutlin, may be advantageous in giving a complete p53-dependent response in tumours.

3.4.6 ANKRD11 function with wild-type p53

ANKRD11 has previously been reported to function as a coactivator of wild-type p53 function, enhancing the ability of p53 to transactivate its target genes (Neilsen et al 2008). These findings have been confirmed and expanded using the EI p53-WT cell line and show that ANKRD11 expression can alter both the transcriptional activity of p53 (Figure 3.8) and the cellular fate of H1299 cells expressing wild-type p53 (Figure 3.9). In the presence of stable ANKRD11 expression, induction of p53 resulted in a cellular apoptotic response (Figure 3.9). This ANKRD11 expression is also correlated with a trend toward preferential enhancement of apoptosis-related gene expression, such as the observed 2.9-fold increase in *NOXA* compared to just 2.3-fold increase in *p21* expression (Figure 3.8). The evidence presented here suggests that the ANKRD11 protein may play an important role in regulating the cellular response to p53 activation, although further work is required to conclusively show an effect of ANKRd11 on the cellular (apoptotic) response to p53.

This may be similar to the previously reported interaction of p53 with the apoptosis stimulating proteins ASPP1 and ASPP2, which has been demonstrated to enhance the p53-mediated apoptotic response (Samuels-Lev et al 2001). Similarities between the ASPP proteins and ANKRD11 are also evident in their expression profiles in tumours and cancer cell lines. ASPP expression is down-regulated in cancer cell lines (Liu et al 2005, Mori et al 2004, Mori et al 2000) and hyper-methylation of the ASPP promoter has also been observed (Liu et al 2005). Similarly, we have shown that ANKRD11 expression is down-regulated in the majority of breast tumours (see Chapter 5, Figure 1) and exhibits promoter methylation (Section 3.3.1.8, Figure 3.7). These data suggest that ANKRD11 and the ASPP proteins may function similarly with wild-type p53, and this is most likely to occur through direct interactions with, and subsequent modifications of, the p53 protein. Together, this supports previous observations that protein-protein interactions play an integral role in the regulation of p53 activity (Braithwaite et al 2006, Zuckerman et al 2009). Furthermore, this data identifies ANKRD11 as a potential therapeutic target to enhance the apoptotic response of cancer cells expressing wild-type p53.

3.4.7 Restoring activity to p53 mutants

Previously it was shown that stable expression of ANKRD11 in a breast cancer cell line harbouring the R273H mutant restores some normal function to the mutant p53, including increased DNA binding ability at a canonical p53-RE and increased p21 expression (Neilsen et al 2008). The ecdysone inducible system was utilised to extend these findings to another common cancer mutant, R248Q. The expression of a range of p53 target genes was increased by 2- to 4-fold in the presence of the R248Q mutant with stable ANKRD11 expression, compared to that observed by R248Q alone

(Figure 3.10). The trend in p53 target gene expression represented in this data indicates that ANKRD11 may be capable of restoring some level of normal function to p53 mutants. However, as loss-of-function encompasses only one aspect of p53 mutation it is important to not only investigate the ability to restore wild-type-like function to p53 mutants, but also to suppress the gain-of-function pathways that play a significant role in mutant p53-mediated tumorigenesis. The ability of ANKRD11 to suppress mutant p53 GOF pathways is investigated in a manuscript in the final stages of review at *Oncogene* (presented as Chapter 5 of this thesis).

The data presented here, coupled with previously published work, suggests that the ANKRD11-p53 interaction may provide a basis to develop therapies for mutant p53-expressing tumours to restore wild-type activity while concurrently suppressing gain-of-function properties of mutant p53. The generation of the CDB3 peptide, based on the ASPP2-p53 interaction, that can restore some function to mutant p53 provides proof-of-principle for this concept (Friedler et al 2002, Issaeva et al 2003). Indeed, an 80 amino acid region of ANKRD11 that may suppress mutant p53 gain-of-function has been identifed (Chapter 5, Figure 7), which provides a platform for the development of new mutant p53-targeted therapies.

CHAPTER 4 – Mutant p53 reprograms the cancer cell transcriptome through a mechanism involving p63

4.1 PREFACE

The inducible system described in Chapter 3 is ideal to investigate the initial events of transcriptional reprogramming by mutant p53 expression. This chapter describes the use of six of the ecdysone-inducible mutant p53 cell lines and the wild-type control to determine key transcriptional targets of mutant p53 that may be potential regulators of the oncogenic function of mutant p53. In addition, a novel mechanism is described in which mutant p53 may regulate transcriptional activity through direct recruitment with p63 to canonical p53/p63 response elements.

Some of the data presented throughout this chapter was contributed by other lab members, but is presented here to maintain a holistic view of the project. I would therefore like to acknowledge Prof. David Callen and Dr. Paul Neilsen for their contribution to data analysis, specifically pertaining to Table 4.1 and Figure 4.1. I would also like to acknowledge Kristen Ho for technical support in the generation of data for Figure 4.8 and Fares Al-Ejeh for performing Ingenuity Pathway Analysis (Table 4.4). This work is currently being continued and will be the basis for a publication to be submitted by the end of 2011.

4.2 INTRODUCTION

4.2.1 Mutant p53 regulated pathways

The *TP53* gene is mutated in approximately 50% of all human cancers, commonly resulting in the expression of a full-length protein with a single amino acid substitution ((Olivier et al 2010, Vogelstein et al 2000); discussed in Chapter 1, Section 1.2). These mutant p53 proteins have been shown to promote tumorigenesis by enhancing the ability of cancer cells to invade and metastasise (Adorno et al 2009, Bossi et al 2006, Bossi et al 2008, Dittmer et al 1993, Heinlein et al 2008, Khromova et al 2009, Muller et al 2009) as well as increasing resistance to chemotherapies (Blandino et al 1999, Irwin et al 2003), promoting genomic instability (Song et al 2007, Xu 2008) and driving multinucleation (see Chapter 5, Figure 2). To-date, the molecular mechanisms harnessed by mutant p53 to drive these oncogenic, gain-of-function (GOF) pathways are largely unknown.

4.2.2 Identification of mutant p53-regulated genes

As previously discussed (see Chapter 1, Section 1.2.3.1) the GOF pathways of mutant p53 involve the aberrant regulation of the cancer cell transcriptome. Specifically, mutant p53 regulated genes have previously been identified through microarray analysis of p53 null cells with stable expression of three common p53 mutants (R175H, R273H and D281G) (Scian et al 2004a, Scian et al 2004b, Scian et al 2005, Weisz et al 2004), as well as through the expression of PonA inducible mutant p53 (R175H) in the p53 null H1299 cell lines (Fontemaggi et al 2009). Other studies harness different methodologies to analyse mutant p53-regulated gene expression, such as silencing the expression of endogenous mutant p53 in cell lines (Yan and

Chen 2009) and over-expressing mutant p53 in cell lines expressing wild-type p53 (Tepper et al 2005). However, due to the broad range of p53 mutants expressed in cancer and the diverse genetic backgrounds of models for mutant p53 function these previous studies have been restricted in their ability to obtain a global understanding of mutant p53 transactivation function.

In general, mutant p53 has not been shown to transactivate normal p53 target genes, and this is supported by the data presented from the inducible cell lines examined in this thesis (see Chapter 3, Figure 3.3). However, mutant p53 has been shown to differentially regulate the wild-type p53 target gene *CD95*/*FAS*, through binding at a site distinct from the canonical p53-RE (Zalcenstein et al 2003). In addition, there is increasing evidence that mutant p53 can regulate gene expression through specific interaction with, and subsequent aberrant regulation of, a range of transcription factors. These include: Ets-1 (Sampath et al 2001), NF-Y (Di Agostino et al 2006), VDR (Stambolsky et al 2010), E2F1 (Fontemaggi et al 2009) and NF-κβ (Scian et al 2005, Weisz et al 2007).

4.2.3 Regulation of gene expression by p63

The p53 family member p63 is a transcription factor which exhibits a high degree of amino acid sequence homology with the p53 protein ((Osada et al 1998); discussed in Chapter 1, Section 1.2.3.3). It has been demonstrated that p63 can bind the canonical p53-RE and subsequently regulate the expression of a number of p53 target genes including *p21*, *BAX* and *MDM2* (Osada et al 1998, Shimada et al 1999). Specific genes involved in apoptotic and developmental pathways which exhibit dual regulation by p53 and p63 are reviewed by Harms *et.al* (Harms et al 2004).

The consensus sequences of the p53 and p63 binding sites are similar but contain subtle differences. A consensus p63 DNA binding site (p63-RE) has been identified that shows a region within the 20 base pair consensus site is more degenerate for p63 binding, relative to that required for p53 binding (Perez et al 2007). This was subsequently confirmed in another study which used a ChIP-seq approach to identify global p63 binding sites relating to genes involved in development (Kouwenhoven et al 2010). These minor changes in the composition of the p53/p63-REs may be sufficient to allow differential regulation of genes by p53 and p63.

A recent study investigated the ability of p53 or its family members to regulate the expression of genes involved in DNA damage repair pathways. A number of genes that were down-regulated in the absence of p53 or its family members, p63 and p73, were identified and these were shown to contain putative p53/p63-REs (due to the degeneracy of the consensus binding sites). Two of these genes (*BRCA2* and *MRE11*) were shown to be regulated by p63 independently of p53 (Lin et al 2009). This indicates that the identification of a p53-RE, due to its high similarity to the p63-RE, may not necessarily correlate with regulation by p53.

Mutant p53 can interact with p63, sequestering it from the DNA and subsequently inhibiting its transcriptional activity ((Gaiddon et al 2001, Strano et al 2002); see Chapter 1, Section 1.2.3.3). This mechanism of action by mutant p53 is reported to result in increased invasive and metastatic potential of cells (Adorno et al 2009, Muller et al 2009). Although mutant p53 has been shown to be recruited to DNA in combination with other transcription factors (Di Agostino et al 2006, Fontemaggi et al

2009, Sampath et al 2001, Scian et al 2005, Stambolsky et al 2010, Weisz et al 2007), this has not as yet been observed with p63.

4.2.4 Utilising an inducible system to investigate gene regulation in response to mutant p53 expression

The panel of H1299 cell lines expressing wild-type and a range of p53 hotspot mutants under the control of an ecdysone-inducible (EI) promoter allows for tightly regulated expression of p53 protein upon addition of the inducing agent (see Chapter 3, Figure 3.1). In this chapter the downstream modulation of gene expression in response to wild-type and mutant p53 expression is investigated through the use of this isogenic panel of cell lines. This chapter reports a number of novel targets of mutant p53, as identified through microarray expression analysis. It is demonstrated that genes can be transcriptionally responsive to both mutant and wild-type p53. Mechanistically, due to the high degree of similarity between the consensus sequences of the p53- and p63-REs, mutant p53 is proposed to interact with p63 on p53/p63-REs to directly modulate the activity of specific target genes.

4.3 RESULTS

4.3.1 Investigation of global transcriptional regulation by mutant p53

Previous studies that investigate the regulation of genes in response to mutant p53 expression have focussed on a limited number of mutants (Bossi et al 2008, Fontemaggi et al 2009, Scian et al 2004a, Scian et al 2004b), been performed in the presence of wild-type p53 (Tepper et al 2005) or in a range of genetic backgrounds (Yan and Chen 2009). To overcome these challenges, and to comprehensively investigate the role of mutant p53 in regulating gene expression, expression microarray analysis (Affymetrix exon arrays) was performed on EI p53-WT and six EI p53-MUT cell lines. The mutants investigated include six hot spot mutants, including both structural (R175H, R249S and R282W) and DNA contact (R248Q, R248W and R273H) mutants. This panel of mutant p53 inducible cell lines provides a sensitive expression platform to capture the initial events of transcriptional reprogramming by mutant p53. Each cell line was cultured in the presence or absence of PonA for 24 hours and analysis performed on two independent biological replicates. Gene expression profiling revealed the ability of mutant p53 proteins to aberrantly regulate transcription in the H1299 cell lines. A core set of 59 genes was shown to be regulated by all six p53 mutants; 57 genes up-regulated and 2 genes down-regulated by an average of ≥1.6-fold compared with the uninduced control (Table 4.1; Figure 4.1A). A threshold of ≥1.6-fold change in gene expression was chosen as this has commonly been used in other studies (Dorer et al 2010, Wurmbach et al 2001), and would provide a more complete list of genes showing regulation by mutant p53 rather than only those that are most responsive. The EI p53-WT cell line was utilised to determine genes specific and unique to mutant p53. Interestingly, the majority of these genes

(54/59) were also regulated by wild-type p53, although this represents only a small subset of the total 1946 genes regulated by wild-type p53 in this system (Figure 4.1A). Furthermore, this system is seen to provide a genuine representation of wild-type p53 transcriptional regulation, as there is considerable overlap between the 1946 wild-type p53-regulated genes and 129 *bone fide* targets of wild-type p53 reported in a review of wild-type p53 regulated genes (Figure 4.1B) ((Riley et al 2008); discussed in Chapter 1, Section 1.1.2.1). This data indicates that mutant p53 function may predominantly occur through aberrant, constitutive activation of wild-type p53-regulated genes, rather than unique mutant p53-specific gain-of-function targets.

4.3.2 Investigation of the relationship between mutant p53 expression profiles

It was expected that expression profiles following mutant p53 induction would cluster relative to the class of mutant p53 i.e. DNA contact or structural. Interestingly however, following clustering of the mutants based on their ability to regulate downstream targets using GenePattern 2.0 (Reich et al 2006), it is evident that there is no consistent relationship between the tertiary structure and classification of mutant p53 and its transcriptional profile (Figure 4.1C). Even two mutants at the same site (R248Q and R248W) exhibit significantly different patterns of gene regulation upon their induction.

FIGURE 4.1

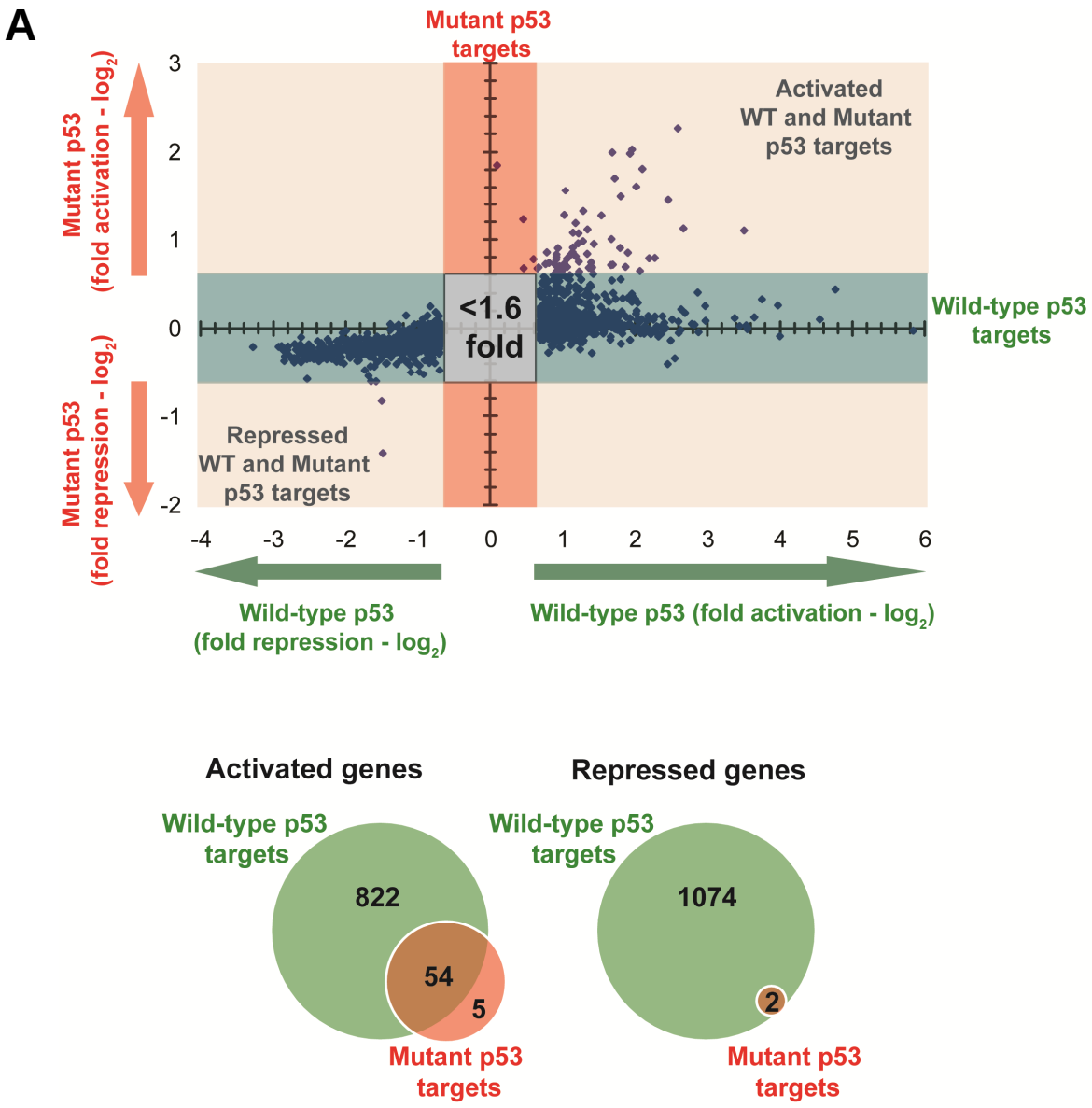

A
Mutant p53 targets
Activated WT and Mutant p53 targets
Repressed WT and Mutant p53 targets
<1.6 fold
Wild-type p53 targets
Mutant p53 (fold activation - log2)
Mutant p53 (fold repression - log2)
Wild-type p53 (fold repression - log2)
Wild-type p53 (fold activation - log2)
Activated genes
Repressed genes
Wild-type p53 targets
822
54
5
Mutant p53 targets
1074
2
B
828
48
61
Published p53 targets
1071
5
15
C
R175H
R249S
R273H
R248Q
R282W
R248W

Figure 4.1 – Expression microarray analysis of inducible wild-type and mutant p53 in H1299 cells

A. Scatterplot and Venn diagrams of expression array data including genes regulated by ≥1.6 fold by wild-type p53 or across all six p53 mutants. Data supplied by Prof. David Callen & Dr. Paul Neilsen.

B. Venn diagram illustrating the overlap between genes regulated by wild-type p53 in this expression microarray analysis as compared with known *bone fide* direct p53 target genes (Riley et al 2008).

C. Hierarchical clustering of transcriptional regulation by each p53 mutant, as determined using Gene Pattern 2.0 (Reich et al 2006). Data supplied by Prof. David Callen.

Table 4.1 Genes regulated by ≥1.6-fold across all six mutants

	Accession Number	Gene Symbol	Gene Name	Fold Change Mut p53	Fold Change WT p53
WT/mut p53 activated	NM_002560	P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4	4.81	6.02
	NM_006528	TFPI2	Tissue factor pathway inhibitor 2	4.08	3.88
	NM_001002236	SERPINA1	Serpin peptidase inhibitor, clade A	3.99	3.21
	NM_002905	RDH5	Retinol dehydrogenase 5	3.95	3.81
	NM_025181	SLC35F5	Solute carrier family 35, member F5	3.50	4.28
	NM_199511	CCDC80	Coiled-coil domain containing 80	3.25	3.29
	NM_001902	CTH	Cystathionase (cystathionine gamma-lyase)	3.05	4.04
	NM_003764	STX11	Syntaxin 11	2.95	2.05
	NM_002599	PDE2A	Phosphodiesterase 2A	2.81	3.48
	NM_012242	DKK1	Dickkopf homolog 1 (Xenopus laevis)	2.74	5.49
	NM_000358	TGFBI	Transforming growth factor, beta-induced	2.51	2.43
	NM_015541	LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1	2.43	2.03
	NM_032169	ACAD11	Acyl-CoA dehydrogenase family, member 11	2.42	2.90
	NM_021947	SRR	Serine racemase	2.28	2.26
	NM_019058	DDIT4	DNA-damage-inducible transcript 4	2.19	6.35
	NM_000332	ATXN1	Ataxin 1	2.17	2.53
	NM_006622	PLK2	Polo-like kinase 2	2.15	11.34
	NM_030762	BHLHE41	Basic helix-loop-helix family, member e41	2.12	2.21
	NM_020946	DENND1A	DENN/MADD domain containing 1A	2.10	2.33
	NM_001957	EDNRA	Endothelin receptor type A	2.01	3.19
	NM_002756	MAP2K3	Mitogen-activated protein kinase kinase 3	1.98	2.43
	NM_003012	SFRP1	Secreted frizzled-related protein 1	1.94	2.69
	NM_021021	SNTB1	Syntrophin, beta 1	1.88	2.20
	NM_005860	FSTL3	Follistatin-like 3	1.88	3.46
	NM_006738	AKAP13	A kinase (PRKA) anchor protein 13	1.88	1.91
	NM_005562	LAMC2	Laminin, gamma 2	1.82	2.20
	NM_003155	STC1	Stanniocalcin 1	1.81	1.72
	NM_001966	EHHADH	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	1.81	3.73
	NM_015046	SETX	Senataxin	1.79	1.88
	NM_024578	OCEL1	Occludin/ELL domain containing 1	1.77	2.13
	NM_015310	PSD3	Pleckstrin and Sec7 domain containing 3	1.76	2.27
	NM_198536	TMEM205	Transmembrane protein 205	1.75	1.88
	NM_033446	FAM125B	Family with sequence similarity 125, member B	1.74	4.83
	NM_006762	LAPTM5	Llysosomal protein transmembrane 5	1.73	4.57
	NM_004780	TCEAL1	Transcription elongation factor A (SII)-like 1	1.72	2.08
	NM_005100	AKAP12	A kinase (PRKA) anchor protein 12	1.72	1.88
	NM_139314	ANGPTL4	Angiopoietin-like 4	1.68	2.62
	NM_015990	KLHL5	Kelch-like 5 (Drosophila)	1.67	1.75
	NM_003619	PRSS12	Protease, serine, 12 (neurotrypsin, motopsin)	1.66	1.84
	NM_003326	TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4	1.66	2.03
	NM_006621	AHCYL1	Adenosylhomocysteinase-like 1	1.66	1.70
	NM_005347	HSPA5	Heat shock 70kDa protein 5	1.65	2.02
	NM_006226	PLCL1	Phospholipase C-like 1	1.65	3.23
	NM_153268	PLCXD2	Phosphatidylinositol-specific phospholipase C, X domain containing 2	1.64	3.18
	NM_021623	PLEKHA2	Pleckstrin homology domain containing, family A member 2	1.63	1.94
	NM_138578	BCL2L1	BCL2-like 1	1.62	1.89
	NM_006379	SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, 3C	1.62	3.65
	NM_016303	WBP5	WW domain binding protein 5	1.62	2.09
	NM_000960	PTGIR	Prostaglandin I2 (prostacyclin) receptor (IP)	1.61	2.00
	NM_001083899	GP6	Glycoprotein VI	1.61	2.65
	NM_001080503	CCDC159	Coiled-coil domain containing 159	1.61	2.57
	NM_002204	ITGA3	Integrin, alpha 3	1.60	2.62
WT/mut p53 repressed	NM_001801	CDO1	Cysteine dioxygenase, type I	-1.77	-2.83
	NM_001771	CD22	CD22 molecule	-2.67	-2.80
Mut p53 activated	NM_152637	METTL7B	Methyltransferase like 7B	3.60	
	NM_005291	GPR17	G protein-coupled receptor 17	2.35	
	NM_020698	TMCC3	Transmembrane and coiled-coil domain family 3	1.72	
	NM_021005	NR2F2	Nuclear receptor subfamily 2, group F, member 2	1.60	
	NM_001098817	INO80C	INO80 complex subunit C	1.60	

4.3.3 Validation of mutant p53 target genes

To validate the expression microarray results (Figure 4.1), the regulation of a selection of these genes was determined by specific real-time RT-PCR analysis using the EI p53-WT, -R248Q, -R175H and -R282W cell lines. The expression of *SERPINA1*, *OCEL1*, *STX11*, *TFPI2*, *BCL2L1*, *DDIT4*, *LAMC2*, *TMEM205*, *DKK1* and *PLK2* were confirmed to be up-regulated in both the wild-type and mutant p53 induced H1299 cells (Figure 4.2A). Furthermore, the negatively regulated gene *CDO-1* was also confirmed to have reduced expression upon induction of both wild-type and mutant p53 (Figure 4.2B).

The ability of mutant p53 to regulate gene expression was subsequently confirmed in two breast cancer cell lines endogenously expressing mutant p53. The expression of the p53 R280K and R273H GOF mutants was silenced in the MDA-MB-231 and MDA-MB-468 breast cancer cell lines, respectively, resulting in a reduction in the basal expression of several of the identified genes (Figure 4.2C).

FIGURE 4.2

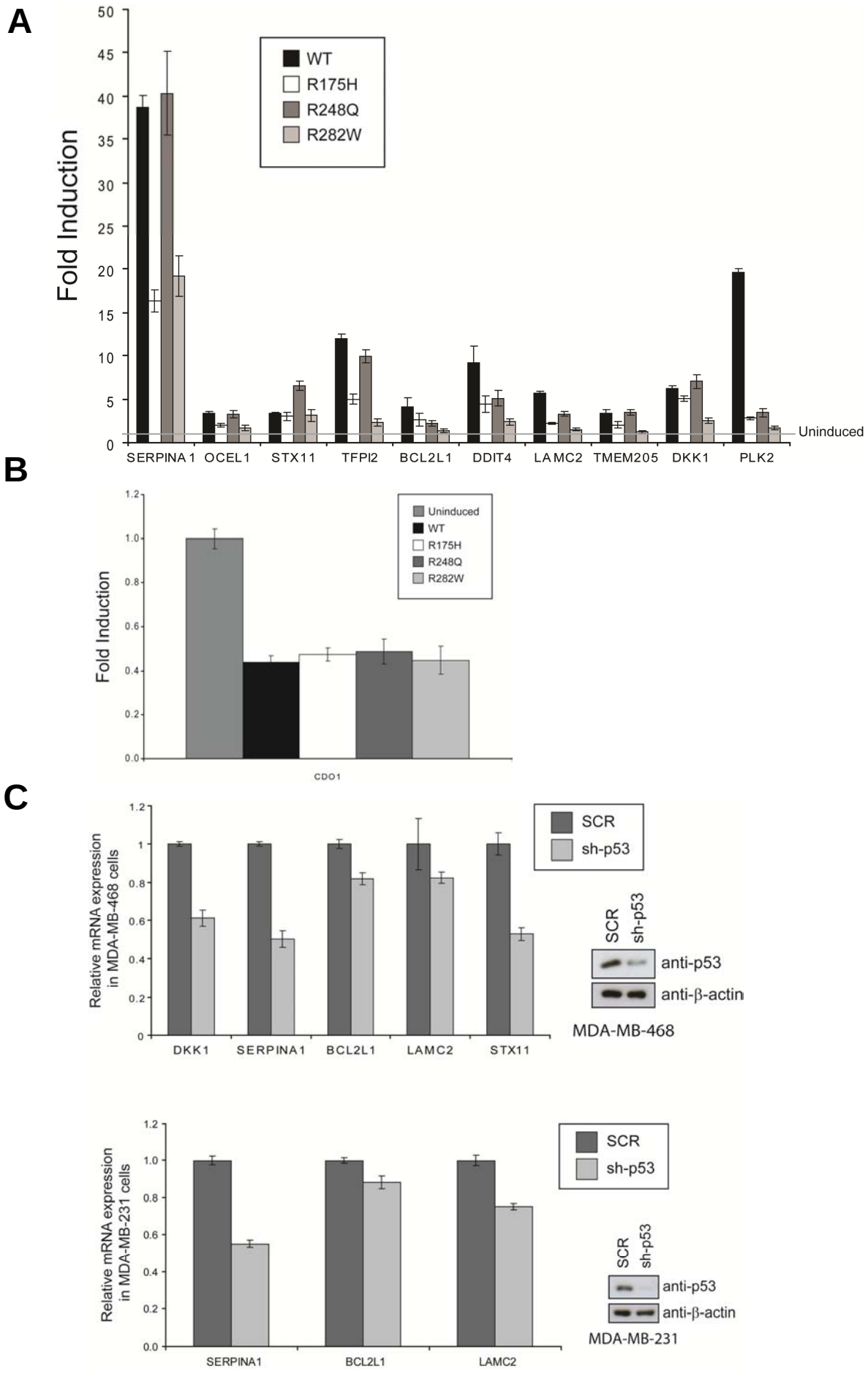

A
Fold Induction
WT
R175H
R248Q
R282W
Uninduced
SERPINA1
OCEL1
STX11
TFPI2
BCL2L1
DDIT4
LAMC2
TMEM205
DKK1
PLK2
B
Fold Induction
Uninduced
WT
R175H
R248Q
R282W
CDO1
C
Relative mRNA expression in MDA-MB-468 cells
SCR
sh-p53
DKK1
SERPINA1
BCL2L1
LAMC2
STX11
anti-p53
anti-β-actin
MDA-MB-468
Relative mRNA expression in MDA-MB-231 cells
SERPINA1
BCL2L1
LAMC2
MDA-MB-231

Figure 4.2 – Validation of target genes identified in EMA

A. EI p53-WT, EI p53-R175H, EI p53-R248Q or EI p53-R282W cells were treated with PonA (or vehicle control) for 24 hours and the mRNA expression of specific target genes was determined by real-time PCR analysis (see Table 2.1 for specific primers). All genes investigated showed an increase in expression following wild-type and mutant p53 induction. Fold induction of target genes is presented relative to the uninduced control in each case (indicated by horizontal line).

B. EI p53-WT, EI p53-R175H, EI p53-R248Q or EI p53-R282W cells treated as in **(A)** showed a reduction in the expression of *CDO-1* mRNA.

C. The ability of endogenous mutant p53 in MDA-MB-468 (expressing p53-R273H) and MDA-MB-231 (expressing p53-R280K) to regulate target gene expression was determined by silencing endogenous mutant p53 by constitutive expression of p53-specific shRNA (sh-p53) (see Chapter 2, Section 2.1.7), followed by real-time PCR analysis of selected genes.

4.3.4 Investigation of the induction kinetics of "mutant specific" target genes

Previous studies investigating mutant p53 gene regulation have identified genes that are repressed (Scian et al 2004b, Scian et al 2005) or unaffected (Fontemaggi et al 2009) by wild-type p53 expression. Similarly, it was expected that this study would identify target genes specific to mutant p53 that may be integral in modulating mutant p53 GOF pathways and therefore provide novel molecular targets to design therapeutics to inhibit mutant p53 GOF. However, only 5/59 genes were revealed as specifically regulated by mutant p53 (see Figure 4.1A). It was of particular interest to investigate if these genes do indeed represent genuine unique mutant p53-specific targets.

To determine if these genes represent *bona fide* mutant p53-specific regulated genes, we examined the kinetics of transactivation by p53 in the EI p53-WT and EI p53-R248Q cell lines. Expression of p53 was induced in each cell line for 0, 8, 24, 48, 72 or 96 hours. At each time point the expression of these genes was determined by specific real-time RT-PCR. It is evident that these genes are up-regulated by both wild type and mutant p53 in a time-dependent fashion (Figure 4.3). Therefore we conclude that up-regulation of *METTL7B*, *GPR17* and *NR2F2* is not specific to mutant p53. However, it is of interest that their expression continues to increase in the presence of mutant p53 significantly above the levels observed in the presence of wild type p53. This was particularly evident for METTL7B and GPR17. Therefore, although these genes were not selectively up-regulated by mutant p53, they may represent genes with high transactivation potential and fast induction kinetics by mutant p53.

FIGURE 4.3

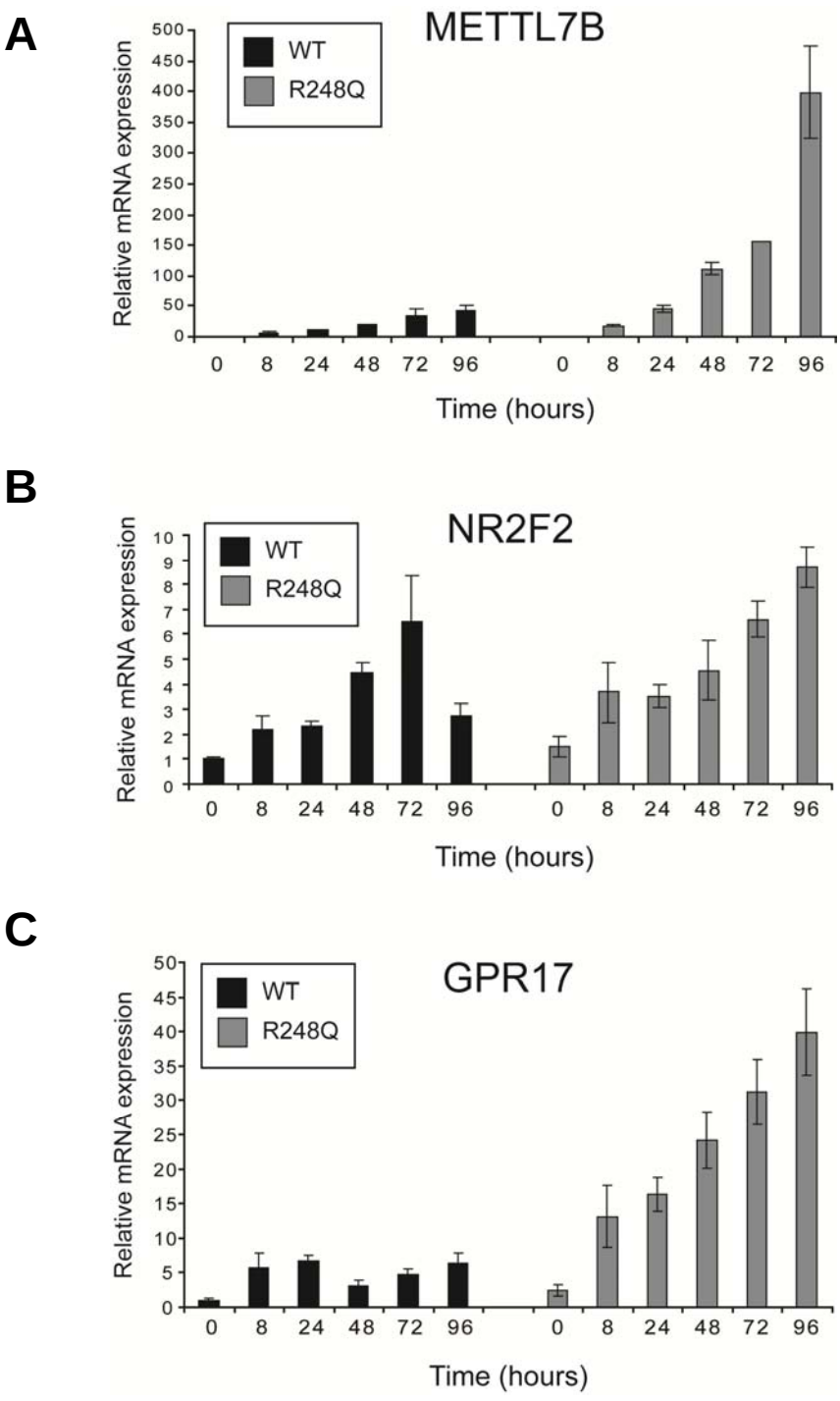


Figure 4.3 – "Mutant specific" target genes are also regulated by wild-type p53

The EI p53-WT and EI p53-R248Q cell lines were treated with PonA to induce p53 protein expression for 0, 8, 24, 48, 72 and 96 hours. The expression of **(A)** METTL7B **(B)** NR2F2 and **(C)** GPR17 was determined by specific real-time PCR analysis. *Analysis of INO80C and TMCC3 is ongoing.*

4.3.5 Identification of p53 response elements in mutant p53 regulated genes

The evidence from the expression microarray (Figure 4.1, Table 4.1) coupled with the subsequent demonstration that the identified mutant p53-specific target genes can in fact also be regulated by wild-type p53 (Figure 4.3) suggest that a subset of genes can be regulated by both wild-type and mutant p53. Although there was significant overlap between wild-type p53 targets identified in this study and those previously identified (see Figure 4.1B), only 3 of the 59 genes regulated by both wild-type and mutant p53 have previously been confirmed as direct wild-type p53 target genes (as reviewed by (Riley et al 2008)). Wild-type p53 up-regulates *DKK1* (Wang et al 2000), *PLK2* (Burns et al 2003) and *DDIT4* (Ellisen et al 2002) expression through direct binding to p53-REs in the promoter regions of these genes. It was hypothesised that more of the 59 identified genes may be directly regulated by wild-type p53. Subsequently, a 10kb region upstream of the transcription initiation site (which includes the classical promoter region), the first intron and 3' UTR of each gene was scanned for putative p53-REs using p53-scan software (Smeenk et al 2008). The results of this p53-scan are presented in Table 4.2. Putative p53-REs were identified in 96% (57/59) of the mutant p53-regulated genes, with only two genes (*TMEM205* and *TCEAL1*) returning no positive hits for a p53-RE. Overall, there was a significant enrichment of putative p53-REs identified in gene regulatory regions. 30% (18/59) had a putative p53-RE located upstream of the transcription initiation site and 24% (14/59) within the first intron or 3' UTR.

Table 4.2 p53-REs identified through *in silico* analysis using p53-scan software

Gene	Genomic Location* Start	End	p53 scan score	Sequence	Spacer	Sequence	Location in gene
ACAD11	32992	33024	14.6	AGGCAGGCCC	CCAGTCCATTGT	GGGCATGTCC	Other
AHCYL1	4732	4752	6.2	TGTGTTGCCC		AGGCTTGTCT	1st intron
AKAP12	23178	23198	7.3	GGGCATGTAC		CACCATGCCT	Other
AKAP12	64720	64740	6.8	AGGCATGCAC		CACCATGCCT	Other
AKAP13	29610	29630	9.1	AAGCAAGTCT		GGTCATACCT	1st intron
AKAP13	103471	103491	10.5	AGGCAAGTCT		GAGCAGGCCC	1st intron
AKAP13	236746	236766	10.8	AGGCATGTGC		CAACATGCCT	Other
AKAP13	290655	290675	11.1	AGACATGCTG		ACACATGCCT	Other
ANGPTL4	-4133	-4113	6.9	AGACCAGCCT		GGACATGGCG	5' to transcriptional start site
ATXN1	22066	22086	10.1	AGACTTGTTT		GTGCTAGTTC	Other
ATXN1	108238	108258	8.1	GGGCATGCCT		GGTCTTGGAT	Other
ATXN1	163817	163837	10.1	AGACTTGTTT		GTGCTAGTTC	Other
ATXN1	250008	250028	8.1	GGGCATGCCT		GGTCTTGGAT	Other
ATXN1	336938	336958	9.2	GAGCTGGTCT		GTACTTGTTT	Other
ATXN1	406299	406319	10.8	AAGCAAGCCC		AGGCCAGTCC	Other
BCL2L1	44063	44083	12.7	GGACTTGTCT		GGACTAGTTG	Other
BHLHE41	-8624	-8604	9.0	GGACAAGCTC		CTGCAAGTCA	5' to transcriptional start site
INO80C	13432	13452	7.6	AAAAAAGTTT		GTACATGTTT	1st intron
CCDC80	10612	10632	8.3	AGACATGTGG		AGGCTAGTAA	Other
CTH	17957	17977	9.0	AAAAAAGTCT		GTACATGTTC	Other
DDIT4	-3155	-3135	14.9	AAGCATGTCT		AGGCAAGCCC	5' to transcriptional start site
DENND1A	86508	86528	7.2	TAACAAGTCC		ATCCATGTTG	Other
DENND1A	154703	154723	7.2	TGGTATGTTC		TGACTTGTGT	Other
DENND1A	237168	237188	8.4	GAGCAAGTCA		CTTCATGTCT	Other
DENND1A	403983	404003	7.7	GTGCATGTGT		GTGCATGTGT	Other
DENND1A	458873	458901	15.5	GGACTTGCCC	CACGTTCC	AGACATGTTA	Other
DENND1A	522573	522593	9.5	AAATATGACA		AGACTTGTCT	Other
DKK1	-3247	-3227	9.9	AGACATGTGT		TAGCATGCTG	5' to transcriptional start site
EDNRA	13130	13150	8.2	AAGCAAGTCA		AATCTTGCAT	Other
EHHADH	43104	43124	9.7	GGACTGGTCT		AGACAAGTGC	Other
FAM125B	30458	30478	11.3	GAACATGCAT		ATGCAAGTCT	Other
FAM125B	133599	133619	8.8	GGTCATGCCA		GGAGATGCCC	Other
FSTL3	5327	5347	5.5	GGACAGGCCT		GGGCCTGGTG	3' UTR
GP6	8713	8733	7.2	GGGCATGGTG		GTGCATGCCT	Other
GPR17	-7623	-7603	5.3	GGACCTGGCA		GGGCTAGTCT	5' to transcriptional start site
HSPA5	3587	3607	8.1	GAACATGACT		TAATATGTCC	Other
ITGA3	-2213	-2193	5.8	GCTTTTGTTT		AGGCATGTCT	5' to transcriptional start site
KLHL5	65333	65353	7.8	TTACAAGTTC		AAGCATATCA	Other
LAMC2	27032	27052	10.8	GTGCAGGTCT		GAACTTGTCA	Other
LAPTM5	9538	9558	13.8	AGACAAGCCC		GGGCCTGTCT	1st intron
LRIG1	13707	13727	9.9	AGACATGTCC		ATACATGGAA	1st intron
LRIG1	62854	62874	9.6	AGACAGGCTT		GGGCACGTTA	Other
CCDC159	-4579	-4559	5.1	AAACAAGACA		AAACAGGCTG	5' to transcriptional start site
MAP2K3	17518	17538	6.7	ACACATCCTT		GGACAAGTTC	Other
METTL7B	-4993	-4973	8.3	ATACATGCTA		CAACATGTGT	5' to transcriptional start site
NR2F2	-9706	-9686	5.6	CAACATGCTC		TGGCTGGCTG	5' to transcriptional start site
OCEL1	-6934	-6914	5.0	AGAGATGCTA		GGACTGGCCC	5' to transcriptional start site
P2RX4	6816	6836	7.6	AGGCGTGTTG		GCGCATGCCT	1st intron
PDE2A	30573	30593	13.7	GAGCATGCCA		GAGCATGCCT	1st intron
PLCL1	57687	57707	9.7	GTACATGCTA		CTACTTGTCT	1st intron
PLCL1	112187	112207	12.9	AGACAAGATT		AGGCATGTTC	1st intron
PLCL1	195614	195634	7.4	ATACATGTAC		AGGTTTGTTA	1st intron
PLCL1	301985	302017	14.0	AGACTAGTCT	ATAAGGAGAGAC	AGACATGTAA	Other
PLCXD2	20869	20889	10.5	AGGCATGCTT		GGCCATGCTT	Other
PLCXD2	191209	191229	10.0	ATGCAAGACA		TGACATGCTC	Other
PLEKHA2	-8602	-8582	10.2	CAGCATGCCC		AGACATGGCA	5' to transcriptional start site
PLK2	-2207	-2187	16.4	AAACATGCCT		GGACTTGCCC	5' to transcriptional start site
PRSS12	34913	34933	7.0	GTGTATGTAC		TTACATGTTT	Other
PSD3	61338	61358	9.6	TGGCAAGCTC		AAACAAGCAT	1st Intron
PSD3	138608	138628	10.1	GGACATGATG		GTGCATGCCT	Other
PSD3	362094	362114	11.6	ATACATGTCT		GGATTTGTCC	Other
PSD3	484223	484243	14.1	TGACATGTCT	GGGACCTC	TGACATGTGT	3' UTR
PTGIR	-6020	-6000	6.8	GGGCAGGTCC		CTGCGTGCCC	5' to transcriptional start site

Gene	Genomic Location* Start	End	p53 scan score	Sequence	Spacer	Sequence	Location in gene
RDH5	455	475	6.1	AGGCAAGCAA		GAAGATGCCT	1st intron
SERPINA1	4742	4762	8.9	GGGAAAGCCA		AGACTTGTTC	Other
SEMA3C	7756	7776	16.5	AGACATGCCT		AAACATGTTT	Other
SEMA3C	145166	145186	9.6	GCACTTGTTT		GAACTTGTAC	Other
SETX	72864	72896	14.6	ATACAAGTTT	GATTTTTTTTTT	AGACATGTTT	Other
SFRP1	42875	42895	9.4	AGGCATGATG		GTGCATGCCT	Other
SLC35F5	42127	42147	7.4	AAGCATGTTT		GTGCAAATTT	3' UTR
SNTB1	79347	79367	10.5	AGGCATGCGC		CAACATGCCC	Other
SNTB1	167434	167454	8.5	ACACATGCAC		ACGCATGTTT	Other
SNTB1	267034	267054	8.8	TGGCATGTGT		TAACATGTAG	Other
SRR	10656	10676	7.2	GGGCATGGTG		GTGCATGCCT	1st intron
STC1	4608	4628	9.1	AGTCATGTAC		ATACATGCAA	Other
STX11	-9986	-9966	6.7	AGGCATGAAC		CATCATGCCT	5' to transcriptional start site
TFPI2	-9250	-9230	5.8	GGACAAGTGC		TGAGATGCTA	5' to transcriptional start site
TGFBI	5076	5096	11.9	ATGCATGTGC		GAACATGTCT	Other
TMCC3	64742	64770	14.0	AGACAAGCAC	AGAGCAAC	AGGCATGTCA	1st intron
TNFSF4	-9836	-9816	11.2	AGACAAGCAC		ATACATGCAT	5' to transcriptional start site
TCEAL1			no site				
TMEM205			no site				
WBP5	-5921	-5901	9.4	ATGCAGGTTT		GTGCATGTTT	5' to transcriptional start site
CD22	11255	11275	10.6	AGACATGCAC		CAGCATGCAT	Other
CDO1	-1850	-1830	5.4	TTGCAAATTT		AAACATGCAC	5' to transcriptional start site

* Location of identified site relative to transcription initiation site in specified gene
# score defined by p53 scan software

4.3.6 Endogenous p63 regulates mutant p53-regulated genes

The discovery of putative p53-REs in the 59 identified genes indicates that these genes may represent direct p53 targets. However, mutant p53 is reportedly unable to bind the canonical p53-RE resulting in a general loss of transactivation function of wild-type p53 target genes (Bullock and Fersht 2001, Joerger et al 2006). Therefore, mutant p53 is likely to be regulating this specific subset of p53 target genes through an alternative mechanism. Mutant p53 has previously been shown to function as an aberrant transcription factor through interactions with other transcriptional regulatory proteins, thereby associating with DNA indirectly (reviewed by (Oren and Rotter 2010)). In addition, mutant p53 binds p63 and subsequently inhibit its function as a transcription factor ((Gaiddon et al 2001, Strano et al 2002); see Chapter 1 Section 1.2.3.3), although a mutant p53/p63 complex associated with DNA has not been reported. Furthermore, a number of p53 target genes are co-regulated by p63 through identified p53-REs (Dohn et al 2001, Osada et al 1998, Shimada et al 1999), including *DDIT4* (Ellisen et al 2002) which was identified in this study. Based on this evidence, it was hypothesised that p63 may also regulate the mutant p53-regulated genes identified in this study.

To investigate this hypothesis, p63 expression was silenced in the immortalised, non-malignant breast epithelial cell line, MCF10A (Figure 4.4A) and the expression of putative target genes was monitored. The majority of the genes examined showed a reduction in expression following silencing of p63 (Figure 4.4B), while *DKK1* expression was significantly elevated (Figure 4.4C), indicating differential gene regulation by p63.

FIGURE 4.4

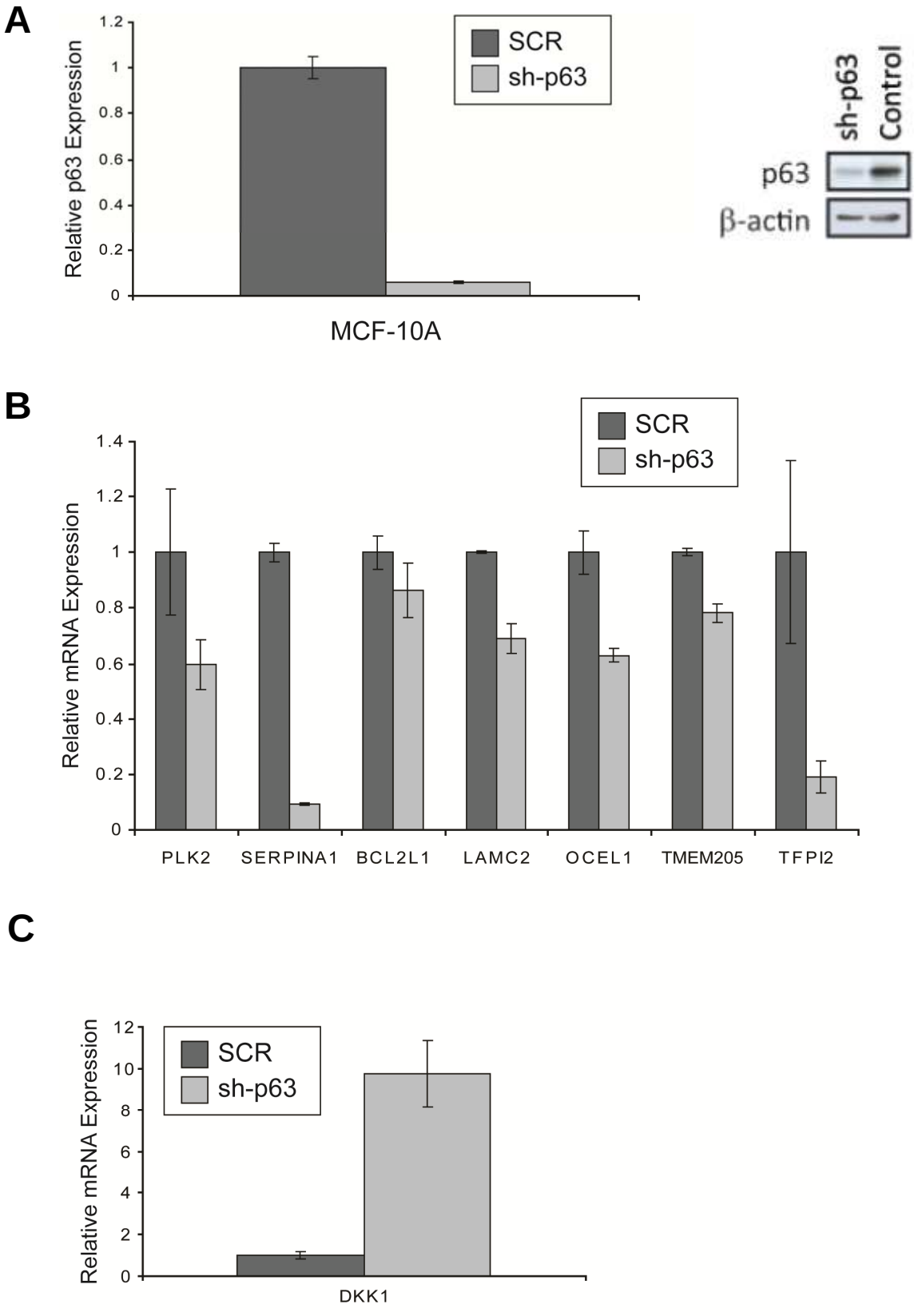

A
Relative p63 Expression
1.2
1
0.8
0.6
0.4
0.2
0
SCR
sh-p63
MCF-10A
sh-p63
Control
p63
β-actin
B
Relative mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SCR
sh-p63
PLK2
SERPINA1
BCL2L1
LAMC2
OCEL1
TMEM205
TFPI2
C
Relative mRNA Expression
12
10
8
6
4
2
0
SCR
sh-p63
DKK1

Figure 4.4 – p63 modulates the expression of mutant p53-regulated genes

A. Endogenous p63 expression was silenced in MCF10A cells using a p63-specific shRNA construct (see Chapter 2, Section 2.2.3.5), as confirmed by real-time RT-PCR and western blot analysis.

B. The basal expression of a selection of mutant p53-regulated genes was determined in the control MCF10A cell line (SCR) by real-time PCR. Changes in gene expression upon silencing of p63 (sh-p63) were subsequently determined, relative to control.

C. As in **(B)**.

4.3.6.1 Identification of putative p63-REs in mutant p53-regulated genes

In order to further investigate the role of p63 in modulating the expression of mutant p53-regulated genes, the *in silico* analysis was expanded to identify putative p63-REs using p63-scan software (Kouwenhoven et al 2010). Results from this scan show that 18 of the previously identified p53-REs were also predicted as p63-REs, 12 of these located within the 5' regions upstream of these genes (Table 4.3). This was not unexpected due to the significant similarity between the defined p63- and p53-RE consensus sequences (Kouwenhoven et al 2010, Perez et al 2007). Interestingly, a putative p63-RE was identified upstream of the *TMEM205* and *TCEAL1* gene coding regions, the two genes which returned negative results from the p53-scan. Four genes were identified with independent p53- and p63-REs within the classical promoter region (specifically *OCEL1*, *PTGIR*, *TFPI2* and *CDO1*). In addition, the *FSTL3* gene contains a putative p63-RE 5' to the transcription initiation site and a p53-RE within the 3' UTR. Taken together, these findings indicate that there may be some redundancy of function between p53 and p63. These sites, along with those identified through the p53-scan, are subsequently referred to as p53/p63-REs as they are potentially responsive to both p53 and p63.

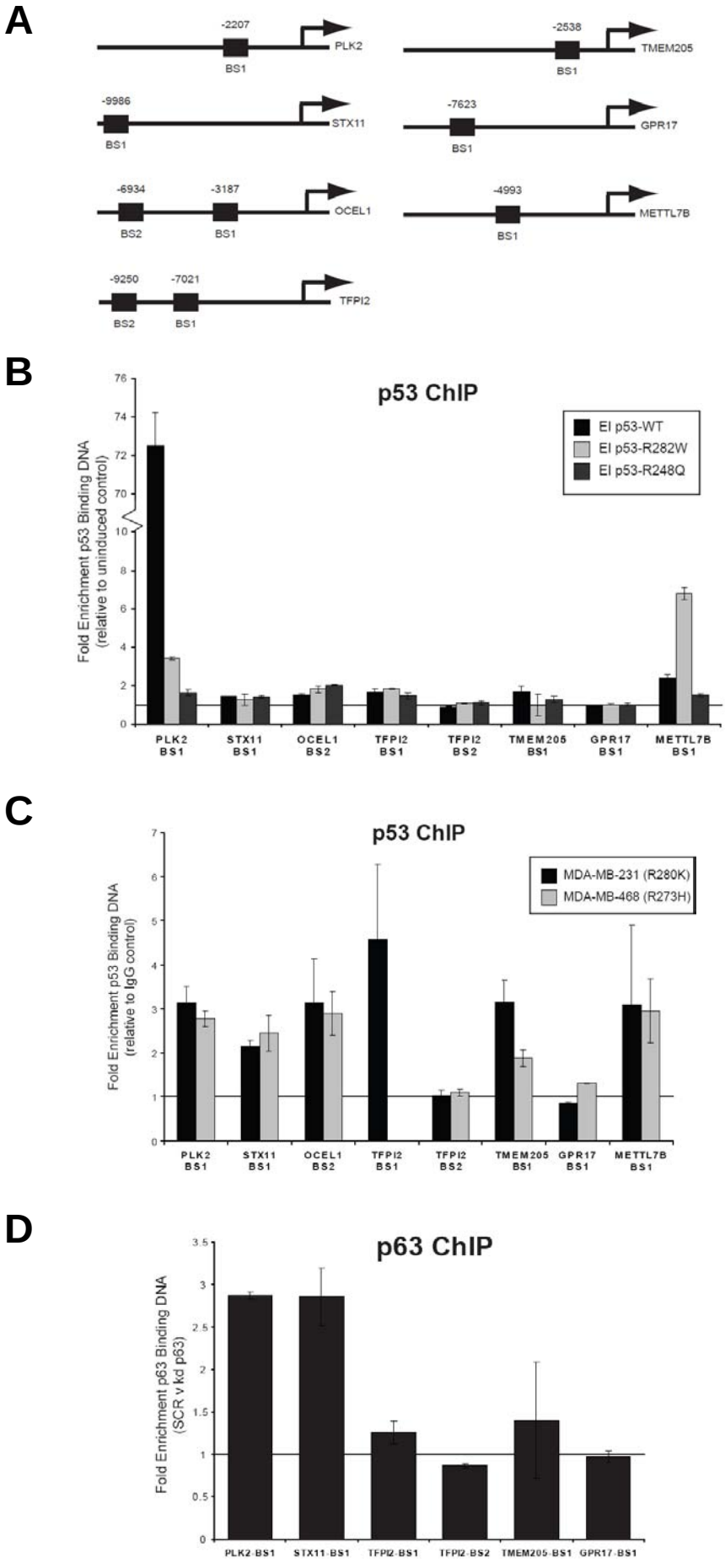
Table 4.3 p63-REs identified through *in silico* analysis using p63-scan software

Gene	Genomic Location Start	End	p63 scan score	Sequence	Sequence	Location in gene	Same site as p53 scan?
AKAP13	1398	1417	8.6	TACAAGTTT	TGTCAAGTTT	1st intron	
ANGPTL4	-4133	-4113	8.3	AGACCAGCCT	GGACATGGCG	Promoter	Y
BHLHE41	-8624	-8604	9.0	GGACAAGCTC	CTGCAAGTCA	Promoter	Y
INO80C	10286	10305	8.7	AACATGTGA	GGACATGAGA	1st intron	
CTH	-5236	-5217	9.1	GACCAGCCT	CGGCCTGCCA	Promoter	
DDIT4	-3155	-3135	11.9	AAGCATGTCT	AGGCAAGCCC	Promoter	Y
DENND1A	6012	6031	9.5	TCCATGTTG	GAGCATGCAT	1st intron	
DKK1	-3246	-3227	10.4	GACATGTGT	TAGCATGCTG	Promoter	Y
FSTL3	-9353	-9334	7.7	GACAGGTGT	GGGCTTGGTT	Promoter	
GPR17	-7623	-7603	9.0	GGACCTGGCA	GGGCTAGTCT	Promoter	Y
HSPA5	-1716	-1697	6.1	GGCATGCAC	CACCACGCCC	Promoter	
LAPTM5	9538	9558	12.0	AGACAAGCCC	GGGCCTGTCT	1st intron	Y
CCDC159	2321	2340	8.5	AGCATGTGT	TTGCCTGCTA	Other	
METTL7B	-4993	-4973	10.4	ATACATGCTA	CAACATGTGT	Promoter	Y
NR2F2	-9706	-9686	9.1	CAACATGCTC	TGGCTGGCTG	Promoter	Y
OCEL1	-3187	-3168	6.3	GGCATGGTG	GCTCATGTCT	Promoter	
P2RX4	6816	6836	9.6	AGGCGTGTTG	GCGCATGCCT	1st intron	Y
PDE2A	30573	30593	12.6	GAGCATGCCA	GAGCATGCCT	1st intron	Y
PLCL1	57687	57707	10.5	GTACATGCTA	CTACTTGTCT	1st intron	Y
PLCXD2	20869	20889	9.5	AGGCATGCTT	GGCCATGCTT	Other	
PLEKHA2	-8602	-8582	10.7	CAGCATGCCC	AGACATGGCA	Promoter	Y
PLK2	-2207	-2187	13.1	AAACATGCCT	GGACTTGCCC	Promoter	Y
PRSS12	5800	5819	9.5	TGCATGCAC	ACGCATGTGT	1st Intron	
PSD3	61338	61358	10.6	TGGCAAGCTC	AAACAAGCAT	1st Intron	Y
PTGIR	-1240	-1221	8.8	TGCAGGCCG	AGGCTGGCCA	Promoter	
RDH5	1925	1944	7.8	GGCATGCGC	CACCATGCCT	Other	
SEMA3C	7756	7776	13.3	AGACATGCCT	AAACATGTTT	2nd intron	
SETX	-4004	-3985	10.8	CACAAGTTC	TGACTTGTCT	Promoter	
SFRP1	42875	42895	10.8	AGGCATGATG	GTGCATGCCT	Other	
SNTB1	79347	79367	11.7	AGGCATGCGC	CAACATGCCC	2nd intron	
SRR	10656	10676	8.2	GGGCATGGTG	GTGCATGCCT	1st intron	Y
STC1	4608	4628	10.2	AGTCATGTAC	ATACATGCAA	Other	
STX11	-9986	-9966	9.5	AGGCATGAAC	CATCATGCCT	Promoter	Y
TFPI2	-7021	-7002	6.7	GTCAAGTTT	GCTCTTGACA	Promoter	
TGFBI	5076	5096	12.1	ATGCATGTGC	GAACATGTCT	Other	
TMCC3	18538	18557	10.2	TGCATGTTG	TTGCATGTAA	Other	
TNFSF4	-9836	-9816	11.1	AGACAAGCAC	ATACATGCAT	Promoter	Y
TCEAL1	-3848	-3829	6.6	GGCTGGACA	GGGCCTGCTG	Promoter	
TMEM205	-2538	-2519	8.3	AGCAGGCAA	ACACATGCTG	Promoter	
WBP5	-5921	-5901	10.7	ATGCAGGTTT	GTGCATGTTT	Promoter	Y
CDO1	-4848	-4829	8.2	ACCATGTCA	ATGCCTTGTCT	Promoter	

4.3.7 Investigation of p53 and p63 binding to specific response elements

To investigate the functionality of the identified p53/p63-REs, ChIP analysis was performed on selected sites (Figure 4.5A). The ability of wild-type or mutant p53 to associate with DNA was investigated using the EI p53-WT, EI p53-R282W and EI p53-R248Q cell lines. The fold enrichment of p53 binding to specific sites was determined by real-time PCR (see Chapter 2, Table 2.2 for specific primers). Both wild-type and mutant p53 binding was confirmed on the majority of sites examined, although no binding was detected on TFPI2-BS2 or GPR17-BS1 (Figure 4.5B). These results were confirmed through ChIP of endogenous mutant p53 in two breast cancer cell lines (Figure 4.5C). In fact, the endogenous mutant p53 showed greater capacity for binding than that observed in the inducible cell lines. The ability of endogenous p63 to bind identified p53/p63-REs was subsequently examined in MCF10A cells (Figure 4.5D) and was seen to correlate with the binding capacity of mutant p53 at the same sites.

FIGURE 4.5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-2207
PLK2
BS1
-2538
TMEM205
BS1
-9986
STX11
BS1
-7623
GPR17
BS1
-6934
-3187
OCEL1
BS2
BS1
-4993
METTL7B
BS1
-9250
-7021
TFPI2
BS2
BS1
B
p53 ChIP
EI p53-WT
EI p53-R282W
EI p53-R248Q
Fold Enrichment p53 Binding DNA
(relative to uninduced control)
PLK2 BS1
STX11 BS1
OCEL1 BS2
TFPI2 BS1
TFPI2 BS2
TMEM205 BS1
GPR17 BS1
METTL7B BS1
C
p53 ChIP
MDA-MB-231 (R280K)
MDA-MB-468 (R273H)
Fold Enrichment p53 Binding DNA
(relative to IgG control)
PLK2 BS1
STX11 BS1
OCEL1 BS2
TFPI2 BS1
TFPI2 BS2
TMEM205 BS1
GPR17 BS1
METTL7B BS1
D
p63 ChIP
Fold Enrichment p63 Binding DNA
(SCR v kd p63)
PLK2-BS1
STX11-BS1
TFPI2-BS1
TFPI2-BS2
TMEM205-BS1
GPR17-BS1

Figure 4.5 – p53 and p63 bind identified p53/p63-REs

A. Schematic of p53/p63-REs location based on the *in silico* analysis of mutant p53-regulated genes.

B. Anti-p53 ChIP of induced wild-type or mutant p53 in the EI p53 cell lines at a range of putative p53/p63-REs. Data is presented as the fold enrichment of p53 binding DNA relative to the uninduced control. Data represents two biological replicates.

C. Anti-p53 ChIP of endogenous mutant p53 in MDA-MB-231 or MDA-MB-468 cells. Fold enrichment of mutant p53 binding to DNA is presented relative to IgG controls.

D. Anti-p63 ChIP was performed in MCF10A-shp63 and control (SCR) cell lines. Data is presented as the relative fold enrichment of p63 binding (SCR vs. sh-p63).

4.3.8 Mutant p53 association with the promoter of identified target genes is decreased by silencing endogenous p63 expression

Based on the observations that both mutant p53 and p63 can regulate gene expression (Table 4.1; Figure 4.4) and p63 binding to specific p53/p63-REs is correlated with the ability of mutant p53 to bind the same sites (Figure 4.5), it was hypothesised that mutant p53 is recruited to the p53/p63-REs through its interaction with p63.

To investigate this, the ability of endogenous mutant p53 to ChIP at promoters following the silencing of p63 expression in the MDA-MB-231 breast cancer cell line (expressing p53-R280K) was examined (Figure 4.6). Mutant p53 exhibits a minimum 2-fold enrichment at PLK2-BS1, STX11-BS1, TFPI2-BS1 and TMEM205-BS1 in the control cell line, while no binding is observed on TFPI2-BS2 or GPR17-BS1, which is consistent with previous results. Following silencing of p63 expression, the ability of mutant p53 to bind DNA was reduced, particularly on TFPI2-BS1 and STX11-BS1. These data indicate that there may be a relationship between the ability of mutant p53 to bind identified sites within the promoter of target genes and p63 expression.

FIGURE 4.6

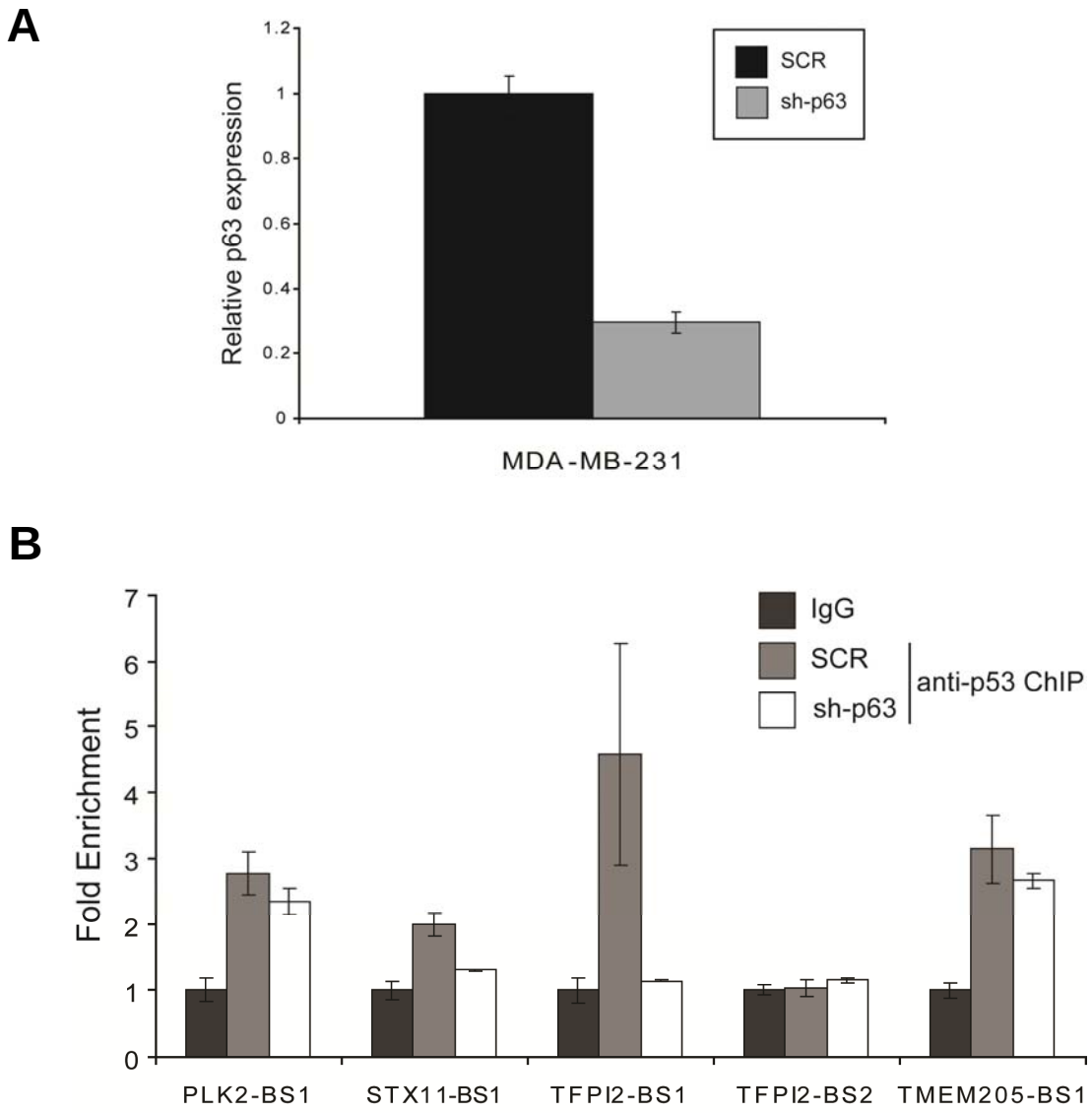

Figure 4.6 – Mutant p53 association with the promoter of its target genes is decreased by silencing p63 expression

A. Silencing of p63 expression in MDA-MB-231 cells was confirmed, relative to a SCR control, by real-time PCR.

B. Anti-p53 ChIP of endogenous mutant p53 in MDA-MB-231 cells in a control cell line (SCR) or with silenced p63 expression (sh-p63) was performed. Binding to specific response elements was determined by real-time PCR (see Chapter 2, Table 2.2 for primers). The fold enrichment of mutant p53 binding to DNA is presented relative to an IgG control.

4.3.9 Targeting a mutant p53 regulated gene product, PLK2

PLK2 is identified as a mutant p53-regulated gene in this study. The anti-cancer activity of a PLK small molecule inhibitor (BI2536) is currently under investigation in Phase II clinical trials (Schoffski et al 2010). It is hypothesised that tumours expressing mutant p53 up-regulate *PLK2* expression, thus sensitising cells to PLK inhibition. To investigate the functional consequences of mutant p53-mediated regulation of *PLK2*, the effect of the PLK inhibitor on the proliferation of H1299 cells expressing mutant p53 was investigated. The EI p53-R175H cell line was treated with PonA or a vehicle control for 24 hours prior to treatment with the PLK inhibitor for 48 hours. Cells were subsequently seeded at low density and their growth monitored over 5 days. The proliferation of the uninduced (p53 null) or induced (R175H expressing) H1299 cells was measured in real time using Incucyte (Essen). There was no difference in the proliferation of p53 null or R175H expressing cells over 96 hours. However, following treatment with 5 or 25 nM PLK inhibitor the growth of the R175H cell line was reduced compared to the p53 null control (Figure 4.7A). This data indicates that mutant p53-expressing tumours may be increasingly sensitive to treatment with a PLK inhibitor due to the increased expression of *PLK2*.

FIGURE 4.7

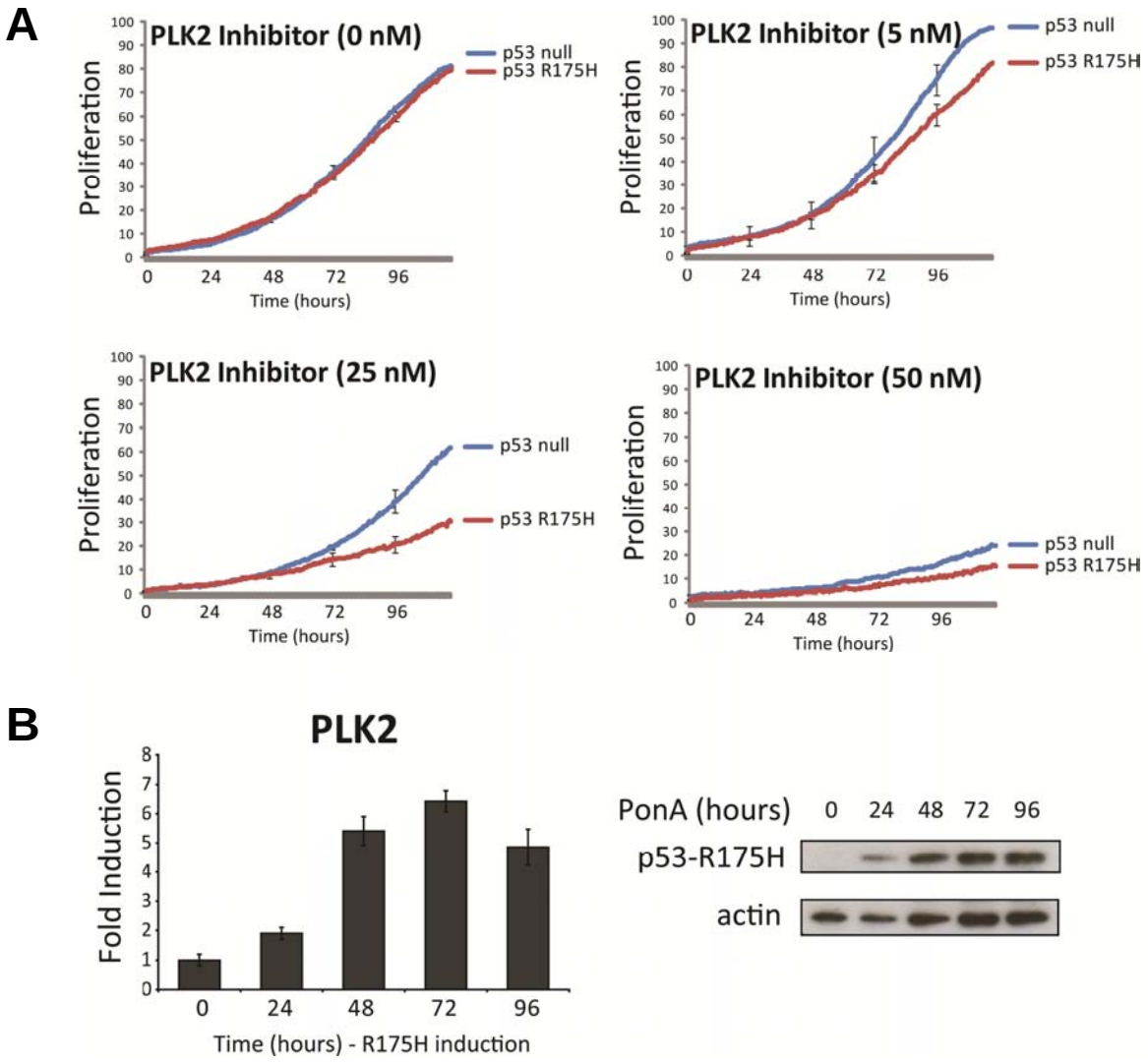

Figure 4.7 – Mutant p53 up-regulates PLK2 and sensitizes cells to PLK inhibition

A. EI p53-R175H cells were induced for 24 hours prior to treatment with the indicated concentrations of the PLK inhibitor (BI2536) for 48 hours. Cells were subsequently seeded at low density (1:30 split) and their growth monitored over 5 days. Proliferation was determined in real-time through the collection of phase contrast images at 30 minute intervals using Incucyte (Essen).

B. The level of PLK2 mRNA expression was determined by real-time PCR analysis at 0, 24, 48, 72 and 96 hours following p53-R175H induction. Induction of p53-R175H was confirmed by western blot analysis.

4.4 DISCUSSION

4.4.1 A system to investigate mutant and wild-type p53 regulated genes

The EI H1299 cell lines described in this thesis are a pivotal resource that can be harnessed to investigate a wide range of wild-type and mutant p53 functions. This chapter specifically describes the investigation of wild-type and mutant p53 transcriptional activity. These inducible cell lines provide ideal tools to define the gene targets of mutant p53 which may in turn attribute to mutant p53 driven oncogenic pathways, and the mechanisms harnessed by mutant p53 to drive these pathways.

Through extensive Affymetrix based expression profiling, it was observed that 54/59 of the genes with significantly increased expression in the presence of mutant p53 also showed similar regulation by wild-type p53 (Table 4.1, Figure 4.1). These findings indicate that mutant p53 may in fact regulate a selection of wild-type p53-regulated genes to drive oncogenic pathways. This is in opposition to previous studies utilising expression microarray of H1299 cells stably expressing mutant p53 which have reported a range of novel, gain-of-function mutant p53-specific target genes. Interestingly, these genes were not identified in the study presented here. In these previous studies the expression of wild-type p53 in H1299 cells was achieved through infection of cells with wild-type p53 adenovirus, compared to the stable transfection and selection of cells for mutant p53 expression (Scian et al 2004b, Scian et al 2005). It is possible that in these studies, comparing downstream regulated genes may be compromised through the use of differentially derived cell lines. Although in the same genetic background, changes due to clonal variation may be present. The inducible

system however allows for wild-type or mutant p53 expression to be investigated in a single system, with control cells undergoing identical treatments, therefore allowing changes in gene expression between cell lines to be directly compared.

It must also be considered that expression microarray analysis (as performed in this study) will not detect all changes that may occur due to mutant p53 expression that drive tumorigenesis. Specifically, epigenetic changes, which are defined as 'heritable changes in gene expression that are not due to any alteration in DNA sequence' (Holliday 1987) will not be determined. Differences in DNA methylation patterns between normal and tumour samples have previously been identified (Feinberg et al 1983, Fraga et al 2004, Esteller 2007, Herman and Baylin 2003), indicating that these changes play a role in tumour progression. Furthermore, the coverage of non-coding RNAs and their relative expression in the presence of mutant p53 is minimal using this system. Non-coding RNAs, such as microRNAs (miRNAs) or small nucleolar RNAs (snoRNAs) have previously been demonstrated to have aberrant expression patterns in cancer (White 2008, Dong et al 2009, Chang et al 2002, Davis and Hata 2010, Lu et al 2005), and provide some usefulness as a prognostic tool (Liao et al 2010, Calin et al 2005, Schetter et al 2008). The inducible system described in this thesis provides an important resource through which the effect of mutant p53 expression on these pathways may be examined in detail.

In addition, the limited numbers of mutants previously investigated does not allow for a comprehensive comparison of gene regulation by individual mutants or indeed classes of mutants. This study suggests that there is no relationship between the predicted tertiary structure of mutant p53, based on the location of its mutation within

the core domain, and its transcriptional profile (Figure 4.1C). Furthermore, it is clear that identified transcriptional targets of mutant p53 should be validated for a range of p53 mutants, as data acquired for a single mutant may not be equivalent across mutants, even of the same structural classification.

4.4.2 Mutant p53-regulated genes are involved in tumorigenesis

There is evidence in the literature that a number of the identified mutant p53-regulated genes may play a role in enhancing tumorigenesis. *ANGPTL4* expression has been correlated with tumour invasion and lymph node metastasis in gastric adenocarcinomas (Nakayama et al 2010) and its expression has been shown to be enhanced by TGFβ in lung metastasis (Padua et al 2008). Mutant p53 has previously been demonstrated to enhance TGFβ-mediated signalling (Adorno et al 2009), therefore *ANGPTL4* may represent an important mutant p53-regulated gene in this process. Furthermore, *EDNRA* expression has been shown to enhance tumorigenesis (Lange et al 2007), while the methylation of the *CDO-1* promoter has been demonstrated to predict metastasis in breast cancer patients (Dietrich et al 2010) corresponding with the ability of mutant p53 to positively and negatively regulate these genes respectively. The up-regulation of the protein kinase MAP2K3 has been associated with tumour progression and invasion (Demuth et al 2007) and a recent study has identified *MAP2K3* as a transcriptional target of endogenous mutant p53 in cancer cell lines (Gurtner et al 2010). *MAP2K3* expression was also found to be up-regulated by wild-type p53 in the microarray described here, although this was not previously described. *LAMC2* expression is detected in tumour cells that have spread from the primary site (Brabletz et al 2005) and has subsequently been described as a biomarker for bladder cancer metastasis (Smith et al 2009). Furthermore, *LAMC2*

expression is shown to be activated by β-catenin (Hlubek et al 2001) which in turn is associated with mutant p53 expression (Hadjihannas et al 2010). Together, these findings support a role for mutant p53-regulated genes in contributing to the invasive GOF and poor prognosis associated with mutant p53 expression in tumours.

4.4.3 Cellular pathways modulated by mutant p53

The wild-type p53 protein has been implicated in a number of pro-survival pathways (reviewed by (Garner and Raj 2008, Janicke et al 2008)). Specifically, an anti-apoptotic function for wild-type p53 has been described (Nantajit et al 2010) which is likely to occur due to the ability of p53 to activate genes involved in growth arrest and DNA repair. Indeed activation of genes in these pathways has specifically been demonstrated to result in a prolonged G2 arrest and subsequently inhibit the activation of apoptotic pathways in response to treatment with chemotherapeutic agents (Lin and Wang 2008).

It was expected that mutant p53 would regulate an independent set of genes compared to wild-type p53, as has been demonstrated previously (Scian et al 2004b, Scian et al 2005), however the majority of genes identified as responsive to mutant p53 expression in this study show similar regulation following induction of wild-type p53 (Figure 4.1). The expression and activity of the wild-type p53 protein is tightly controlled, with activation and protein stabilisation occurring in response to cellular stresses (Liu and Chen 2006, Vogelstein et al 2000). This is in direct comparison to the constitutively high expression of mutant p53 in tumours (Goh et al 2011). Due to the known role of wild-type p53 in regulating pro-survival pathways, it is conceivable

that the differences in wild-type and mutant p53 protein regulation may lead to differential outcomes, despite their ability to regulate a common set of genes.

In addition, Ingenuity Pathway Analysis (IPA) of the genes identified as regulated by both wild-type and mutant p53 showed enrichment for metabolic and bone resorption pathways (Table 4.4). A role for wild-type p53 in the regulation of cellular metabolism, the de-regulation of which has been shown to play an integral role in cancer cell growth, has recently been established (Kroemer and Pouyssegur 2008, Vousden and Ryan 2009), although the role of mutant p53 in these pathways has not been verified. Findings from this analysis indicate that mutant p53 may in fact be involved in regulating metabolic pathways, although further investigation is required to determine the consequences and significance of this.

Table 4.4 Ingenuity Pathway Analysis of mutant p53 regulated genes

Pathway	*p* value
Selenoacid metabolism	0.002
Role of osteoblasts, osteoclasts and chrondrocytes in rhematoid arthritis	0.005
Role of macrophages, fibroblasts and endothelial cell sin rhematoid arthritis	0.02
Acute phase response signalling	0.03
Wnt-β-catenin signalling	0.03
Sulphur metabolism	0.04
cAMP-mediated signalling	0.05

4.4.4 A mutant p53-regulated gene linked to arthritis and bone disease

Upon analysis of the global pathways regulated by mutant p53 the role of mutant p53 in regulating bone resorption pathways was evident. One gene in this pathway is of particular interest for further study. DKK1, a secreted glycoprotein, has previously been identified as a p53-regulated gene that functions to antagonise Wnt signalling

pathways in cancer cell lines (Wang et al 2000). However, there are a number of studies that demonstrate that high serum levels of DKK1 are correlated with decreased bone mineral density due to the inhibition of osteoblast formation (Butler et al 2011, Morvan et al 2006, Yaccoby et al 2007). DKK1 has also been identified as a potential prognostic marker in tumours, due to its high expression in breast, lung and kidney cancers and its association with the presence of bone metastases (Forget et al 2007, Tian et al 2003, Voorzanger-Rousselot et al 2007). The available data relating to DKK1 and its role in the inhibition of osteoblasts, coupled with its identification in this study as a mutant p53-regulated gene, provides a novel mechanism through which mutant p53 may mediate bone metastasis. Importantly, a DKK1 specific antibody has been demonstrated to increase bone mineral density in an animal model (Yaccoby et al 2007), indicating the potential value of DKK1-targeted therapies. Further investigation of DKK1 and its role in bone formation and metastasis is required to discern the significance of these findings.

4.4.5 p63 co-regulation of p53 target genes

The p53 family member p63 is known to bind the consensus p53-REs of endogenous p53 target genes and regulate their expression (Attardi et al 2000, Dohn et al 2001, Ihrie et al 2005, Osada et al 1998, Reczek et al 2003, Shimada et al 1999). Furthermore, p53 family member consensus sites have been identified in a number of genes involved in DNA damage repair pathways that have subsequently been shown to respond to p63 but not to p53 (Lin et al 2009). It is therefore evident that there is some redundancy in function between p53 and p63. This is most likely due to the high degree of homology between the DNA binding domains of the two proteins (Harms and Chen 2006, Osada et al 1998) and the subsequently similar consensus response

elements. In addition, the data presented within this chapter suggests that there may also be cross-talk between p63 and mutant p53 in relation to activation of target genes. This is evident through the identification of genes responding to mutant p53 expression which were subsequently shown to exhibit some p63-mediated regulation (Figure 4.4) as well as the identification of p53/p63-REs within the regulatory regions of a number of these genes, which were subsequently shown to bind mutant p53 and p63 (Figure 4.5).

4.4.5.1 Does a mutant p53/p63 complex exist on DNA?

To-date, although mutant p53/p63 complexes have been described ((Gaiddon et al 2001, Strano et al 2002); see Chapter 1 Section 1.2.3.3), this complex has not been demonstrated to physically occur on the DNA and has not been shown to remain transcriptionally active. Data accumulated in this study led to the hypothesis that p63 can bind p53/p63-REs and subsequently recruit mutant p53 to regulate gene expression. Indeed, the data presented here demonstrates that there is some correlation between p63 expression and the ability of endogenous mutant p53 to bind promoters in a breast cancer cell line (Figure 4.6B). The variations observed across the response elements tested can be explained by residual p63 expression, as a knockdown of only 80% was achieved (Figure 4.6A), or by different binding affinities (of mutant p53 or p63) to promoters, which has not been investigated to-date. It is also of interest to note that the two binding sites showing the most significant reduction in mutant p53 binding following silencing of p63 are located within the TFPI2 and STX11 genes (Figure 4.6B), which are among the top 8 most highly up-regulated genes in the presence of mutant p53 (see Table 4.1).

Further investigation is required to comprehensively determine if mutant p53 and p63 complex together on DNA, and this work is currently ongoing. The inducible H1299 system could be utilised to determine the mechanism of recruitment to the promoter. A p63-ChIP in H1299 cells was unable to determine any p63 binding at the identified binding sites (data not shown), however, by performing p63-ChIP following induction of mutant p53 in H1299 cells, it would be possible to determine if p63 may in fact be recruited to the promoter by mutant p53.

CHAPTER 5

Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11

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Oncogene 2011; submitted paper

CHAPTER 5 – Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11

5.1 PREFACE

This chapter has been submitted as a complete manuscript and is currently in the final review stage at *Oncogene*. A role for ANKRD11 in the regulation of p53 activity has previously been described (Neilsen, 2008). The ecdysone-inducible system was utilised to investigate the gain-of-function properties of mutant p53 expression and the ability of ANKRD11 to suppress these functions.

ABSTRACT

Mutations of p53 in cancer can result in a gain-of-function (GOF) associated with tumour progression and metastasis. We show that inducible expression of several p53 “hotspot” mutants promote a range of centrosome abnormalities including centrosome amplification, increased centrosome size and loss of cohesion, which lead to mitotic defects and multinucleation. These mutant p53 expressing cells also exhibit a change in morphology and enhanced invasive capabilities. Consequently, we sought for a means to specifically target the function of mutant p53 in cancer cells. This study has identified ANKRD11 as a key regulator of the oncogenic potential of mutant p53. Loss of expression of ANKRD11 with p53 mutation defines breast cancer patients with poor prognosis. ANKRD11 alleviates the mitotic defects driven by mutant p53 and suppresses mutant p53-mediated mesenchymal-like transformation and invasion. Mechanistically, we show that ANKRD11 restores a native conformation to the mutant p53 protein and causes dissociation of the mutant p53•p63 complex. This represents the first evidence of an endogenous protein with the capacity to suppress oncogenic properties of mutant p53.

INTRODUCTION

The p53 protein is a tumour suppressor that predominantly functions as a sequence-specific transcription factor regulating the expression of various target genes involved in cell-cycle arrest, apoptosis, senescence, DNA repair, and inhibition of angiogenesis and metastasis, in response to a variety of cellular insults (Lane 1992, Liu and Chen 2006). Approximately 50% of all human cancers contain a mutation in the *TP53* gene (Hollstein et al 1991, Vogelstein et al 2000) with the majority of these mutations occurring within the DNA-binding domain (DBD) (Bullock and Fersht 2001, Olivier et al 2010). Mutations at six specific residues in the DBD have been identified as mutational "hotspots" and are classified into two categories: DNA contact mutants (R248Q, R248W, R273C and R273H) involving residues which specifically interact with DNA, and structural mutants (R175H, G245S, R249S and R282W) which exhibit local or global distortions of the p53 protein structure (Cho et al 1994).

Mutant p53 proteins exhibit both loss- and gain-of-function characteristics. Loss-of-function is largely due to the inability of mutant p53 to bind the canonical wild-type p53 binding site, resulting in an inability to transactivate its target genes (Kato et al 2003, Scian et al 2004), an attenuated tumour suppressive function and consequently, deregulated cellular growth and apoptosis. In contrast, gain-of-function (GOF) characteristics of mutant p53 are critical for tumour progression and metastasis (Brosh and Rotter 2009, Oren and Rotter 2010). A variety of GOF phenotypes of mutant p53 have been reported including increased invasive and metastatic potential of cells, resistance to chemotherapies, regulation of pro-inflammatory and anti-apoptotic

pathways and increased genomic instability (Oren and Rotter 2010), all of which give mutant p53 expressing cells a selective growth and survival advantage.

Various strategies have been developed to target mutant p53 in cancers including small molecules that aim to restore the native conformation to the unfolded mutant p53 proteins (Bykov et al 2002), or target their interactions with family members (Di Agostino et al 2008, Kravchenko et al 2008). Using a novel protein-based approach, we have previously shown that ANKRD11 can restore normal transactivation potential to a p53 hotspot mutant in a breast cancer cell line (Neilsen et al 2008), and represents the first endogenously-expressed protein with a capacity to rescue mutant p53 function. In this study we utilised a panel of ecdysone-inducible cell lines in a p53 null background to express various p53 hotspot mutants to investigate cellular processes that are driven by mutant p53 GOF and which can be suppressed by ANKRD11.

MATERIALS AND METHODS

Cell Lines and Antibodies

H1299, MDA-MB-231, MCF-7 and SK-BR-3 cells were maintained in DMEM or RPMI with 10% FCS. Ecdysone-inducible derivatives of the H1299 cells were generated by stable transfection of pVgRXR and selection in zeocin (Invitrogen, CA) at 100 µg/ml, followed by stable transfection of pI-TK-Hygro-p53-wt/mut plasmid linearised with *Xba*I and subsequent selection of clones in Hygromycin B (Sigma Aldrich, St. Louis, MO) at 600 µg/ml. Stable cell lines expressing GFP-ANKRD11-Myc were generated through retroviral transduction using pLNCX2 vector as described previously (Neilsen et al 2008) and selected in G418 (Invitrogen) at 500 µg/ml. Antibodies used were: mouse α-Myc, mouse α-FLAG, mouse α-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rat α-HA (Roche), rabbit α-β tubulin (Abcam), mouse α-β actin (Sigma Aldrich), mouse α-p21 (Thermo Scientific), mouse α-ZO-1 (Zymed), mouse α-Centrin, rabbit α-γ tubulin (Sigma Aldrich), mouse α-MPM2 (Upstate), α-mouse IgG HRP linked (GE Healthcare), α-rabbit IgG HRP linked (GE Healthcare), α-rat IgG HRP linked (Dako, Carpinteria, CA), mouse light chain specific HRP linked (Millipore, Temecula, CA).

Plasmids

To generate epitope tagged fragments of ANKRD11, specific regions of ANKRD11 were PCR amplified using primers 1 to 8 (Table S1) from pLNCX2-ANKRD11-Myc (Neilsen et al 2008) and cloned in-frame into mammalian expression vectors pCMV-Tag2 or pCMV-Myc. Myc-p53-FL were generated as previously described (Neilsen et al 2008). Specific fragments of p53 were PCR amplified using primers 9 to 14 (Table

S1) and cloned in frame into pCMV-Myc. Deletion constructs of Myc-p53 were generated through overlap PCR using the primers 15 to 19 (Table S1).

Breast tumour analysis, expression microarray analysis and real-time RT-PCR

Breast tumour patient samples and real time PCR are as previously described (Kumar et al 2005, Pishas et al 2010). Expression profiling was performed using Affymetrix Human Gene 1.0 ST array as per manufacturer's protocol.

Co-Immunoprecipitation Assays

H1299 cells (5×10^5) were seeded into 60 mm dishes and transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen). For the interaction domain mapping studies, cells were collected 24 hours post-transfection, resuspended in lysis buffer 1 (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100) supplemented with 1× complete protease inhibitor cocktail (Roche), sonicated and centrifuged. Clarified lysates were incubated with FLAG-M2 agarose (Sigma Aldrich) for 2 hours at 4ºC with rotation. Beads were washed twice with lysis buffer 1, twice with wash buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and twice with 20mM Tris-HCl pH 7.5. Protein complexes were eluted with FLAG peptide (200 ng/mL). For the p63/p73 interaction studies, cells were sequentially transfected with the indicated plasmids and harvested 48 hours post-transfection in 300μL lysis buffer 2 (20 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl, 1 mM DTT, 10% glycerol), sonicated and centrifuged. Clarified lysates were incubated with 200 ng α-p53 (DO-1) for 1 hour at 4ºC with rotation, followed by addition of 10 μL Protein G sepharose beads (GE Healthcare) for 1 hour. Beads were washed four times with 400 μL lysis buffer 2 and protein

complexes were eluted with 1×SDS loading buffer at 95ºC for 5 min. Western blot analysis of inputs and co-immunoprecipitated protein complexes was performed as described previously (Kumar et al 2005).

Centrosome defects and multinucleation assays

To measure centrosome defects, unsynchronised cells were plated at 10% confluency and treated with 2.5 μg/mL PonA for the indicated times. Multinucleation, number of centrosomes and centrosome size was measure in interphase cells. Distance between centrosomes was measured in S and G2 cells. 50 cells were counted per condition. For mitotic assays, cells were plated at 10% confluency, synchronised by double thymidine block and harvested at 11 hours post-release. 2.5 μg/mL PonA was added 24 hours before harvesting. 100 cells were counted per condition.

Colony development assays

EI p53-mut cell lines were treated with or without 2.5 μg/mL PonA for 72 hrs. Cells were subsequently collected and plated in duplicate in a 6-well format at 1000 cells/well in DMEM ± PonA. Cells were grown for 10 days, fixed for 5 minutes with methanol and stained with Giemsa Stain (Sigma Aldrich) as per manufacturer's protocol.

Immunofluorescence

For ZO-1 staining, 2000 cells were plated on glass coverslips in a 6-well and allowed to form small colonies of 4-6 cells (~2 days). PonA was added to wells at a final concentration of 2.5 μg/mL for 0, 16 or 40hrs. IF protocols were based on those previously described (Neilsen et al 2008).

For mitotic staining, cells were synchronised by addition of 2.5 mM thymidine for 16 hours then washed three times in PBS. Cells were incubated for a further 10 hours before the thymidine block was repeated. Cells were fixed with ice-cold methanol at 11 hours post-release from the second thymidine block. PonA was added at 24 hours prior to harvesting. Where metaphase cells were required 10 μM MG132 was added at two hours prior to harvesting. Cells were blocked in 3% BSA in PBS for 20 minutes followed by incubation with the indicated antibodies in 03% BSA/PBS solution (30 minutes, RT), and incubation with the indicated Alexa-Fluor-conjugated secondary antibodies (30 minutes, RT). Coverslips were mounted in mowiol containing 2.5% DABCO. Cells were visualised using a DeltaVision personal DV deconvolution microscope. Images underwent restoration deconvolution and were analysed using softWoRx version 3.6.1. Fluorescent images represent projections of z-stacks. Images were cropped and resized using Adobe Photoshop (San Jose, CA, USA).

Invasion Assays

Real-time invasion assays were performed using the xCelligence Real-Time Cell Analyzer (RTCA) DP (Roche), as per the manufacturer's protocol. Ecdysone-inducible H1299 derivatives, EI-H1299-p53-R175H or EI-H1299-p53-R175H-ANKRD11 were grown in the presence or absence of PonA at 2.5μg/ml for 24 hours. Following induction, sub-confluent cell cultures were collected in serum free media and plated at 2×10^4 cells per well in the top chamber of a CIM-Plate 16 pre-coated with 5% Matrigel (BD Biosciences, San Jose, CA). DMEM containing 10% FCS was used as a chemo-attractant. Real-time migration assays were performed using

Incucyte (Essen). Phase images were taken every 30 minutes and wound closure and cell confluence calculated using specific Incucyte software.

Live cell imaging

Live cell imaging was conducted using a DeltaVision Core microscope using softWoRx version 3.6.1. Cells were plated at 10% confluency, synchronised by double thymidine block and 2.5 μg/mL PonA added 24 hours before imaging. Bright field images were taken every five minutes and the movies analysed using ImageJ version 1.43u.

Statistical Testing

Significance testing for breast tumour data was performed using a two-tailed Student's t-test. Two-Way ANOVA and Bonferroni posttests were performed using GraphPad Prism (v5, CA, USA).

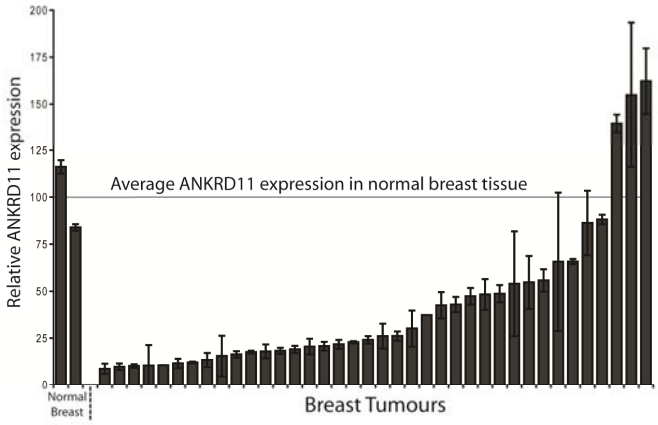
RESULTS

p53 mutation and loss of ANKRD11 expression define cancer patients with poor prognosis

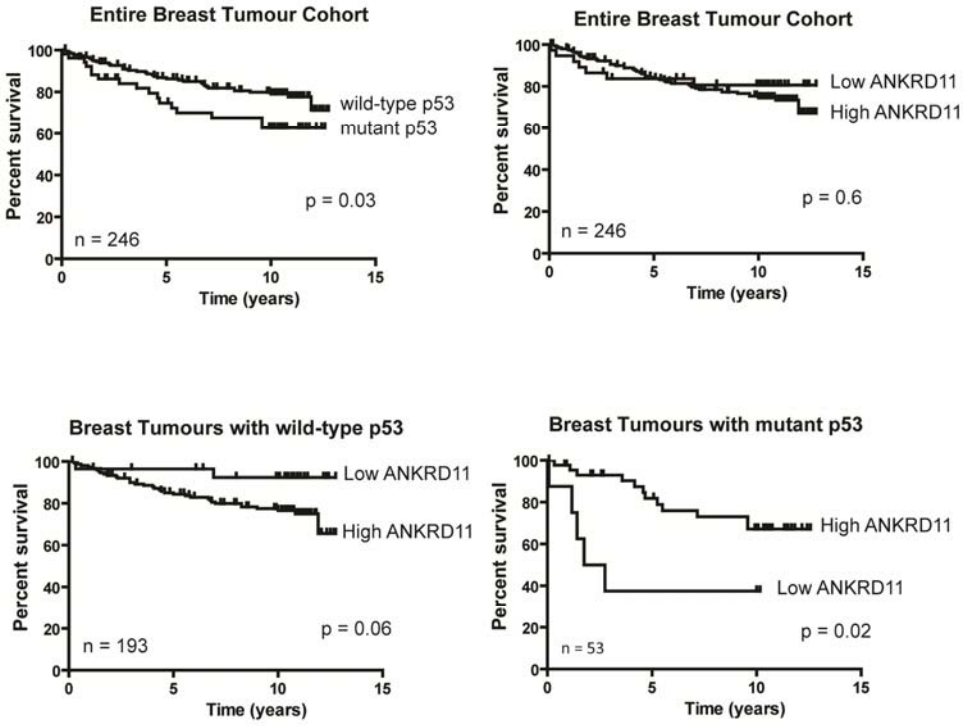
Previously, we established that ANKRD11 could restore normal transactivation potential to the p53-R273H mutant (Neilsen et al 2008) and thus we explored a wider role for ANKRD11 in the suppression of the oncogenic functions of mutant p53. ANKRD11 was robustly expressed in the normal breast epithelium, however was down-regulated in the majority of breast tumours (Fig. 1A). Analysis of the expression profiling of a cohort of breast tumours (Miller et al 2005) revealed that the combination of p53 mutation and loss of ANKRD11 expression defined a subset of patients with poor outcomes (Fig. 1B). Low ANKRD11 expression was also significantly associated with poor survival in cohorts of bladder and lung tumours (Supp. Fig. S1). Furthermore, loss of ANKRD11 expression was significantly associated with invasion to the lymph nodes in breast tumours expressing mutant p53 (Fig. 1C). Collectively, the data support a role for ANKRD11 as a suppressor of the oncogenic potential of mutant p53. We subsequently sought to understand the mechanism of this suppression.

Figure 1

A



B



C

Breast tumours with mutant p53

	Lymph node negative	Lymph node positive
Low ANKRD11	1	7
High ANKRD11	32	13

Fisher's exact test

p = 0.003

n = 53

Figure 1 – ANKRD11 is down-regulated in breast tumours and is predictive of poor clinical outcome

A. ANKRD11 expression levels were determined by quantitative real-time PCR in 38 breast tumours and 2 normal breast tissues.

B. Kaplan-Meier curves derived from publically available survival data associated with a cohort of 246 breast cancer patients with known p53 status (GSE3494; (Miller et al 2005)). Expression of ANKRD11 was sourced from expression microarray analyses. Tumours were ranked by ANKRD11 expression, with the lowest 15% of these tumours defined as 'Low ANKRD11'.

C. Two-by-two test (Fisher's exact test) showing a correlation between lymph node status of a cohort of 53 patients with mutant p53 expressing breast tumours (GSE3494; (Miller et al 2005)) and ANKRD11 expression (as defined in B).

Mutant p53 drives centrosome abnormalities and multinucleation that can be suppressed by ANKRD11

Several biological pathways were examined to assess mutant p53 GOF. These assays were based on the inducible expression of mutant p53 in the H1299 p53-null genetic background. In these cell lines, p53 can be expressed dose-dependent to the inducing agent, Ponasterone A (PonA). Furthermore, the inducible expression of wild-type p53 was equivalent to DNA damage induced activation of the p53 pathway, and mutant p53 levels comparable to endogenous mutant p53 expression in cancer cell lines (Supp. Fig. S2).

Induction of mutant p53 in H1299 cells resulted in a time-dependent increase in multinucleation, with approximately 25% of cells exhibiting more than one nuclei following 96 hours of mutant p53 expression (Fig. 2A). Giant multinucleated H1299 cells were observed following prolonged expression of mutant p53 (Supp. Fig. S3A). Co-staining cells for α-tubulin and γ-tubulin, markers for microtubules and centrosomes respectively, showed induction of mutant p53 was associated with a small but significant increase in the number of centrosomes per cell (Fig 2B). The limited size of the increase was most likely due to the small number of cell cycles observed. Centrin immunofluorescence studies demonstrated that these aberrant centrosomal frequencies were not due to centriole splitting (Supp. Fig. S3B). However, there was a significant increase in average centrosome size (Fig. 2C), which was not due to increased γ-tubulin expression (Supp. Fig. S3B) A significant increase in the average distance between centrosomes was also observed (Fig. 2D). These observations are demonstrated as *bone fide* mutant p53 GOF pathways as multinucleation and centrosomal aberrations were not observed in cells with induced

wild-type p53 (Fig. 2A-D), and importantly multinucleation of EI-H1299 cells was not observed following treatment with PonA (Supp. Fig. S4). Furthermore, at 24 hours post-induction cells were observed with centrosomal amplification without multinucleation, suggesting that the defects in centrosomal duplication are driving the multinucleation phenotype and not mitotic failure.

To examine how the restored expression of ANKRD11 would impact upon the ability of mutant p53 to disrupt normal mitotic fidelity, ANKRD11 was stably-expressed in EI p53 mutant cells (which exhibit undetectable ANKRD11 expression) to approximate physiological levels, as judged by the levels observed in primary epithelial cells (Supp. Fig. S5). Restoring ANKRD11 expression completely abolished the ability of induced p53-R175H to drive multinucleation in EI p53-R175H cells (Fig. 2A). A similar result was also seen following induction of p53-R248W in the presence of ANKRD11 expression (Supp. Fig. S6). Furthermore, induction of p53-R175H failed to modulate centrosome size, separation or cohesion in the presence of restored ANKRD11 expression (Fig. 2B-D). Importantly, ANKRD11 did not influence p53-R175H mutant protein levels following exposure to the inducing agent, PonA (Fig. 2E). Together these findings illustrate the capacity of ANKRD11 to suppress the oncogenic potential of a p53 GOF mutant.

Figure 2

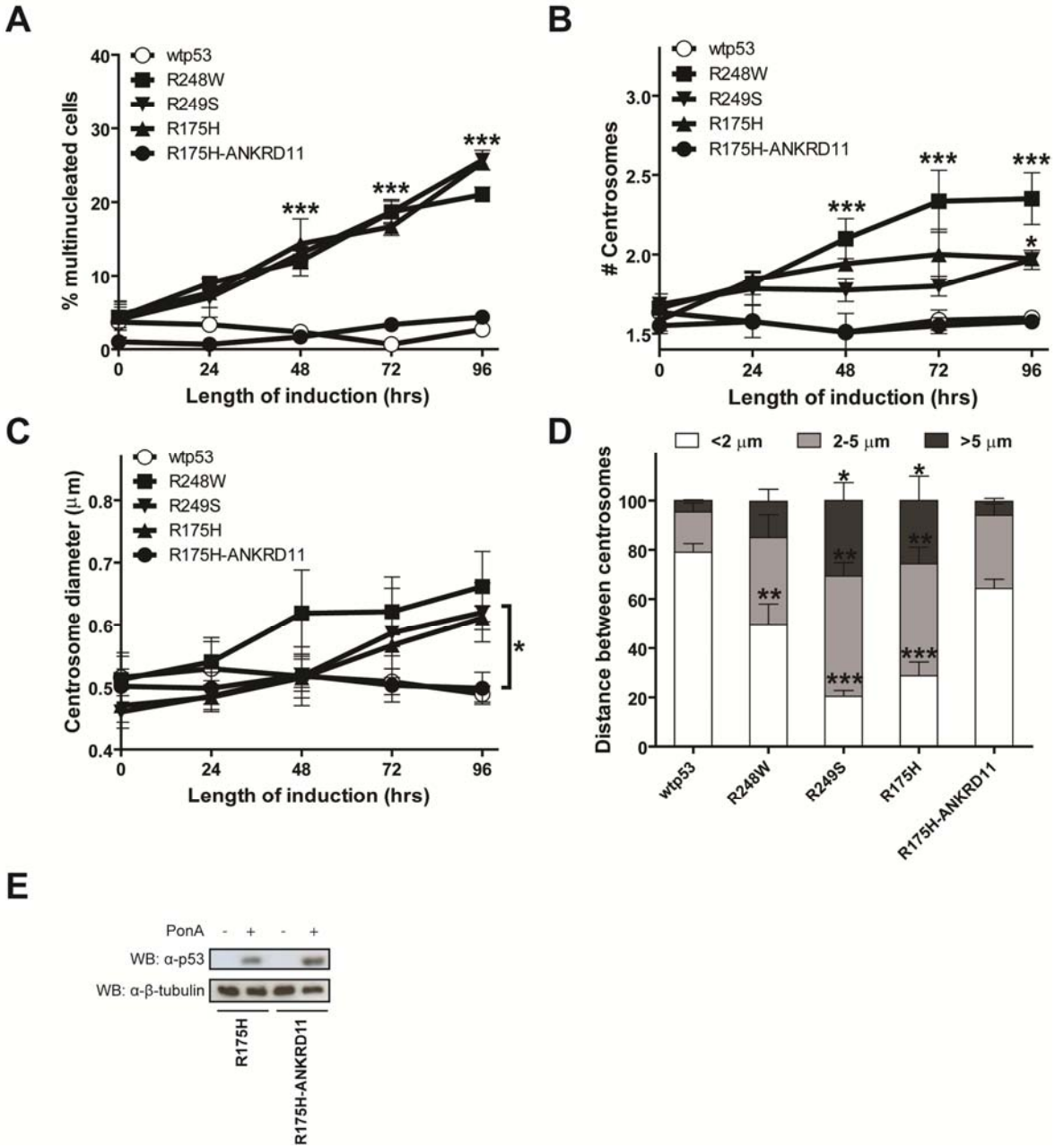

A
wtp53
R248W
R249S
R175H
R175H-ANKRD11
% multinucleated cells
Length of induction (hrs)
B
Centrosomes
Length of induction (hrs)
C
Centrosome diameter (μm)
Length of induction (hrs)
D
<2 μm
2-5 μm
>5 μm
Distance between centrosomes
wtp53
R248W
R249S
R175H
R175H-ANKRD11
E
PonA - + - +
WB: α-p53
WB: α-β-tubulin
R175H
R175H-ANKRD11

Figure 2 – Mutant p53 expression promotes multinucleation and centrosome abnormalities that are suppressed by ANKRD11

EI p53-WT, EI p53-R248W, EI p53-R175H, EI p53-R249S and EI p53-R175H-ANKRD11 were plated at 10% confluency and treated with 2.5µg/mL PonA for 0, 24, 48, 72 or 96 hours. Multinucleation and centrosome characteristics were subsequently analysed. γ-tubulin was used as a centrosome marker for centrosome measurements. Error bars represent the SEM of three independent experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

A. The percentage of multinucleated cells was determined using DAPI and α-tubulin staining. For each experiment 100 cells were counted per condition.

B. The average number of centrosomes per cell was determined in wt and p53 mutants at 24 hour intervals over 96 hours. For each experiment 40 cells were counted per condition.

C. The average centrosome size was determined in wt and p53 mutants at 24 hour intervals over 96 hours. For each experiment 40 cells were counted per condition. Where two or more centrosomes were present the largest centrosome was measured.

D. The average distance between centrosomes was determined in wt and p53 mutants at 24 hour intervals over 96 hrs. For each experiment 40 cells were counted per condition. Where three or more centrosomes were present the largest distance between two neighbouring centrosomes was measured.

E. The level of wild-type or p53-R175H protein expressed following 24 hour induction in the presence or absence of ANKRD11 re-expression was determined by western blot analysis, with β-actin protein levels used as a loading control.

Mutant p53 expression is associated with chromosome segregation defects that can be suppressed by ANKRD11

As centrosome abnormalities have previously been linked to aberrant mitotic progression (Mailand et al 2002, Wonsey and Follettie 2005), we next examined the mutant p53 expressing cells ability to undergo mitosis. The defects in centrosome duplication and segregation in the induced mutant p53 cells was associated with a 20 to 30% increase in the frequency of abnormal mitotic spindles (Fig. 3A & B), and a significant increase in cells with lagging chromosomes (Fig. 3C) and anaphase bridges (Fig. 3D) compared with uninduced cells. The duration of mitosis, as assessed by live cell imaging, showed mutant p53 cells slowed the progression through mitosis to 72-75 mins ($p<0.03$) compared with 57-58 minutes in uninduced H1299 cells (Fig. 3E). These findings show that mutant p53 expression does impact upon the normal execution of mitosis.

In the presence of restored ANKRD11 expression, the ability of induced p53-R175H to drive the formation of abnormal spindles, lagging chromosomes and anaphase bridges was completely ablated (Fig. 3A-D). Furthermore, the induced EI p53-R175H cells expressing ANKRD11 exhibited an average of 60 minutes in mitosis as compared to 75 minutes for induced p53-R175H alone (Fig. 3E). These observations indicate that p53 mutants cannot drive tumourigenesis in the presence of physiological levels of ANKRD11.

Figure 3

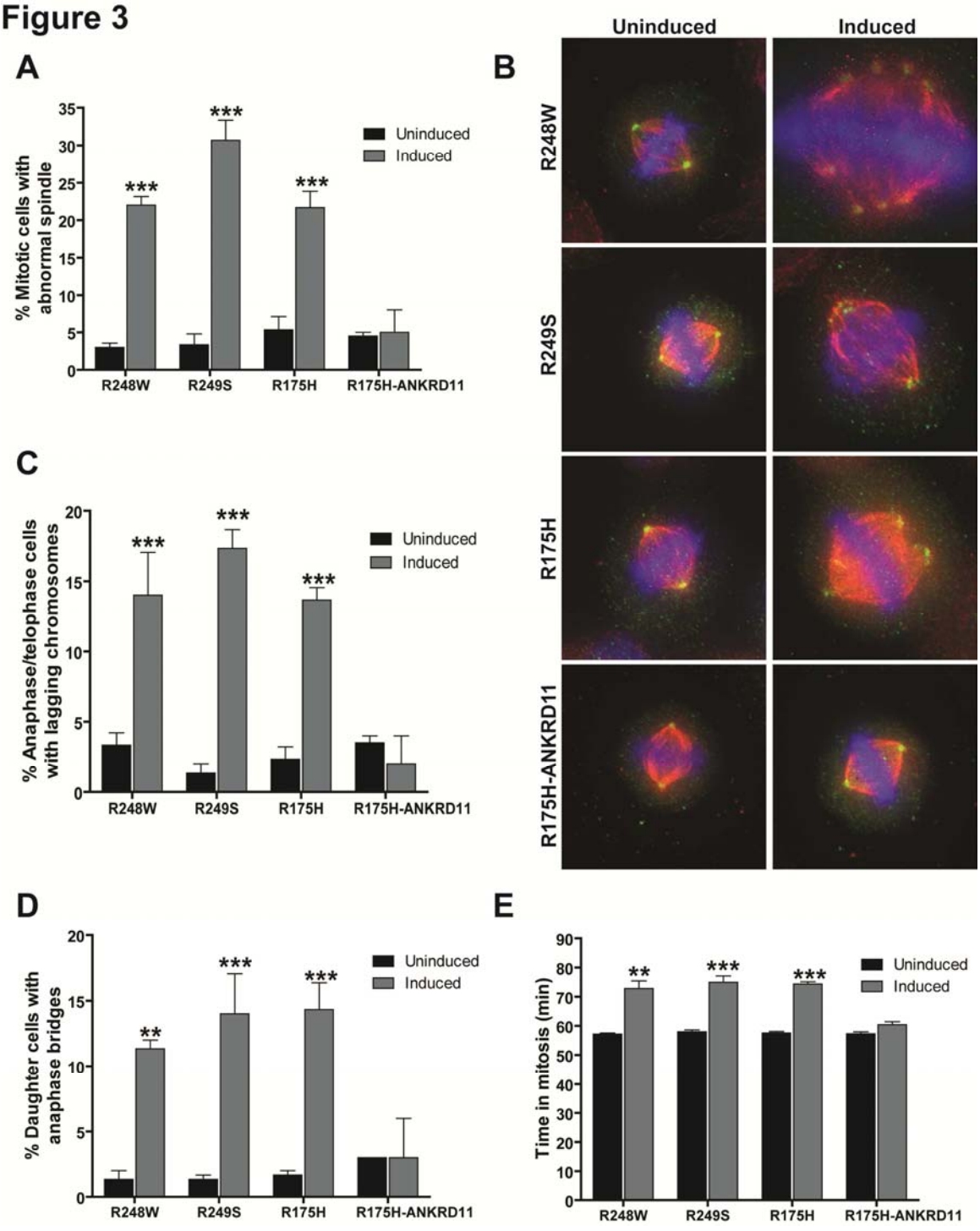

A
% Mitotic cells with abnormal spindle
Uninduced
Induced
R248W
R249S
R175H
R175H-ANKRD11
B
Uninduced
Induced
R248W
R249S
R175H
R175H-ANKRD11
C
% Anaphase/telophase cells with lagging chromosomes
D
% Daughter cells with anaphase bridges
E
Time in mitosis (min)

Figure 3 – Induction of mutant p53 results in mitotic defects that are alleviated by ANKRD11 expression

EI p53-R248W, EI p53-R175H, EI p53-R249S and EI p53-R175H-ANKRD11 cells were plated at 10% confluency and synchronised by double thymidine block and harvested at 11 hours post-release. PonA was added at 24 hours before harvesting. For each experiment 100 cells were counted per condition. Error bars represent the SEM from three independent experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

A. The average percentage of metaphase cells with abnormal spindles was determined using α-tubulin as a marker of the mitotic spindle. Cells were synchronised in metaphase by the addition of 10 μM MG132 at two hours prior to harvesting.

B. Representative images of the spindle morphology uninduced and induced cells. Cells were stained for α-tubulin (red), γ-tubulin (green) and DAPI (blue). Scale bar represents 20 μm.

C. The average percentage of anaphase/telophase cells with lagging chromosomes was determined using DAPI as a marker of the DNA.

D. The average percentage of daughter (newly divided) cells with anaphase bridges was determined using DAPI as a marker of the DNA.

E. The average time taken for cells to undergo mitosis. Synchronised cells were followed by time lapse microscopy and imaged every five minutes. Time in mitosis was defined as the time from nuclear envelope breakdown until the point at which two daughter cells could be seen. For each experiment 50 cells were counted per condition.

Mutant p53 induces a mesenchymal morphology that is suppressed by ANKRD11

Gene expression arrays were used to determine the specific genes and pathways regulated upon induction of the p53-R175H mutant (Table 1). Following analysis of microarray expression data, Ingenuity Pathway Analysis revealed that many of the genes regulated by p53-R175H were shared across multiple biological pathways, which converged towards a network involved in cell motility and cell-to-cell signaling/interaction (Table 1, Supp. Fig. S7). Consequently, we investigated if induction of mutant p53 affected colony formation and morphology of epithelial H1299 cells. Control H1299 cells grew from single cells as tight colonies with defined boundaries, typical of epithelial cells (Fig. 4A, left). Upon induction of a structural (R175H) or DNA contact (R248Q) mutant, cells acquired an elongated mesenchymal-like morphology and exhibited disordered growth and decreased colony density (Fig. 4A, middle). In addition, ZO-1 immunofluorescence of EI p53-R175H cells showed that cells dissociated from each other within 16 hours of p53-R175H induction (Fig. 4B), which is indicative of an invasive phenotype. As we had already shown that ANKRD11 could alleviate the mitotic defects caused by mutant p53 induction, we next aimed to determine if the mesenchymal-like morphology of mutant p53-expressing cells could also be suppressed by ANKRD11. Physiological ANKRD11 expression in EI p53-R175H cells suppressed the mesenchymal-like phenotype, as demonstrated by a retention of tight colony formation following p53-R175H induction (Fig. 4A, right & Fig. 4B). Stable ANKRD11 expression in EI p53-R248Q cells also resulted in a restoration of the epithelial-like phenotype (Fig. 4A, right).

Table I. Genes regulated by induction of p53-R175H

Category	Function	p-value	Gene Names		Molecules
			Down-regulated	Up-regulated	
Cellular Movement	migration	1.69E-05	CD22, DLX2,	ANGPTL4, ANPEP, DKK1, ID2, ITGA3, ITGB3, KRT6A, LAMC2, MAP2K3, NR2F2, OPHN1, PDE2A, PPARG, RAC2, RALB, SEMA3C, SERPINA1, SFRP1, STC1, TGFBI, TNFSF4, TP53	24
Cancer	tumorigenesis	3.45E-05	CD22, CD274, SOCS2, STC1, TUBB2C, WEE1	ABCC3, AHCYL1, AKAP12, AKAP13, ANGPTL4, ANPEP, BCL2L1, CDA, CDH3, CPA4, DDIT4, DECR1, DKK1, EDNRA, EHHADH, FSTL3, ID2, ITGA3, ITGB3, KRT6A, LAMC2, RALB, SCD, SEMA3C, SERPINA1, SFRP1, SLC46A3, LRIG1, MAP2K3, MT1X, OGG1, PDE2A, PLK2, PPARG, PRDM1, PRSS12, PSD3, PTX3, TFPI2, TP53	46
Cell Death	cell death	7.01E-05	ATG12, CD22, CD274, DLX2, WEE1	AKAP12, ANGPTL4, ANPEP, ATXN1, BCL2L1, CTH, DDIT4, DECR1, DKK1, FSTL3, GLIPR1, ID2, ITGB3, LRIG1, MAP2K3, MT1X, OGG1, P2RX4, PLK2, PPARG, PRDM1, RAC2, RALB, SCD, SERPINA1, SFRP1, SRR, TFPI2, TGFBI, TP53, UQCRFS1, VIPR2	37
Cell-To-Cell Signaling and Interaction	activation	2.17E-03	CD22, SOCS2, CD274	BCL2L1, CD63, ITGA3, ITGB3, MAP2K3, PPARG, , STC1, TNFSF4, TP53, VIPR2	13
Cellular Growth and Proliferation	proliferation	2.92E-03	CD22, CD274, PTX3, SOCS2	AKAP13, ANGPTL4, BCL2L1, CTH, DECR1, DKK1, EDNRA, ID2, ITGA3, ITGB3, LRIG1, PDE2A, PPARG, PRDM1, RAC2, RALB, ROMO1, SERPINA1, SFRP1, STC1, TFPI2, TGFBI, TNFSF4, TP53, ZMIZ1	29
Gene Expression	transcription	1.04E-02	DLX2, SOCS2	AKAP5, AKAP13, ATXN1, BCL2L1, BHLHE41, DKK1, FSTL3, HIVEP3, ID2, ITGB3, MAP2K3, NR2F2, PLK2, PPARG, PRDM1, SFRP1,TCEA2, TCEAL1, TP53, ZMIZ1	22

FIGURE 4

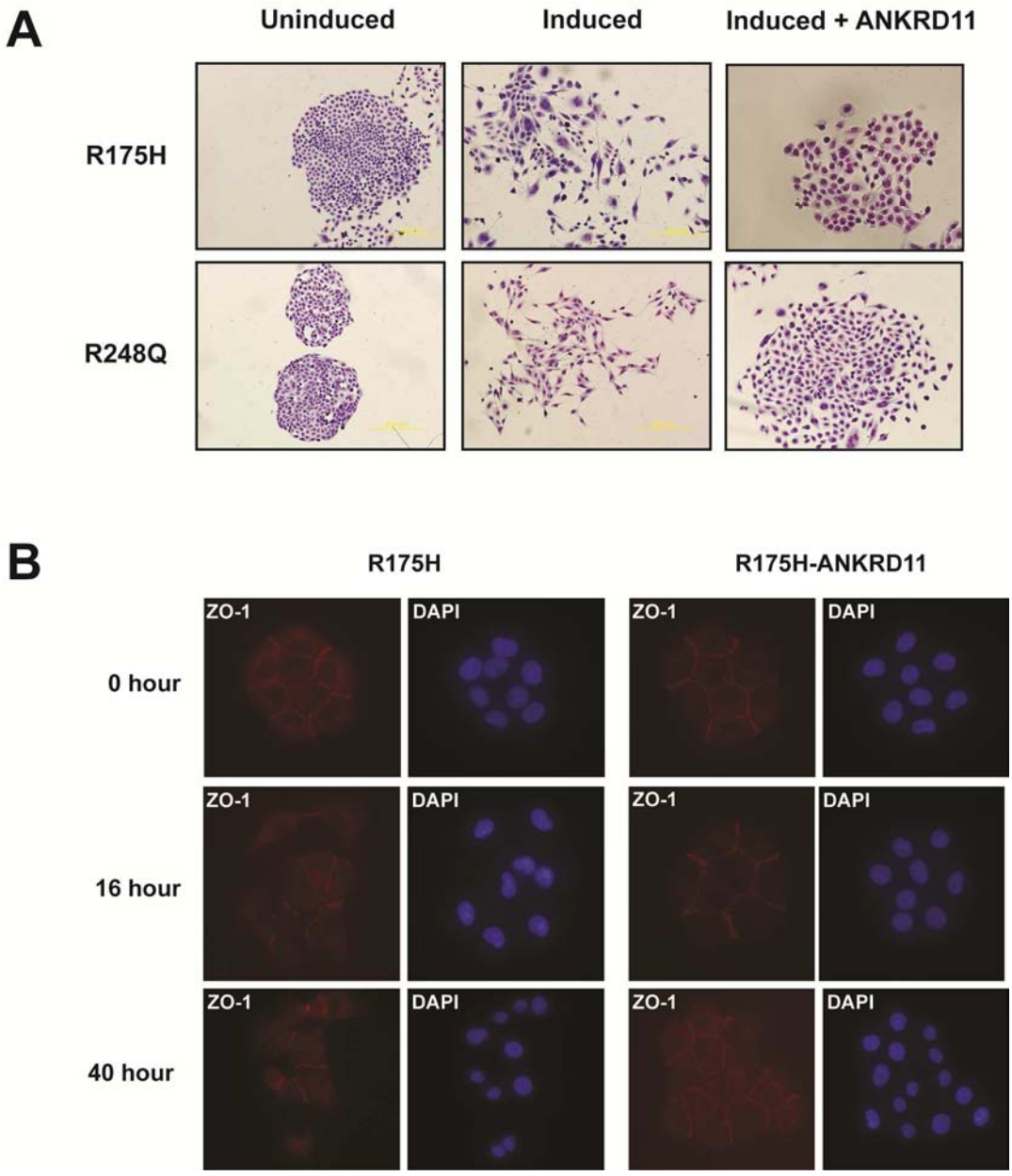

A
Uninduced
Induced
Induced + ANKRD11
R175H
R248Q
B
R175H
R175H-ANKRD11
ZO-1
DAPI
0 hour
16 hour
40 hour

Figure 4 – Induction of mutant p53 in H1299 cells results in an altered cellular phenotype, which is suppressed by ANKRD11

A. EI p53-R175H and EI p53-R248Q cells were untreated (left) or treated (middle) with 2.5 µg/mL PonA and plated at single cell density. Colonies were fixed and stained 10 days post-plating. Pictures show representative colonies. Each cell line with re-expressed ANKRD11 was treated in a similar manner (right).

B. The EI p53-R175H cell line were seeded at single cell density and allowed to develop into colonies for 72 hours prior to the addition of PonA (2.5 µg/mL) as indicated. Colonies were stained for the presence of cell-cell junctions, as indicated by ZO-1 staining (red).

ANKRD11 suppresses mutant p53 invasive GOF

Mutant p53 GOF plays a key role in tumour progression, particularly through migration, invasion and metastasis. As induction of p53-R175H gave rise to an invasive phenotype, we aimed to determine if this was associated with an increased invasive capacity. The ability of uninduced or induced cells to invade through matrigel toward a chemo-attractant was measured in real-time using the Xcelligence system. Induction of p53-R175H did indeed result in an enhanced invasive capacity of H1299 cells, as demonstrated by a 2.5-fold ($p<0.05$) increased rate of invasion (Fig. 5). Stable expression of ANKRD11 in this system also ablated the ability of the induced R175H mutant to drive invasion (Fig. 5). Importantly, we also demonstrate that ANKRD11 can influence the tumorigenic properties of an additional mutant p53-expressing cancer cell line, MDA-MB-468 (p53 R273H). Expression of ANKRD11 in this cell line was shown to slow the rate of migration of the MDA-MB-468 breast cancer cell line in a mutant p53-dependent manner (Supp. Fig. S8).

Figure 5

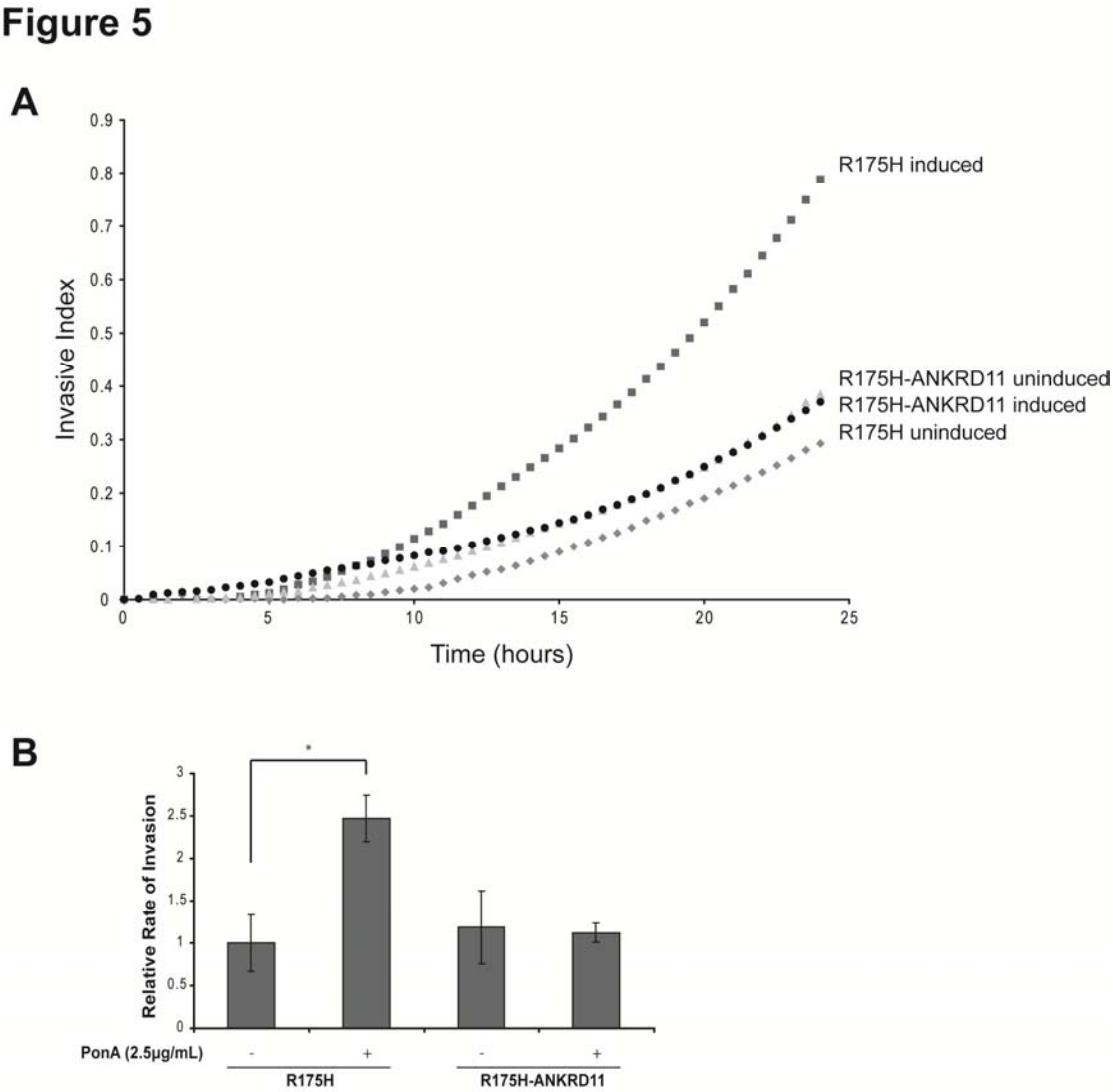


Figure 5 – ANKRD11 suppresses p53-R175H mediated invasion

A. EI p53-R175H or EI p53-R175H-ANKRD11 cells were treated as indicated with 2.5 µg/mL PonA for 24 hrs prior to plating in the upper chamber of a CIM-16 plate coated with 5% matrigel. Real-time invasion of cells was measured on an Xcelligence RTCA DP analyser.

B. The rate of invasion during the linear phase (between 15 and 24 hours) as determined by the gradient (n=4; * $p<0.05$).

ANKRD11 restores a wild-type conformation and function to the p53-R175H mutant protein

We speculate that ANKRD11 can restore normal p53 function to p53 mutants as a consequence of its ability to directly interact with the mutant p53 proteins and revert the protein to a wild-type p53 conformation. We have previously shown the ankyrin repeat domain ($ANKRD11^{144\text{-}288aa}$) to directly interact with wild-type p53 (Neilsen et al 2008). We confirmed through co-immunoprecipitation assays that $ANKRD11^{144\text{-}288aa}$ could interact with both wild-type and eight p53 hotspot mutants (Fig. 6A). Further to these findings, we identified the C-terminus of p53 ($p53^{325\text{-}393aa}$) as the minimal region required for interaction with the ankyrin domain of ANKRD11 (Supp. Fig. S9).

To test the ability of ANKRD11 to affect mutant p53 tertiary structure, we utilised the conformation specific p53 antibody PAb1620 which recognises the wild-type p53 native protein structure under non-denaturing conditions (Milner et al 1987). In the presence of ANKRD11, induced p53-R175H exhibited an enhanced binding to the PAb1620 antibody, in comparison to that observed in the absence of ANKRD11 (Fig. 6B). These data suggest the association of ANKRD11 with p53-R175H restores the mutant protein conformation to more closely resemble the wild-type protein, thus facilitating recognition by the PAb1620 antibody. This newly adopted ‘wild-type’ conformation of the mutant p53 protein was also associated with restoration of p53 target gene expression by the p53 mutant to a level similar to that achievable by wild-type p53 (Supp. Fig. S10). This restoration of transcriptional activity to the mutant p53 protein is also accompanied by a reduction in the percentage of cells undergoing mitosis, as demonstrated by MPM2 staining. Induction of wild-type p53 leads to a

65% reduction in cells undergoing mitosis ($p<0.01$) which is not seen following induction of various mutant p53 proteins. However, in the presence of ANKRD11, induction of the p53-R175H mutant leads to a significant ($p<0.01$) reduction in cells in mitosis (Supp. Fig. S11).

Figure 6

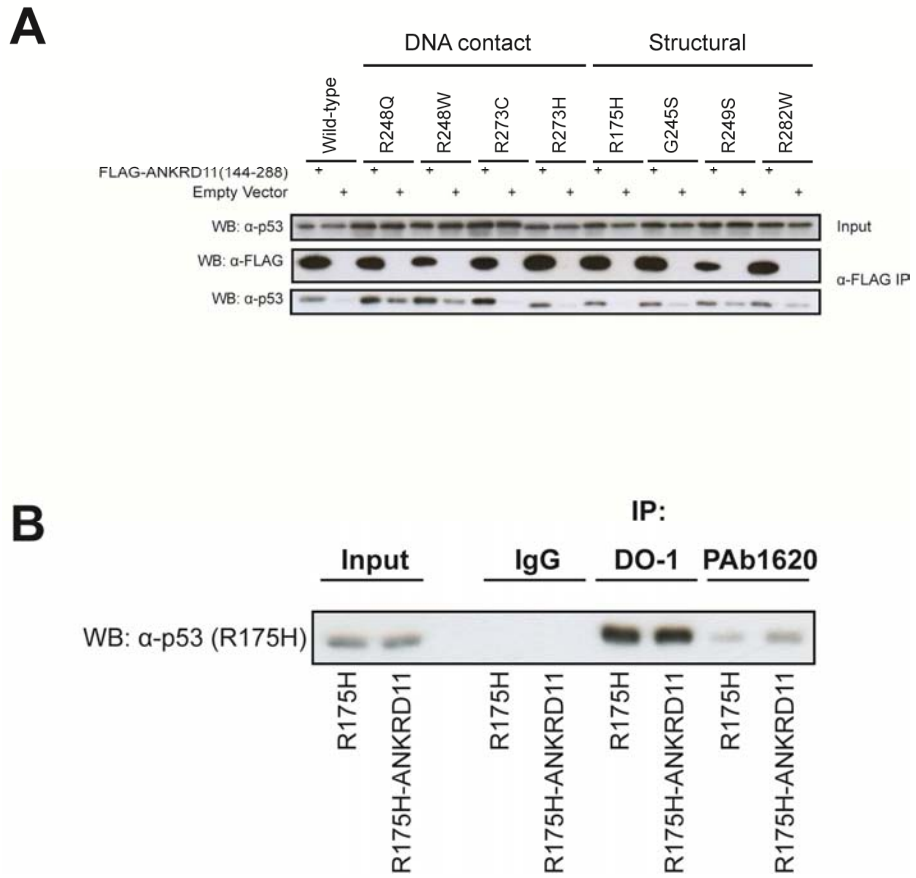


Figure 6 – ANKRD11 restores a native conformation to a p53 mutant

A. H1299 cells were sequentially transfected with 4 µg FLAG-ANKRD11$^{144\text{-}288aa}$ and 0.5 µg wild-type or mutant p53 expression constructs, followed by immunoprecipitation with an α-FLAG antibody. Inputs and immunoprecipitated complexes were subjected to western blot analysis with α-p53 and α-FLAG antibodies. All p53 mutants investigated are shown to co-precipitate with ANKRD11$^{144\text{-}288aa}$ above the level of non-specific, background levels seen in empty vector control lanes.

B. EI p53-R175H and EI p53-R175H-ANKRD11 cells were treated with 2.5 µg/mL PonA for 24 hours, immunoprecipitated with indicated antibody and detected by western blot analysis using α-p53 (DO-1) and α-mouse light chain-specific secondary antibody.

ANKRD11 impedes mutant p53-p63 and mutant p53-p73 complex formation

Previous studies have established that wild-type p53 does not interact with p63 and p73 while p53 mutants, including R175H, R249S and R273H exhibit GOF interactions with p63 and p73 (Gaiddon et al 2001, Strano et al 2000, Strano et al 2002). Immunoprecipitation in H1299 cells of co-transfected TAp63 and p53 mutants, including the eight p53 hotspot mutants, encompassing both DNA contact (R248Q, R248W, R273C and R273H) and structural (R175H, G245S, R249S, R282W) mutants suggest that p63 was primarily sequestered by p53 structural mutants (Fig. 7A). The DNA contact mutant p53-R273H also weakly co-precipitated with p63, which is consistent with previously published reports, while the remaining three DNA contact mutants did not interact (Fig. 7A). The same subset of p53 mutants that interacted with p63 were also shown to form a complex with TAp73 (Fig. 7B). Since the interaction between mutant p53 and p63 contributes to the invasive GOF of mutant p53 in H1299 cells (Muller et al 2009) we speculated that the suppressive functions of ANKRD11 are due, in part, to an interference with mutant p53•p63 complex formation.

Immunoprecipitation results demonstrated the formation of an R175H•p63 complex in the absence of ANKRD11 (Fig. 7C, lane 7). However, expression of the complete ankyrin domain ($ANKRD11^{144\text{-}288aa}$) resulted in dissociation of the R175H•p63 complex by up to 62 % (Fig. 7C, lane 8). $ANKRD11^{144\text{-}288aa}$ was also co-expressed with p53-R175H and p73 in the same system, and the interaction between R175H and p73 was reduced to 56% of the base-line (Fig. 7D). These results indicate that the association of ANKRD11 with mutant p53 disrupts its ability to interact with both p63 and p73. Further immunoprecipitation experiments with various regions

encompassing the five ankyrin repeats present in ANKRD11 enabled the region of the domain that disrupts the mutant p53•p63 interaction to be defined. Ankyrin repeats one to three dissociated the p53-R175H•p63 complex by approximately 54% (Fig. 7C, lane 9). The R175H•p63 interaction was, however, maintained in the presence of ankyrin repeats four and five (Fig. 7C, lane 10). Expression of the first ankyrin repeat did not dissociate the R175H•p63 complex, however expression of repeats two and three resulted in a 21% decrease in the observed interaction between p53-R175H and p63 (Supp. Fig. S12). It was therefore inferred that an 80 amino acid region of ANKRD11 (ANKRD11$^{144\text{-}288aa}$) dissociated the R175H•p63 complex, with a slight reduction in complex formation also observed following co-expression of a 50 amino acid region (ANKRD11$^{176\text{-}225aa}$). These results are summarised in Figure 7E.

Figure 7

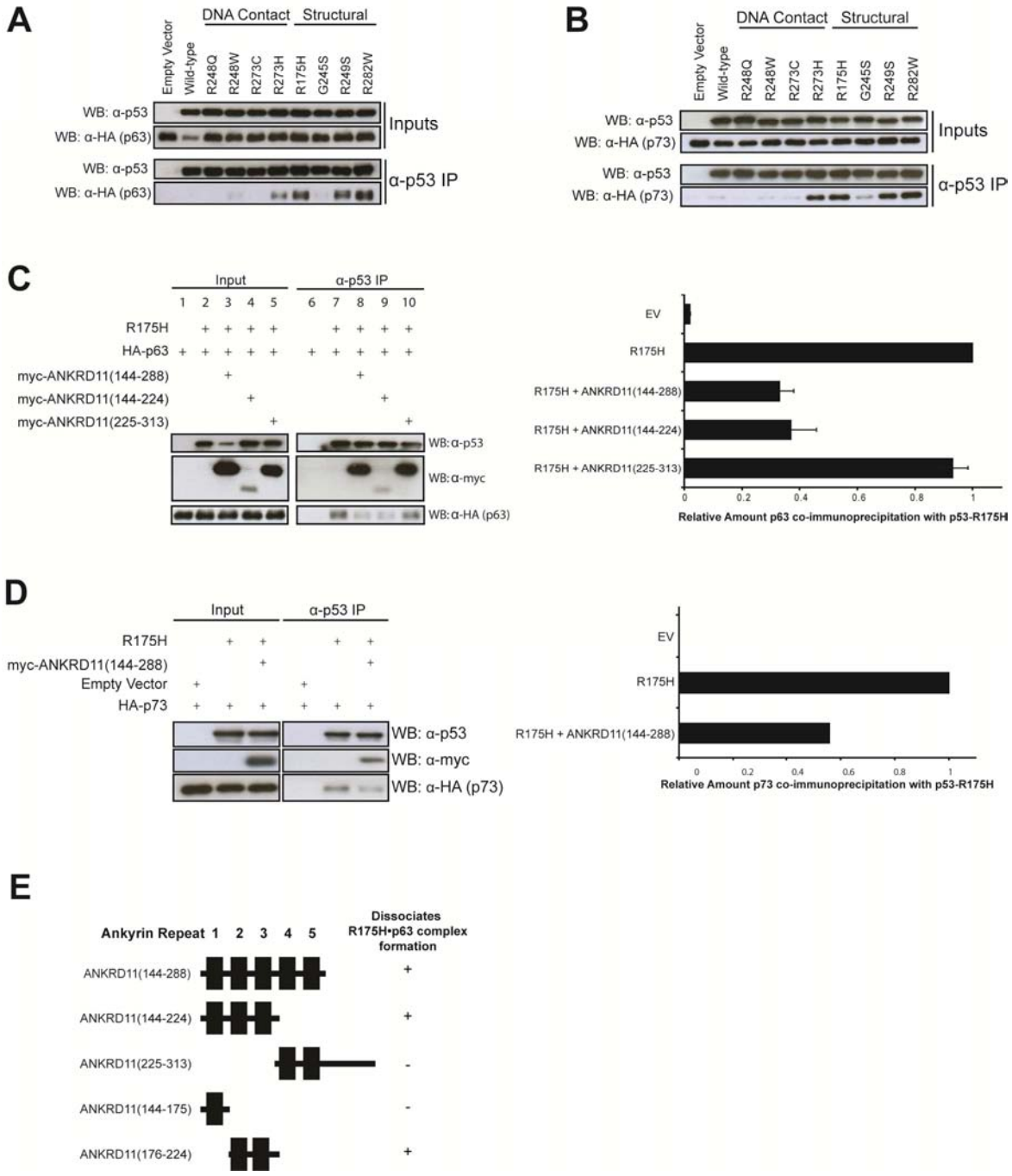

A
DNA Contact
Structural
Empty Vector
Wild-type
R248Q
R248W
R273C
R273H
R175H
G245S
R249S
R282W
WB: α-p53
WB: α-HA (p63)
Inputs
α-p53 IP
B
DNA Contact
Structural
WB: α-p53
WB: α-HA (p73)
Inputs
α-p53 IP
C
Input
α-p53 IP
R175H
HA-p63
myc-ANKRD11(144-288)
myc-ANKRD11(144-224)
myc-ANKRD11(225-313)
WB: α-p53
WB: α-myc
WB: α-HA (p63)
EV
R175H
R175H + ANKRD11(144-288)
R175H + ANKRD11(144-224)
R175H + ANKRD11(225-313)
Relative Amount p63 co-immunoprecipitation with p53-R175H
D
Input
α-p53 IP
R175H
myc-ANKRD11(144-288)
Empty Vector
HA-p73
WB: α-p53
WB: α-myc
WB: α-HA (p73)
EV
R175H
R175H + ANKRD11(144-288)
Relative Amount p73 co-immunoprecipitation with p53-R175H
E
Ankyrin Repeat 1 2 3 4 5
Dissociates R175H•p63 complex formation
ANKRD11(144-288) +
ANKRD11(144-224) +
ANKRD11(225-313) -
ANKRD11(144-175) -
ANKRD11(176-224) +

Figure 7 – The ankyrin domain of ANKRD11 dissociates the p53-R175H•p63/p73 complexes

A. H1299 cells were co-transfected with 2 µg wild-type or mutant p53 expression constructs and 4 µg HA-p63. Cellular extracts were subjected to immunoprecipitation with α-p53 (DO-1) antibody. Inputs and immunoprecipitates were subjected to western blot analysis using α-p53 and α-HA antibodies.

B. Cells were treated as described above (A) but p53 was co-transfected with HA-p73.

C. H1299 cells were sequentially transfected with 2 µg p53-R175H, 4 µg HA-p63 and 2 µg various Myc-ANKRD11 fragment expression constructs as indicated followed by immunoprecipitation with α-p53 (DO-1) antibody. Inputs and immunoprecipitates were subjected to western blot analysis with α-p53, α-Myc and α-HA antibodies. The amount of p63 co-precipitated with p53-R175H was determined by densitometry.

D. Cells were treated as above (C) but with HA-p73 and Myc-ANKRD11$^{144\text{-}288aa}$ expression constructs.

E. Schematic diagram illustrating the region of ANKRD11 capable of dissociating the mutant p53-p63 complex.

DISCUSSION

Our current understanding of the GOF properties of mutant p53 is largely based on studies involving stable expression of p53 mutants in a p53 null background (Dong et al 2009, Muller et al 2009) or mutant p53 knock-in transgenic animal models (Song et al 2007). These systems have proven useful to functionally characterise the persistent, long-term oncogenic properties of mutant p53 such as invasion, metastasis and large-scale genetic abnormalities. However, using the ecdysone-inducible system, we have been able to directly investigate the primary pathways that are initiated by mutant p53 expression and which ultimately lead to its GOF, as well as identify ANKRD11 as an endogenously expressed protein capable of suppressing mutant p53 GOF.

Knockdown of endogenous mutant p53 in MDA-MB-468 cells results in reduced metastasis in a mouse model (Adorno et al 2009). Expression of ANKRD11 in MDA-MB-468 cells also significantly reduced their ability to form colonies on plastic (Neilsen et al 2008). Furthermore, loss of ANKRD11 expression with p53 mutation defines breast cancer patients with invasive tumours and poor prognosis (Fig. 1). Low ANKRD11 expression is also correlated with poor 5-year survival in bladder and lung cancer patients (Supp. Fig. S1). Loss-of-heterozygosity of chromosome 16q24 (the genomic location of the *ANKRD11* gene) is associated with good prognosis in breast cancer patients (Hansen et al 1998), therefore the observation that survival is higher in the wild-type p53 expressing breast tumours with low ANKRD11 expression may be an artefact of the loss of the 16q genomic region and not specific to ANKRD11. The correlation of low ANKRD11 expression and poor survival in mutant p53 expressing breast tumours support a role for ANKRD11 as a

suppressor of the oncogenic potential of mutant p53. Using the ecdysone-inducible system, we established three biological assays for investigating mutant p53 GOF and the effect of ANKRD11 on these processes. Specifically we investigated mitotic defects arising from centrosome abnormalities, cellular morphology changes and invasion.

Taking this approach, we show inducible expression of three p53 'hotspot' mutants result in multinucleation, centrosome aberrations, and a significant increase in the percentage of cells with abnormal spindles (Fig. 2 & 3). Previous studies link aberrant centrosomes to both defective mitosis (Mailand et al 2002) and multinucleation (Wonsey and Follettie 2005), both of which we show to occur following mutant p53 induction. The increased distance observed between centrosomes in mutant p53 expressing cells may be due to de-regulation of β-catenin as wild-type p53 inhibits β-catenin, with high β-catenin levels associated with mutant p53 expression and centrosome splitting (Hadjihannas et al 2010, Sadot et al 2001).

Our findings are consistent with observations of mutant p53 GOF in mouse models where the expression of the mouse R172H mutant (equivalent to human R175H) results in tumours exhibiting centrosome amplification and an associated aneuploidy (Caulin et al 2007, Hingorani et al 2005, Murphy et al 2000). However, these previous reports are confounded as they use a K-Ras^{G12D} mutation as an initiating oncogenic event (Caulin et al 2007, Hingorani et al 2005). Our data shows that mutant p53 alone is sufficient to drive the observed GOF phenotype. These mitotic defects would provide a likely origin for chromosomal instability, a common

feature of many human tumours, which promotes metastases and is correlated with poor patient prognosis (Kuukasjarvi et al 1997, Rajagopalan and Lengauer 2004).

This study has uncovered several novel genes and biological processes regulated by mutant p53 that regulate cellular movement and cell-to-cell signaling and interaction (Table 1). R175H has previously been shown to reprogram the cellular transcriptome through recruitment to novel target genes by various transcription factors, such as NF-Y (Di Agostino et al 2006), VDR (Stambolsky et al 2010) and NF-κβ (Schneider et al 2010). Indeed, our pathway analysis also showed that the NF-κβ signaling pathway was strongly up-regulated upon induced expression of p53-R175H (Figure S5B), further confirming a role for NF-κβ in the GOF of mutant p53 (Weisz et al 2007). Interestingly, several integrins (ITGA3 and ITGB3) were also up-regulated upon induction of p53-R175H consistent with a previously observed role for integrin recycling in the oncogenic GOF of mutant p53 (Muller et al 2009).

It has previously been shown that H1299 cells with inducible expression of p53-R175H exhibit a mesenchymal-like phenotype following TGFβ treatment (Adorno et al 2009). We observe this morphological change upon induction of both structural (R175H) and DNA contact (R248Q) p53 mutants with no additional treatment (Fig. 4A). Variations in cell density may account for this contradiction to previous data, as cells plated at higher densities did not exhibit this phenotype following mutant p53 induction (data not shown).

For each of the assays described, the expression of ANKRD11 with induced mutant p53 restores the cellular phenotype to that seen in the uninduced state i.e.

ANKRD11 suppresses the GOF of mutant p53. We have also established that ANKRD11 can restore wild-type p53 activity to p53 mutants. These data represent the first reported evidence of an endogenously expressed protein that has the capacity to suppress mutant p53 GOF as well as restore transcriptional activity to a p53 mutant (Neilsen et al 2008). This has broad implications for the development of new cancer therapies designed to target this pathway, as these may result not only in suppression of the invasive and metastatic properties of mutant p53 tumours, but also prevent tumour growth through a restoration of wild-type p53 activity.

We propose that ANKRD11 suppresses mutant p53 GOF by directly interacting with mutant p53 proteins resulting in a native wild-type p53 conformation (Fig. 6). This unique property of ANKRD11 is similar to that reported for small molecules and peptides PRIMA-1 (Bykov et al 2002) and CDB-3 (Friedler et al 2002, Issaeva et al 2003) which reportedly restore a native conformation to mutant p53. We also show that expression of the ANKRD11 ankyrin domain is sufficient to dissociate the p53-R175H•p63 and p53-R175H•p73 complexes (Fig. 7). The ability of mutant p53 to drive tumourigenesis is reported to be partially based on its ability to sequester the p63 and p73 proteins, thus disrupting their function to suppress metastasis (Di Como et al 1999, Gaiddon et al 2001). It is likely that ANKRD11 is suppressing mutant p53 GOF through an interaction with the C-terminus of p53 (Supp. Fig. S9) coupled with its ability to enhance the acetylation of the DNA binding domain (Neilsen et al 2008), thus leading to a stabilised, active conformation of p53 and subsequent dissociation of the mutant p53•p63/p73 complexes. Alternatively, the C-terminus of p53 has been shown to be required for mutant p53 invasive activity

(Muller et al 2009), therefore we speculate that ANKRD11 may be out competing the recruitment of another unknown oncogenic protein required for mutant p53 GOF.

Studies defining mutant p53 interactions with p63 and p73 have included different subsets of mutants with the predominant focus on p53-R175H and p53-R273H (Gaiddon et al 2001, Strano et al 2000, Strano et al 2002). In this study we present a comprehensive comparison of the interaction of eight p53 hotspot mutants with p63 and p73, and show the structural class of mutants preferentially interact with both p63 and p73 with R273H the only DNA contact mutant shown to interact (Fig. 7), which is consistent with previous studies (Gaiddon et al 2001, Strano et al 2002). A previously reported interaction between p63 and R248W (Gaiddon et al 2001) was not observed in this study. These data and others suggest a correlation between the structural integrity of the p53 protein and its ability to interact with p63/p73 (Gaiddon et al 2001). Interestingly, the endogenous p53-R273H mutant is reported to be immunoprecipitated from cell lines with the mutant specific antibody, PAb240 (Muller et al 2008), suggesting p53-R273H exhibits some structural perturbations. Furthermore, no interaction with p63 or p73 was observed for p53-R273C, suggesting that the amino acid at codon 273 plays an integral role in this interaction. In contrast to other structural mutants, G245S did not interact with p63 or p73 (Fig. 7). However, the p53-G245S protein is only local distorted in comparison to p53-R175H and p53-R282W that are globally distorted (Brosh and Rotter 2009, Wong et al 1999). These findings suggest that the extent of mutant p53 protein distortion may also dictate its ability to sequester p63 and p73.

The DNA contact mutants R248W and R248Q are shown to have a GOF in relation to multinucleation (Fig. 2A) and cellular morphology (Fig. 4A) respectively, however these mutants did not interact with p63 or p73. Therefore these mutants acquire their GOF independently of p63 or p73, presumably through interactions with other regulatory proteins. Possible candidates include the DNA repair complex protein MRE11. The specific interaction between MRE11 and the R248W mutant is reported to inactivate ATM, thereby inducing genetic instability (Liu et al 2010, Song et al 2007).

This study provides novel insight into the complex oncogenic processes that are driven by mutant p53. The discovery of ANKRD11 as an endogenously expressed protein with the capacity to suppress various mutant p53 functions as well as restore wild-type activity to p53 mutants, opens a novel avenue to indirectly restore tumour suppressor activity to the guardian of the genome.

Figure S1

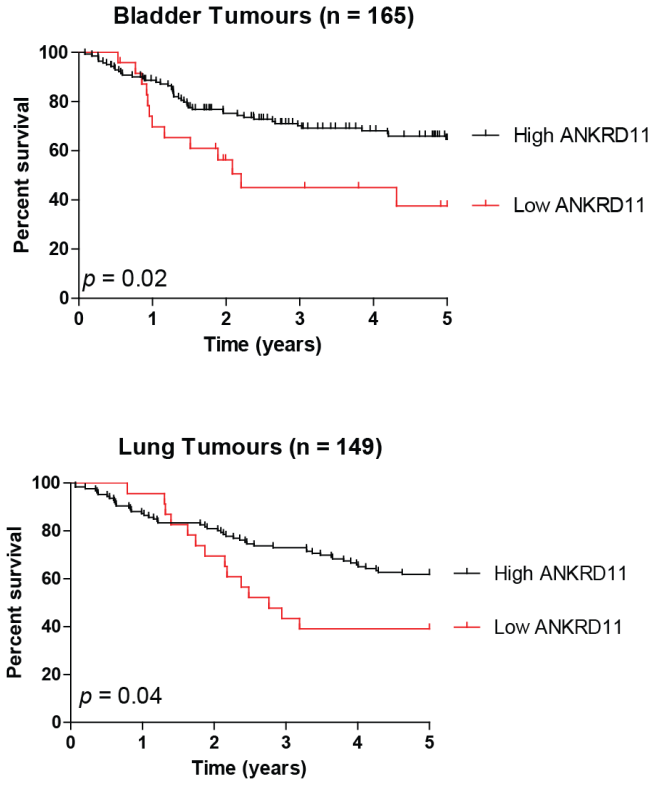


Figure S1 – Down-regulation of ANKRD11 in bladder and lung tumors is predictive of poor clinical outcome

Kaplan-Meier curves derived from publically available survival data associated with cohorts of 165 bladder cancer patients (GSE13507) or 149 lung cancer patients (GSE11969). Expression of ANKRD11 was sourced from expression microarray analyses. Tumours were ranked by ANKRD11 expression, with the lowest 15% of these tumours defined as 'Low ANKRD11'.

Figure S2

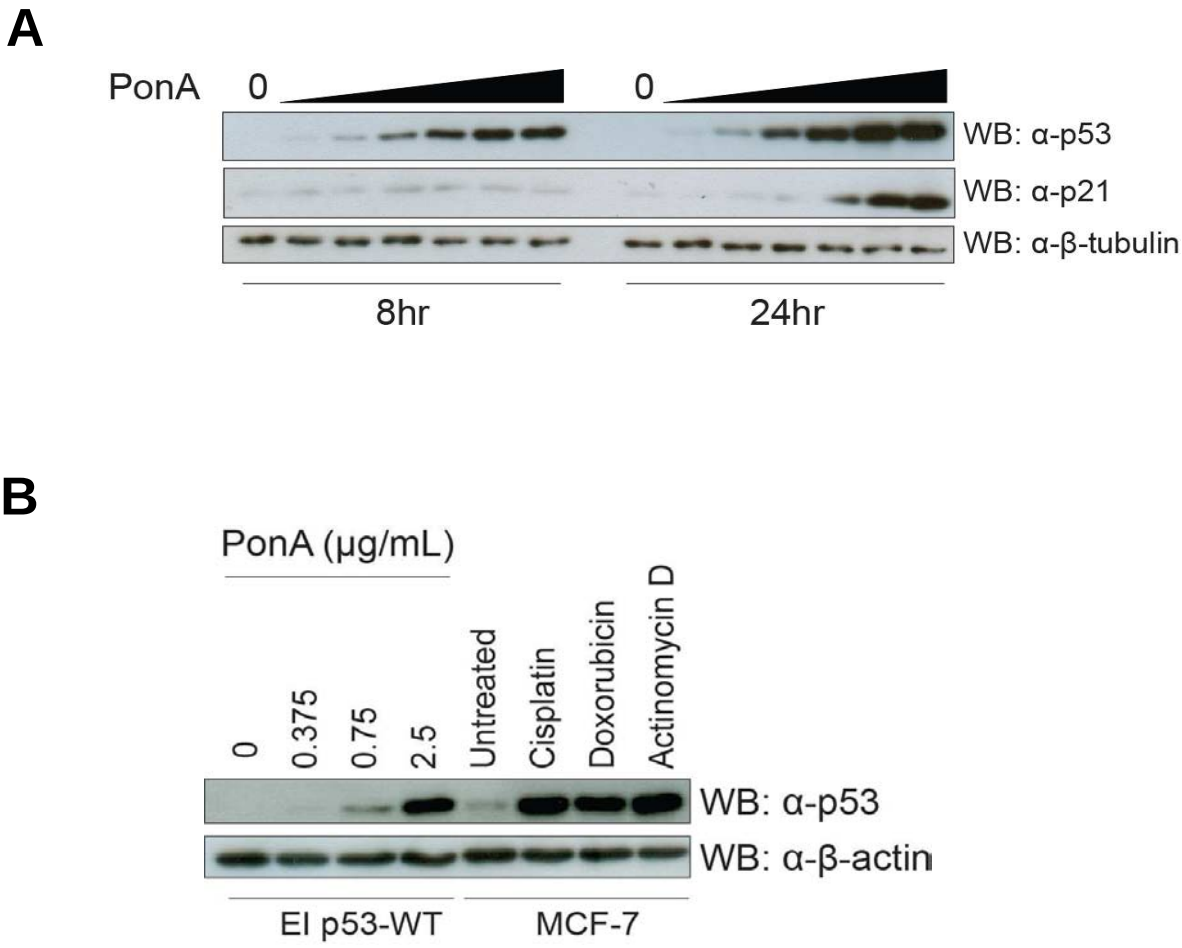

A
PonA
0
0
WB: α-p53
WB: α-p21
WB: α-β-tubulin
8hr
24hr
B
PonA (μg/mL)
0
0.375
0.75
2.5
Untreated
Cisplatin
Doxorubicin
Actinomycin D
WB: α-p53
WB: α-β-actin
EI p53-WT
MCF-7
C
WT
R175H
R249S
R248W
R248Q
MDA MB-231
SKBR3
PonA (2.5μg/mL)
- + - + - + - + - +
p53
β-actin

Figure S2 – Characterisation of ecdysone-inducible H1299 cell lines

A: The EI p53-WT cell line was treated with 0, 0.125, 0.25, 0.5, 1, 1.5 or 2.5 µg/mL PonA for 8 or 24 hours. Expression of p53 and p21 was determined by western blot analysis, with β-tubulin levels included as a loading control.

B: The level of wild-type p53 induced in EI p53-WT cells was compared to p53 protein levels following activation of the pathway in MCF-7 cells by DNA damage. MCF-7 cells were treated with 20 µg/mL cisplatin, 20 µg/mL doxorubicin or 100 ng/mL actinomycin D for 16 hours.

C: Inducible H1299 cell lines expressing WT, R175H, R249S, R248W and R248Q were untreated or treated with 2.5 µg/mL PonA for 24 hours prior to western blot analysis for p53 protein levels. Levels of p53 expression were compared to the expression of endogenous mutant p53 in cell lines MDA-MB-231 (R280K) and SKBR3 (R175H), with β-actin levels included as a loading control.

Figure S3

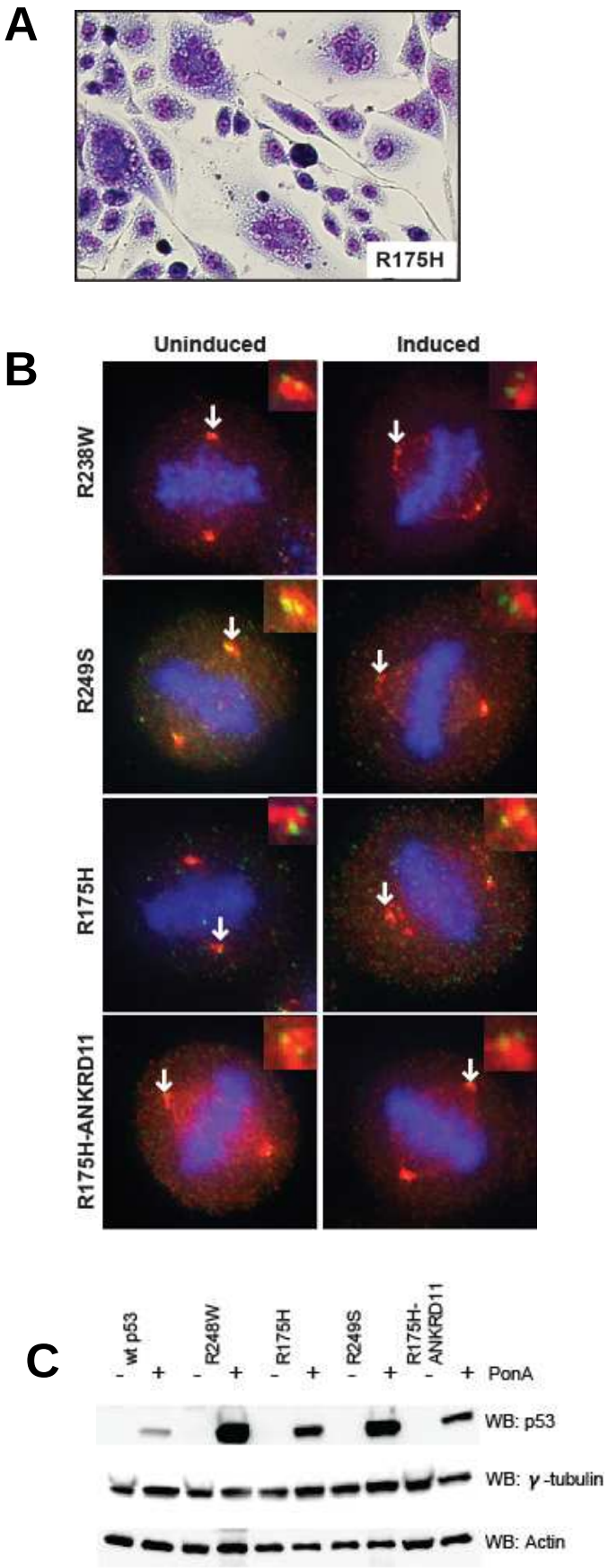

A
R175H
B
Uninduced
Induced
R238W
R249S
R175H
R175H-ANKRD11
C
- wt p53
+
- R248W
+
- R175H
+
- R249S
+
- R175H-ANKRD11
+
PonA
WB: p53
WB: γ-tubulin
WB: Actin

Figure S3 – The centrosome defects are not due to centrosome splitting or changes in γ–tubulin levels.

A: Giant multinucleated cells were observed in EI p53-R175H cells grown from single colonies following PonA treatment (2.5 µg/mL) for 10 days.

B: EI p53-R248W, EI p53-R175H, EI p53-R249S and EI p53-R175H-ANKRD11 cells were plated at 10% confluency and synchronised by double thymidine block and harvested at 11 hrs post-release. PonA was added at 24 hrs before harvesting. Cells were co- stained for the centriole marker, Centrin (green) together with γ–tubulin (red) and DAPI (blue). Inset: enlargements of the spindle poles denoted by the white arrows.

C: EI p53-WT, EI p53-R248W, EI p53-R175H, EI p53-R249S and EI p53-R175H-ANKRD11 were plated at 10% confluency and treated with 2.5µg/mL PonA for 96 hours and subjected to western blot analysis for p53, γ-tubulin, and Actin.

Figure S4

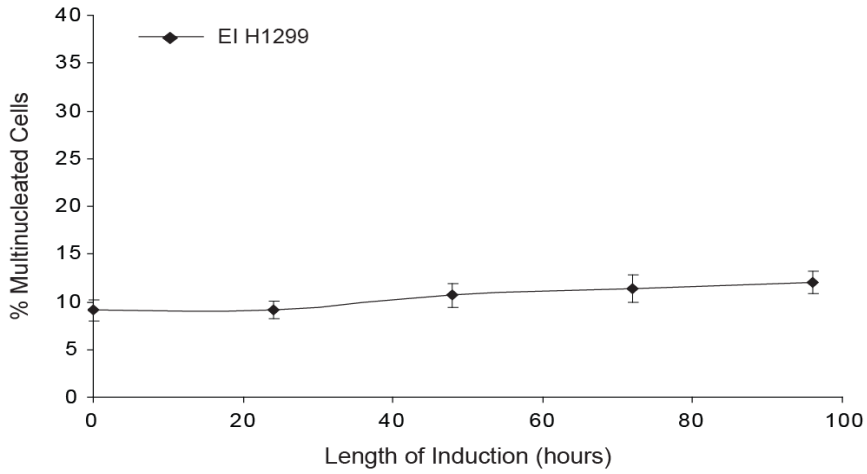


Figure S4 – EI H1299 cell do not respond to PonA

The base EI H1299 cells were plated at 10% confluency and treated with 2.5µg/mL PonA for 0, 24, 48, 72 or 96 hours. Cells were stained with DAPI (nuclear) stain and the percentage of multinucleated cells was counted at each timepoint.

Figure S5

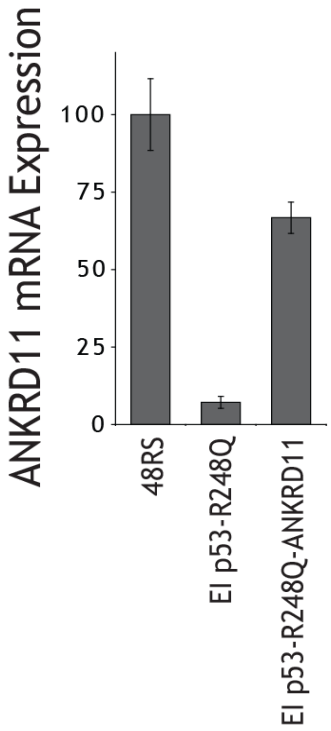


Figure S5 – Restored ANKRD11 expression levels in EI p53 mutant cell lines is comparable to ANKRD11 expression in primary breast epithelial cells.

The level of ANKRD11 mRNA expression in 48RS cells (a primary breast epithelial cell line), EI p53-mutant and EI p53-mutant-ANKRD11 cell lines was determined by real time RT PCR.

Figure S6

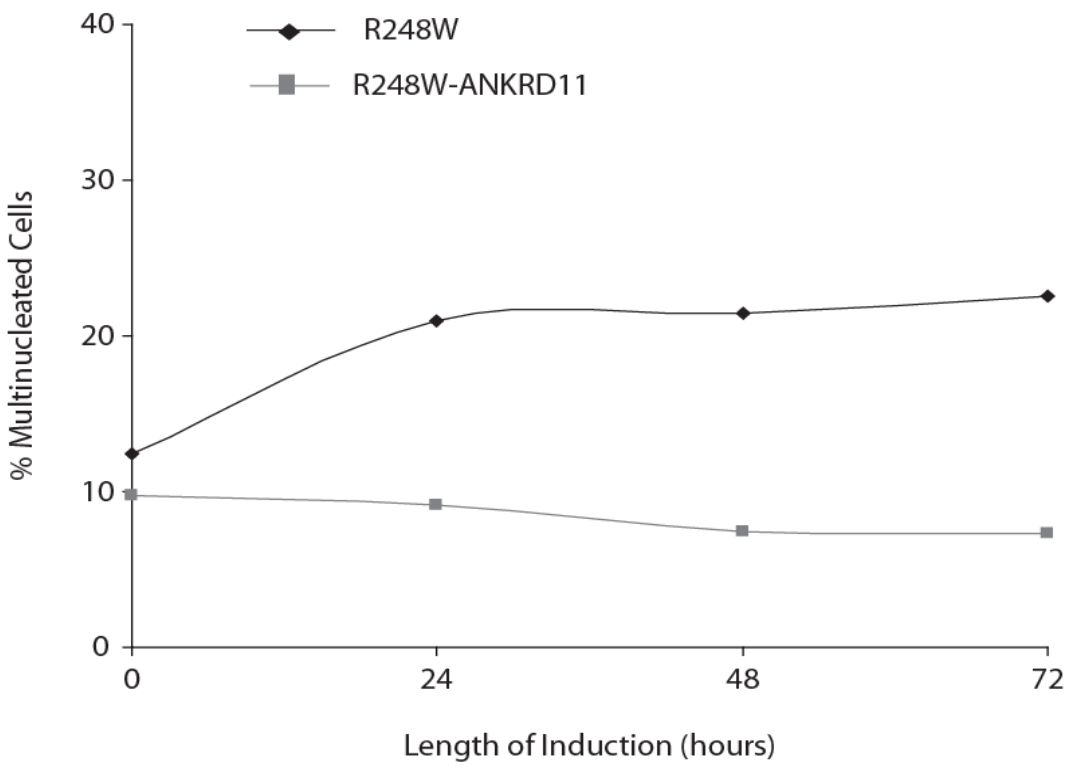


Figure S6 – ANKRD11 suppresses p53-R248W induced multinucleation.

EI p53-R248W and EI p53-R248W-ANKRD11 cells were plated at 10% confluency and treated with 2.5µg/mL PonA for 0, 24, 48 or 72 hours. Cells were stained with DAPI (nuclear) stain and the percentage of multinucleated cells were counted at each timepoint.

Figure S7

A

IPA 8.8

ID	Molecules in Network	Score	Top Functions	Focus Molec
1	↑**ABCD3**, AKAP, ↑**AKAP5**, ↑**AKAP12**, ↑**AKAP13**, Alpha catenin, ↑**ANGPTL4**, ↑**ANPEP**, ↑**CD63**, ↓**CD274**, Collagen type I, Collagen(s), ↑**DKK1**, FSH, hCG, Immunoglobulin, Integrin alpha 4 beta 1, ↑**ITGA3**, ↑**ITGB3**, ↑**LAMC2**, Lh, ↑**MAP2K3**, ↑**MT1X**, NFkB (complex), ↑**NR2F2**, ↑**PLK2**, ↓**PTX3**, ↑**SFRP1**, ↑**STC1**, ↑**TFPI2**, ↑**TGFBI**, Tnf, ↑**TNFSF4**, Vegf, ↓**WEE1**	48	Infection Mechanism, Cellular Movement, Cell-To-Cell Signaling and Interaction	23
2	↑**ABCC3**, ↑**ACAD11**, ↓**ATG12**, BCKDHA, CCL18, ↓**CDO1**, CREG1, ↑**DECR1**, ENPP2, ERCC1, MCAM, MMP9, MT1L, ↑**NAV2 (includes EG:89797)**, ↑**NEU1***, ↑**OPHN1**, ↑**PDE2A**, PDYN, phosphatidylethanolamine, ↑**PLK2**, RAC1, ↑**RALB**, retinoic acid, ↑**SEMA3C**, SERPINE2, ↑**SFRP1**, SLC27A1, SNCA, SNCB, ↑**TFPI2**, THBS2, TNF, TNFAIP2, ↑**UQCRFS1**	28	Gene Expression, Cellular Movement, Hematological System Development and Function	15
3	14-3-3, 26s Proteasome, ↑**AHCYL1**, ↑**ATXN1**, ↑**BCL2L1**, BCR, Cbp/p300, ↑**CCDC80**, ↓**CD22**, ↑**EHHADH**, ERK1/2, IFN Beta, Ige, IgG2a, IL12 (complex), ITPR, ↑**LAPTM5**, LDL, N-cor, NADPH oxidase, Nfat (family), NGF, ↑**OGG1**, P38 MAPK, PDGF BB, PLC gamma, ↑**PLEKHA2 (includes EG:59339)**, ↑**PPARG**, ↑**PRDM1**, ↑**RCN1**, ↑**SCD**, ↑**SERPINA1**, ↓**SOCS2**, ↑**SRR**, STAT5a/b	27	Cellular Function and Maintenance, Hematological System Development and Function, Humoral Immune Response	16
4	Akt, Alp, AMPK, Caspase, Ck2, ↑**CTH**, ↑**DDIT4**, ↑**EDNRA**, ERK, ↑**GLIPR1**, Gpcr, ↑**GPR17 (includes EG:2840)**, Histone h3, Histone h4, ↑**HIVEP3**, ↑**ID2**, IL1, Insulin, Integrin, Jnk, Mapk, ↑**P2RX4**, Pdgf, PI3K (complex), Pka, Pkc(s), PP2A, ↑**PPM1J**, ↑**RAC2**, Ras homolog, Rb, RNA polymerase II, ↓**TM4SF18**, ↑**TP53**, ↑**ZMIZ1**	22	Cell Cycle, Connective Tissue Development and Function, Hematological Disease	13
5	ADH5 (includes EG:128), ARPC1B, ↑**BHLHE41**, CCDC99, ↑**CDA**, CDC14B, ↑**CDH3**, cholecalciferol, DHCR24, dihydrotestosterone, DRAM1, ERBB2, ↑**FCHO2**, HDC, ↑**INO80C**, KLK2, ↑**LRIG1**, MIR124, MIR124-1 (includes EG:406907), PMEPA1, PODXL, PPP1R13L, progesterone, ↑**RDH5**, ↑**RIN2**, SP1, SULT1E1, ↑**TCEAL1**, ↑**TGFBI**, THBD, TMSB4X, TRIM29, UHRF1, ↑**WBP5**, ↓**ZDHHC3**	21	Reproductive System Development and Function, Cell Cycle, Tissue Development	12
6	AK1, ANXA8, CCNG2, CDC7, CDKN2A, CLIC4, ↑**CPA4**, CTCF, ↓**DLX2**, ERCC3, ↑**FAM125B**, ↑**FSTL3**, GTF2I, ↓**HIST2H2BF**, KRT8, ↓**LMO7**, ↑**MAN2B2 (includes EG:23324)**, MDM4, MORC3, NEDD4L, PMEPA1, RAD51AP1, RFC4, RPS7, S100A6, ↓**SEMA6A**, ↑**SETX**, SMAD2, STRAP, SUZ12, TGFB1, ↑**TRIM52**, ↓**TUBB2C**, YWHAZ, ZHX2	19	Cell Cycle, Respiratory System Development and Function, DNA Replication, Recombination, and Repair	11
7	↑**ACO1**, ASS1, BAI1, BAI3, BDKRB2, ↑**C10ORF125**, CALCR, CXCR1, FFAR3, FZD6, FZD7, GART, Gpcr, GPI, GPR87, ↑**GPR116**, ↑**GPR17 (includes EG:2840)**, IFI202B, IFNG, ↑**KLHL5**, ↑**KRT6A**, L-triiodothyronine, LY6A, MLH1, MYC, PEG3, ↑**PSD3***, RPL23, ↑**STX11**, TAF5L, TAF6L, ↑**TCEA2**, TNFSF9, TRAF2, ↑**VIPR2**	16	Cell Cycle, Cellular Growth and Proliferation, Tissue Morphology	10

B

Infection Mechanism, Cellular Movement, Cell-To-Cell Signaling and Interaction

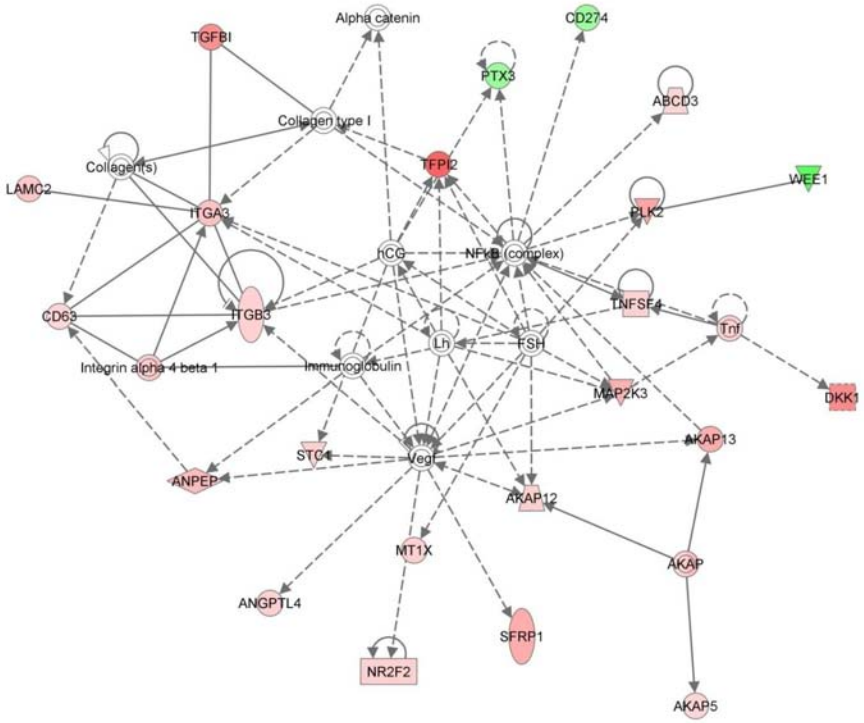


Figure S7 – Induction of p53-R175H drives genes that converge upon cell movement and cell-cell signalling networks.

A: EI p53-R175H cells were cultured in the presence or absence of PonA (2.5 µg/mL) for 24 hours, with expression profiling performed using Affymetrix Human Gene 1.0 ST arrays. Duplicate independent experiments were performed. Genes specifically regulated by p53 R175H (Table 1) were subjected to a pathway analysis (Ingenuity Systems), with the most significantly enriched networks listed.

B: Schematic diagram illustrating the regulation of genes by R175H that are involved in the network 'Infection Mechanism, Cellular Movement, Cell-To-Cell Signaling and Interaction'.

Figure S8

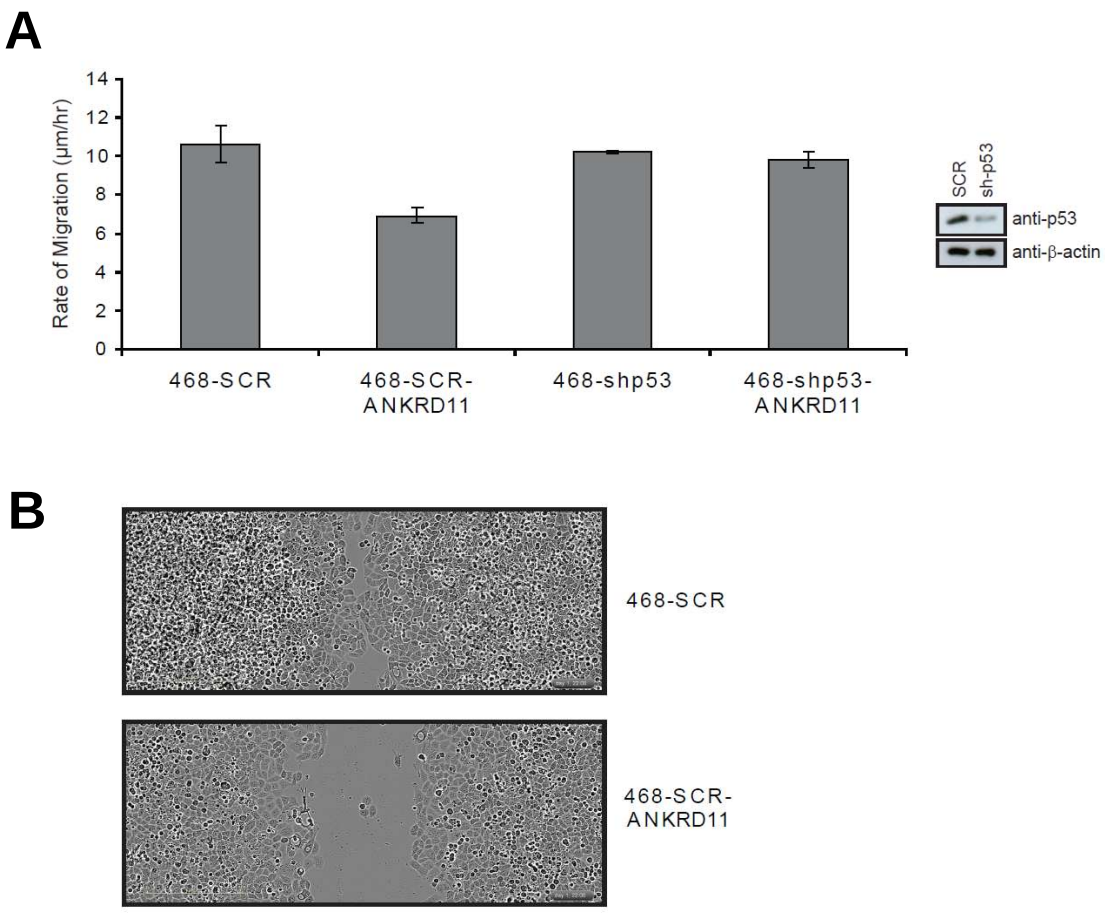


Figure S8 – ANKRD11 suppresses migration of MDA-MB-468 cells in a mutant p53-dependent manner

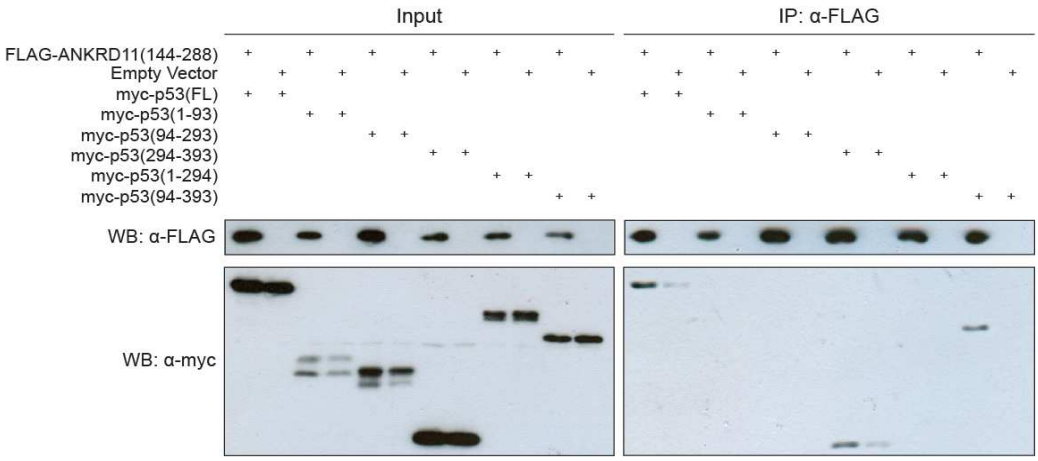
MDA-MB-468 cells were integrated with stable ANKRD11 expression (or vector control) in the presence or absence of a short-hairpin RNA to silence endogenous mutant p53 (R273H) expression. Ability of cells to migrate was determined by a scratch-wound assay in real-time using Incucyte (Essen).

A: The average rate of migration for each cell line was determined over 75 hours.

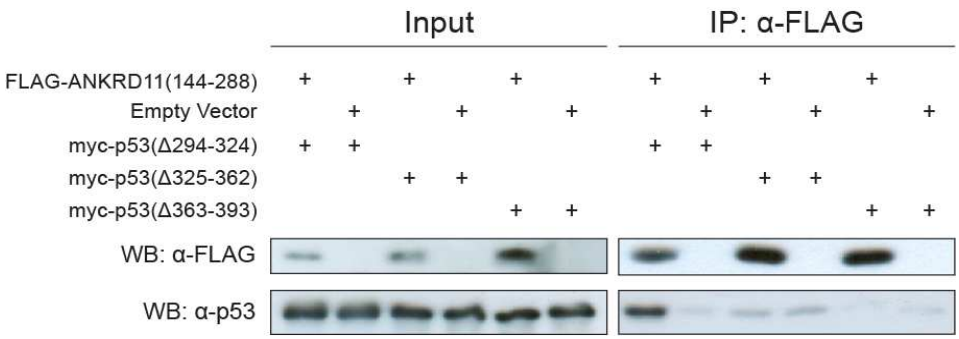
B: Representative screen shots taken from Incucyte show MDA-MB-468-SCR cells in the presence or absence of stable ANKRD11 expression at 46 hours, show an increased rate of wound closure in the absence of ANKRD11.

Figure S9

A



B



C

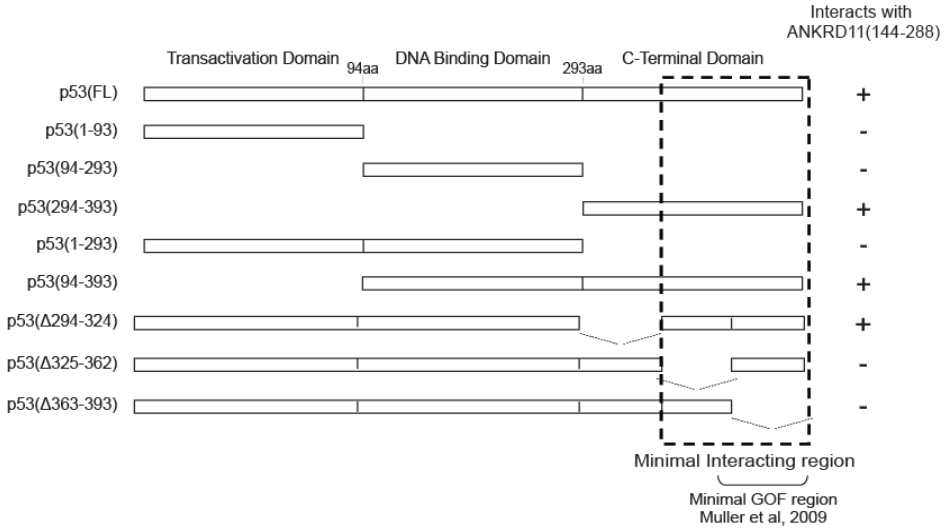


Figure S9 – The ankyrin domain of ANKRD11 interacts with the C-terminus of p53

A: Cellular extracts from H1299 cells co-transfected with FLAG-ANKRD11$^{144\text{-}288aa}$ (4 µg) and various Myc-tagged regions of p53 (0.5 µg) were immunoprecipitated with an α-FLAG antibody, followed by detection of inputs and immunoprecipitates by western blot analysis, using α-FLAG and α-Myc antibodies.

B: Cells were treated as above (A), with co-transfection of FLAG-ANKRD11$^{144\text{-}288aa}$ and Myc-tagged p53 deletion constructs followed by western blot analysis using α-FLAG and α-p53 antibodies. Some non-specific, background binding of p53 to the beads is observed, however interacting regions are seen above the level of background binding.

C: A schematic representation of regions of p53 shown to interact with ANKRD11$^{144\text{-}288aa}$ in immunoprecipitation studies. The region required for invasive function of mutant p53 is illustrated (Muller et al 2009).

Figure S10

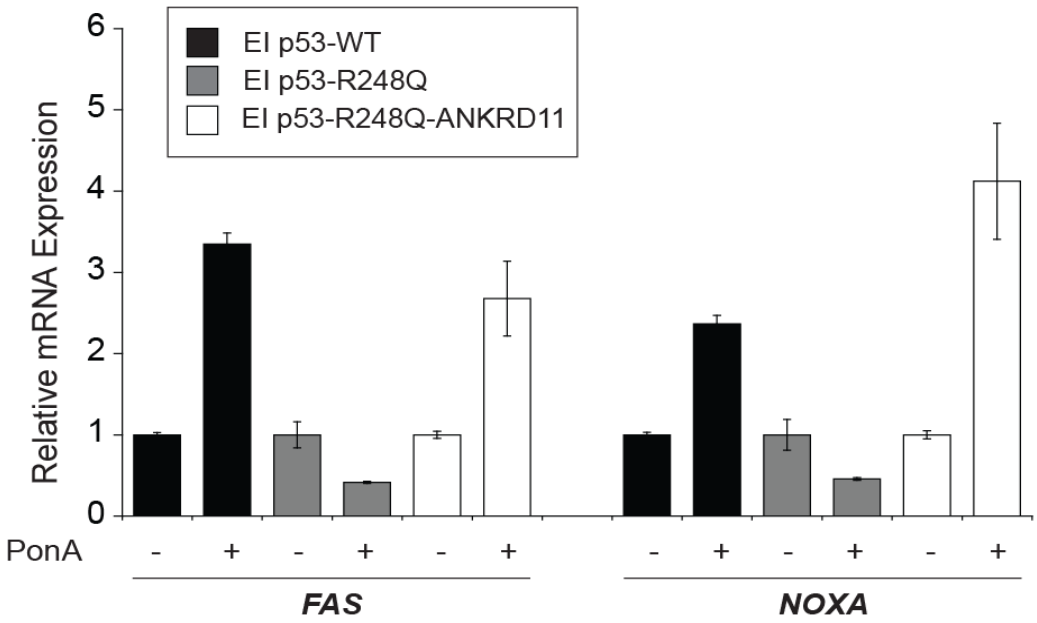


Figure S10 – ANKRD11 restores transactivation potential to a p53 mutant

EI p53-WT, EI p53-R248Q and EI p53-R248Q-ANKRD11 cells were treated in the presence or absence of PonA (2.5 µg/mL) for 24 hours. The expression of specific target genes was subsequently determined by qRT-PCR.

Figure S11

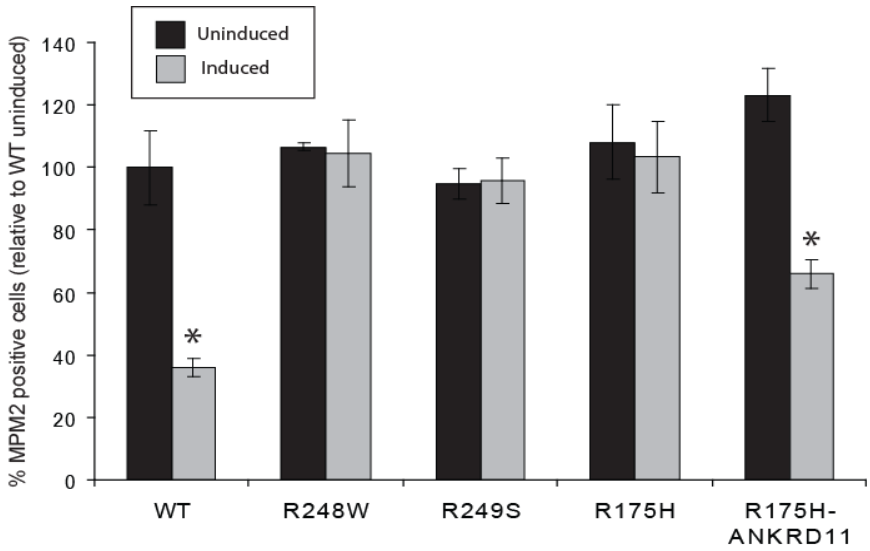


Figure S11 – ANKRD11 restores wild-type function to a p53 mutant

EI p53-WT, EI p53-R248W, EI p53-R249S, EI p53-R175H and EI p53-R175H-ANKRD11 cells were treated in the presence or absence of PonA (2.5 µg/mL) for 72 hours. The percentage of cells undergoing mitosis was determined by MPM2 staining and is graphed as percentage of MPM2 positive cells relative to the uninduced EI p53-WT cell line (* p<0.01; n=3).

Figure S12

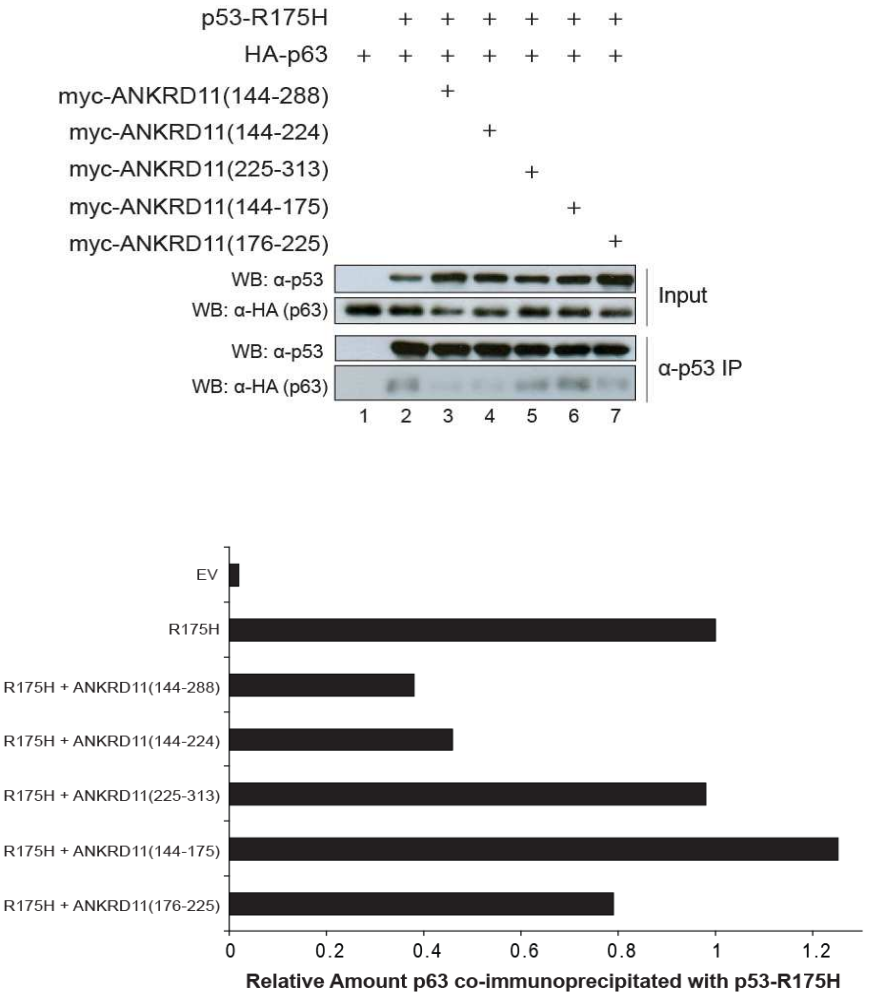


Figure S12 - The ankyrin repeat domain of ANKRD11 dissociates the p53-R175H•p63/p73 complexes.

Cellular extracts from H1299 cells sequentially co-transfected with p53-R175H (2 µg), HA-p63 (4 µg) and various Myc-ANKRD11 fragment expression constructs (2 µg) as indicated, were immunoprecipitated with an α-p53 antibody. Inputs and immunoprecipitates were subjected to western blot analysis with α-p53, α-Myc and α-HA antibodies. The amount of p63 co-precipitated with R175H was determined by densitometry.

Supplementary Table I

Number	Primer Sequence (5’ – 3’)
1	ACCATGTCTACAGTGTGTCAGAAGGGAAC
2	TTAGCCTTTGCCTAACAGGAGGTTCAC
3	TTATGCAGCCAGCAGCTGCTTCGCGACGT
4	TGCGGAGGTGAACACCAAGGGCCTA
5	TTAATCGATACTGGAAGGTGCGAAGGATGGTG
6	ATCTACAGTGTGTCAGAAGGGAAC
7	TTATCGGTGCAGGCGGGTCTCTCCACGCT
8	AGCCGCCATCCGCGGGGACGCCCGGCGCAT
9	ATGGAGGAGCCGCAGTCAGATCCTAG
10	TTACAGGGGCCAGGAGGGGGCTGGTGCA
11	TCATCTTCTGTCCCTTCCCAGA
12	TTACCCTTTCTTGCGGAGATTCTCTTC
13	AGAGCCTCACCACGAGCTGCCCCCA
14	TCAGTCTGAGTCAGGCCCTTCTGTCTTG
15	AGAATCTCCGCAAGAAAGGGGGAGAATATTTCACCCTTCA
16	TGAAGGGTGAAATATTCTCCCCCTTTCTTGCGGAGATTCT
17	CAAAGAAGAAACCACTGGATCGTGCGCACTCCAGCCACCT
18	AGGTGGCTGGAGTGCGCACGATCCAGTGGTTTCTTCTTTG
19	TCAGCTCCCCCCTGGCTCCTTCCCAGCCT

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CHAPTER 6

Mutant p53 drives invasion in breast tumours through a pathway involving miR-155 and ZNF652

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Text in manuscript

CHAPTER 6 - Mutant p53 drives invasion in breast tumours through a pathway involving miR-155 and ZNF652

6.1 PREFACE

The work presented in chapters 3-5 of this thesis has utilised the inducible p53 system to investigate mutant p53 function. This has culminated in a focus on identifying mechanisms through which mutant p53 gain-of-function can occur in cancer cells and mechanisms to suppress these functions. This chapter describes the investigation of yet another mechanism through which mutant p53 functions to enhance EMT and invasion in cancer cells. The data presented here is novel in that it presents evidence linking mutant p53 to the expression of a miRNA involved in breast tumour metastasis.

This chapter is presented as a text in manuscript, which is currently in the final stages of preparation for submission. Prior to submission, it will be necessary to further characterise the mechanism through which mutant p53 up-regulates miR-155 expression, via inhibition of p63-mediated repression (see Figure 4). Furthermore, miR-155 expression levels must be examined *in vivo*, similarly to that described for ZNF652 (see Figure 8).

ABSTRACT

Loss of p53 function is a critical event during tumorigenesis, with half of all cancers harbouring mutations within the *TP53* gene. Such events frequently result in the expression of a mutated p53 protein with gain-of-function properties that drive invasion and metastasis. Here, we show that the expression of miR-155 is up-regulated by mutant p53 to drive invasion. The miR-155 host gene was directly repressed by p63, providing the molecular basis for mutant p53 to drive miR-155 expression. Significant overlap was observed between miR-155 targets and the molecular profile of mutant p53-expressing breast tumours *in vivo*. A search for cancer-related target genes of miR-155 revealed ZNF652, a novel zinc finger transcriptional repressor. ZNF652 directly repressed several genes involved in invasion and metastasis, and silencing of ZNF652 in epithelial cancer cell lines promoted invasion into matrigel. Importantly, loss of ZNF652 expression in primary breast tumours was significantly correlated with increased local invasion and defined a population of breast cancer patients with a poor outcome. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53-expressing tumours.

MANUSCRIPT

Disruption of the function of p53 is arguably a universal requirement for the development of cancer. The p53 tumour suppressor is a DNA binding transcription factor which plays a pivotal role in driving proliferation, apoptosis and senescence. Approximately 20% of breast cancers possess somatic mutations of *TP53* and such tumours are typically aggressive and associated with a poor patient prognosis (Miller et al 2005, Olivier et al 2006). These mutations of p53 are frequently missense mutations and commonly occur within the DNA binding domain, resulting in abrogation of normal p53 function, consistent with a role of p53 as a tumour suppressor. In addition to the loss of normal p53 function as a transcription factor, these cancer-associated p53 mutants acquire a gain-of-function (Oren and Rotter 2010). Subtle changes to the p53 protein allow for the acquisition of new properties that are currently an area of intense research interest.

Mutant p53 has been shown to acquire the ability to sequester p63 and p73, and this is thought to promote tumour invasion and metastasis (Adorno et al 2009, Muller et al 2009). It is of interest to determine the key downstream target genes of p63 and p73 that are deregulated to promote tumorigenesis in cancer cells expressing mutant p53. Recent studies have identified the metastasis suppressors *Sharp2* and *Cyclin G2* as critical downstream targets of mutant p53 that are deregulated through loss of p63 signalling (Adorno et al 2009). The expression of these two genes alone defined a subset of particularly aggressive breast tumours associated with poor patient outcome (Adorno et al 2009). Such examples demonstrate the utility of downstream mutant p53 targets as robust clinical prognostic tools and ideal therapeutic targets in

mutant p53-expressing tumours. The mechanisms and pathways exploited by mutant p53 to drive tumorigenesis however remain poorly understood. For example, the role of mutant p53 in the regulation of microRNAs (miRNAs) is yet to be explored. This study reports that mutant p53 can regulate the expression of miR-155 and its downstream targets to promote invasion and metastasis.

MiRNAs are small endogenous RNA molecules that specifically target mRNAs to reduce their levels (Guo et al). Mir-155 is considered an oncomiR in breast cancer due to its up-regulated expression (Iorio et al 2005) which is associated with the promotion of tumourigenesis (Jiang et al). Mutant p53 (Adorno et al 2009) and miR-155 expression (Kong et al 2008) are associated with invasive properties of breast tumours *in vivo*, which prompted us to investigate if they both promote malignant transformation through a similar oncogenic axis. We initially determined if miR-155 can phenocopy mutant p53 in its ability to drive invasion and mensenchymal transformation in H1299 cells (Noll et al 2011). Expression of miR-155 in H1299 cells significantly enhanced their invasive capabilities and drove a mesenchymal phenotype with close resemblance to that observed upon mutant p53 expression (Figure 1). These observations indicate that miR-155 and mutant p53 share the common ability to drive invasion and EMT in the same genetic background.

FIGURE 1

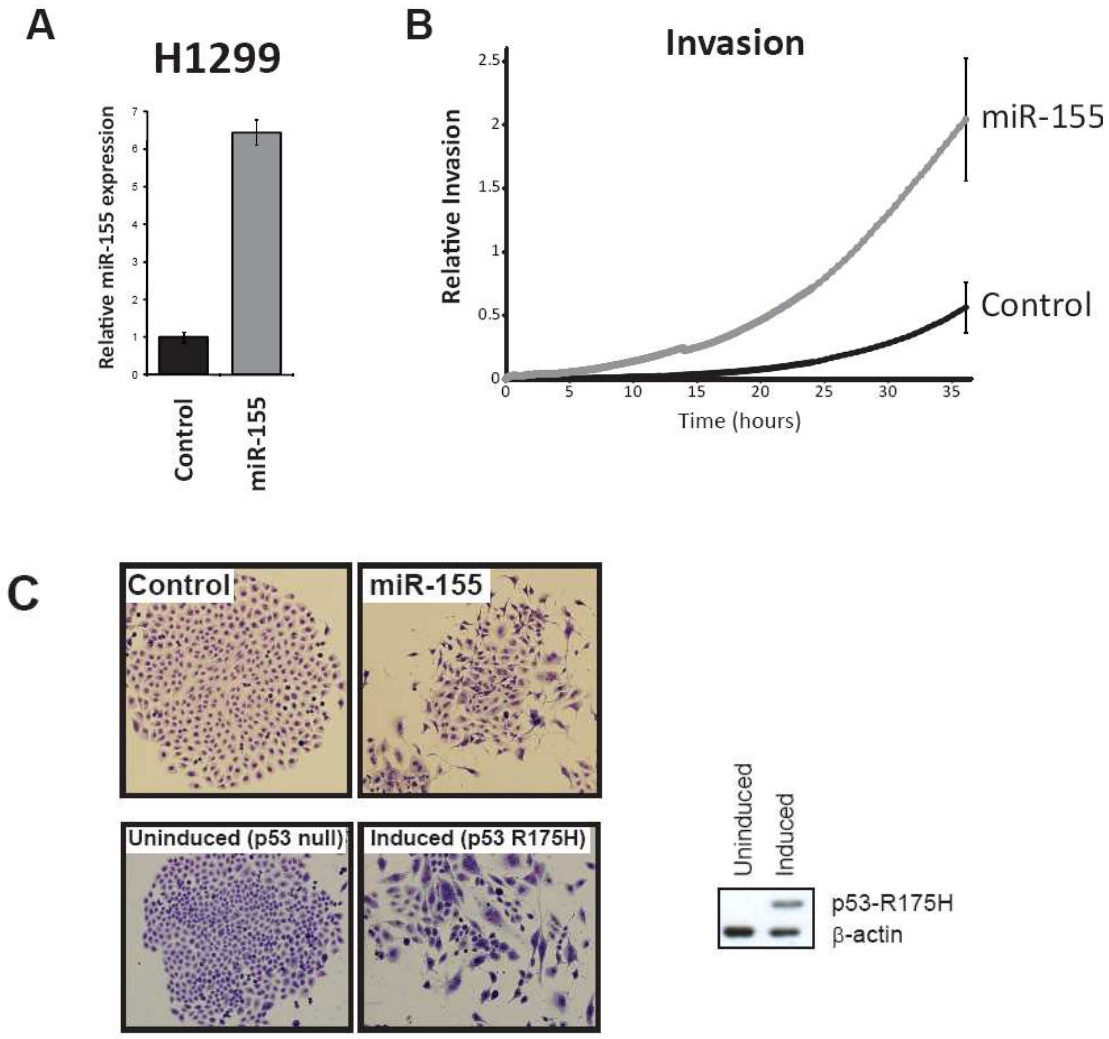


Figure 1. miR-155 expression phenocopies mutant p53 expression in H1299 cells.

A. The relative expression of miR-155 was determined in control and miR-155 expressing H1299 epithelial cells.

B. H1299 cells (control or miR-155) were plated in the upper chamber of a CIM-16 plate coated with 5% matrigel. Real-time invasion was measured in on an Xcelligence RTCA DP analyser.

C. H1299 cells (as indicated) were seeded at single cell density and colonies were fixed and stained after 10 days. Pictures show representative colonies. Western blot shows expression of p53-R175H specific to the induced cell line.

To investigate the potential role of miR-155 in invasion and mesenchymal transformation, we initially screened a panel of established breast cancer cell lines of epithelial (luminal) and mesenchymal (basal B) origin (Blick et al 2008) for the expression of miR-155. Indeed, miR-155 expression was almost absent in epithelial lines, whilst the majority (4 out of 5) of mesenchymal lines showed high expression (Figure 2A), suggesting an up-regulation of miR-155 during the EMT process. To gain further insight into the ability of miR-155 to drive invasion in breast tumours, miR-155 was stably expressed in the ZR-75-1 epithelial breast cancer cell line. ZR-75-1 cells represent an ideal model for a primary breast epithelial tumour due to their slow proliferative rates, non-invasive capacity, poor clonogenic properties and corresponding low levels of miR-155. Therefore, a polyclonal population of ZR-75-1 cells stably expressing miR-155 was generated. The expression of miR-155 dramatically enhanced the ability of ZR-75-1 cells to migrate (Figure 2B) and invade through matrigel (Figure 2C) and induced an amoeboid transformation associated with enlarged filamentous cytoplasm with frequent protrusion of lamellipodia (Figure 2D). Lamellipodia are usually found on highly mobile and invasive cells (Laura 2008), properties not frequently associated with the epithelial ZR-75-1 cell line. The amoeboid and mesenchymal transitions of epithelial tumour cells represent two distinct modes of migration and invasion (Sahai 2005), with our findings suggesting that miR-155 able to drive both phenotypes in a cell-line specific manner.

FIGURE 2

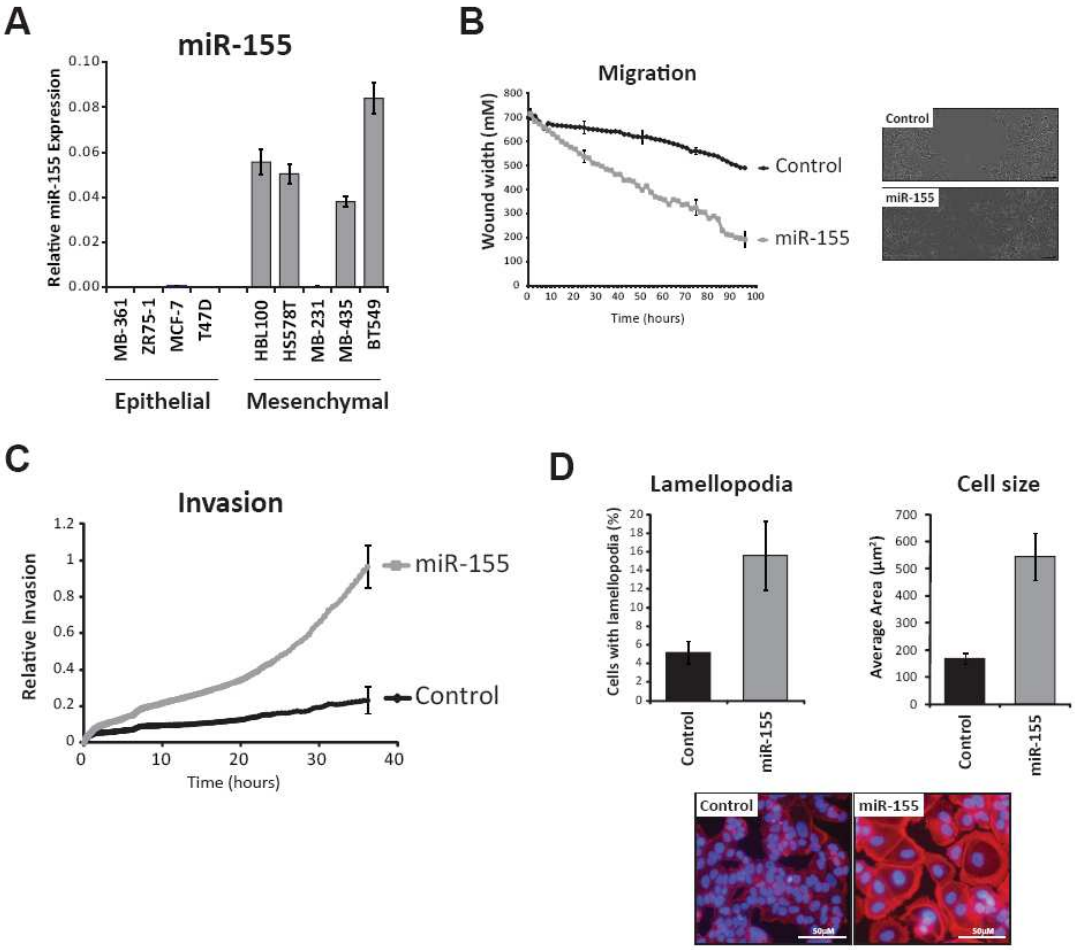


Figure 2. miR-155 expression drives an invasive phenotype in ZR-75-1 cells

A. The relative expression levels of miR-155 were determined in a panel of epithelial and mesenchymal breast cancer cell lines (as indicated).

B. The ability of ZR-75-1 cells (control or miR-155) to migrate was determined by a scratch-wound assay using Incucyte (Essen). Phase images were taken every 15 minutes and wound width (µm) was calculated in real-time using specific Incucyte software.

C. ZR-75-1 cells (control or miR-155) were plated in the upper chamber of a CIM-16 plate coated with 5% matrigel. Real-time invasion was measured in on an Xcelligence RTCA DP analyser.

D. ZR-75-1 cells (control or miR-155) were seeded at 10% confluence and grown for 12 days. Cells were stained for actin using a phalloidin antibody and lamellopodia and cell size were scored as described in Materials and Methods.

To examine the altered transcriptional events associated with this cellular transformation, EMT expression profiling was performed on ZR-75-1 cells expressing miR-155 through expression analysis of 84 EMT-related genes by real-time PCR. Indeed, expression of miR-155 in ZR-75-1 cells was associated with a significant up-regulation of numerous mesenchymal markers (Figure 3A). Interestingly, much of the transcriptional reprogramming by miR-155 converges upon the TGF-β signalling pathway, which is consistent with a previous study whereby miR-155 accelerated TGF-β mediated EMT of the NMuMG mouse mammary epithelial cell line (Kong et al 2008). TGF-β has a complex role throughout tumour development, functioning as a tumour suppressor during the initial events of oncogenesis whilst driving invasion and metastasis in advanced malignancies (Leivonen and Kähäri 2007). This prompted us to investigate the effects of TGF-β treatment on ZR-75-1 cells. Indeed, ZR-75-1 cells exposed to TGF-β were highly invasive and underwent similar morphological changes to cells expressing miR-155, involving enlarged cytoplasm and increased frequency of lamellipoidia (Figure 3B). These data indicate that miR-155 functions through the TGF-β pathway to enhance cellular invasion and promote an EMT.

FIGURE 3

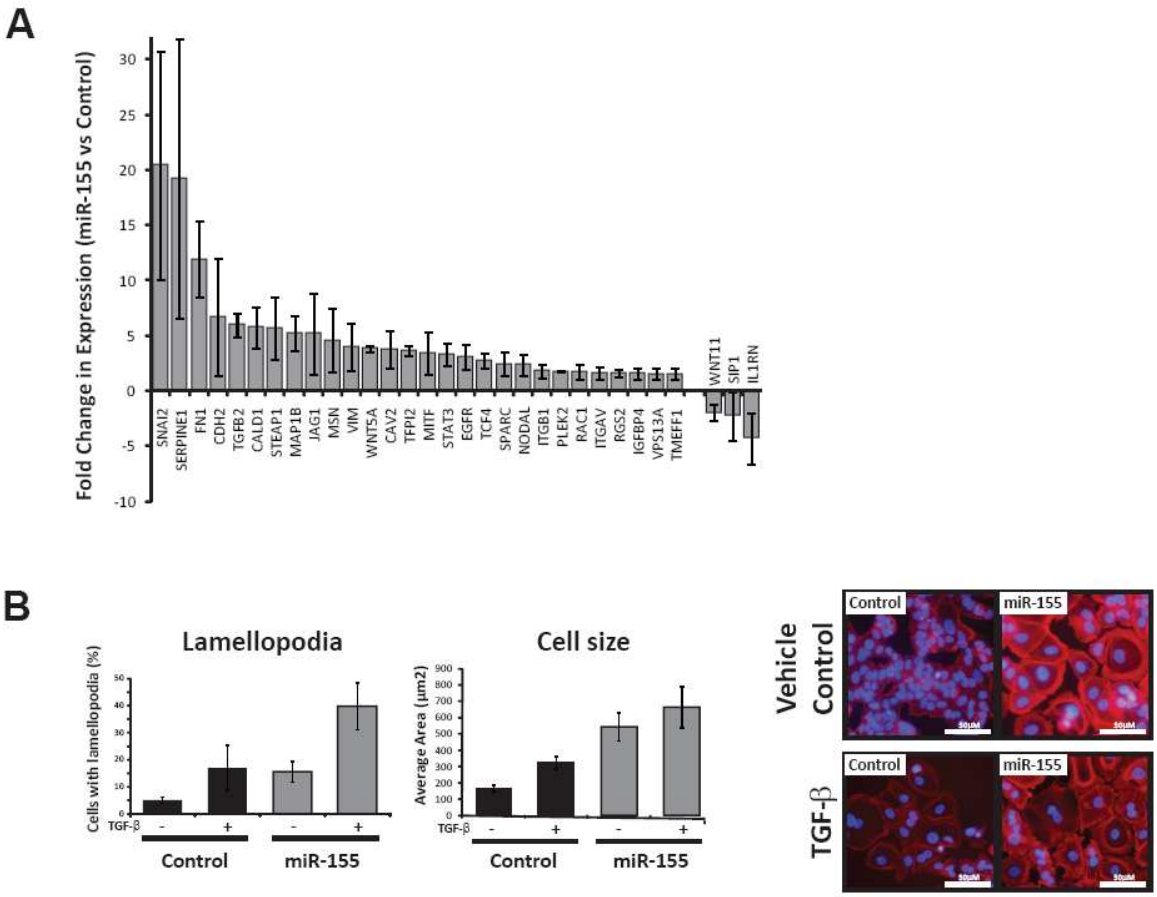


Figure 3. miR-155 regulates EMT related genes in the TGF-β pathway

A. The expression of EMT related genes in ZR-75-1 miR-155 expressing cells were determined using a low density PCR array. Data is presented as a fold change in expression relative to a control cell line.

B. ZR-75-1 cells (control or miR-155) were seeded at 10% confluence and grown for 12 days in the presence of 2 ng/mL TGF-β (or vehicle control). Cells were stained for actin using a phalloidin antibody and lamellopodia and cell size were scored as described in Materials and Methods.

Mutant p53 drives invasion and metastasis through aberrant control of gene transcription by interaction with and inactivation of transcription factors such as p63 (Adorno et al 2009, Muller et al 2009). Therefore, we examined a role for p53 and p63 in the transcriptional control of *MIR155HG* (miR-155 host gene), the precursor transcript for miR-155. Previous observations have shown that miR-155 is repressed by wild-type p53 through an unknown mechanism (Brosh et al 2008). Our studies confirm this, as MIR155HG expression is repressed following induction of wild-type p53 in H1299 cells (Figure 4A). It was of interest to note that induction of a common p53 cancer mutant, R248Q, resulted in up-regulation of MIR155HG expression (Figure 4A). The ability of mutant p53 to up-regulate miR-155 expression was confirmed in the mesenchymal cell line BT549, expressing the 'hotspot' mutant, R249S. Knockdown of this endogenous p53 mutant in BT549 cells resulted in a significantly reduced level of miR-155 (Figure 4B), implying that this microRNA is indeed a downstream target of mutant p53. Furthermore, it was demonstrated that silencing of endogenous p63 expression results in up-regulation of *MIR155HG* in MCF10A, a non-malignant breast epithelial cell line that expresses wild-type p53 (Figure 4C) indicating that p63 constitutively represses miR-155 expression. In addition, our studies provide mechanistic insight into the repression of miR-155 by wild-type p53 and p63, as chromatin immunoprecipitation (ChIP) experiments demonstrate that induced wild-type p53 in H1299 cells and endogenous p63 in MCF10A are directly recruited to a consensus p53 response element (RE) in the 3rd exon of *MIR155HG* in MCF10A (Figure 4D). These findings suggest a redundancy of function between p53 and p63 in the repression of miR-155. We speculate that mutant p53 drives miR-155 expression through both a loss of wild-type p53-mediated repression and inactivation of p63 function.

FIGURE 4

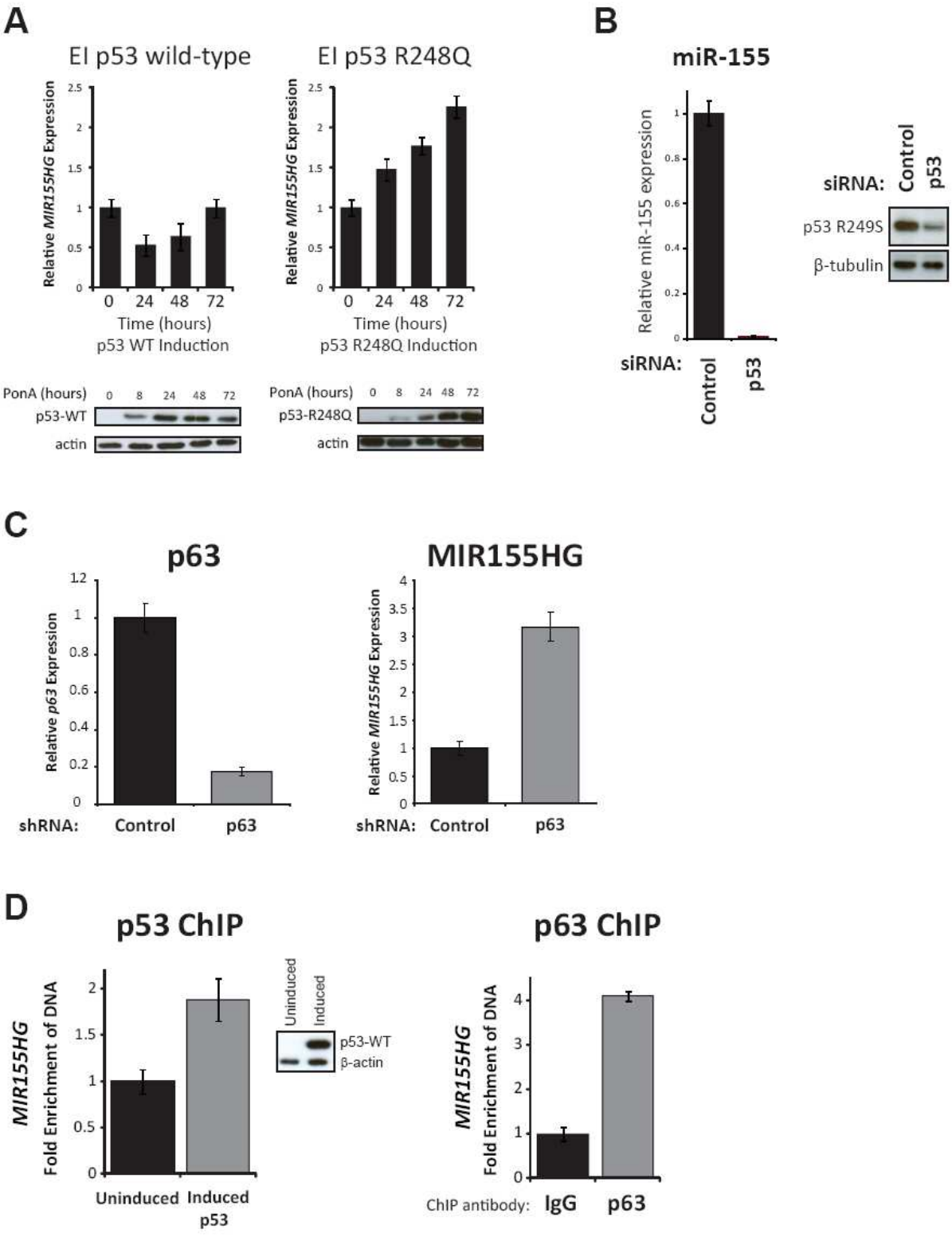

A
EI p53 wild-type
EI p53 R248Q
Relative MIR155HG Expression
Time (hours)
p53 WT Induction
p53 R248Q Induction
PonA (hours) 0 8 24 48 72
p53-WT
p53-R248Q
actin
B
miR-155
Relative miR-155 expression
siRNA: Control p53
p53 R249S
β-tubulin
C
p63
MIR155HG
Relative p63 Expression
Relative MIR155HG Expression
shRNA: Control p63
D
p53 ChIP
p63 ChIP
MIR155HG
Fold Enrichment of DNA
Uninduced Induced
p53
p53-WT
β-actin
ChIP antibody: IgG p63

Figure 4. miR-155 expression is regulated by p53 and p63

A. EI p53-WT or EI p53-R248Q cells were treated with 2.5 µg/mL PonA for 0, 8, 24, 48 or 72 hours to induce p53 protein expression. Relative *MIR155HG* expression levels were determined by specific RT-PCR. Induction of p53 protein was determined by western blot, actin is a loading control.

B. Endogenous mutant p53 (R249S) expression was silenced by transient transfection of a specific siRNA targeted to p53 (see Materials and Methods). Relative miR-155 expression was determined by an ABI Taqman miRNA assay.

C. Endogenous p63 expression was silenced in MCF10A cells by stable, lentiviral-mediated integration of a specific shRNA (see Materials and Methods). Relative expression of p63 and MIR155HG was determined by specific RT-PCR.

D. Fold enrichment of induced wild-type p53 (relative to uninduced) or endogenous p63 in MCF10A (relative to IgG control) binding to a p53-RE within the MIR155HG was determined by ChIP analysis.

Having established miR-155 as a downstream oncomiR of mutant p53, we sought to identify the specific targets of the miR-155/mutant p53 axis that drive invasion in breast tumours. To obtain a global understanding of miR-155 targets, a comprehensive list of *bone fide* miR-155 targets was collated from the literature (data not shown). Selection criteria for these published miR-155 targets included (i) regulation of the endogenous transcript by miR-155 and (ii) validation of the miR-155 seed sequence through a reporter assay. This list contains several known tumour suppressors, and we propose that mutant p53 may be indirectly down-regulating the expression of these genes through aberrant up-regulation of miR-155. Thus, an *in vivo* relationship between the defined miR-155 targets and mutant p53 in breast cancer was examined through analysis of the publically available transcript profiles of 251 p53-sequenced primary breast tumours (Miller et al 2005). There was a remarkably strong correlation between the expression of these defined miR-155 targets and p53 status, with 42 of the 140 miR-155 targets (30%) showing a significantly lower expression in breast cancers with mutant p53 compared with those cancers with wild type p53 (Figure 5A; Table 1). These 42 genes define those most likely to play critical roles in the suppression of breast cancer invasion and metastasis through the mutant p53•miR-155 axis. Hence, we evaluated the potential of the expression of these 42 genes to predict breast cancer metastasis. Analysis of a publically available dataset of expression profiling in 78 breast tumours (Minn et al 2005) shows that low expression of ZNF652, PDCD4, TCF12 or IL17RB translated into poor metastasis-free survival (Figure 5B). We speculated that these four miR-155•mutant p53 targets collectively drive pathways that prevent invasion and metastasis. Consistent with this hypothesis, PDCD4 and IL17RB have established roles in the suppression of breast oncogenesis. PDCD4 has been previously shown to inhibit motility and invasion of breast cancer

cell lines *in vitro* (Nieves-Alicea et al 2009, Santhanam et al) and is down-regulated in invasive breast carcinomas (Ito et al 2006, Wen et al 2007). Likewise, loss of IL17RB expression is a strong biomarker for clinical outcome in breast cancer patients receiving tamoxifen monotherapy (Ma et al 2004). However, the role of ZNF652 and TCF12 as suppressors of invasion and metastasis is unclear.

Table 1. Targets of the mir-155 • mutant p53 axis

Accession Number	Gene Name	*p* value
NM_001083919.1	C10orf26	0.000000000002
NM_014456.3	PCD4	0.00000013
NM_005903	SMAD5	0.0000011
NM_024077.3	SECISBP2	0.0000022
NM_001042646.1	TRAK1	0.0000026
NM_015335.4	MED13L	0.0000033
NM_000685	AGTR1	0.0000060
NM_024632.5	SAP30L	0.000013
NM_016210.4	C3orf18	0.000017
NM_015084.2	MRPS27	0.000021
NM_033285	TP53INP1	0.000026
NM_019087.2	ARL15	0.000031
NM_001025105.1	CSNK1A1	0.000042
NM_015093	LNX2	0.00055
NM_001145365.1	ZNF652	0.00084
NM_001161661.1	WWC1	0.00095
NM_014904.2	RAB11FIP2	0.001
NM_001482.2	GATM	0.0012
NM_012257.3	HBP1	0.0014
NM_015041.1	CLUAP1	0.0017
NM_020314.4	C16orf62	0.0017
NM_207036.1	TCF12	0.0018
NM_199320.2	PHF17	0.0026
NM_005900	SMAD1	0.0029
NM_014247.2	RAPGEF2	0.0036
NM_015990.4	KLHL5	0.0036
NM_025134.4	CHD9	0.0041
NM_007199	IRAK3	0.0063
NM_183075.2	CYP2U1	0.0074
NM_001668	ARNT	0.0076
NM_021946.4	BCORL1	0.0076
NM_001105549.1	ZNF83	0.0093
NM_002734.3	PRKAR1A	0.012
NM_018725.3	IL17RB	0.016
NM_002009	FGF7	0.017
NM_014647.3	KIAA0430	0.024
NM_012141.2	INTS6	0.036
NM_001099412.1	MYST3	0.044
NM_014431.2	KIAA1274	0.044
NM_153686.7	LCORL	0.046
NM_203487.2	PCDH9	0.048
NM_022474.2	MPP5	0.049

FIGURE 5

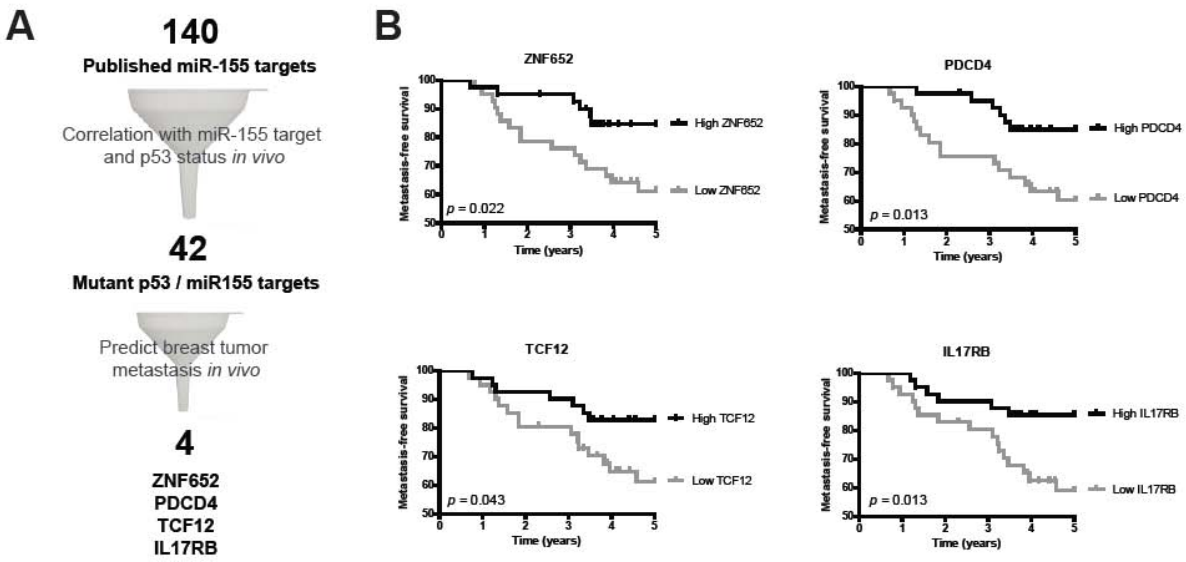


Figure 5. Four genes commonly regulated by the mutant p53/miR-155 axis are associated with poor metastasis-free survival

A. Schematic representing the filtering of 140 miR-155 target genes to 42 genes correlated with mutant p53 status *in vivo* (Miller et al 2005) (see Table 1) and 4 genes that predict metastasis *in vivo*, as determined from a publically available dataset of 78 breast tumours (Minn et al 2005).

B. Kaplan-Meier plots derived from publically available survival data associated with a cohort of 78 breast tumours with metastasis-free survival data (Minn et al 2005). Expression of ZNF652, PCDC4, TCF12 and IL7RB was sourced from expression microarray analyses. Tumours were ranked for gene expression, with those tumours below the median expression level of the cohort defined as 'Low Expression' and those tumours above the median expression level of the cohort defined as 'High Expression'.

ZNF652 was considered an excellent candidate as this gene was originally identified in a breast expression library (Kumar et al 2006) and encodes a classical zinc-finger DNA binding transcription factor that functions as a transcriptional repressor (Kumar et al 2008), potentially regulating downstream pathways of the miR-155•mutant p53 axis. Initially we demonstrated that ZNF652 protein (Figure 6A) and transcript (Figure 6B) levels are highly expressed in breast cancer epithelial cell lines, compared to mesenchymal cell lines. The robust negative correlation between miR-155 (Figure 2A) and ZNF652 levels in these cell lines is indicative of ZNF652 as a direct target of miR-155. Indeed, over-expression of miR-155 in ZR-75-1 markedly reduced endogenous ZNF652 protein levels (Figure 6C). ZNF652 expression was further investigated using gene expression microarray data of 51 breast cancer cell lines (Neve et al 2006) classified into luminal (epithelial) and basal B (mesenchymal) (Blick et al 2008). The luminal (epithelial) cell lines have a significantly higher expression of ZNF652 compared with the basal B (mesenchymal) cell lines ($p<0.0001$). Furthermore, the expression of ZNF652 is negatively correlated with vimentin expression, a well-known marker of mesenchymal cells ($r^2=0.33$, $p<0.0001$) (Figure 6D). This data defines ZNF652 as a novel marker for breast epithelial cells and indicate that ZNF652 expression is down-regulated during mesenchymal transformation, presumably in response to up-regulation of miR-155.

FIGURE 6

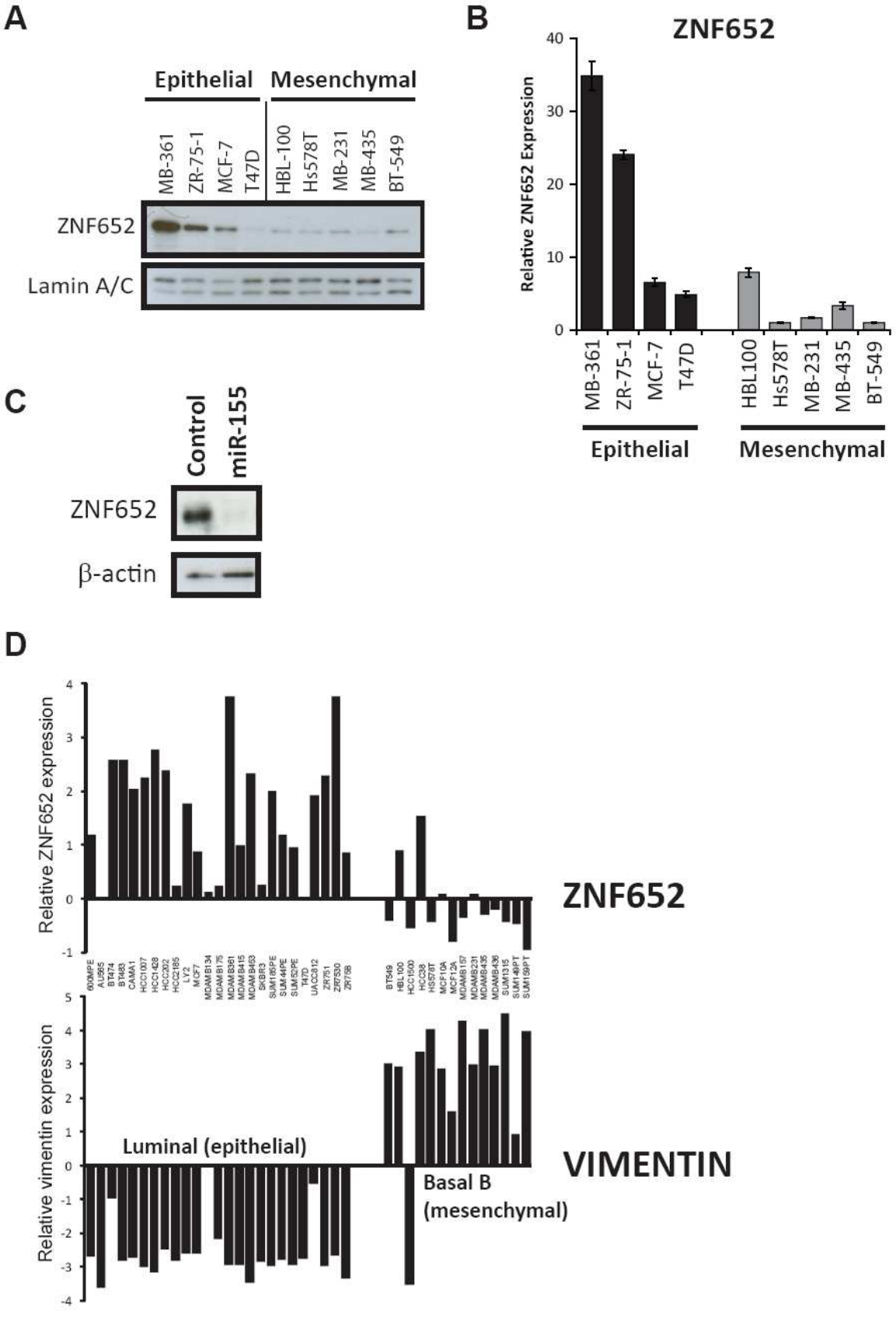

A
Epithelial
Mesenchymal
MB-361
ZR-75-1
MCF-7
T47D
HBL-100
Hs578T
MB-231
MB-435
BT-549
ZNF652
Lamin A/C
B
ZNF652
Relative ZNF652 Expression
40
30
20
10
0
MB-361
ZR-75-1
MCF-7
T47D
HBL100
Hs578T
MB-231
MB-435
BT-549
Epithelial
Mesenchymal
C
Control
miR-155
ZNF652
β-actin
D
Relative ZNF652 expression
ZNF652
Relative vimentin expression
Luminal (epithelial)
Basal B
(mesenchymal)
VIMENTIN

Figure 6. ZNF652 is an epithelial cell marker which is down-regulated in EMT by miR-155

A. Nuclear extracts from a panel of epithelial and mesenchymal breast cancer cell lines were screened for ZNF652 protein expression with a specific polyclonal anti-ZNF652 antibody (Kumar, 2006). Equal loading was determined by Lamin A/C.

B. Relative mRNA expression of ZNF652 was determined in a panel of cell lines by specific RT-PCR.

C. Over-expression of miR-155 is seen to significantly reduce the expression of ZNF652 in ZR-75-1 cells.

D. Expression microarray data from a cohort of 51 breast cancer cell lines representing both luminal (epithelial) and basal b (mesenchymal) cell types (Neve, 2006) shows that ZNF652 expression is up-regulated in epithelial cell lines and inversely correlated with the expression of the mesenchymal marker, vimentin.

ZNF652 is a repressor of gene transcription (Kumar et al 2006), and we hypothesised that this transcription factor may suppress invasion and metastasis through constitutive repression of key drivers of mesenchymal transformation. Identification of the key ZNF6562 targets will further elucidate the downstream regulatory network responsible for metastasis in mutant p53/miR-155 expressing breast tumours. It is expected that the elevated miR-155 levels observed during EMT would result in a loss of ZNF652 expression with a subsequent de-repression of the ZNF652 target genes. Therefore, we initially focused our attention towards the EMT genes which were elevated upon expression of miR-155 (Figure 3A), as these may represent direct ZNF652 target genes that are de-repressed upon silencing of ZNF652 by miR-155.

These putative ZNF652 target genes were screened for the presence of a consensus ZNF652 DNA binding sequence (AnGGGTTAA) (Kumar et al 2008). This *in silico* screen revealed consensus binding sequences in the first intron of *TGFB2* and *EGFR* and the 3' UTR of *VIM*. *TGFB2*, *VIM* and *EGFR* have been shown to play important roles in the promotion of mesenchymal transformation and invasion (Do et al 2008, Mitsudomi and Yatabe 2010, Vuoriluoto et al , Yu and Stamenkovic 2004, Zhu et al , Zuo et al 2011). Direct recruitment of endogenous ZNF652 to the putative binding sites within *TGFB2*, *VIM* and *EGFR* regulatory regions was subsequently demonstrated through ChIP analysis (Figure 7A). Other candidate genes involved in the TGF-β signalling pathway were also demonstrated to recruit ZNF652 to putative ZNF652 binding sites (Figure 7A). In addition, silencing of ZNF652 expression in ZR-75-1 cells using a specific siRNA resulted in a de-repression of *TGFB2* and

TGFBR2 expression (Figure 7B). Collectively, these data suggest that these genes are direct targets that are constitutively repressed by ZNF652 in epithelial cells.

Interestingly, the ZNF652 target genes are involved in the TGF-β signalling pathway. The oncogenic role of the TGF-β signalling pathway is mediated by activation of SMAD proteins to enable transcription of downstream targets that promote mesenchymal transformation and invasion. Activation of SMAD proteins by TGF-β is also required for mutant p53 to sequester p63, driving invasion and metastasis (Adorno et al 2009). We therefore hypothesize that ZNF652 may suppress invasion through direct repression of the TGF-β signalling pathway. Indeed, forced expression of ZNF652 ablated the ability of exogenous TGF-β to activate SMAD proteins, as demonstrated using a SMAD reporter assay (Figure 7C). Furthermore, silencing of ZNF652 expression results in an enhanced invasive capacity of H1299 cells (Figure 7D). Collectively, these data suggest that ZNF652 is a master repressor of the EMT gene network, specifically repressing genes involved in the TGF-β signalling pathway, and subsequently suppressing invasion.

The ability of ZNF652 to suppress invasion *in vivo* was evaluated in human breast tumours by staining tumour microarrays of paraffin embedded formalin fixed tissues with our affinity purified polyclonal anti-ZNF652 antibody. There is a highly significant association of low ZNF652 levels and increased propensity for tumour invasion (Figure 8).

FIGURE 7

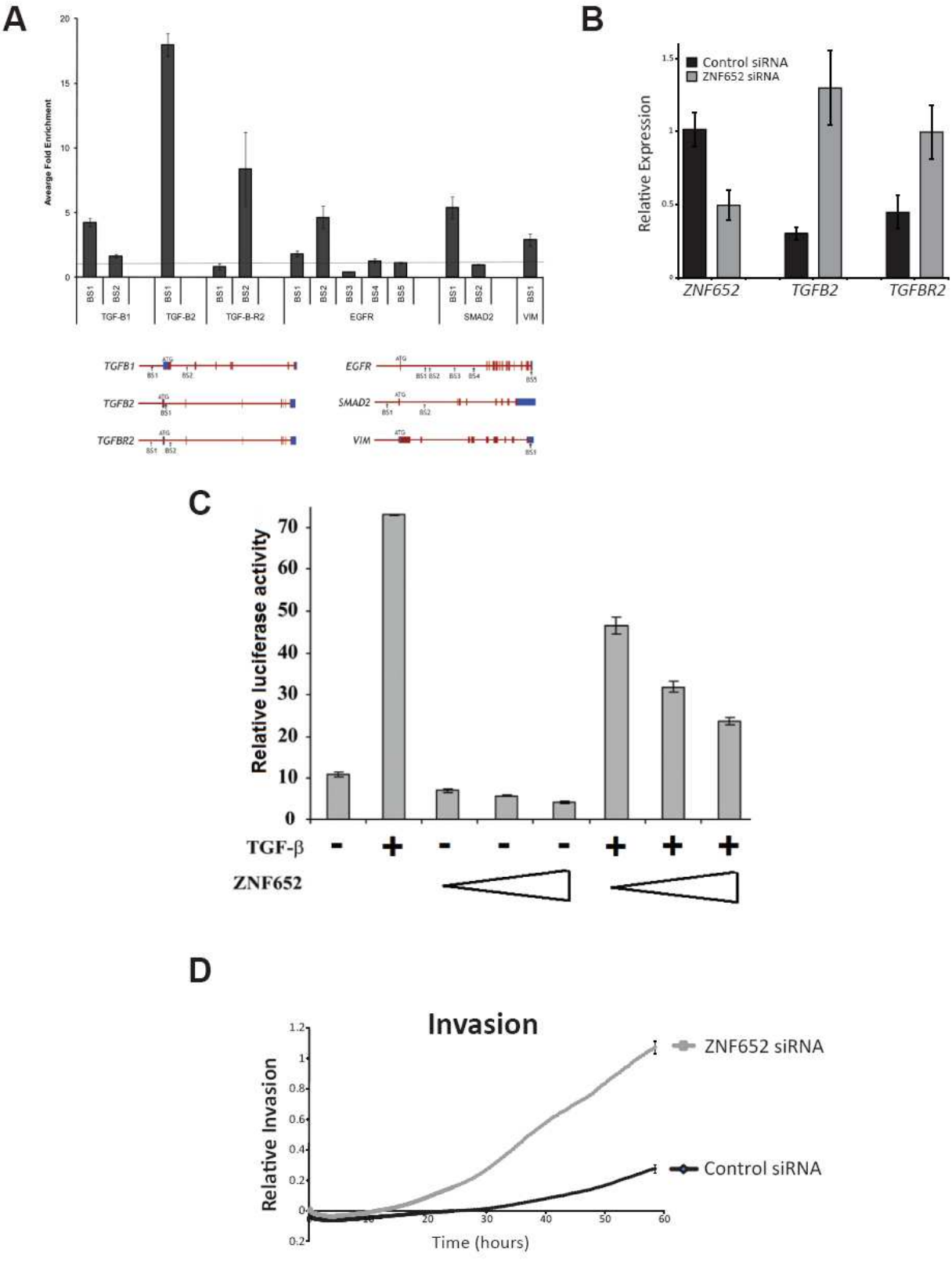

A
Avearge Fold Enrichment
BS1
BS2
TGF-B1
TGF-B2
TGF-B-R2
BS3
BS4
BS5
EGFR
SMAD2
VIM
TGFB1
TGFB2
TGFBR2
B
Control siRNA
ZNF652 siRNA
Relative Expression
ZNF652
TGFB2
TGFBR2
C
Relative luciferase activity
TGF-β
ZNF652
D
Invasion
Relative Invasion
Time (hours)
ZNF652 siRNA
Control siRNA

Figure 7. ZNF652 directly represses EMT-related genes resulting in the suppression of invasion

A. ChIP analysis was performed in ZR-75-1 cells using a specific ZNF652 antibody. The fold-enrichment of ZNF652 bound at identified ZNF652 binding sites was determined relative to an IgG control.

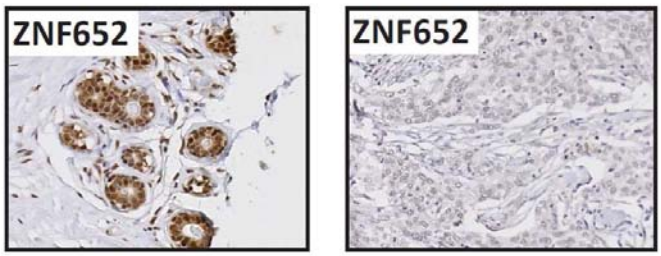
B. ZNF652 expression was silenced in ZR-75-1 cells through the use of specific siRNAs (see Materials and Methods) and the relative mRNA levels of ZNF652 and its putative target genes, TGB2 and TGFBR2 were determined by specific RT-PCR.

C. A SMAD reporter assay shows that increased ZNF652 expression ablates the ability of TGF-β to activate SMAD.

D. Knockdown of ZNF652 expression resulted in an enhanced ability of H1299 cells to invade into matrigel, as determined in real-time using an Xcelligence RTCA DP analyser.

FIGURE 8

A



Normal Breast Tissue Malignant Breast Tissue

INVASION

	Low	Medium	High *
Low ZNF652	25	17	8
High ZNF652	49	13	0

B

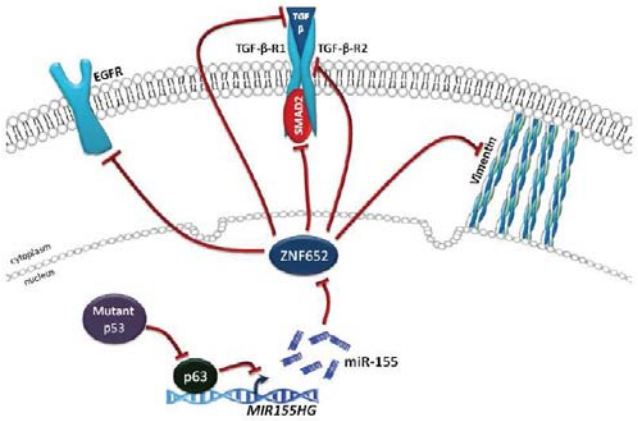


Figure 8. Loss of ZNF652 expression is associated with increased tumour invasion *in vivo*

A. IHC was performed on breast tissue microarrays to determine ZNF652 expression *in vivo*. ZNF652 expression was significantly associated (* indicates $p<0.001$, Fisher exact test) with tissue invasion as assessed by the TNM staging (where 'low' indicates tumour invades muscalaris propria; 'medium' indicates tumour invades through muscalaris propria; 'high' indicates tumour invades other organs and structures).

B. Schematic diagram representing the proposed mechanism of mutant p53 driving EMT and tumour cell invasion through a pathway involving miR-155 and ZNF652.

Based on our evidence, we propose that epithelial cancers expressing mutant p53 acquire enhanced invasive and metastatic potential through up-regulation of mir-155. This oncomiR drives invasion by directly targeting the transcript of ZNF652, resulting in the de-repression of the EMT gene network and acquisition of an invasive phenotype. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53 expressing breast tumours. Further identification of the downstream targets of the mutant p53•miR-155 axis will continue to reveal suppressors of invasion and metastasis that, like ZNF652, may have clinical implications as prognostic tools.

MATERIALS AND METHODS

Cell culture, plasmids and treatments

H1299 cells were maintained in DMEM supplemented with 10% FBS. ZR-75-1 and BT549 cells were maintained in RPMI supplemented with 10% FBS and 1mM sodium pyruvate. H1299 cells with inducible expression of mutant p53 are as described previously (Noll et al 2011). H1299- and ZR-75-1-SCR and -miR-155 over-expressing epithelial cell lines were generated by retroviral mediated over-expression of miR-155 (pMSCV-Puro-GFP-miR-155 retroviral expression construct was obtained from Erik Flemington) or a non-targeting SCR control (Open Biosystems), followed by selection in 500ng/mL puromycin (Sigma Aldrich).

To silence gene expression in cell lines, cells were transfected with 10 μM specific siRNA molecules. P53 siRNA sequence: GCA UGA ACC GGA GGC CCA UdT dT / AUG GGC CUC CGG UUC AUG CdT dT (QIAGEN). ZNF652 siRNA sequence: GUA GAG AAA GUC AGC GUU AUU / PUA ACG CUG ACU UUC UCU ACU U; GAG AAG UGC UUU CGG GUG AUU / PUC ACC CGA AAG CAC UUC UCU U. Briefly, cells were seeded at 50% confluence in 800 μL antibitotic-free media. siRNA molecules were diluted (final concentration 1μM) in OPTI-MEM reduced serum medium (Invitrogen) and incubated with 25 μM lipitoid transfection reagent (diluted in OPTI-MEM) at room temperature for 10 minutes. Transfection reagent was added to cells and 1mL media added 24 hours post-transfection. Cells were incubated for a total of 48 hours post-transfection prior to collection and analysis. Alternatively, MCF10A cells with silenced p63 expression were generated using a GIPZ lentiviral shRNAmir system (Open BioSystems). HEK-

293T cells were seeded at 50% confluence and transfected using the Translentiviral GIPZ packaging system (Open BioSystems). Briefly (for 1× 6-well), 5 μL Expression Arrest™ Trans-Lentiviral Packaging Mix was added to 250 μL OPTI-MEM reduced serum medium (Invitrogen) with 1.5 μg DNA mixed with 10 μL Arrest-In Transfection Reagent with 250 μL OPTI-MEM and incubated at room temperature for 20 minutes. 1 mL OPTI-MEM was added prior to addition of transfection mix to 293T cells. Growth medium was changed after 4-6 hours and cells incubated at 37°C with 5% CO_2 for 48 hours. Recipient cells were seeded at 50% confluence, filtered media containing lentivirus added and incubated for 48 hours. Growth medium was subsequently changed and cells were selected in 1 μg/mL puromycin.

For colony formation assays, cells were plated in duplicate in a 6-well format at 1000 cells/well in DMEM. Cells were grown for 10 days, fixed for 5 minutes with methanol and stained with Giemsa Stain (Sigma Aldrich) as per manufacturer's protocol.

Immunofluorescence (Actin staining)

Cells were plated at 10% confluence on glass cover slips and grown for 12 days. Where indicated, cells were treated with 2ng/mL TGF-β. Media was refreshed every 3 days. For actin staining, media was removed from cells followed by 1 × wash with warmed PBS. Cells were fixed with warmed 4% PFA for 15 minutes and blocked in 1% BSA/PBS for 20 minutes. Cells were washed 1 × PBS and permeabilised for 5 minutes with 0.1% Triton-X/PBS. Phalloidin antibody (diluted 1:500 in 0.1% Triton-X/PBS) was added and incubated for 1 hour at room temperature in the dark. Cells were washed 2 × with PBS and mounted in Vectashield

mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged using Olympus IX70 inverted microscope.

To score cell size, the major and minor axis of the cells was measured. To score lamellopodia, cells with lamellopodia (visualised with actin stain) were counted. Total cell numbers were determined by DAPI stain. Analysis was performed using ImageJ software over ≥4 fields of view. A minimum of 100 cells were counted per condition.

Isolation of RNA, RT-PCR and miR analysis

Briefly, total RNA was extracted from cells using RNeasy mini kit (Qiagen) according to manufacturer's protocol. Complimentary DNA (cDNA) was synthesized from 1 μg RNA using random primers (Promega) and RNase H- reverse transcriptase (Promega) as per manufacturer's protocol. Real-time PCR reactions were performed on a BioRad iCycler (BioRad) using IQ SYBR Green Supermix (BioRad) as previously described (Pishas et al 2010). Changes in the mRNA expression of specific genes was subsequently determined by the ΔΔCT method (Livak and Schmittgen 2001), with the levels of gene expression normalised to the average Ct value of the peptidylpolyl isomerise G (PPIG), β-actin or cyclophilin housekeeping genes. Gene specific primers – miR155HG (Forward: 5' – AGC AAG CGC GGG GAA CCA AGG – 3'; Reverse: 5' TCC ATT GGG TGG GAG AGC CAA GG – 3'), ZNF652 (Forward: 5' – CTT CAC CAG CAA ACA GAC TGT GAA - 3'; Reverse: 5' – TTC TTT TCT GCA TAT CCA TGG ACG - 3'), TGFB2 (Forward: 5' – GAA TGC TTC CAA TTT GGT GAA AG – 3'; Reverse: 5' – GCT GGG TTG GAG ATG TTA AAT C – 3'), TGFBR2 (Forward: 5' – GGG GAA ACA ATA CTG GCT GAT CA – 3';

Reverse: 5' – GAG CTC TTG AGG TCC CTG TGC A – 3'). Low density PCR arrays were performed using the Human Epithelial to Mesenchymal Transition RT^2Profiler PCR Array System (SABiosciences) as per manufacturer's protocol. MiR-155 analysis was performed using the ABI Taqman miRNA assay for miR-155, normalising levels to U6, U48 and miR-16 according to the standard protocol.

Western blot analysis and antibodies.

Nuclear extracts of cells were prepared. Briefly, cells were resuspended in Buffer A (10mM Hepes, 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% glycerol) supplemented with 25 × protease inhibitor (Roche). Triton X-100 was added to a final concentration of 0.1% followed by incubation on ice for 8 minutes. Nuclei were pelleted by centrifugation for 5 minutes (1,300 × *g*, 4 ºC). Nuclear pellets were subsequently lysed in lysis buffer (50mM Tris-HCl (pH 7.5), 250mM NaCl, 1mM EDTA, 50mM NaF, 0.5% Triton-X 100, 0.1mM Na_3VO_4) with 1× protease inhibitor cocktail. Lysates were sonicated 1× 15 seconds at 25% amplitude and clarified (13,200 rpm, 4ºC) for 15 min. Western blot analysis was performed as previously described (Kumar, 2005). Antibodies used for western blot were: mouse α-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse α-β actin (Sigma Aldrich), mouse α-Lamin A/C, rabbit α-ZNF652 (Kumar et al 2006), α-mouse IgG HRP linked (GE Healthcare), α-rabbit IgG HRP linked (GE Healthcare).

Invasion/Migration Assays

Real-time invasion assays were performed using the xCelligence Real-Time Cell Analyzer (RTCA) DP (Roche), as per the manufacturer's protocol. Briefly, sub-confluent cell cultures were collected in serum free media and plated at 2×10^4 cells

per well in the top chamber of a CIM-Plate 16 pre-coated with 5% Matrigel (BD Biosciences, San Jose, CA). DMEM containing 10% FCS was used as a chemo-attractant. Real-time migration assays were performed using Incucyte (Essen). Phase images were taken every 15 minutes and wound closure and cell confluence calculated using specific Incucyte software.

Chromatin Immunoprecipitation (ChIP)

Cells (as indicated) were collected and DNA and proteins were cross-linked by addition of 1% formaldehyde for 9 min with rotation at RT. To stop cross-linking, 625mM cold glycine was added, mixed and centrifuged for 5 minutes at 300 *g*. Cells were subsequently washed twice with 50 mL cold PBS. Cell pellets were lysed in 400 μL SDS Lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) with protease inhibitors, followed by sonication (6 × 15 sec; 30% amplitude). Following clarification, lysates were diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) and inputs taken. Lysates were precleared with Protein A sepharose beads with BSA and sonicated salmon sperm DNA (ssDNA) at 4ºC with rotation for 2 hours. Lysates were subsequently incubated with 4 μg rabbit α-ZNF652, rabbit α-p63 H-129 (Santa Cruz) or rabbit IgG at 4ºC with rotation overnight. Immune complexes were precipitated with Protein A sepharose with ssDNA at 4ºC with rotation for 2 hours. Beads were washed once each with low salt immune complex wash buffer (20mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), high salt immune complex wash buffer (20mM Tris-HCl pH 8, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), LiCl immune complex wash buffer (10mM Tris-HCl pH 8, 1mM EDTA, 0.25M LiCl, 1% NP-40, 1% sodium deoxycholate) and twice with TE buffer

(10mM Tris-HCl pH 8, 1mM EDTA). Specific immune complexes were eluted in 250µL SDS Elution Buffer (1% SDS, 0.1M $NaHCO_3$). Cross-links were reversed by addition of 10µL 5M NaCl and heating at 65ºC for 16 hours, followed by addition 10µL 0.5M EDTA, 20µL 1M Tris-HCl pH 6.5 and 4µL 10mg/mL Proteinase K and heating at 45ºC for 1 hour. DNA was extracted using standard PCR purification kit (Qiagen). Levels of specific promoter DNAs were determined by real-time PCR using specific primers (see Table 2). Three independent biological replicates were performed.

Table 2. Primers used for ChIP analysis

Primer Name	Forward (5'→3')	Reverse (5'→3')
TGFB2-BS1	TTGCTCCGATTGGCGTGGAGC	CGGGTTTTGTCTCGGCGATGC
TGFBR2-BS1	AGTACTGCAAGGACATGCTCCAAAAT	TGTAGAACCACACTCCGTGCTCT
TGFBR2-BS2	TCCACTGACGCCTTGGCATGGA	AGACAGCGATCAGGTGGGTAGCCA
EGFR-BS1	TAGAGACATGGAGGACTCCCGACC	GCACATTTTTCCATGCAGATGAGCAGC
EGFR-BS2	ACAAGTGAGCTGCGCAGGGTG	TCTTGAAAATGGGAAACTGGCTGTCA
EGFR-BS3	GGCAGCTGCTTGCACCGAAAG	ACGGGCCTGCATCTGGAAACT
EGFR-BS4	GCTAAGCCATCCCCAAGTTTGTCG	AGCCTGCTGGAAATGCAGCCC
EGFR-BS5	GCGGGATATAGTTCTCTTTATGTAGCACTG	ACCAAGCTAAATGTGCTTTCCATCATCT
SMAD2-BS1	ACCCTCAGGCTCTTTCCAGAAGC	AGGCACATAGAGCAGGGATTGCT
SMAD2-BS2	ACGACCTACTGTTGACTGGAAGCCT	AGACCTTCATGATGATCCACTTCCACT
VIM-BS1	TGCCACTGGGCTCTAAGCAGTGT	AGGAGTGGAGGCTTTGACAGAGT
MIR155HG-BS1	TCATGTCATTCTTAATTGCAGGTTTTGGC	ACCTGGGGGAAAGTACCAGTTTCT

TMAs and IHC

Breast tissue microarrays BR951, BR961 and BR963 were sourced from US Biomax Inc.. Immunohistochemistry was performed to determine ZNF652 expression. Briefly, slides were heated at 50˚C for 2 hours followed by dewaxing 3 × 5 min with xylene, rehydration 3 × 5 min with 100% ethanol and 2 × 3 min with PBS. Slides were subsequently treated with 1:100 dilution H_2O_2 in PBS for 5 min at RT followed by 2 × 3 min wash in PBS. Slides were immersed in citrate buffer and microwaved on high for 3 min (until boiling) followed by 15 min on low. Cooled slides were washed 2 × 3 min in PBS. Slides were blocked in 5% rabbit serum in PBS at RT for 30 min

and incubated with an affinity purified rabbit polyclonal antibody against ZNF652 (Kumar et al 2006) overnight at 4˚C. Slides were washed 2 × 3 min in PBS followed by addition of biotinylated rabbit specific secondary antibody at 1:400 in blocking solution and incubated 1 hr at RT in humid chamber. 1:500 dilution streptavidin in PBS was added and incubate 1 hr at RT in humid chamber. Add DAB and H_2O_2 solution and incubate at RT 6 min. Counterstain with Lillie-Mayer haematoxylin for 15-30 sec. Rinse with water and dehydrate and clear slides; 3 × 5 min with 100% ethanol and 3 × 5 min with xylene. Slides were mounted in DPX and visualised.

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CONCLUSION

The *TP53* gene is the most commonly mutated gene in human cancer. Wild-type p53 functions to control cell growth, however mutations in p53 result in a loss of wild-type function. Mutant p53 also acquires oncogenic, gain-of-function properties that drive tumour progression and metastasis. The study of mutant p53 and its role in the promotion of tumourigenesis is of extreme importance for the development of new cancer therapeutics to target novel pathways that drive tumour progression.

This thesis has described the generation and applications of a panel of inducible cell lines that express a variety of common p53 'hot spot' mutants. It is evident that this system provides an important resource to investigate various aspects of mutant p53 function including transcriptional activity and biological responses to new and existing chemotherapeutic agents (Chapter 3). Utilising this system, a role for mutant p53 in regulating a subset of p53 target genes has been identified and a novel mechanism for mutant p53 transcriptional activity is proposed (Chapter 4). This addresses a key area in current research, to identify mutant p53-regulated pathways that may be targeted in cancer therapies.

The identification of novel mechanisms of mutant p53 function allow for the potential development of strategies to target these processes, with an eventual aim of inhibiting mutant p53 gain-of-function. ANKRD11 is identified as an endogenous protein with the capacity to suppress mutant p53 gain-of-function (Chapter 5), suggesting that the ANKRD11-p53 interaction may provide the basis for the design of therapies to target mutant p53-expressing tumours. Future work will be focussed toward identifying a

peptide that can mimic the ANKRD11 interaction with mutant p53 and subsequently act to inhibit mutant p53-dependent tumour cell growth and invasion. In addition, a novel relationship has been established between mutant p53 and a microRNA, which results in the enhanced invasive and metastatic potential of breast cancer cells (Chapter 6). Collectively, the identification of key mechanisms and novel pathways through which mutant p53 drives tumourigenesis enhances our understanding of the molecular processes leading to tumour development and progression as well as providing potential targets for the development of new cancer therapies.

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