The Regulation of p53-dependent microRNA Expression in Response to Genotoxic Stress

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

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DECLARATION

The accompanying thesis submitted for the degree of Doctor of Philosophy, entitled *"The Regulation of p53-dependent microRNA Expression in Response to Genotoxic Stress"* is based on work conducted by the author in the Department of Biochemistry at the University of Leicester during the period October 2008 and April 2013. All of the work recorded in this thesis is original unless otherwise acknowledged in the text or references. None of the work has been submitted for another degree in this or any other university.

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ABSTRACT

INTRODUCTION: miR-16 and miR-26a have been identified as key effectors of the p53 pathway in response to genotoxic stress. This work is focused on preliminary elucidation of regulatory mechanisms by which p53 controls expression of miR-16 and miR-26a and characterisation of their gene targets involved in the p53 network.

METHODS: Microarray expression analysis of miR-16 and miR-26a was followed by Q-PCR to confirm these miRNAs dependence on p53. We analysed the transcriptional regulation of these miRNAs by p53 via luciferase assay and ChIP assay. We investigated these miRNAs contribution to p53-dependent response to genotoxic stress. To validate miR-16 and miR-26a targets (Cyclin E, CHK1, and WEE1) we employed Q-PCR, western blotting and luciferase assay. We also analysed the transcriptional regulation of Cyclin E by SET9 via luciferase assay.

RESULTS: High miR-16 and miR-26a expression are associated with increased cancer survival. p53-dependent and -independent regulatory mechanisms exist for miR-16 and miR-26a, and p53 controls expression of miRNAs on several levels. p53 recruits Drosha complex to miR-16 and miR-26a to facilitate the processing of these miRNAs. miR-26a cooperates with p53 to induce apoptosis and miR-16 enhances p53-mediated cell cycle arrest. miR-16 and miR-26a regulates CHK1 and WEE1, in the presence or absence of p53. miR-16 also reduces Cyclin E levels, in the presence and absence of p53. SET9 controls expression of Cyclin E on a transcriptional level.

CONCLUSIONS: Our results showed that in response to DNA damage, miR-16 and miR-26a expression levels are controlled by p53-dependent and p53-independent mechanisms, potentially involving other stress-response transcription factors such as E2F1. Our data also confirms that miR-16 and miR-26a directly target Cyclin E, CHK1, and WEE1 for down-regulation. Additonally, SET9 directly controls Cyclin E expression. Reduced CHK1 and WEE1 levels leads to decreased G2/M arrest, and reduced Cyclin E levels results in increased G1/S arrest. As a consequence, apoptosis occurs.

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CONTENTS

TABLES AND FIGURES

ABBREVIATIONS

%	Percent
°C	Degrees Celsius
17p 13.1	Chromosome 17 at position 13.1
A	Adenine
A15/16/26a/34	Anti-microRNA
ABL	Abelson murine leukemia viral oncogene homolog
Ad. p53-DC	Adenovirus-transduced dendritic cell-based p53-modified vaccine
Adr	Adriamycin
AGO	Argonaute
AIP4	Aryl hydrocarbon receptor interacting protein
AKT	Protein Kinase B
AMO	Antisense oligodeoxyribonucleotide
Amp^r	Ampicillin resistance
APAF1	Apoptotic protease activating factor 1
APS	Ammonium persulphate
ARC	Caspase recruitment domain
Arg	Argenine
ASPP	Apoptosis-stimulating of p53 protein
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad-3 related
β	Beta
BAD	BCL2-associated death promoter
BAK	BCL2 homologous antagonist killer
BAX	BCL2-associated X
BBC3	BCL2-binding component 3
B.C.	Before Christ
BCL2	B-cell lymphoma 2
$BCLX_L$	B-cell lymphoma-extra large
BER	Base excision repair

BID	BH3 interacting-domain death agonist
BL	Burkitt lymphoma
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
Bp	Base pair
BRCA2	Breast cancer 2
BRDU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	Cytosine
C.elegans	*Caenorhabditis elegans*
C-terminus	Carboxyl-terminus/carboxy-terminus
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
CCND1	Cyclin D1
CD	Core domain
$CD4^+/CD5^+/CD8^+$	Cluster of differentiation 4/5/8
CDC2	Cell division cycle 2
CDC25C	Cell division cycle 25C
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CH_2/CH_3	Ethyl/methyl
CHK1/2/CHEK	Checkpoint kinase
CK	Casein kinase
CLL	Chronic lymphocytic leukaemia
Cm	Centimetre(s)
Cm^2	Square centimetre(s)
CO_2	Carbon dioxide
COOH	Carboxylic acid
CTD	Carboxy-terminal domain
CTDSP2	Carboxy-terminal domain small phosphatase 2

CUL5	Cullin-5
Δ	Delta
ΔNp63/ΔNp73	N-terminally truncated protein 63/N-terminally truncated protein 73
Δp53	Delta-protein 53
Δ40p53/Δ40p53-β/Δ40p53-γ	Truncated protein 53 mutant (deletion of the first 40 amino acids)
Δ133p53/Δ40p53-β/Δ40p53-γ	Truncated protein 53 mutant (deletion of the first 133 amino acids)
D.melanogaster	*Drosophila melanogaster*
DEPC	Diethylpyrocarbonate
DHFR	Dihydrofolate reductase
DLG5	Discs large homolog 5
DMEM	Dulbeccos's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT3A	DNA methyltransferase 3A
dNTP (dATP, dCTP, dGTP, dTTP)	Deoxyribonucleotide triphosphates
DQTSFQKENC	Synthetic p53 peptide
DR	Death receptor
dT	Deoxythymine
DTT	Dithiothreitol
E1B55	Early-1B 55
E2F	E2 factor
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell

EST	Expressed sequence tag
EZH2	Enhancer of zeste homolog 2
F1 ori	F1 phage origin
FACs	Fluorescence-activated cell sorting
FACT	Facilitates chromatin transcription
FBS	Foetal bovine serum
Fig	Figure
γ	Gamma
G	G-force/Guanine
G1/S	Gap 1/Synthesis
G2/M	Gap 2/Mitosis
GADD45	Growth arrest and DNA damage 45
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
Gly	Glycine
GTP	Guanosine triphosphate
H3K4me3	Trimethylation of Lys4 of histone 3
H3K9/14Ac	Acetylation of Lys9/14 of histone 3
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HDAC1	Histone deacetylase
HESC	Human embryonic stem cell research
HIF	Hypoxia-inducible factor
HIPK2	Homeodomain-interacting protein kinase 2
HMGA2	High mobility group AT-hook 2
HMT	Histone methyltransferase
HOXD10	Homeobox D10
HPV	Human papillomavirus
Hr	Hour(s)

HRP	Horseradish peroxidase
HSP	Heat-shock protein
HTERT	Human telomerase reverse transcriptase
HZF	Hematopoietic zinc finger
iASPP	Inhibitor of apoptosis-stimulating protein of p53
IBID	Interferon-binding domain
IC	Intracellular
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRS1	Insulin receptor substrate 1
JNK	C-Jun N-terminal kinase
K	Lysine
K4R	p53 mutant (lysine residue acetylation and methylation sites 320, 372, 373 and 382 replaced with arginine residues)
Kb	Kilobase
KCl	Potassium chloride
KD	Knockdown
kDa	Kilodalton
KLF4	Kruppel-like factor 4
L4	Larval stage 4
LB	Luria broth
LFS	Li-Fraumeni syndrome
LiCl	Lithium chloride
LIF	Leukemia inhibitory factor
LNA	Locked nucleic acid
LSD1	Lysine-specific histone demethylase
LUC/Luc^{+}	Luciferase gene
Lys	Lysine
M	Molar
MAD2L1	Mitotic arrest deficient 2, yeast, human homolog

	like-1
MAP	Mitogen-activated protein
MCL1	Myeloid cell leukaemia 1
MCM	Mini-chromosome maintenance
MDM2	Mouse double minute 2 homolog
MDMX/MDM4	Mouse double minute X homolog/Mouse double minute 4 homolog
Me	Methylated
MetA	Methylcholanthrene
µg	Microgram(s)
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
Min	Minute(s)
miR	microRNA
miRISC	microRNA-induced silencing complex
miRNA	microRNA
µl	Microlitre(s)
Ml	Millilitre(s)
MLH1	MutL homolog 1
MLLDLRWCYFLINSS	p53 synthetic peptide
µM	Micromolar
mM	Millimolar
mRNA	Messenger RNA
MTDH	Metadherin
MTF	Myelin transcription factor
MyoD	Myoblast determination
N-terminus	Amino-terminus, NH_2-terminus, amine-terminus
Na_2HPO_4	Sodium phosphate (dibasic)
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NaH_2PO_4	Sodium phosphate (monobasic)

NaN_3	Sodium azide
NEB	New England Biolabs
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NER	Nucleotide excision repair
NES	Nuclear export signal
NF-Y	Nuclear factor Y
Ng	Nanogram(s)
NH_2	Amine
NLS	Nuclear localisation signal
nM	Nanomolar
NP40	Nonyl phenoxypolyethoxylethanol-40
NRD	Negative regulatory domain
NSCLC	non-small cell lung cancer
OCT4	Octamer-binding transcription factor 4
OD_{260}	Optical density at 260nm
Oligo	Oligonucleotide
ONPG	O-nitrophenyl-β-d-galactopyranoside
p	P-value
P15/16/26a/34	Precursor-microRNA
p16	Protein 16
p21	Protein 21
p38	Protein 38
p53	Protein 53
p53-β/p53-γ	Full-length p53 isoforms (Beta and gamma)
p53BP2	Tumour suppressor p53-binding protein 2
p53i9	p53 isoform formed by alternative splicing of the intron 9
p53-SLP	long synthetic p53-derived peptide
p63	Protein 63
p73	Protein 73

PAGE	Polyacrylamide gel electrophoresis
PBC	Primary biliary cirrhosis
PBS	Phosphate-buffered saline
pCAF	p300/CBP-associated factor
PC4	Proprotein convertase 4
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pen/Strep	Penicillin-streptomycin
PERP	p53 apoptosis effector related to PMP-22
pH	Power of hydrogen
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIC	Preinititation complex
PIDD	p53-induced death domain
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PIRH2	p53 induced RING-H2
PIWI	P-element induced wimpy
pM	Picomole(s)
PML	Progressive multifocal leukoencephalopathy
PMS2	Postmeiotic segregation increased 2
PMSF	Phenylmethanesulfonyl fluoride
PNACL	Protein Nucleic Acid Chemistry Laboratory
PolyA/Poly(A)	Polyadenylated
POU5F1	POU domain, class 5, transcription factor 1
PP2A	Protein phosphatase 2A
pRb	Retinoblastoma protein
PRD	Proline-rich domain
Pre	Precursor
Pri	Primary

PRIMA1	Proline-rich membrane anchor 1
PRMT1	Protein arginine methyltransferases
Pro	Proline
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
PXXP	Proline-rich motif
Q-PCR	Quantitative polymerase chain reaction
R175H	p53 mutant (arginine residue 175 in the DNA-binding domain replaced with a histidine residue)
R273H	p53 mutant (arginine residue 273 replaced with a histidine residue)
rAd-p53	recombinant adenovirus that expresses p53
Rb	Retinoblastoma
RE	Response element
REG	Regulatory domain
Rho	Ras homolog
RHOC	Ras homolog gene family, member C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNA pol II/III	RNA polymerase II/III
RNase	Ribonuclease
ROS	Reactive oxygen species
RPA	Replication protein A
Rpm	Revolutions per minute
RRRCWWGYYY	p53 consensus sequence motif
RT	Reverse transcriptase
S	Serine
S100	Soluble in 100%
SAM	Sterile alpha motif
SAP	Shrimp alkaline phosphatase

SCCHN	Squamous cell carcinoma of head and neck
SCF	Stem cell factor
SCLC	Small cell lung cancer
Scr	Scramble(d)
SDN	Small RNA degrading nuclease
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	Second(s)
Seq	Sequencing
Ser	Serine
Sh	Small hairpin
SH3	SRC homology 3-like
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SIRT1	Silent mating type information regulation 2 homolog
SMC4	Structural maintenance of chromosomes protein 4
SMYD2	SET and MYND domain containing 2
SOX2	SRY-box 2
SSB	Single-strand binding
ssDNA	Single-stranded DNA
SUMO1	Small ubiquitin-related modifier 1
SV40	Simian virus-40
T	Threonine/Thymine
TAD	Transactivation domain
TAE	Tris base, acetic acid and EDTA
TAF	(TBP)-associated factor
TAp63	Full-length protein 63
TAp73	Full-length protein 73
TAZ	Tafazzin

TBP	TATA-binding protein
TBS	Tris-buffered saline
TBST/TTBS	Tris-buffered saline with Tween-20
TCF3	Transcription factor 3
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TET	Tetramerisation domain
TFIIA/TFIIB/TFIID/TFIIE/TFIIF/TFIIH	Transcription factor II
TFAM	Mitochondrial transcription factor A
TGF	Transforming growth factor
Thr	Threonine
TIP60	Tat interacting protein 60
TP53	Tumour protein p53
TRBP	TAR RNA binding protein
TU	Transcription unit
U	Unit(s)
UBC9	Ubiquitin-conjugating Enzyme 9
UNG	Uracil-DNA glycosylase
UTR	Untranslated region
UV	Ultraviolet
V	Volt(s)
v/v	Volume to volume ratio
w/v	Weight to volume ratio
WIP1	Wild-type p53-induced phosphatase 1
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis protein
XRN2	Exoribonuclease 2
YB1	Y-box binding protein 1
YY1	Ying yang 1

INTRODUCTION

After 25 years of rapid advances, cancer research has produced a great body of knowledge, showing cancer to be a disease involving key alterations in the genome. Cancer is a complicated genomic disease that can be defined as the uncontrollable division and spread of abnormal cells to other healthy tissues (Bhadauria *et al.*, 2012), and it is the second most common cause of death after heart disease (Jemal *et al.*, 2007). The earliest written account of cancer was scripted circa 3000 B.C. It was deduced that a bulging breast tumour was a serious disease and there was no cure for it (Breasted 1930). Abnormalities associated with cancer are both expression- and structure-related (Tsafrir *et al.*, 2006) and the disease is caused by damage to genomic DNA by mutation (Bartkova 2005). The main gene classes implicated in alterations leading to cancer are oncogenes and tumour suppressors (Osada & Takahashi 2002). Oncogenes, which are mutated forms of proto-oncogenes which allow for the normal growth and division of cells, promote continuous and uncontrollable cell division. In turn, this encourages the survival and growth of cancer cells (Foster 2008). Examples of well-known proto-oncogenes include *RAS*, *WNT*, and c-*MYC* (Croce 2008). On the other hand, tumour suppressor genes are activated on stress to challenge oncogenic activity and therefore limit cell proliferation, growth, and survival (Campisi 2001). Examples of well-known tumour suppressor genes include *p53*, *pRb*, and *PTEN*. Progression made in cancer research is revealing the molecular mechanisms of tumourigenesis, resulting in the development of targeted therapies which impede the growth and spread of cancer cells, whilst not affecting normal cells (Sawyers 2004). As the majority of human cancers present altered p53, the notion to restore p53 for cancer therapy is very appealing. Animal models showing that reactivation of wild-type p53 resulted in tumour regression, for example regression of liver carcinoma and lymphoma (Ventura *et al.*, 2007).

p53 the tumour suppressor gene

Of all the tumour suppressors, the nuclear protein p53, encoded by the *TP53* gene (Matlashewski *et al.*, 1984), is one of the most important (Jochemsen 2012).

The discovery of p53

Known as the 'guardian of the genome', as stated by David Lane in 1992, or 'cellular gatekeeper' (Naccarati *et al.*, 2012), p53 has a key role in maintaining stability by the prevention of genomic mutation (Chiang 2012). In addition to its function in normal development and growth, p53 has a crucial role in tumourigenesis (Brož & Attardi 2010). Since it was discovered in 1979, the role of p53 in cancer has been greatly studied and it is one of the most explored tumour suppressors in cancer research (Ozaki & Nakagawara 2010). It was shown that the overexpression of a 53 kDa protein occurred in SV40-transformed murine cells, as well as in uninfected embryonal carcinoma cells (Linzer & Levine 1979). It was uncovered that the large T-antigen of simian virus 40 (SV40) co-precipitated with and formed an oligomeric complex with the 53 kDa protein which was shown to change during tumourigenesis (De Leo *et al.*, 1979; Lane & Crawford 1979; Linzer & Levine 1979). This unknown protein was later named p53 and it was then predicted that this protein could be encoded by the cellular genome. The *p53* gene was initially believed to be an oncogene because it was thought to associate with oncogenic RAS to transform normal embryonic cells, and that SV40 infection or transformation of mouse cells stimulated the synthesis or stability of the protein (Eliyahu *et al.,* 1984). It was later uncovered that this gene was the mutant form of p53 and promoted transformation by abolishing the tumour suppressive nature of the wild-type *p53* gene (Baker *et al.*, 1989; Vogelstein *et al.*, 1989). It was also shown that the humoral immune response of mice to methylcholanthrene-induced tumour cell lines was associated with the p53 protein (Fig. 1.1). It was found that these mice produced an immune response specifically against p53. In 1982, antibodies against human p53 protein were found in nearly 10% of breast cancer patient sera.

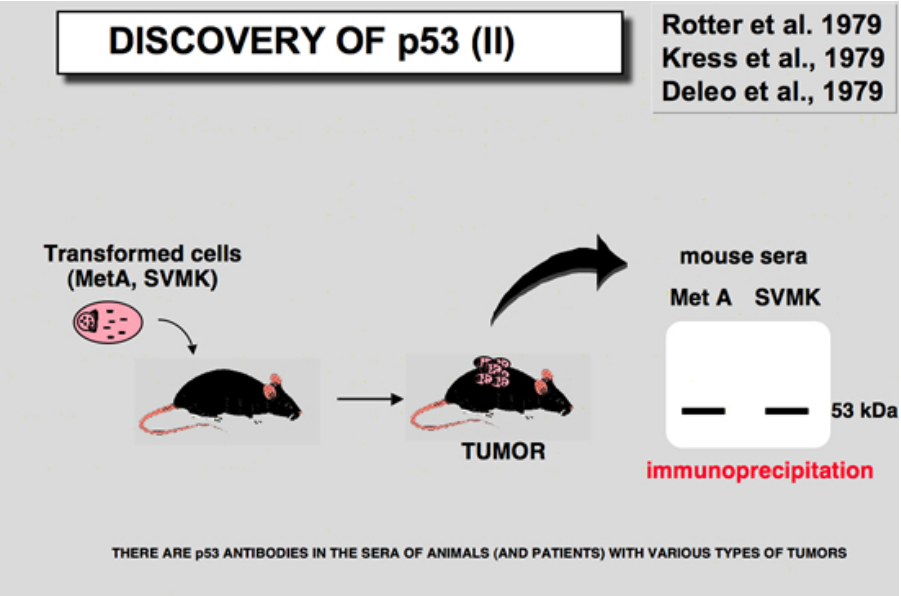


Figure 1.1 Discovery of p53 – humoral immune response
De Leo *et al.* showed that the humoral response of mice to many methylcholanthrene-induced tumour cell lines was against the p53 protein. Following on from this, it was shown that animals exhibiting several tumour types stimulated an immune response specifically for p53.
Taken from p53.free.fr.

It was later confirmed that p53 was a tumour suppressor when it was found that Li-Fraumeni patients possessed a germ-line *p53* mutant allele that could be inherited (Malkin *et al.*, 1990). In the 1960s, many extremely cancer-prone families were identified in the United States and Europe (Li & Fraumeni 1969). Mutations in the *p53* gene were shown to be the underlying genetic defect in the majority of Li-Fraumeni patients. Li-Fraumeni syndrome (LFS) was later shown to be caused by germline mutations in *p53* (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Sufferers of LFS, a rare autosomal-dominant disorder and a cancer predisposition syndrome, have a 25 times increase in primary onset cancer development (Senzer *et al.*, 2007). This syndrome is characterised by a broad spectrum of tumour types, such as breast cancer, sarcomas, and other neoplasms, found in a wide age range (Berger *et al.*, 2005). After 30 years of intense research, further knowledge of the p53 pathway has been accumulated and its vast complexity has been unveiled (Levine & Oren 2009). p53 is central to a network where it processes incoming signals from different pathways which sense various forms of genotoxic and cytotoxic stress and either block propagation of damaged cells or induces their death. Accordingly, p53 elicits expression of cell cycle inhibitory genes

and/ or pro-apoptotic genes. Mutation in or loss of the *p53* gene could affect genomic stability and therefore promote neoplasia (Murphy & Rosen 2000).

p53 structure

The tumour suppressor p53 is at the centre of several signaling pathways that control the cell cycle and maintain the integrity of the human genome (Kastan *et al.*, 1992). The structure of p53 is complex. In the mid-1990s, numerous structure-function studies unveiled structural details of individual domains of the p53 protein, including the DNA-binding domain and the tetramerisation domain (Joerger & Fersht 2010). This provided the basis to help understand the effect of p53 mutants that are common in cancers. In the following decade, much less progress was made in unravelling the structural basis of p53 function or its inactivation in cancer. Recently, we have begun to comprehend how p53 works as a whole by analysing simultaneously structural biology, protein engineering techniques, and computational methods (Xu *et al.*, 2011).

The *p53* gene has been mapped to the short arm of human chromosome 17 at position 17p 13.1, a region prone to deletion in human cancer (Isobe *et al.*, 1986; McBride *et al.*, 1986; Miller *et al.*, 1986) and is comprised of 11 exons spanning 16-20 kb of DNA (Masuda *et al.*, 1987). p53 exercises its role as a tumour suppressor mainly as a nuclear sequence-specific transcription factor and it is biologically active as a homotetramer made up of 4 x p53 monomers (Wei *et al.*, 2006). Evidence has been accumulated suggesting that p53 regulates transcription by several mechanisms: by functional variety between p53 mutants, cofactor variation, variations in DNA binding motifs, and differing stress-specific post-translational modifications. Additionally, promoter architecture differs between varying p53 response genes. Similar to other transcription factors, p53 has a modular protein domain structure (Fig. 1.2), which is of a flexible nature (Fernandez-Fernandez & Sot 2011).

p53 has a modular protein domain structure. It consists of 3 main domains - an NH_2-terminal transactivation domain (amino acids 1-42), a central core and folded DNA-binding domain (amino acids 98-299), and a COOH-terminal basic regulatory domain (amino acids 363-393), which are flanked by intrinsically disordered regions at the N- and C-termini (Kato *et al.*, 2003). The N-terminal domain also contains a SRC homology 3-like (SH3) domain, which is a proline-rich domain (amino acids 63-97). The C-terminal also contains nuclear localization (amino acids 300-323) and export (amino acids 356-

364) signals (NLS and NES, respectively), and a tetramerisation domain (amino acids 324-355) (Okorokov *et al.*, 2006).

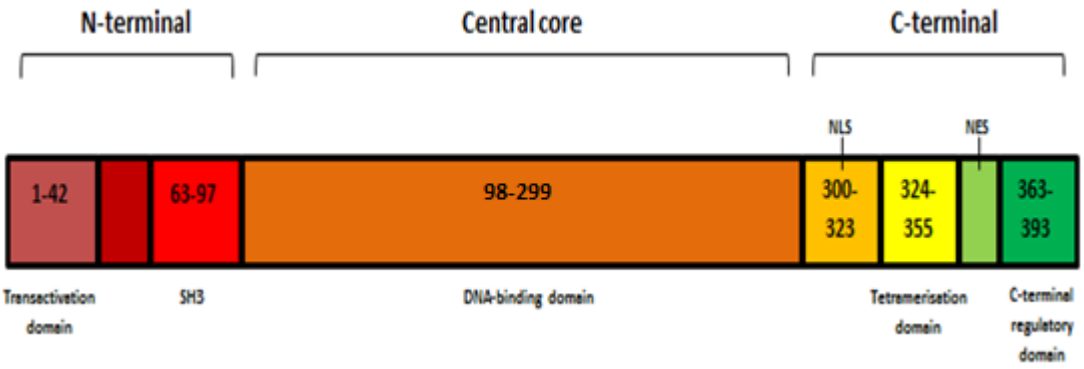


Figure 1.2 Structure of p53 protein domains
p53 has a modular protein domain structure. It consists of 3 main domains - an NH_2-terminal transactivation domain, a central core and folded DNA-binding domain, and a COOH-terminal basic regulatory domain. The N-terminal domain also contains a SRC homology 3-like (SH3) domain, which is a proline-rich domain. The C-terminal also contains nuclear localization and export signals (NLS and NES, respectively), and a tetramerisation domain.
Adapted from Bode & Dong 2004.

The transcriptional activity of p53 relies on its N-terminus domain (Matas *et al.*, 2001). The transactivation domain (TAD), which is subdivided into TAD1 and TAD2 subdomains, is essential for the functionality of p53 as a transcription factor (Ohki *et al.*, 2007). The interaction properties of p53 TAD are modulated by post-translational modifications, as well as direct binding to p53 transcriptional co-activators and components of the basal transcription machinery. Transcriptional co-activators and co-repressors have an effect on the transcriptional activity of p53 by affecting p53 binding to its target promoters, or by provoking or blocking transcriptional machinery assembly (Laptenko & Prives 2006; Beckerman & Prives 2010). Also, transcriptional co-activators and co-repressors aid in regulating the p53 transcriptional response by changing the chromatin structure close to p53 response elements (Laptenko & Prives 2012). For example, p53 recruits PIN1 to chromatin where it enhances acetylation of p53 by CBP/p300 coactivator (Mantovani *et al.*, 2007). GADD45 has been shown to serve in chromatin remodelling to allow access to sites of DNA damage (Smith *et al.*, 2000). BRN3B, ASPP1, and ASPP2 co-factors alter the loading of p53 onto chromatin at cell cycle and apoptotic target promoters, which enhances p53 apoptotic activity rather than cell cycle arrest, whereas BRN3A, iASPP (an evolutionary-conserved

inhibitory member of the ASPP family), and HZF have an opposing effect (Millau *et al.*, 2012). Other proteins involved in chromatin remodelling include the methyltransferases PRMT1 and CARM1, the histone deacetylase HDAC1, and the co-repressor MSIN3A. On stress, transcription is heightened at the transactivation domain by the recruitment of basal transcriptional machinery components such as the TATA-binding protein TBP, and TBP-related proteins TAFs, and coactivator complexes such as the histone-modifying enzymes CBP/p300 acetyltransferase and PRMT1 (Beckerman & Prives 2010), by the complementary transcriptional activation domains TAD1 and TAD2 to p53 target gene promoters (Brady & Attardi 2010). The p300 domains TAZ2/CH3, TAZ1/CH1, KIX, and IBID bind to the full TAD, with the TAZ2/CH3 domain having the highest affinity (Teufel *et al.* 2007). But the TAD1 subdomain also binds strongly to the negative regulators murine double minute 2 (MDM2) and MDMX (also known as MDM4) (Shvarts *et al.*, 1997). Under normal physiological conditions, MDM2, which has an intrinsic E3 ubiquitin protein ligase activity, negatively controls p53 protein levels and sustains it at minimal levels in an inactive state when p53 is not needed, by promoting p53 degradation through the ubiquitin-dependent proteasome pathway (Marine *et al.*, 2006; Toledo & Wahl 2006).

The proline-rich domain, which links the TAD to the core domain, contains five repeats of the amino acid PXXP motif in its sequence, where P is proline and X is any amino acid (Dornan *et al.*, 2003), and it is crucial for cell cycle arrest and apoptosis (Zhu *et al.*, 2000; Chipuk & Green 2004). It is in the proline-rich domain where lies the most commonly described p53 polymorphism at codon 72, where either arginine or proline is encoded (Chen *et al.*, 2008), and it is commonly associated with breast cancer (Alawadi *et al.*, 2011), cervical cancer (Klug *et al.*, 2009), colon cancer (Wang *et al.*, 2011), lung cancer (Piao *et al.*, 2011), pancreatic cancer (Sonoyama *et al.*, 2011), and squamous cell carcinoma of head and neck (SCCHN) (Yu *et al.*, 2011). The p53 Pro72 isoform is not as efficient at inducing apoptosis compared to the Arg72 isoform, due to the increased locality of the p53 Arg72 isoform at mitochondria and its low affinity for the iASPP protein, an inhibitor of p53 at the proline-rich domain (Bergamaschi *et al.*, 2006). One of two major pathways that apoptosis occurs through is the intrinsic mitochondrial pathway (Kroemer *et al.*, 2007). At the mitochondria, p53 impedes the

functions of BCL2 family members, preventing them from hindering apoptosis (Haupt *et al.*, 2003).

The DNA-binding domain is required for the sequence-specific binding activity of the p53 tetramer (Kern *et al.*, 1991), consisting of four oligomerised p53 molecules, to p53 response elements in DNA located close to promoters of p53 target genes. These response elements are defined as a consensus sequence motif consisting of tandem 10 base pair elements of RRRCWWGYYY (R is a purine, W is adenine or thymine, and Y is a pyrimidine), separated by a 1-13 nucleotide spacer between half-sites (Menendez *et al.*, 2010). This definition has recently been further refined by a genome-wide mapping of p53 binding sites, indicating that most p53 response elements have consecutive half-sites (Wei *et al.*, 2006), and by orderly measurements of the effect of every base-pair substitution within a palindromic half-site once p53 is bound (Veprintsev & Fersht 2008). In addition to DNA, the DNA-binding domain is similarly bound by p53 cofactors, such as ASPP1 and ASPP2, which positively regulate p53 activity. iASPP allows cells to bypass the tumour suppressor functions of p53 and the ASPP proteins (Bergamaschi *et al.*, 2003). The majority of tumour-related mutations are located in the DNA-binding domain, which disturb specific DNA binding (Van Oijen & Slootweg 2000), which may lead to decreased binding of p53 to target genes, resulting in serious alterations in the p53 transcriptional response. Frequently mutated codons ("hotspot" residues) in this region that are commonly linked to cancer include Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282 (Joerger *et al.*, 2005). Formation of mixed tetramers of impaired activity between wild-type and mutant p53 is believed to be the molecular basis of the "dominant–negative effect" of mutant p53 in heterozygous cells (Kern *et al.*, 1992; Chan *et al.*, 2004; Dong *et al.*, 2007; Junk *et al.*, 2008). For example, *in vitro*, the p53 mutant R273H (arginine to histidine substitution at codon 273) forms hetero-tetramers with wild-type p53 of weakened DNA-binding affinity in comparison with wild-type homo-tetramers (Natan *et al.*, 2009). Also, the N-terminally truncated p53 isoforms ΔNp63 and ΔNp73 can act in a dominant–negative manner towards p53, and also towards full-length isoforms TAp63 and TAp73 (Melino *et al.*, 2003; Benard *et al.*, 2003).

The DNA-binding and oligomerisation domains are connected via a flexible-linker region. The oligomerisation state of p53 is regulated via its tetramerisation domain.

The tetramer consisting of a dimer of dimers that p53 forms relies on the presence of its oligomerisation domain in its C-terminus, giving rise to its high binding ability to bind DNA and activate transcription (Weinberg *et al.*, 2004), with a ratio of one p53 molecule per consensus five nucleotides (Jordan *et al.*, 2008). The significance of tetramerisation, which is the predominant conformation of p53, for p53 in its role as a tumour suppressor has been emphasised by showing that the oligomerisation domain alone is required for efficient Ser15, Ser20, and Ser33 phosphorylation induced by DNA damage (Shieh *et al.*, 1999). There is increasing evidence that the equilibrium of p53 is modulated via an intricate network of accessory proteins, which can have either positive or negative regulatory effects. For example, direct binding of apoptosis repressor with caspase recruitment domain (ARC) to the p53 tetramerisation domain in the nucleus, inhibits p53 tetramerisation and promotes its nuclear export (Foo *et al.*, 2007). In contrast, *in vitro*, binding of 14-3-3σ proteins to the p53 carboxyl terminus, which is strengthened on phosphorylation of the latter, enhances formation of p53 tetramers (Rajagopalan *et al.*, 2008).

The carboxy-terminal regulatory domain (CTD) follows the tetramerisation domain. The basic C-terminal domain, especially the last 30 amino acids, is an essential domain for regulating the activity of p53 (Harms & Chen 2005). The C-terminus of p53 also functions as a negative regulatory domain, and has also been implicated in induction of cell death. According to this model, the C-terminal tail of p53 may regulate the ability of its core DNA binding domain to lock the DNA binding domain as a latent conformation. Also, the C-terminus by itself is able to bind DNA in a non-specific manner, which may be required for the function of p53 in DNA repair when the protein is scanning for damaged nucleotides (Ahn & Prives 2001). Various posttranslational modifications occur at the six carboxy-terminal lysines (370, 372, 373, 381, 382, and 386), comprising acetylation, methylation, ubiquitination, sumoylation, and neddylation, as well as phosphorylation of serine and threonine residues (e.g. Ser366, Ser378, Thr387, and Ser392). These modifications play essential roles in modulating p53 function and regulating its cellular protein levels, (Toledo & Wahl 2006; Kruse & Gu 2009; Hamard *et al.*, 2012) by heightening p53 sequence-specific DNA binding, as well as its transcriptional activity on stress (Ryan *et al.*, 2001). It has been shown that nearly all the residues in the C-terminus can be post-translationally modified (Feng *et*

al., 2005). It is post-translational modifications and protein-protein interactions that regulate the stability of p53-specific DNA complexes (Fig. 1.3). Binding partner proteins, including MDM2 and p300, are multidomain proteins similar to p53 with structurally disordered regions, and they make multipoint interactions with p53. Additionally, they are the two most common proteins to interact with the transactivation domain of p53. Oncogenic MDM2 and MDMX bind to the N-terminal transactivation domain of p53 and destabilises and inactivates p53, thus blocking its transactivation function by preventing the recruitment of vital components of basal transcriptional machinery and key co-factors here. In this way, p53 is quickly degraded via the ubiquitin-proteasome pathway. MDM2 is also observed at the DNA-binding domain, as well as the BCL2-family members $BCLX_L$ and BAK. 14-3-3σ proteins also commonly interact with the p53 C-terminus (Waning *et al.*, 2010).

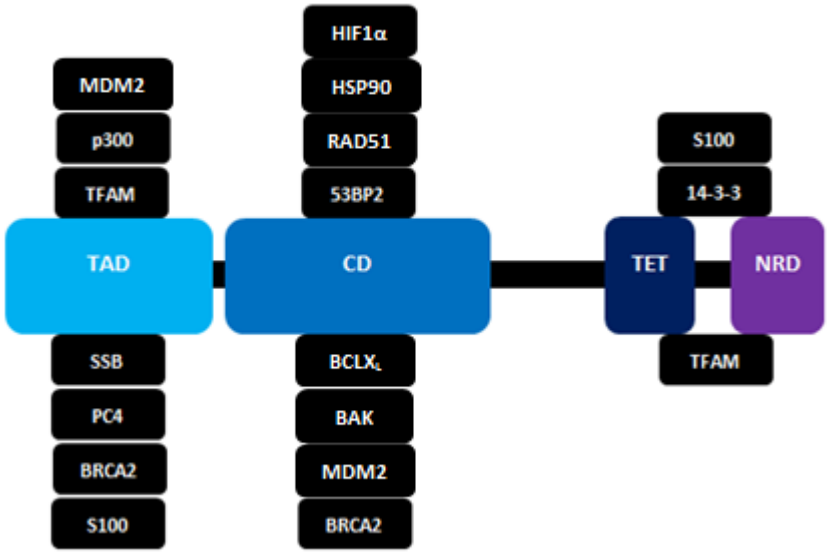


Figure 1.3 The protein binding partners of p53
Binding partner proteins bind p53 at its N-terminal transactivation domain (TAD), the DNA-binding core domain (CD), the tetramerisation domain (TET) or the C-terminal negative regulatory domain (NRD).
Adapted from Fernandez-Fernandez & Sot 2011.

p53 homologues

Two p53-related genes, *p63* and *p73*, were first identified in 1997 (Kaghad *et al.*, 1997; Yang *et al.*, 1998). These two proteins share significant structural and functional homologies with p53, particularly in the DNA binding domain. These proteins are also similar in their domain architecture with p53. These traits enable p63 and p73 to form homo- and hetero-oligomers, bind to p53 DNA-binding sites, leading to the

transactivation of p53-responsive genes, and therefore induce cell cycle arrest, senescence, or apoptosis, as a response to DNA damage (Fig. 1.4) (De Laurenzi & Melino 2000). Alongside p53, they form a transcription factor family without being completely functionless, and the main role of each p53 family member shows that each protein has its own unique functions, further confirmed by the differing phenotypes shown in *p53*, *p63*, and *p73* knockout mice (Murray-Zmijewski *et al.*, 2006). p63 and p73 occur as multiple protein variants, with similar or opposing activities, as a result of alternative splicing. Six mRNA variants due to alternative splicing are expressed by the *p63* gene to encode six different p63 protein isoforms. 35 mRNA variants due to alternative splicing are expressed by the *p73* gene which can encode 28 different p73 protein isoforms. Full-length isoforms TAp63 and TAp73 have similar functions to p53, relating to overlapping target promoters and biological functions. The N-terminally truncated isoforms ΔNp63 and ΔNp73 can transactivate through sterile alpha motif (SAM) domains but different sets of genes. SAM domains in the C-termini of p63 and p73 are implicated in protein-protein interactions. TAp63 and TAp73 bind to p53REs and induce the expression of p53 target genes through their transactivation domain (Melino *et al.*, 2003; Benard *et al.*, 2003). ΔNp63 and ΔNp73 isoforms also specifically bind to p53REs and directly activate specific target genes (Dohn *et al.*, 2001; Wu *et al.*, 2003; Liu *et al.*, 2004). In stressful conditions, p63 and p73 cooperate with p53 to regulate tumourigenesis. However, the p53 family members are mostly implicated in differentiation and development regulation, in the absence of stress. For example p63 is involved in squamous epithelia development, whereas p73 is important for neuronal differentiation (Yang & McKeon 2000).

The structure of the *p53* gene is much simpler compared to p63 and p73. Similar to *p63* and *p73* genes, the *p53* gene can transcribe six different mRNAs which encode six p53 protein isoforms. Studies using sensitive methodology uncovered at least 10 isoforms of p53: Wild-type p53, p53-β, p53-γ, Δ40p53, Δ40p53-β, Δ40p53-γ, Δ133p53, Δ133p53-β, Δ133p53-γ, and Δp53 (Fig. 1.5). Three mRNA splice variants that can be transcribed from the human *p53* gene include p53, p53i9, and Δ40p53. p53i9 is defective in transcriptional and DNA-binding activity. Δ40p53 has an N-terminal truncation and is still able to activate gene expression, due to its partial transactivation domain. Δ40p53 can inhibit the transcriptional activity of p53, as well as p53-mediated

apoptosis, and also alter the cellular location of p53 and inhibit its MDM2-mediated degradation. Altogether, the human *p53* gene can express nine different forms of the p53 protein, containing different domains of the protein because of alternative splicing, usage of an alternative promoter, and alternative initiation of translation (Bourdon *et al.*, 2005). Several clinical studies report the expression of the p53 homologues in numerous types of cancer, clarifying that the p53 homologues are expressed both at the mRNA and protein levels. Moreover, the abnormal expression of the p53 isoforms in different cancer types suggests that their differing expression may disrupt the p53 response and result in tumour formation. Furthermore, it may provide an explanation to the difficulties in numerous clinical studies to link the status of p53 to cancer prognosis and treatment (Bourdon *et al.*, 2011).

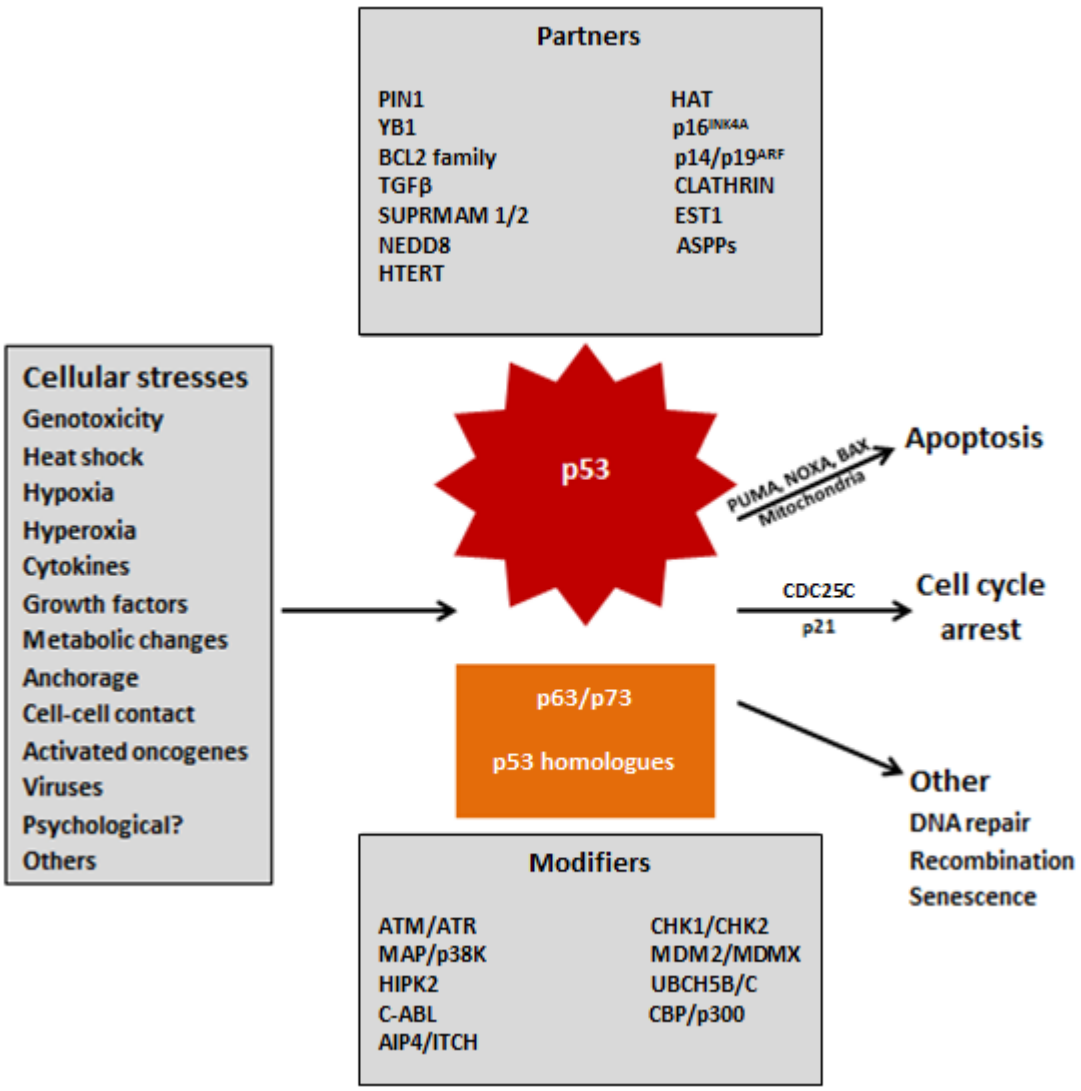


Figure 1.4 p53/p63/p73 pathway
p63 and p73 proteins are similar in their domain architecture with p53. These traits enable p63 and p73 to form homo- and hetero-oligomers, bind to p53 DNA-binding sites, leading to the transactivation of p53-responsive genes, and therefore induce cell cycle arrest, senescence, or apoptosis, as a response to DNA damage.
Adapted from Braithwaite *et al.*, 2005.

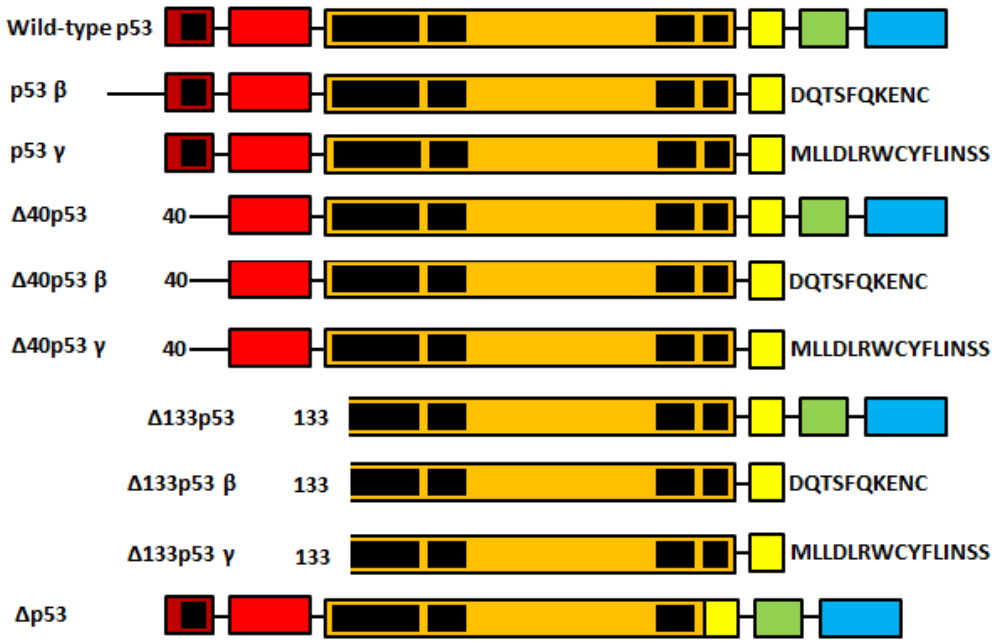


Figure 1.5 Human p53 isoforms
Studies using sensitive methodology uncovered at least 10 isoforms of p53: Wild-type p53, p53-β, p53-γ, Δ40p53, Δ40p53-β, Δ40p53-γ, Δ133p53, Δ133p53-β, Δ133p53-γ, and Δp53.
Adapted from p53.free.fr.

Evolutionary conservation of p53

The *p53* ancestral gene is believed to be over one billion years old and to originate from descendants of single cell choanoflagellates and the early metazoan sea anemone. This gene is most closely related to a combined *p63*/*p73*-like gene. The function of this ancestral gene in the sea anemone is to protect against DNA damage, a function which is also seen in clams, humans, insects, vertebrates, and worms. In sea anemones, clams, fruit flies, roundworms, zebrafish, mice, or humans, the *p53*, *p63*, and p73 genes appear to retain these functions. However in higher organisms, they have additional traits. Huge diversity and selective change in *p53*, *p63*, and *p73* genes has been seen through a billion years of evolution, particularly in vertebrates. Indications of positive selection of alleles in the *p53*, *p63*, and *p73* genes have been found in the late stages of vertebrate evolution. In early vertebrate lineage, duplication of an ancestral gene most closely related to the *p53* gene was produced, and functioned as a tumour suppressor. A further duplication of the parent *p63*/*p73* hybrid gene arose, creating the *p53* gene, which evolved to have functions of somatic stem cell surveillance of genomic instability and other stressful responses. This led to the functionality of the *p53* gene as a tumour suppressor (Belyi *et al*., 2010). Some of the

p53 family-member-regulated genes themselves have developed sexually dimorphic roles; i.e. *LIF*, *WIP1*, and *MDM2* are estrogen- and p53-regulated genes and *PERP* is a progesterone- and p53/p63-regulated gene that produces a tetraspanin protein important for skin cell adhesion (Attardi *et al.*, 2000; Ihrie *et al.*, 2005; Hu *et al.*, 2007a; Hu *et al.*, 2007b). The structural and functional conservation of the p53 family of transcription factors, consisting of these three sister genes, portrays the key roles of these proteins in biology through evolution.

p53 mutations

p53 is one of the most commonly mutated tumour suppressors in a wide variety of human cancer types (Hollstein *et al.*, 1991). One of the most frequently occurring mutations in cancer is the direct inactivation of the *p53* gene, which occurs in over 50% of all human tumours carry non-functional DNA mutations in the *p53* gene (Sigal & Rotter 2000). The majority of the remaining malignancies exhibit mutations upstream or downstream of the p53 regulatory network which lead to deactivation of the p53 pathway (Olivier *et al.*, 2010). It is believed that more than 80% of tumours have dysfunctional p53 signalling (Hinkal & Donehower 2007). 95% of p53 mutants originating from tumours have a missense mutation, usually a point mutation (commonly found in arginine amino acids 102-292) within the genomic region which encodes its DNA-binding domain (Cho *et al.*, 1994). Of all the mutations that occur in the DNA-binding domain, approximately a third occur within the six "hotspot" residues (Fig. 1.6). This leads to the disruption of the conformation of the DNA-binding domain caused by a single residue change (Ho 2005).

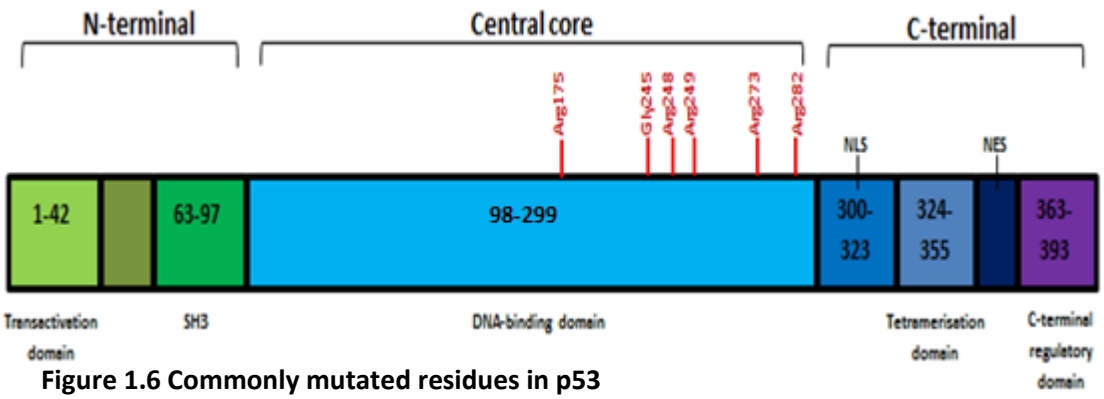


Figure 1.6 Commonly mutated residues in p53
Of all the mutations that occur in the DNA-binding domain, approximately a third occur within the six "hotspot" residues (Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282). This leads to the disruption of the conformation of the DNA-binding domain caused by a single residue change. As a result, p53 mutants have changed or abolished affinity for their target genes, leading to a binding deficit and therefore a deficiency in the sequence-specific transactivation ability of p53, and they may also exhibit oncogenic characteristics.
Adapted from Bode & Dong 2004.

As a result, p53 mutants have changed or abolished affinity for their target genes, leading to a binding deficit and therefore a deficiency in the sequence-specific transactivation ability of p53 (Ozaki & Nakagawara 2010), and they may also exhibit oncogenic characteristics (Sigal & Rotter 2000). While wild-type p53 under normal, unstressed conditions is a very short-lived protein, these missense mutations lead to the production of full-length altered p53 protein with a prolonged half-life (Strano *et al.*, 2007). This is probably due to the fact that the mutated form of p53 is not able to transactivate *MDM2*. Many of these stable mutant forms of p53 can exert a dominant-negative effect on the remaining wild-type allele, serving to abrogate the ability of wild-type p53 to inhibit cellular transformation, particularly when the mutant protein is expressed in excess of its wild-type counterpart (Brosh & Rotter 2009; Oren & Rotter 2010). Such dominant-negative activity may be affected by either formation of mutant or wild-type p53 hetero-tetramers (Chan *et al.*, 2004) or the incorporation of wild-type p53 into mutant p53 supratetrameric aggregates (Xu *et al.*, 2011). In a heterozygous situation, the tumour suppressor function of wild-type p53 is antagonised by mutant p53, in a dominantly negative manner, whereby mutant p53 interferes with the DNA-binding domain of wild-type p53. The first report of this gain-of-function by mutant p53 was the observation that transfection of mutant p53 into p53-null cells enhanced tumour formation in mice (Oren & Rotter 2010). Numerous other studies have confirmed this finding (Dittmer *et al.*, 1993; Van Oijen & Slootweg 2000). These observations, among others, have led to the ‘‘gain-of-function’’ hypothesis, which states that mutation of p53 is not equivalent to simply losing wild-type p53 function; rather, the strong selection for maintained expression of a select group of mutant p53 proteins suggests a positive role for certain p53 mutants in tumourigenesis (Freed-Pastor & Prives 2012).

In Li-Fraumeni patients, sporadic breast tumours are commonly associated with mutations of the Thr81/Pro82 motif, as well as mutations in p53 “hotspot” residues (Berger *et al.*, 2005). A mouse model in which *p53* was disrupted by homologous recombination revealed that although $p53^{-/-}$ mice were developmentally normal but they were very cancer-prone. They were found to be mostly susceptible to the spontaneous development of sarcomas and lymphomas before reaching 6 years of age (Donehower *et al.*, 1992; Attardi & Jacks 1999).

It is now widely acknowledged that *p53* mutations are the most common genetic event in human cancer (Levine & Oren 2009). While at least half of all tumours exhibit mutation of p53, in those that retain wild-type p53, its activity can be attenuated by several other mechanisms. For example, many DNA tumour viruses encode proteins that can inactivate p53; SV40 large T-antigen, adenovirus E1B55kDa protein, and the E6 oncoprotein of human papilloma virus (HPV) types 16 and 18, all bind to p53 and either destabilise the protein or inactivate its function (Levine 2009; Lin *et al.*, 1994).

Transcription initiation by p53

p53 can modulate transcription initiation and elongation at RNA polymerase II (RNA pol II)-transcribed loci. The ability of p53 to stimulate transcription in this way is the most studied function of the tumour suppressor. When chromatin is modified and remodelled (Li *et al.*, 2007), components of the preinititation complex (PIC) are recruited or altered to allow for transcription initiation. Under basal conditions, p53 directs PIC assembly at key target gene promoters, and at other promoters only responding to stress. TFIID is recruited to the promoter's TATA region to nucleate PIC formation, followed by TFIIB, and then by the assembly of the other transcription initiation factors, including TFIIF, TFIIE, and TFIIH, complexed with unphosphorylated RNAPII (Orphanides *et al.*, 1996; Woychik & Hampsey 2002). p53 assists in recruiting several PIC components to the promoter, such as TBP and its associated factors (Seto *et al.*, 1992; Chen *et al.*, 1993; Liu *et al.*, 1993; Thut *et al.*, 1995; Farmer *et al.*, 1996), as well as TFIIA and TFIIH (Ko & Prives 1996; Xing *et al.*, 2001). This process involves the ordered recruitment of histone methyltransferases (HMTs), histone acetyltransferase (HATs), and other co-regulators in the locality of the p53 response elements, to open up chromatin so that RNA Pol II and its associated general transcription factors can bind to the transcription start site of the locus. The most well-described p53-dependent histone modification is acetylation. Once p53 has bound its recognition site, a number of HATs such as CBP/p300 (Avantaggiati *et al.*, 1997; Gu & Roeder 1997; Lill *et al.*, 1997; Scolnick *et al.*, 1997), pCAF (Scolnick *et al.*, 1997; Barlev *et al.*, 2001), GCN5 (Candau *et al.*, 1997), or TIP60 (Gevry *et al.*, 2007), are recruited in a p53-dependent manner, to acetylate the histones within the vicinity of p53 response elements (REs). Importantly, these HATs acetylate both histones, as well as p53, correlating with increased target transactivation (Avantaggiati *et al.*, 1997; Gu &

Roeder 1997; Scolnick *et al.*, 1997). However, acetylation is a later event of transcriptional activation. On DNA damage, first, p53 becomes phosphorylated in its amino-terminal region, and these damage-inducible modifications enhance CBP/p300-mediated acetylation at the C-terminal (Lambert *et al.*, 1998; Sakaguchi *et al.*, 1998). Additionally, MDM2 and MDMX disrupt the interaction between p300 and p53, and therefore hinder p53 acetylation by CBP/p300, by competing with p300 for binding the p53 N-terminal (Grossman *et al.*, 1998; Ito *et al.*, 2002; Wadgaonkar & Collins 1999). When complexed with MDM2, CBP/p300 serves as an E4 ubiquitin ligase for p53 (Grossman *et al.*, 2003), leading to its degradation. These results show that p300 can positively and negatively control p53 function, suggesting that CBP/p300 can regulate the balance from p53 degradation to stabilisation following DNA damage.

Transcription elongation by p53

In response to specific stimuli, p53 can also modulate transcription elongation via functional and physical interactions with various elongation factors. The initiation phase of transcription was originally believed to be the stage at which most regulatory events occur. Recent findings show transcription elongation is just as an important point of control as initiation of transcription (Sims *et al.*, 2004). After RNA Pol II clears the promoter, numerous elongation factors function to repress the stalling and pausing of the polymerase. p53 interacts with many of these factors, such as CDK9 (Claudio *et al.*, 2006; Radhakrishnan & Gartel 2006), FACT (Keller *et al.*, 2001), several components of the mediator complex (Gu *et al.*, 1999; Zhang *et al.*, 2005), and ELL (Shinobu *et al.*, 1999). Many recent studies have shown a role for p53 and its upstream signalling pathways in the stimulus and locus-specific control of transcription elongation (Gomes *et al.*, 2006; Donner *et al.*, 2007; Mattia *et al.*, 2007; Hill *et al.*, 2008; Beckerman *et al.*, 2009). Transcription elongation regulation has arisen as an essential control mechanism in the fine-tuning of the p53 response.

The regulation of p53

p53 protein levels are regulated by several methods. These include post-translational control of the p53 protein half-life, allosteric regulation as a result of post-translational modifications, and by binding of key viral proteins, the most famous being SV40. These have all been shown to impact on the regulation of the p53 pathway responding to various stress stimuli (Bai & Zhu 2006).

Genotoxic stress - DNA damage signalling

The p53 protein responds to numerous stress signals, including DNA damage. These detected signals are then conveyed to p53 through various enzymes that bring about post-translational modifications to p53. In turn, this leads to an increased p53 half-life resulting in p53 protein accumulating in cells (Appella & Anderson 2001). The first stress type discovered to activate p53 was DNA damage (Efeyan & Serrano 2007). The progression of several human cancers can be enhanced by exposing to DNA damaging agents, including gamma radiation, ultraviolet light, chemotherapeutic agents, and chemical carcinogens (Liu & Kulesz-Martin 2001; Maltzman & Czyzyk 1984), or endogenously-derived processes associated with replication and metabolism (De Bont & Van Larebeke 2004). Premature termination of replication fork advancement results in replication stress, which may lead to fork breakdown and damaged DNA (Branzei & Foiani 2010). Also, reactive oxygen species (ROS), derivatives of normal oxidative metabolism, may result in nucleotide oxidation (Achanta & Huang 2004). DNA damage may affect the integrity of the genome by generating double-strand breaks and by introducing mutations in DNA, leading to potential rearrangements in the genome or loss of genetic information (Khanna & Jackson 2001). It has been shown that the DNA damage signalling response is constantly stimulated to prevent advancement in human cancer, in premalignant lesions. This implies that transformation on a cellular level is producing constant damage to DNA. This kind of DNA damage in cancer cells can be a result of high ROS turnover, as well as replication stress in DNA originating from abnormal firing of DNA replication origins (Burhans & Weinberger 2007). The response to DNA damage converges on p53 via the initiation of post-translational modifications, and a reduction in p53 turnover (Appella & Anderson 2001).

Stability control of p53

Dis-regulated p53 activity can be harmful to the viability of the cell and therefore the organism. Thus there are several mechanisms to help maintain p53 levels in check, including post-translational modifications to MDM2 and p53, which disturb the MDM2-p53 interaction. For example, continuous mono- or polyubiquitination by MDM2, whose overexpression is seen in various cancers, is synergistically enhanced by oncogenic MDMX (Badciong & Haas Arthur 2002). Polyubiquitinylation of p53, unlike mono-ubiquitinylation, leads to its degradation by 26S proteasome in the cytoplasm (Waning *et al.*, 2010). Monoubiquitination of p53 occurs when MDM2 levels are low, and p53 is therefore transported into the cytoplasm for additional ubiquitinylation and subsequent degradation. However, a large fraction of mono-ubiquitinylated p53 is targeted to the mitochondria. p53 undergoes deubiquitination by HAUSP which renders p53 active to carry out its apoptotic function in mitochondria. High levels of MDM2 result in polyubiquitination of p53. This procedure also involves various proteins including Yin Yang 1 (YY1) transcription factor, p300, and gankyrin (Brooks & Gu 2004). In addition to ubiquitinylation, the ubiquitin-like protein NEDD8 associates with Lys370, Lys372, and Lys373 of p53. This is mediated by MDM2, in order to inhibit p53 transcriptional activity (Xirodimas *et al.*, 2004). The association between MDM2 and HDAC1 facilitates the deacetylation of p53 and therefore its degradation (Ito *et al.*, 2002). p53 is quickly stabilised and accumulated in the cell by MDM2 inhibition and degradation in response to stress signals via numerous mechanisms, such as the sequestering of MDM2 by the ARF tumour suppressor to the nucleolus. As a result of stress signals, ARF tumour suppressor positively controls the stability and activation of p53. The interplay between YY1 and DM22 is disrupted and MDM2 is sequestered to the nucleolus by ARF, leading to enhanced p53 stability, as a result of reduced MDM2 levels (Sui *et al.*, 2004; Zhang *et al.*, 1998). In contrast, high p53 levels are also implicated in a negative feedback loop with ARF, reducing ARF levels and increasing MDM2 binding to *p53*. Stress signals activate ATM/CHK2 and ATR/CHK1 kinases to phosphorylate p53, MDM2, and MDMX. In contrast to p53, phosphorylation of MDM2 results in its de-stabilisation. Hence, p53 is stabilised by inhibition of MDM2 binding to *p53*, as a result of phosphorylation of p53 at Ser15 by Ataxia-telangiectasia mutated kinase (ATM) and at Ser20 indirectly by CHK2. The Ser15, Thr18, and Ser20 residues are

situated in the regions where p53 binds to both MDM2 and MDMX. Relying on early Ser15 phosphorylation, Thr18 and Ser20 phosphorylation take place later on following DNA damage, and is believed to encourage stabilisation of p53 by impeding p53 and MDM2 interplay. Phosphorylation at Ser15 has been shown to augment CBP/p300 interactions, as well as possibly having an effect on the binding of MDM2. Phosphorylation of Ser20 by CHK2 is essential for encouraging the dissociation of MDM2 from the MDM2/p53 complex, further activating and stabilising the tumour suppressor (Appella & Anderson 2001). p53 stabilisation is additionally enhanced by MDM2 auto-ubiquitination leading to degradation of phosphorylated MDM2 and MDMX, as well as a reduced HAUSP association (Alarcon-Vargas & Ronai 2002). USP10, a newly discovered ubiquitin-specific protease and regulator of p53, impedes the MDM2 and p53 interplay, by provoking p53 de-ubiquitination (Yuan *et al.*, 2010). p53 directly activates the transcription of the *MDM2* gene at its promoter by binding to two adjacent p53-responsive elements, which in turn, targets p53 for degradation. This provides a negative autoregulatory feedback loop to control *p53* expression and restrain p53 activity in normal cells, in the absence of stress (Moll & Petrenko 2003). In contrast, *MDMX* is not transcriptionally regulated by p53 and its regulation is mostly via HAUSP interactions (Lenos & Jochemsen 2011). Comparable to MDM2, p53-inducible and PIRH2 E3 ubiquitin ligases are involved in a similar negative feedback loop with p53 (Fig. 1.7) (Moll & Petrenko 2003).

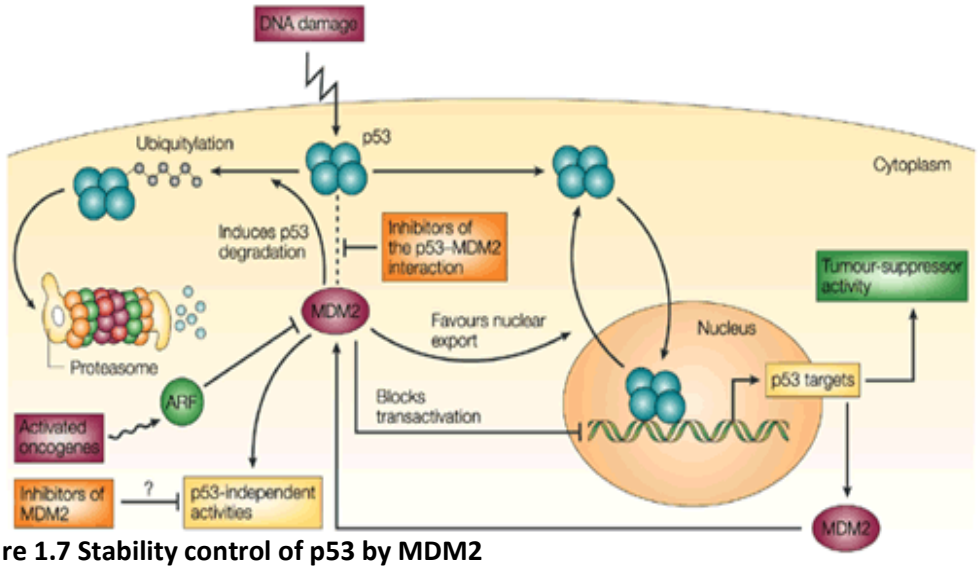


Figure 1.7 Stability control of p53 by MDM2
Under normal physiological conditions, MDM2, which has an intrinsic E3 ubiquitin protein ligase activity, negatively controls p53 protein levels and sustains it at minimal levels in an inactive state when p53 is not needed. On the contrary, p53 is quickly stabilised and accumulated in the cell by MDM2 inhibition and degradation in response to stress signals via numerous mechanisms, such as the sequestering of MDM2 by the ARF tumour suppressor to the nucleolus. As a result, ARF tumour suppressor positively controls the stability and activation of p53.
Taken from Chène 2003.

Subcellular localisation

While a loss of p53 function by inactivating mutations was the most extensively studied (Fojo 2002), a relatively recently emerged area in the p53 field is its regulation through intracellular localisation. In the 1980s it was believed that in normal, unstressed cells, p53 was mostly cytoplasmic and its localisation was dependent on MDM2 shuttling between the cytoplasm and the nucleus (Liang & Clarke 2001). p53 is usually found in the nucleus of transformed cells with stress (Rotter *et al.*, 1983), where it principally exerts its role as a transcription factor (Ozaki & Nakagawara 2011). For p53 to function in growth inhibition (Prabha *et al.*, 2012) and apoptosis (Haupt *et al.*, 2003), its nuclear import and retention is essential (O'Brate & Giannakakou 2003). The aberrant localisation of p53 to the cytoplasm results in it being non-functional and is often found in specific human tumour cells, such as neuroblastoma, colorectal and breast carcinomas, and retinoblastoma (RB) (Nikolaev & Gu 2003). For nucleo-cytoplasmic transport to occur, nuclear localisation signals (NLS) and nuclear export signals (NES) are required (O'Keefe *et al.*, 2003). A cluster of three NLSs in the C-terminus of p53 have been identified that mediate the migration of p53 into the nucleus of the cell (Nikolaev & Gu 2003). The most active of the NLSs in directing nuclear export encompasses the amino acids 316-322 of p53 (Ostermeyer *et al.*, 1996); the remaining NLSs incorporating amino acids 370-384 are not as important for nuclear export (Shaulsky *et al.*, 1990). The two nuclear export signals of p53 encompass amino acids 11-27 and 340-351 (Thompson *et al.*, 2005). p53 oligomerisation has been shown to control nuclear export by having an effect on the accessibility to these NESs (Liang & Clarke 2001). Also, interactions with the molecular motor dynein and network of microtubules are crucial for p53 nuclear import (Moseley *et al.*, 2007).

Post-translational regulation of p53

The importance of a specific modification for regulating p53 function is not clear as there are numerous conflicting reports. Responding to numerous cellular stresses, induction and activation of p53 in the nucleus is regulated both by protein stabilisation and transcriptional competency. Both these tasks are largely exerted through multiple post-translational modifications (Fig. 1.8). As well as occurring in response to stress, these post-translational modifications take place to maintain homeostasis (Karve &

Cheema 2011). Over 36 differing amino acids have been revealed to be altered in p53 by post-translational modifications, and following the triggering of differing pathways upstream, these modifications are the resulting product. The majority of these covalent post-translational modifications occur at both the N- and C-termini of p53. These post-translational modifications influence p53 stability and activation, and may therefore enhance its capacity as a transcription factor. There is increasing evidence that differing post-translational modifications may have an influence on each another. The most well-known post-translational modifications that occur in p53 include serine and threonine phosphorylation, and lysine acetylation, ubiquitination, methylation, sumoylation, and neddylation (Meek & Anderson 2009).

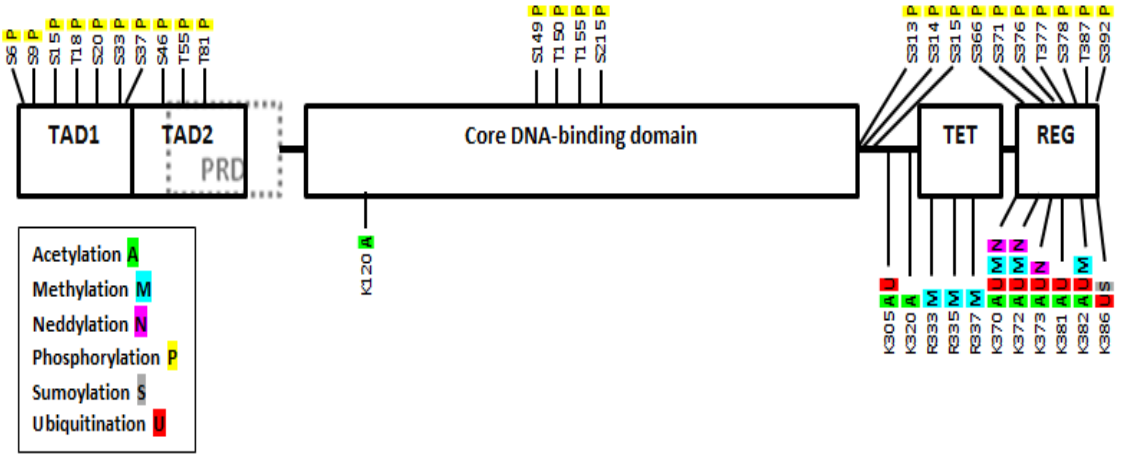


Figure 1.8 Post-translational modifications to p53
Over 36 different amino acids are altered in p53 by post-translational modifications, and the majority of these covalent post-translational modifications occur at its N- and C-termini. The most well-known post-translational modifications that occur in p53 include serine and threonine phosphorylation, and lysine acetylation, ubiquitination, methylation, sumoylation, and neddylation.
Adapted from Maclaine & Hupp 2009.

Of all modifications to p53, N-terminal phosphorylation has been studied the most as well as being the best described, and it has been shown to be a key player in the stabilisation of p53. In response to numerous stress types, including DNA damage, all threonines and serines of the N-terminal of p53 in its first 89 residues are phosphorylated or dephosphorylated. Following on from DNA damage, p53 phosphorylation occurs via several kinases, such as ATM, ATR, CHK1, CHK2, CK1, CK2, ERK, JNK, and p38. The best characterised phosphorylations of p53 occur at its N-terminal Ser15, Ser20 and Ser46, and Thr18 in its transactivation domain. ATM

phosphorylates p53 at Ser15, leading to p53 stabilisation and activation. Ataxia-telangiectasia and Rad-3 related kinase (ATR) sustains the phosphorylation of Ser15 for several hours. p53 has a higher transcriptionally activity following on from Ser15 phosphorylation, leading to its induction of cell cycle arrest and apoptosis. p53 has a short half-life of around 30 minutes. The half-life of p53 is increased around 7-fold to 200 minutes after DNA damage, leading to p53 accumulation in cells (Appella & Anderson 2001).

It has been shown that p53 phosphorylation on Ser15, Thr18, and Ser20 triggers its subsequent acetylation (Appella & Anderson 2001). HIPK2 is the best described kinase to phosphorylate p53 at Ser46, which is believed to help specify the promoter binding activity by p53 and in pro-apoptotic gene induction, and therefore provoke cell death (Hofmann *et al.*, 2002; Puca *et al.*, 2009). Phosphorylation here also occurs through ATM and it is essential for CBP/p300-induced acetylation of Lys382 in p53. Ser46-phosphorylated p53 has been shown to be liberated from sites of double-strand breaks in order to induce pro-apoptotic genes (Smeenk *et al.*, 2011). In contrast, it has been shown that p53 can be stabilised regardless of its phosphorylation. Nutlins stabilise unphosphorylated p53, which executes its role as a transcription factor just as effectively as its phosphorylated form, showing that if certain p53 modifications are lost, other mechanisms can compensate for this (Thompson *et al.*, 2004). On DNA damage, various histone acetyltransferases, including PCAF and CBP/p300, provoke p53 acetylation at numerous lysine residues. p53 oligomerisation occurs prior to its acetylation and provides docking sites in the C-terminal of p53 for these acetyltransferases (Itahana *et al.*, 2009). It is believed that the lysine residues fine-tune p53 reactions to stress. It has been shown that p53 was the first non-histone protein to undergo acetylation and deacetylation, provoked by cellular stress, including DNA damage. p53 acetylation, combined with its phosphorylation, leads to full activation of p53 as a transcription factor, by enhancing co-factor recruitment. p53 acetylation augments its stability, DNA binding ability, and transcriptional activity. Stress signals are linked with target gene promoters bound with acetylated p53, further illustrating the effect of acetylation on the transcriptional activity of p53. It is believed that p53 acetylation reduces its levels of ubiquitination by competition between acetylation and ubiquitination. Acetylation of p53 by CBP/p300 and PCAF occurs at the C-terminal

lysine residues 372, 373, 381, and 382, and 320 respectively, which are also all ubiquitinylation sites. As a consequence, acetylation of p53 increases p53 stability, by hindering degradation by proteasomes (Li *et al.*, 2002). Deacetylation by SIRT1 or HDAC1 aids in the downregulation of both p53 stability and activity (Brooks & Gu 2003).

Newer p53 modifications that were discovered more recently include methylation, sumoylation, and neddylation, and modify lysine residues. It has been shown that on DNA damage, methylation of p53 is essential for its successive acetylation, leading to p53 protein stabilisation (Ivanov *et al.*, 2007). There are two main locations where p53 methylation takes place, which result in contrasting p53 functionality. SET7/9 (SET9) methyltransferase, has been shown to methylate histones as well as non-histone proteins including p53. Methylation of p53 by SET9 at Lys372 in its C-terminus leads to p53 stabilisation, containing it in the nucleus, and transcriptional activation (Chuikov *et al.*, 2004). On the contrary, methylation of p53 at Lys370 by SMYD2 methyltransferase inhibits p53 activity on a transcriptional level. On DNA damage, methylation at Lys372 leads to the inhibition of Lys370 methylation, and therefore allowing p53 to bind DNA (Huang *et al.*, 2006). p53 methylation has also been shown to fine-tune p53 binding to DNA and aid in p53 protein-protein interactions. Histone LSD1 demethylase, which is lysine-specific, also provokes repression of p53 transcriptional activation (Dai & Gu 2010). SUMO1, which is a ubiquitin-like protein, becomes covalently linked to Lys386 to bring about p53 sumoylation. It has been shown that there is interplay between acetylation and sumoylation in the regulation of p53 DNA binding and therefore transcriptional activity. Sumoylation has been shown to both induce and repress p53 transcriptional activity. p53 sumoylation hinders its consequent acetylation by p300. Sumoylation of p53 by UBC9 E2 ligase at Lys386 inhibits the sequence-specific DNA binding ability of p53, which therefore leads to a loss in its transcriptional activity (Wu & Chiang 2009). Neddylation has been shown to occur on Lys370, Lys372, and Lys373 p53 residues which are also all ubiquitinated (Xirodimas *et al.*, 2004).

<u>p53-based cancer therapy</u>

Normal cells possess tight regulatory mechanisms that enable p53 to maintain cellular homeostasis without impeding normal growth and function. These regulatory mechanisms are disrupted in cells within most cancers that lack wild-type p53 activity.

These observations led to the notion that sudden reactivation of p53 may trigger lethality or permanent growth arrest in p53-deficient cancer cells, and might lead to the development of successful anticancer treatments. The almost universal loss of p53 activity in tumours has spurred an enormous effort to develop new cancer treatments based on this fact, and it represents a vital cellular drug target (Lane *et al.*, 2010). As the majority of human cancers exhibit alterations in p53, so the idea of restoring p53 for cancer therapeutics is very appealing. p53 regulation for cancer therapy has been an active area of research for years. Several animal models have shown that activation of the p53 response in the most advanced tumours can be curative. Wild-type p53 reactivation has resulted in significant regression of tumours, such as lymphoma (Martins *et al.*, 2006; Ventura *et al.*, 2007) and hepatocarcinoma regression (Xue *et al.*, 2007).

In 1996, the first gene therapy based on p53 was used. The wild-type *p53* gene, contained in a retroviral vector controlled by an actin promoter, was administered intravenously into non-small cell lung tumours (Roth *et al.*, 1996). The p53 protein level is raised in many tumours by virtue of an increase of the protein's half-life and this tumour specific alteration in p53 processing has attracted tumour immunologists, who are now testing a number of p53-based vaccines conjugated to toxins to selectively kill cells with overexpressed mutant p53 in cancer patients (Buonaguro *et al.*, 2011).

Gendicine was the first type of gene therapy approved for use clinically in humans. Several studies demonstrated that delivery of recombinant adenovirus engineered to express wild-type p53 (rAd-p53), such as Gendicine, triggers a dramatic apoptotic response in cell culture and tumour regression in rodents with subcutaneously and orthotopically implanted cancers, e.g. gliomas. The adenovirus is engineered for treating patients with tumours that have mutated p53 genes. The clinical potential of Gendicine therapy has been approved by the Chinese State Food and Drug Administration for the treatment of head and neck squamous cell carcinoma. The US equivalent of Gendicine, Advexin, employs the same gene strategy and has identical cancer targets. It has shown activity in number of clinical trials and it was tested in phase I studies for the treatment of patients with recurrent malignant gliomas (Räty *et al.*, 2008).

A different approach had entered early phase I/II clinical trials, employing a vaccination of p53-derived peptides. Due to overexpression and frequent mutation of p53 in human cancers, such as colorectal cancer, p53 is immunogenic, as cancer patients produce p53 antibodies and p53-reactive $CD4^+$ and $CD8^+$ T cells. Therefore, p53 may serve as a target for immunotherapy. The most advanced clinical trials validation includes a vaccine consisting of a mixture of long synthetic p53-derived peptides, p53-SLP, for the treatment of metastatic colorectal cancer. The immunogenicity and safety of the p53-SLP vaccine was also investigated in patients suffering with the same cancer (Speetjens *et al.*, 2009). In 90% of patients p53-specific immune responses were detected (Cheok *et al.*, 2011).

p53 gene mutations is associated with small cell lung cancer (SCLC), leading to overexpression of the tumoral protein and recognition by p53-specific cytotoxic T cells. INGN-225, an adenovirus-transduced dendritic cell-based p53-modified vaccine (Ad. p53-DC) against SCLC, has also undergone trials. INGN-225 triggered a p53-specific immune response in around 40% of SCLC patients, who were subsequently sensitised to chemotherapy (Chiappori *et al.*, 2010).

Another approach of p53-targeted therapy is to restore wild-type function of mutant p53 protein by small molecules. PRIMA1 was identified as a compound that specifically inhibited the growth of p53 mutant tumour cells, but restores the sequence-specific DNA binding ability of mutant p53, as well as its active conformation. PRIMA1 also triggers the mitochondria-dependent intrinsic apoptosis program in human tumour cells via activation of caspase 2. Xenograft studies using lung and osteosarcoma cell lines have documented potent antitumourigenic activity of PRIMA1 alone or in combination with cisplatin, without adverse systemic side effects (Bykov *et al.*, 2002).

Other small molecules can be used to restore the p53 function in cancer cells. In this respect, it was shown that MDM2 could be targeted in order to stabilise p53, for instance by nutlins, to antagonise the interplay between p53 and MDM2 (Vassilev *et al.*, 2004). Importantly, nutlin 3 shows synergism with conventional chemotherapeutic drugs, including doxorubicin and cytarabine, radiation, TRAIL, and inhibitors of XIAP, γ-secretase, CDKs, JNK, PI3K, and aurora kinases, pointing to the possibility of combinatorial therapies for the treatment of advanced stage blood, brain and bone cancers. Importantly, p53 inhibition with small molecules may safeguard normal cells

through radiation therapy or genotoxic chemotherapy to dampen severe side effects of inflammation (Komarov *et al.*, 1999).

Despite intensive research and new drug discoveries, the conventional p53-based therapeutic strategies have met with limited success. Numerous obstacles have been identified, including unwanted side effects in normal tissues and the development of p53-resistant tumours. As pointed out by Desilet *et al.*, whether p53 will fulfil its promise of playing a leading role in cancer therapy remains to be seen.

p53 target gene products

In normal unstressed cells, the p53 protein is maintained at low steady-state levels that restrict its impact on cell fate. The first kind of stress uncovered as activating the p53 pathway was DNA damage (Zhang *et al.*, 2011). Genotoxic stress triggers a series of posttranslational modifications on p53 that contribute to its stabilisation, nuclear accumulation and biochemical activation. p53 responds to a variety of cellular stress signals including DNA damage, hypoxia, ribonucleotide triphosphate depletion, mitotic spindle damage, nutritional starvation, ribosome biogenesis inhibition, metabolic deficiency, telomere erosion, viral infection and deregulated oncogene activation (Anderson *et al.*, 2011). Activated p53 functions through the regulation of the transcription of its target genes. These target gene protein products are the last executors to induce reversible cell cycle arrest, senescence, or apoptosis, which are all implicated in tumour suppression (Fig. 1.9). Importantly, this type of regulation provides a coordinated manner to execution of the gene expression programme. For example, p53 binds more avidly to promoters of cell cycle and DNA repair genes compared to the promoters of pro-apoptotic genes (Vousden & Lu 2002). Therefore if high levels of genotoxic stress persists and cannot be repaired, then the p53-dependent apoptotic or senescence response is induced. The functionality of p53 in this way helps to protect against neoplasia. On the other hand, p53 stimulates protecting, pro-survival responses including DNA repair, protective antioxidant protein production due to amplified ROS levels, and temporary cell cycle arrest in order to maintain the integrity of the genome in cells that encounter damage that is at a low level and that can be repaired. Cell cycle arrest allows DNA to be repaired to permit cell survival (Pellegata *et al.*, 1996). p53 has also been shown to regulate additional cellular processes such as autophagy, fertility, metabolism, and differentiation of stem cells (Spike & Wahl 2011). Aside from its role in cancer, p53 is also implicated in other diseases including atherosclerosis, Huntington's disease, and Parkinson's disease (Gudkov & Komarova 2010).

Cell cycle arrest genes

As mentioned earlier, the p53 protein suppresses tumour formation not only by inducing apoptosis but also by causing cell cycle arrest. On DNA damage, p53 stops cell cycle progression at G1/S and G2/M phases (Ceribelli *et al.*, 2006; Kastan & Kuerbitz

1993). G1 cell cycle arrest is mainly mediated by the upregulation of the cyclin-dependent kinase inhibitor *p21*^{WAF1} (Agami & Bernards 2000), which was the first transcriptional p53 target identified (Bieging & Attardi 2011), and this process is well known and has been extensively studied. In this way, G1 arrest is elicited due to augmented *p21* ^{WAF1} because of inhibition of the cyclins D and E (Bartek & Lukas 2001). Cell cycle arrest genes regulated by p53, whose protein products are involved in G2/M transition following DNA damage, include *p21*^{WAF1}, *GADD45*, and *14-3-3σ* (Luk *et al.*, 2012). *p21*^{WAF1} provokes G2 arrest by the blockage of PCNA at replication forks during DNA synthesis and repair (Soria *et al.*, 2008). p53 can block cell entry into mitosis by inhibition of CDC2. CDC2 needs to bind to cyclin B1 in order to function. Repression of cyclin B1 by p53 also arrests cells in G2. The G2 arrest is mostly executed by *GADD45* and *14-3-3σ* cooperatively, where *14-3-3σ* impedes CDC2 and cyclin B1 nuclear import by sequestering them into the cytoplasm (Hermeking 2003), while *GADD45* disrupts CDC2/Cyclin B complexes (Jin *et al.*, 2000). It is still debated whether p53 is always present at certain promoters, such as *p21*^{WAF1}, or if it only binds upon genotoxic stress (Barlev *et al.*, 2001; Espinosa & Emerson 2001).

Cellular senescence also plays an important role in p53-mediated tumour suppression, and it can be defined as permanent cell growth arrest. Cellular senescence provokes ATM and ATR to activate cell cycle checkpoints through p53 and CHK1/CHK2, with the aid of RB, p16, and p21. p53 induces p21 and growth arrest soon after senescence stimulation, and the maintenance of growth arrest is by p16 (Larsson 2005). A vital regulator of p53 which is implicated in senescence is PML, which promotes acetylation of p53 by p300 at Lys382 following oncogenic *RAS* expression. Inhibition of p53- and PML-induced senescence is brought about by SIRT1, a member of NAD-dependent Sirtuins protein family, which de-acetylates p53 (Yi & Luo 2010). Oncogenic *RAS* expressed in human and rodent primary cells results in cellular senescence, which is due to the accumulation of p53 and tumour suppressor p16 (Serrano *et al.*, 1997). Cellular senescence induced by p53 is important not only for cancer prevention but also for the anti-cancer effect induced by any wild-type p53 introduced in established tumours (Suzuki & Matsubara 2011).

DNA repair genes

Recently the mismatch repair genes *PMS2* and *MLH1* have been shown to respond to the activation of p53 following DNA damage (Chen & Sadowski 2005). p53 protein directly participates in DNA repair (Offer *et al.*, 2002), for example the final 30 amino acids in the C-terminus of p53 can identify various structures subjected to DNA damage, including insertion/deletion mismatches and free DNA ends (Liu & Kulesz-Martin 2001). p53 has also been shown to have a direct role in base excision repair (BER), nucleotide excision repair (NER), and double strand break repair (Zhang *et al.*, 2009). Nucleotide excision repair (NER) is transcriptionally regulated by p53, possibly by easing access to chromatin to the repair machinery (Rubbi & Milner 2003), whereas p53 regulates base excision repair (BER) via interactions with BER-specific factors (Adimoolam & Ford 2003). p53 has also been shown to inhibit homologous recombination as a result of replication fork stalling. In this situation, p53 impedes double-strand break accumulation (Janz & Wiesmüller 2002). Blockage of DNA replication leads to p53 phosphorylation at Ser15 and its association with essential homologous recombination enzymes (Dmitrieva *et al.*, 2001). Once DNA repair is accomplished, then cells go back into the normal cell cycle (Offer *et al.*, 2002). If damage to DNA is beyond the capacity of cells to repair then powerful stress signals can drive p53 to initiate apoptosis, or cellular senescence via transcriptionally activating p21 (Hinkal *et al.*, 2009).

Apoptotic genes

One of the most dramatic responses to p53 activation is the induction of apoptosis. The regulation of p53-dependent apoptosis is complex and numerous reports have described the mechanism by which p53 induces apoptosis. The cue of events can be divided into three parts: what signals p53 to induce apoptosis, the mechanisms involved, and what the downstream effects are. p53 triggers apoptosis to prevent stressed or damaged cell outgrowth, which could progress to cancer (Haupt *et al.*, 2003). Apoptosis occurs through either one of two major pathways described as either the intrinsic mitochondrial or extrinsic death receptor pathway (Kroemer *et al.*, 2007). The transcriptional induction of *p53* mostly leads to up-regulation of pro-apoptotic members of the BCL2 family BAK, BAX, PUMA, NOXA, and BID. Their protein products induce the depolarisation of mitochondria, from which cytochrome C is released into

the cytoplasm. Cytochrome C forms a complex with APAF1 to make up the apoptosome, which activates a cascade of several caspases (caspases 3, 6, 7 and 9) which bring about apoptosis (Haupt *et al.*, 2003). Caspase 6 has been shown to provoke p53 phosphorylation at Ser46 in order to enhance the pro-apoptotic activity of p53 (Mayo *et al.*, 2005). For many years it was thought that, like induction of cell cycle arrest, induction of apoptosis by p53 was due solely to the transactivation of pro-apoptotic genes. However, transactivation-independent p53-dependent apoptosis does occur in tumour cells (Fig. 1.14). It is at the mitochondria where p53 also physically impedes the functions of members of the BCL2 family of proteins BCL2 and $BCLX_L$, preventing them from hindering BAK and BAX oligomerisation. BAK and BAX oligomerisation results in a transmembrane pore required for cytochrome C release from the mitochondria. p53 complexes with BAD to encourage apoptosis, by activation and oligomerisation of BAK (Haupt *et al.*, 2003). Also, p53 directly acts on BAK which allows its dissociation from BCL2 family member MCL1 (Gélinas & White 2005). *PUMA* (also known as *BBC3*) is another p53-induced gene, whose transcription leads to activation of BAX, by releasing p53 from its inhibitory complex with $BCLX_L$ (Ming *et al.*, 2006). The importance of PUMA and NOXA to p53-mediated apoptosis became obvious when it was reported that certain cell types from *PUMA* knockout mice showed almost complete impairment to apoptosis via p53 (Jeffers *et al.*, 2003). In other cells, apoptosis was only partially affected, and NOXA appeared to contribute to this effect. Activation by p53 of the extrinsic pathway occurs by death receptor transcription. The death receptors PIDD, PERP, KILLER/DR5, DR4, and FAS, situated on the plasma membrane, cooperate to respond to cell death signals. These death receptors of the tumour necrosis factor receptor family form complexes with their cognate ligand, which also activates a cascade of various caspases (caspases 3 and initiator caspase 8) to eventually induce apoptosis. p53 overexpression enhances cell surface levels of FAS by promoting its trafficking from the Golgi complex (Bennett *et al.*, 1998). In addition, p53 activates DR5, the death domain-containing receptor for TRAIL; DR5 is induced in response to DNA damage (Wu *et al.*, 1997) and, in turn, promotes cell death through caspase 8. The transactivation of BID by p53 presents a link between the intrinsic and extrinsic pathways, as initiation of the extrinsic pathway promotes BID activation by caspase 8, then it is translocated to the mitochondria to

activate BAX and therefore initiate the intrinsic pathway. Alternatively, p53 can also trigger apoptosis by repression of anti-apoptotic genes, such as *SURVIVIN,* thus promoting caspase activation (Hoffman *et al.*, 2002). Higher p53 levels result in apoptosis, and therefore mask the cell-cycle response (Vousden & Lu 2002).

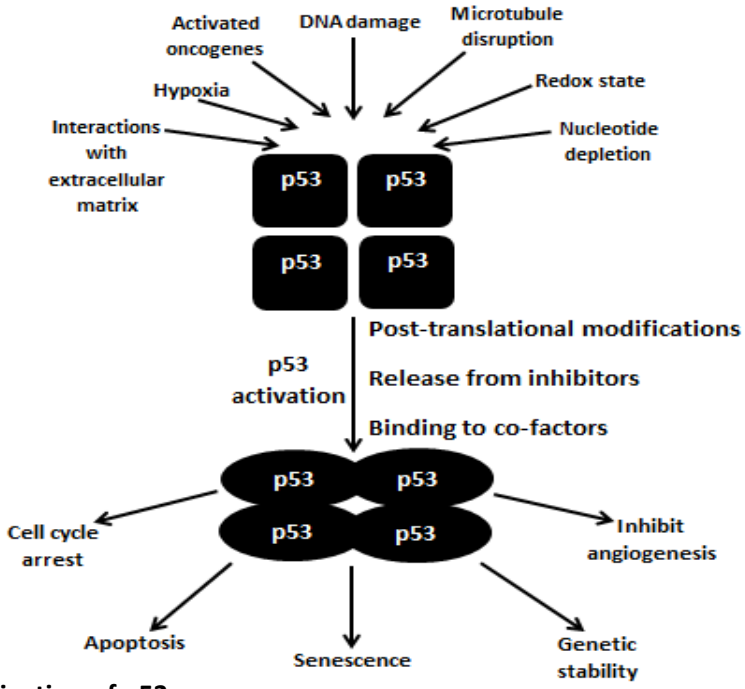


Figure 1.9 Activation of p53
Genotoxic stress triggers a series of posttranslational modifications on p53 that contribute to its stabilisation, nuclear accumulation and biochemical activation. p53 responds to a variety of cellular stress signals including DNA damage, hypoxia, ribonucleotide triphosphate depletion, mitotic spindle damage, nutritional starvation, ribosome biogenesis inhibition, metabolic deficiency, telomere erosion, viral infection and deregulated oncogene activation. Activated p53 functions through the regulation of the transcription of its target genes. These target gene protein products are the last executors to induce reversible cell cycle arrest, senescence, or apoptosis, which are all implicated in tumour suppression.
Adapted from www.breast-cancer-research.com.

p53 and microRNAs

Recently, it has been demonstrated that miRNAs play an instrumental role in the p53 network. p53 has been shown to regulate the transcription of miRNA genes, which also aid in the processes of cell cycle arrest and apoptosis. Both p53 and miRNAs are implicated in human disease, particularly cancer, and this kind of cross-regulation adds another level of complexity to cellular responses. The determination of the functions of the p53 pathway in cancer could be clarified by identifying regulatory associations

between p53 and miRNAs, and the downstream targets of the latter (Schetter *et al.*, 2010; Feng *et al.*, 2011).

MicroRNAs

Over the past decade, it has become progressively more clear that a large class of small-noncoding RNAs, known as miRNAs, function as important regulators of a wide range of cellular processes by modulating gene expression. Within 10 years of research, we have gone from discovering the existence of miRNAs in mammals to exploring their therapeutic applications in numerous diseases. Recently we have begun to understand the scope and diversity of these regulatory molecules. Of all the small non-coding RNAs, miRNAs are the best described. miRNAs are a group of small (20-25 nucleotides) non-coding RNA molecules which are expressed endogenously in cells and that regulate gene expression at the level of post-transcription. Within an RNA-induced silencing complex (RISC) complex, miRNAs hybridise to complementary sequences in the 3'-untranslated regions (UTRs) of target protein-coding messenger RNAs (mRNAs) via their 5' proximal 'seed' region (nucleotide positions 2-7) by sequence-specific base pairing (Fig. 1.10), resulting in mRNA destabilisation or translation inhibition, leading to gene silencing (Bartel 2004).

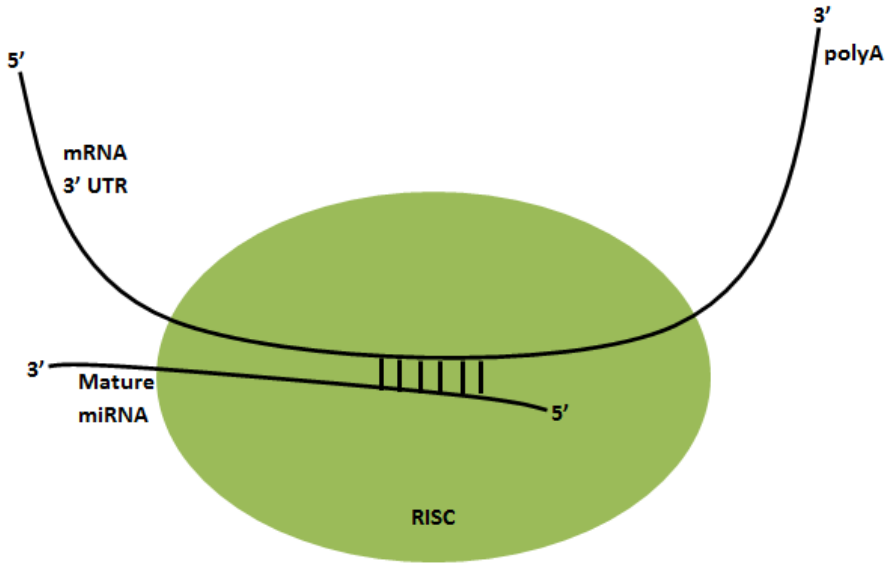


Figure 1.10 Mechanism of action of microRNAs
Within a RISC complex, miRNAs hybridise to complementary sequences in the 3' UTRs of target protein-coding mRNAs via their 5' proximal 'seed' region by sequence-specific base pairing, resulting in mRNA destabilisation or translation inhibition, leading to gene silencing.
Adapted from www.dzne.de.

The discovery of microRNAs

The discovery of miRNAs and the identification of its role in cancer pathogenesis provide promise in improvements to cancer management. In 1993 the first miRNA was discovered by Ambros, Feinbaum, and Lee. The group were conducting studies in *Caenorhabditis elegans* on post-embryonic development and temporal control abnormalities. They uncovered that the *lin-4* gene in *C.elegans* produced non-coding RNA transcripts that regulate post-embryonic development timing by translational repression of lin-14 (Lee *et al.*, 1993). Furthermore, the RNA sequence of lin-4 was shown to have partial complementary to the lin-14 3'UTR, which once bound, regulates the expression of lin-14 by protein synthesis control. Let-7 was the next miRNA to be uncovered in a study carried out in *C. elegans*. Let-7 was shown to regulate the developmental progression from the L4 larval stage to the adult stage. Let-7 was shown to hinder lin-41 and lin-57 protein synthesis using the same mechanism as lin-4. In contrast to lin-4, let-7 is conserved in metazoan evolution, and let-7 was also found to be conserved in many species, indicating the existence of a wider phenomenon. Since the discovery of let-7, thousands of miRNAs have been identified in organisms as diverse as viruses, worms, and primates through random cloning and sequencing or computational prediction. The identified miRNAs are currently curated and annotated at miRBase, hosted by the Sanger Institute as a repository open to the public (He & Hannon 2004). Soon after, around 100 miRNAs were reported to have been discovered in differing laboratories (Bartel 2004). In the last 10 years, over 4000 miRNAs have been discovered in a wide variety of species, including 150 in *C. elegans* and 150 in *Drosophila melanogaster* (Ibáñez-Ventoso *et al.*, 2008). More than 700 miRNAs have been identified in humans to date and around 1000 are predicted to exist (Li *et al.*, 2009). miRNAs can control the expression of hundreds of protein-coding genes and therefore regulate various cellular regulatory pathways, including apoptosis, development, differentiation, metabolism, and proliferation (Bueno *et al.*, 2008). Due to their capacity to target and regulate the expression of several mRNAs, approximately a third of protein-coding genes and most genetic pathways are regulated by numerous miRNAs (Berezikov *et al.*, 2005; Lim *et al.*, 2005; Griffith-Jones *et al.*, 2006). This clearly emphasises the key role that miRNAs play in regulating gene expression. In closely related species, almost all miRNAs are

conserved and distant species are often the source of several miRNA homologs. This further implies an evolutionary conservation of miRNAs with regards their function through animal lineages (Berezikov 2011).

Genomic location of microRNAs

It was initially thought that most miRNA genes were located in intergenic regions (Macfarlane & Murphy 2010). However, recent analyses of miRNA gene locations showed that the majority (70%) of mammalian miRNA genes (161 out of 232) are located in defined transcription units (TUs). Using a combination of up-to-date genome assemblies and expressed sequence tag (EST) databases, Rodriguez *et al.* demonstrated that many miRNA genes (117 out of 161) were found in the introns in the sense orientation, which is more than previously expected. It has been shown that human miRNA genes are specifically located in all chromosomes but the Y chromosome. The genomic location of miRNA genes can help to categorise them. Intronic miRNAs are located either in protein-coding transcription units (60%) or in non-coding transcription units (20%), and exonic miRNAs are found in non-coding transcription units (20%) (Kim & Nam 2006). The majority of miRNAs are found in the introns of protein-coding and non-coding genes (Ozsolak *et al.*, 2008). Many of the miRNAs implicated in the p53 network are located either in intergenic or intronic DNA regions (Table 1.1):

microRNA	Genomic location
miR-16	intron
miR-26	intron
miR-29	intergenic
miR-122	intergenic
miR-125	intergenic/intron
miR-143	intergenic
miR-145	intergenic
miR-372	intergenic
miR-373	intergenic
miR-449	intron
miR-504	intron

Table 1.1 Genomic location of miRNAs
Many of the miRNAs implicated in the p53 network are located either in intergenic or intronic DNA regions.

Intronic miRNA expression is mediated by RNA pol II from the host gene promoter, whereas intergenic miRNA genes have their own transcription regulatory elements, such as terminal signals and promoters (Monteys *et al.*, 2010).

Regulation of microRNA genes

While currently many studies focus on defining the targets of miRNAs (Filshtein *et al.*, 2012; Freedman & Tanriverdi 2013), fewer are directed towards how miRNA genes are transcriptionally regulated (Xiao *et al.*, 2013). Recent studies of miRNA transcription have elucidated RNA pol II as the major polymerase of miRNAs (Lee *et al.*, 2004), but little is known of the structural features of miRNA promoters, especially those of mammalian miRNAs. It was observed that insertion of RNA Pol II enhancer induced miRNA expression in the case of bantem RNA in *D.melanogaster* (Brennecke *et al.*, 2003). This led to the findings by Lee's group in 2004, supported by direct experimental evidence, that miRNA genes are transcribed by RNA pol II. However, the restriction of miRNA transcription to RNA pol II may have been true for the miRNA defined at the time, but many more miRNAs have been identified and the identity of correspondent RNA polymerases remains untested (Borchert *et al.*, 2006; Haurie *et al.*, 2010). Together, this has led to the consensus that intragenic miRNAs (within introns or exons of protein-coding genes) on the same strand as their host gene are co-transcribed by Pol II, while intergenic miRNAs are transcribed from their own RNA pol II or RNA pol III promoter (Corcoran *et al.*, 2009). Researchers identified several histone modifications, such as trimethylation of Lys4 of histone 3 (H3K4me3), and acetylation of Lys9/14 of histone 3 (H3K9/14Ac), as valuable markers of transcriptionally active promoters (Pokholok *et al.*, 2005). With this knowledge, determining the transcription factors and regulating the process of miRNA expression becomes a process of mapping binding sites of known transcription factors through computational methods of bioinformatics (Saini *et al.*, 2007). Using this protocol, the occupancy of several embryonic stem cell (ESCs) transcription factors (POU5F1, SOX2, NANOG, and TCF3) was mapped to the promoters of 55 distinct miRNA transcription units, which included three clusters of polycistrons. Altogether, these transcription factors regulate approximately 20% of annotated mammalian miRNAs in ESCs. This number is roughly equal to the number of protein-coding genes that are also regulated by these transcription factors in ESCs (Marson *et al.*, 2008). p53 was shown to repress c-MYC expression via induction of

miR-145 tumour suppressor. c-MYC is directly targeted and specifically silenced by *miR-145*, which is transcriptionally up-regulated by p53 (Sachdeva *et al.*, 2009). In addition to regulation of miRNAs via such a linear pathway, recent reports have shown that certain environmental conditions influence miRNA expression. Specifically, cells undergoing hypoxic stress have demonstrated the up-regulation of miR-210. Huang *et al.* demonstrated that miR-210 was regulated by HIF1A interaction. miRNAs have been described to have multiple mRNA targets, and with the emergence of these studies detailing HIF1A regulatory control over multiple miRNAs, we are beginning to appreciate the possibility that key factors may regulate many miRNA products as well. Further study of the regulatory interaction between miRNAs and these factors may help illustrate their role in normal function and disease progression.

Biogenesis of microRNAs

The mechanism of miRNA biosynthesis is evolutionarily conserved. The biogenesis of miRNAs is an intricate procedure (Fig. 1.11). Firstly, transcription of the *miRNA* gene mainly by RNA pol II (Lee *et al.*, 2004) in the nucleus leads to the formation of a long non-coding primary transcript termed primary (pri)-miRNA, which is normally 1-3 kb in size (Kim & Nam 2006). This pri-miRNA, which contains several hairpin structures, is further processed via and stabilised by polyadenylation and capping, and cleaved at the hairpin stem by the RNAse III endonuclease Drosha to form a smaller hairpin precursor (pre)-miRNA termed pre-microRNA, which is usually 60-100 bps in size (Lee *et al.*, 2003). In humans, the microprocessor complex, which carries out this initial maturation step, comprises Drosha associated with DGCR8, a cofactor which is believed to aid Drosha in recognising its substrate (Han *et al.*, 2004). DGCR8 interacts with pri-miRNAs at the base of its stem structure, whilst Drosha provokes its cleavage. DGCR8 and Drosha interplay allows DGCR8 to govern the exact location at which the pri-miRNA is cleaved. The two proteins that make up this big 650 kDa complex are only conserved in metazoans. Following on from nuclear cropping, the pre-miRNA stem loop is exported by the RAN-dependent nuclear transport receptor Exportin-5 from the nucleus across the nuclear membrane into the cytoplasm (Bohnsack *et al.*, 2004), where it is further processed and cleaved by the evolutionary-conserved RNAse III endonuclease Dicer to liberate a mature miRNA duplex that is 20-25 bps in length (Ketting *et al.*, 2001). Via its conserved PAZ domain, Dicer binds the 3’ end of the

double-stranded miRNA. Dicer also associates with a cofactor, namely TRBP, to aid in miRNA processing as well as the assembly of RISC (Chendrimada *et al.*, 2005). Dicer has also been shown to associate with PACT and Loquacious proteins, which stabilise the Dicer protein itself, the dicing process, and establishing of the RISC complex. To complete the maturation of miRNA, one strand from the short-lived miRNA duplex, known as the guide strand, is incorporated into the effector complex RISC, where this mature miRNA aligns with and recognises target mRNA (Schwarz *et al.*, 2003). Degradation of the passenger strand occurs at a quick rate. This final stage of miRNA processing and the assembling of RISC are executed by the RISC loading complex, which is comprised of Dicer, TRBP, PACT, and Argonaute (AGO) proteins. AGO proteins are the primary component of the RISC complex, and the effectors of miRNA-mediated repression of target mRNAs. AGO proteins have been shown to be accountable for removing the passenger strand from miRNA duplexes through their slicer endonucleolytic activity. The human genome contains eight AGO family proteins; AGO1-4 and PIWI1-4. While all of the AGO proteins have the ability to interact with miRNAs and siRNAs, AGO2 is the only one with RNA cleavage activity and is thought to play a critical role in miRNA-mediated mRNA silencing. AGO2 provokes the cleavage of the passenger strand at its 3' end (Davis & Hata 2009). Immunohistochemical analysis of 68 gastric and colorectal tumours indicates decreased AGO2 protein in 40% and 35% of gastric and colorectal cancers, respectively (Davis-Dusenbery & Hata 2010).

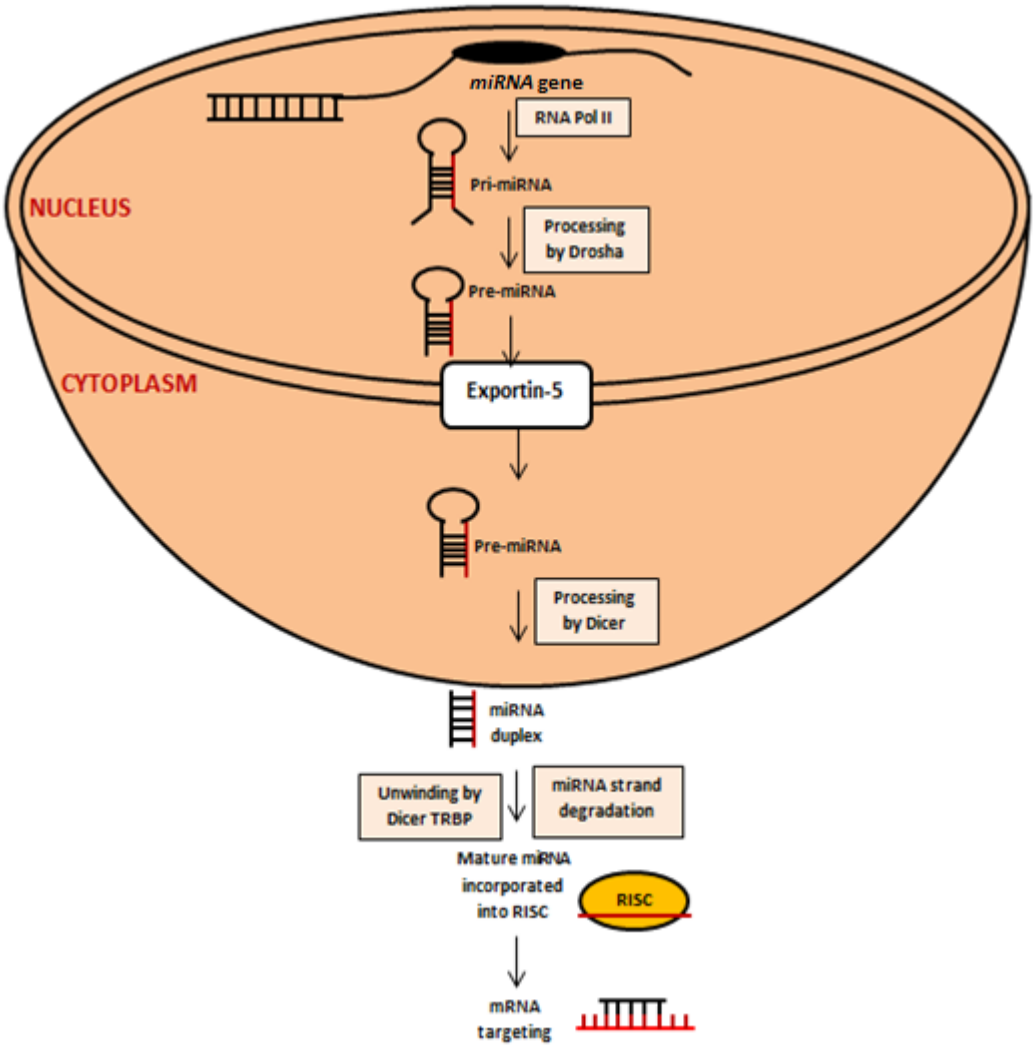


Figure 1.11 Biogenesis of microRNAs
The biogenesis of miRNAs is an intricate procedure. Firstly, transcription of the *miRNA* gene mainly by RNA polymerase II in the nucleus leads to the formation of pri-miRNA. This pri-miRNA is further processed by polyadenylation and capping, and by the endonuclease Drosha to form pre-miRNA. Pre-miRNA stem loop is exported by exportin5 from the nucleus across the nuclear membrane into the cytoplasm where it is further processed and cleaved by endonuclease Dicer to liberate a mature miRNA duplex. One strand from the short-lived miRNA duplex, guide strand, is incorporated into RISC, where this mature miRNA aligns with and recognises target mRNA.
Adapted from Dalmay 2008.

microRNA turnover regulation

Turnover of mature miRNA is required for rapid changes in mRNA expression profiles. During miRNA maturation in the cytoplasm, uptake by the Argonaute protein is thought to stabilise the guide strand, while the passenger strand is preferentially destroyed. AGO proteins may prefer to retain miRNAs with many targets over miRNAs with few or no targets, resulting in degradation of the non-targeting molecules. Decay

of mature miRNAs in *C.elegans* is mediated by the 5´ to 3´ exoribonuclease XRN2 (Chatterjee *et al.*, 2011). In plants, small RNA degrading nuclease (SDN) family members degrade miRNAs in the 3' to 5' direction. Similar enzymes are encoded in animal genomes, but their roles have not yet been described. Several miRNA modifications affect miRNA stability. As indicated by work in *Arabidopsis thaliana*, mature plant miRNAs appear to be stabilised by the addition of methyl moieties at the 3' end. The 2'-O-conjugated methyl groups block the addition of uracil residues by uridyltransferase enzymes, a modification that may be associated with miRNA degradation. However, uridylation may also protect some miRNAs; the consequences of this modification are not completely understood (Rogers & Chen 2013). Uridylation of some animal miRNAs has also been reported (Heo *et al.*, 2008). Both plant and animal miRNAs may be altered by addition of adenine residues to the 3' end of the miRNA. An extra 'A' added to the end of mammalian miR-122, a liver-enriched miRNA important in Hepatitis C, stabilises the molecule , and plant miRNAs ending with an adenine residue have slower decay rates (Kai & Pasquinelli 2010).

microRNA mechanism of action

The future of mRNAs bound by miRNAs is determined by the level of complementarity between the miRNA and its target mRNA (Fig. 1.12). The efficiency of miRNAs binding to their targets via their seed sequence is increased by an adenine residue at position 1 or 9 or a uracil residue at position 9. miRNAs target mRNAs for protein translational repression at the initiation or elongation step (imperfect complement) or cleavage as a result of deadenylation (perfect complement), and thus negatively regulate gene expression (Brennecke & Cohen 2003). It has been shown that miRNAs are guides by their associated protein factors. Cleavage of a phosphodiester bond in target mRNA is directed by mature miRNAs when they perfectly base-pair to their target mRNA. Counting from the miRNA 5' end, the cut is found between the residues paired to nucleotide residues 10 and 11. This mRNA cleavage mediated by AGO2 proteins, can be evaded if mismatches or protrusions appear in the middle of the miRNA-mRNA duplex (Pillai 2005). Existing evidence shows that miRNAs provoke translational repression at both the levels of initiation and post-initiation. miRNAs have been shown to repress translation initiation by inhibiting the eukaryotic initiation factor 4E (EIF4E), the function of the poly (A) tail, and ribosome assembly (Humphreys *et al.*, 2005).

EIF4E promotes translation initiation by recognising the mRNA 5' terminal cap. AGO2 competes with EIF4E in this mRNA region to bring about repression of translation initiation. The miRNA/RISC complex (miRISC) has also been shown to facilitate deadenylation by GW182 of the mRNA tail, thus preventing the ability of mRNA recircularisation by impeding the interaction between its 3' tail and the 5' cap (Eulalio *et al.*, 2009). Additionally, ribosomes can be prevented from assembling at the start codon by AGO associating with the 60S ribosomal subunit, which prevents it from joining with 40S preinitiation complex to commence elongation. At the level of post-initiation, translational repression has been shown to occur by miRNAs via disengagement of ribosomes during translation elongation through miRISC (Petersen *et al.*, 2006). Also, there is the possibility for the newly-forming polypeptide to undergo degradation during its translation (Valencia-Sanchez *et al.*, 2006). As well as deadenylation, miRNA-mediated mRNA degradation occurs by exonucleolytic digestion and decapping. mRNAs have also been shown to be sequestered to cytoplasmic P bodies, which are short-term places to store mRNAs which have been repressed by miRNAs, so that they may be sheltered from translation and are therefore degraded (Fabian *et al.*, 2010).

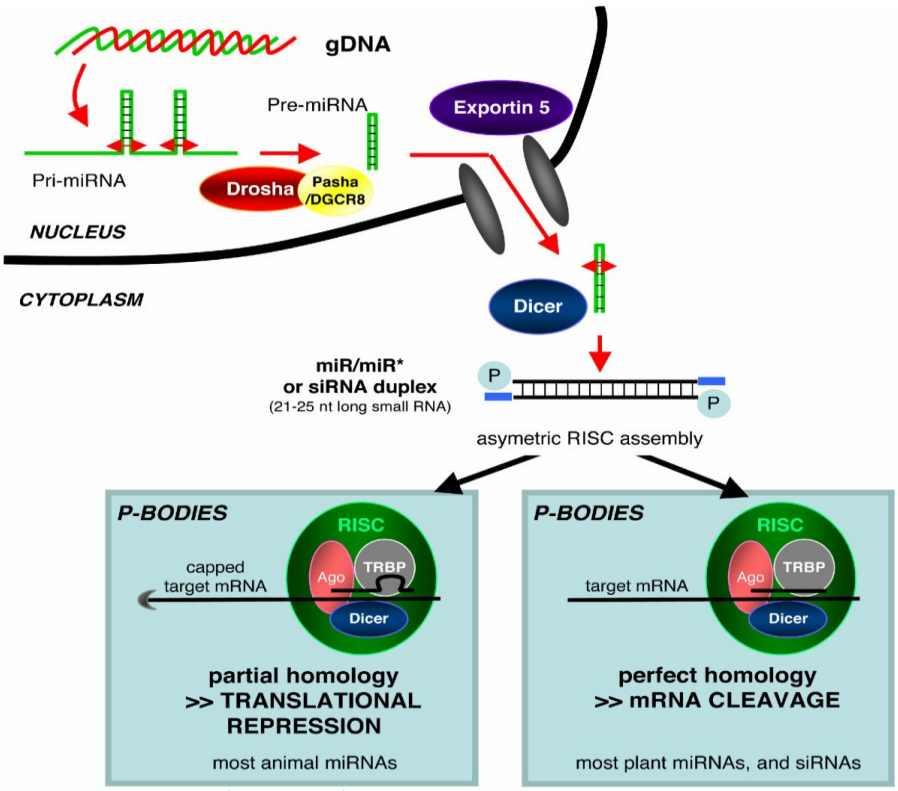


Figure 1.12 Mechanism of action of microRNAs
miRNAs target mRNAs for protein translational repression at the initiation or elongation step (imperfect complement) or cleavage as a result of deadenylation (perfect complement), and thus negatively regulate gene expression. mRNAs have been shown to be sequestered to cytoplasmic P bodies, short-term places to store mRNAs which have been repressed by miRNAs, so that they may be sheltered from translation and are therefore degraded.
Taken from Saumet & Lecellier 2006.

microRNA functions

Since they were first discovered, miRNAs have attracted a lot of attention due to their unique functional significance and modes of action, providing a new dimension of, and the latest addendum to, the central dogma of molecular biology. Among these small RNAs, miRNAs are the most phylogenetically conserved and function post-transcriptionally to regulate many physiological processes, including embryonic development. Investigations on the phenotypic effects of lin-4 and let-7 miRNA mutations and mutations of their targets have provided evidence for the functions and targets of other miRNAs. Several computer softwares, including TargetScan, miRanda, DIANA-microT, and PicTar, have been generated to predict most of the miRNA targets, which also offers clues to the function of miRNAs. The majority of biological processes and cellular pathways, such as housekeeping roles and environmental stress responses, may be regulated by miRNAs (Bartel 2009). Altered miRNA expression or the effect of miRNAs on their targets has been shown to correlate with various human diseases, including heart failure (Divakaran & Mann 2008), diabetes (Tang *et al.*, 2008), and cancer (Sessen *et al.*, 2008). Several neurological syndromes, such as Parkinson's disease and Alzheimer's disease, have also been shown to exhibit altered miRNA or their target expression. Numerous syndromes that miRNAs are linked to include DiGeorge syndrome, distinguished by a 22q11.2 chromosomal region deletion that codes for DGCR8, and Downs syndrome (Hébert & De Strooper 2008). The discovery of various virus-encoded miRNAs, has linked miRNAs to such viruses as retroviruses and herpesviruses. SV40 virus provided the first defined viral miRNAs, decreasing viral T antigens by targeting viral mRNAs (Sullivan *et al.*, 2005).

microRNAs and cancer

Important observations early in the history of miRNAs suggested a potential role in human cancer. The earliest miRNAs discovered in *C. elegans* and *D.melanogaster* were shown to regulate cell proliferation and apoptosis. As miRNAs controls numerous cellular processes which are dis-regulated in cancer, including apoptosis, differentiation and proliferation, it is believed that mutations which have an effect on miRNAs or on their control of their target oncogenes and tumour suppressor genes may promote tumourigenesis. Mutations can have an effect on mature miRNA sequences which can eliminate their capacity to bind their target mRNAs and alter

their specificity, and mutations in the seed-match sequences of target mRNAs can prevent their repression by a specific miRNA and enable their repression by other miRNAs (Wiemer 2007). Malignant tumours and tumour cell lines were noted to have extensive deregulated miRNA expression in comparison with normal tissues (Tahiri *et al.*, 2013).

Alterations in miRNA expression are commonly associated with cancer and are directly involved in the process of carcinogenesis as oncogenes or tumour suppressors (Esquela-Kerscher & Slack 2006). Alterations in miRNA transcriptional control can be due to alterations in transcription factors that control these miRNAs (Calin & Croce 2006). However, the causes of altered miRNA expression are not well understood.

The earliest report indicating a possible role of miRNAs in cancer was of a miR-15a/miR-16-1 deletion at 13q14, which is commonly associated with chronic lymphocytic leukaemia (CLL), in addition to prostate cancer and mantle cell lymphoma. These two miRNAs function as tumour suppressors, and target oncogenic BCL2, WNT3A, and CCND1, to regulate proliferation, invasion, and apoptosis (Bonci *et al.*, 2008).

In general, most miRNAs are downregulated in cancer, as suggested by the first obtainable signatures of miRNA expression in tumours (Lu *et al.*, 2005). For example, c-MYC-induced miRNA transcriptional silencing proposed a model whereby miRNAs are down-regulated in transformed cells on a global scale. However, evidence has shown that there are miRNAs that are overexpressed in tumours (Volinia *et al.*, 2006). Thus, currently it is not clear whether down-regulation of miRNAs is a hallmark of cancer.

Changes in miRNA expression patterns are frequently found in tumours, and have been seen in many cancer types including breast and colorectal cancers (Volinia *et al.*, 2006). Over half of human miRNA genes are situated at common breakpoints, fragile sites, loss of heterozygosity, and regions of amplification in the genome (Calin *et al.*, 2004). Mapping of the chromosomal location of miRNAs indicated that several miRNA genes are found in places that are commonly implicated in chromosomal changes and deletions in human cancers (Croce 2009).

For instance, let-7 displays the activity of a tumour suppressor, proposed by several let-7 family members found mapped to chromosomal areas frequently deleted in tumours and absent in many cancers, such as cervical, breast, and lung. Furthermore,

let-7 has been shown to control *HMGA2*, *c-MYC* and *RAS* oncogene levels. Colorectal cancers have been linked to reduced *miR-143* and *miR-145* expression. In contrast, miR-155 and the miR-17-92 cluster, the first oncogenic miRNAs to be discovered and whose overexpression is linked to several tumours, map to chromosomal regions of amplification in mostly human B-cell lymphoma such as Burkitt's lymphoma (Yanaihara *et al.*, 2006). Lymphoma progression undergoes acceleration when miR-17-92 cluster works together with c-MYC to impede apoptosis, enhance proliferation, and provoke tumour angiogenesis. A report illustrated an increase in miR-10b levels in patients positive for metastasis, compared to metastasis-free patients. The TWIST transcription factor induces miR-10b to impede HOXD10 mRNA translation, leading to pro-metastatic *RHOC* gene expression, and then tumour cell invasion and metastasis. miR-126 and miR-335 have been shown to suppress metastasis, and low levels of these miRNAs have been associated with minimal metastasis-free survival in breast cancer (Santarpia *et al.*, 2010).

miRNA signatures could have key diagnostic and prognostic significances as biomarkers in various cancer types, providing vital information for prognosis and therapy efficacy (Waldman & Terzic 2008). miRNAs have been shown to help pinpoint the tissue of origin of poorly differentiated cancers. The expression profiling of miRNAs has also been used to identify the origin of tumours with an undetermined origin, and to distinguish between differing tumour subtypes (Wittmann & Jäck 2010). A miRNA expression signature differentiating cancerous tissues from normal tissues has been established, profiling several miRNAs which exhibit changed expression in differing tumours (Calin & Croce 2006). Therefore, frequently de-regulated pathways in cancer could have these miRNAs as downstream targets.

As miRNA deregulation is a classic trait in cancer, it is predicted that these miRNAs would be key targets for therapy. Potential therapeutic approaches include treatments that inhibit miRNA expression, including antagomirs. miRNAs bind these artificial complementary RNAs, which in turn, impedes the ability of the miRNA to bind their target mRNA (Krützfeldt *et al.*, 2005). A type of antagomir called anti-miRNA oligonucleotides (AMO) have been produced to directly rival endogenous miRNAs with oncogenic characteristics (Weiler *et al.*, 2006). Examples of AMOs include locked-nucleic-acid (LNA) antisense oligonucleotides (Stenvang *et al.*, 2008). An alternative

way to reduce the association of miRNAs with their targets is by using miRNA sponges, which are artificial mRNAs with several binding sites for endogenous miRNAs (Ebert *et al.*, 2007). It has to be noted that miRNAs can also execute tumour suppressor functions. For example, the delivery of miR-26a in mice has been shown to lead to c-MYC induced hepatocellular carcinoma regression. Liposomal (e.g. cationic) or viral (e.g. adenovirus) mechanisms of delivery can be used to increase miRNA expression associated with tumour suppression (Budhu *et al.*, 2010). miRNA mimics, which imitate endogenous mature miRNAs, have also been applied to augment miRNA levels (Akinc *et al.*, 2008).

The p53 pathway and microRNAs

p53, its regulators, as well as its target genes, comprise an intricate p53 network consisting of a vast array of genes and their products (Fig. 1.14), which control various physiological responses to pressures of a cancer origin. Recent studies have shown that close interactions between miRNAs and p53 occur at multiple levels (Feng *et al.*, 2011). Initial findings showed a difference in expression of several miRNAs in $HCT116^{p53+}$ colon cancer cells versus $HCT116^{p53-}$. These same miRNAs also contained p53 response elements in their promoters, suggesting that these miRNAs are direct transcriptional targets of p53 (Barsotti *et al.*, 2011; Chang *et al.*, 2007).

Transcriptional regulation of microRNAs by p53

The interactions linking p53 and miRNAs were initially shown by identifying numerous miRNAs as direct p53 target genes. Recently it was shown that p53 induces the transcriptional expression of a set of miRNAs which exhibit a tumour suppressive function, by binding to p53REs in these miRNA promoters. In turn, RNA products of these genes help in p53 functioning as a tumour suppressor (He *et al.*, 2007). In 2007, several papers identified the miR-34 family members, consisting of the homologous miRNAs miR-34a, miR-34b, and miR-34c, as the first verified miRNAs that were directly targeted by p53 (Chang *et al.*, 2007; Corney *et al.*, 2007; He *et al.*, 2007; Raver-Shapira *et al.*, 2007; Tazawa *et al.*, 2007). miR-34a is encoded by an individual transcript and expressed in a majority of tissues, and miR-34b and miR-34c share a common primary transcript and are mainly expressed in lung tissues. These reports mainly concentrated on the analysis of global miRNA expression profiles and the correlation between these expression patterns and the status of p53. Expression of *miR-34a* promotes p53-dependent apoptosis, G1 cell cycle arrest and senescence, in the presence of genotoxic stress (Fig. 1.13). Cell type plays a significant role in the effects of miR-34. For instance, overexpression of miR-34 in human lung fibroblastic cells leads to nearly $2/3^{rd}$ cells displaying changed characteristics associated with cellular senescence. The miR-34 family members aid in the tumour suppressive function of p53 by directly repressing the expression of hundreds of mRNA targets implicated in regulating the cell cycle. Various genes involved in the progression of the cell cycle, which have been verified as being miR-34 targets, include *Cyclins E2* and *E2F3*, *CDK4*, and *CDK6*. Genes implicated in promoting cell proliferation and survival, such as anti-apoptotic *BCL2* and *SIRT1*

deacetylase, have also been proved to be targeted by miR-34 (Weeraratne *et al.*, 2011). Several human cancers, including lung and breast cancers, have displayed miR-34 deletions suggesting that loss of miR-34 could promote tumourigenesis (Peurala *et al.*, 2011). These findings, for the first time, strongly suggest that in addition to many protein-encoding genes, miRNAs, the non-coding genes, can also be regulated by p53 as a new class of p53 target genes.

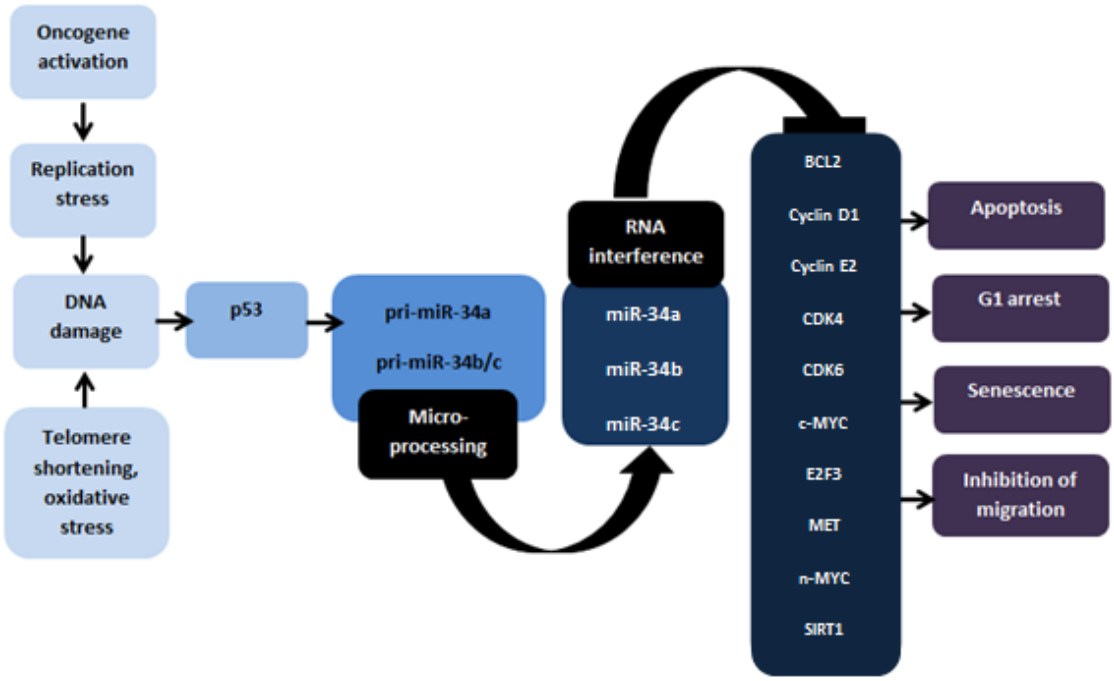


Figure 1.13 miR-34 family mechanism of action
The miR-34 family members, consisting of the homologous miRNAs miR-34a, miR-34b, and miR-34c, were identified as the first verified miRNAs that were directly targeted by p53. Expression of *miR-34a* promotes p53-dependent apoptosis, G1 cell cycle arrest and senescence, in the presence of genotoxic stress. Various genes involved in the progression of the cell cycle, which have been verified as being miR-34 targets, include *cyclins E2* and *E2F3*, *CDK4*, and *CDK6*. Genes implicated in promoting cell proliferation and survival, such as anti-apoptotic *BCL2* and *SIRT1* deacetylase, have also been proved to be targeted by miR-34.
Adapted from www.pathologie.med.uni-muenchen.de.

After discovery of miR-34, several other p53-dependent miRNAs were identified and characterised: miR-107, miR-145, miR-192, and miR-215, were shown to be directly regulated by p53 on a transcriptional level (Shi *et al.*, 2010; Feng *et al.*, 2011; Hermeking 2012). miR-107, which is down-regulated in human stomach, colon, and pancreas cancers, aids p53 in its role in anti-angiogenesis and hypoxic signalling

regulation (Yamakuchi *et al.*, 2010). HIF1 complex, comprised of hypoxia inducible factor 1beta (HIF1β) and 1alpha (HIF1α) subunits, is a transcription factor that mediates the transcriptional response to hypoxia and plays an important role in tumourigenesis. Hypoxia signalling is reduced by miR-107 by its inhibition of HIF1β expression, resulting in suppressed tumour angiogenesis and therefore tumour growth observed in human colon cancer cells in mice. miR-145 has been shown to connect the p53 and oncogenic c-MYC pathways, and therefore has a significant role in c-MYC repression brought about by p53. miR-145 post-transcriptionally regulates c-MYC by targeting it directly for silencing, resulting in inhibited tumour cell growth (Sachdeva *et al.*, 2009). The homologous miRNAs miR-192 and miR-215, which are down-regulated in colon cancer, suppress tumour formation. A set of downstream targets of miR-192 and miR-215 were identified, which include a number of regulators of DNA synthesis and the G1 and G2 cell cycle checkpoints in cells, such as CDC7, MAD2L1 and CUL5 (Georges *et al.*, 2008). Similar to miR-34a, the p53-dependent upregulation of miR-192 and miR-215 can lead to G1 and G2 cell cycle arrest due to increased p21 levels (Braun *et al.*, 2008). Overexpression of miR-192 and miR-215 in tumour cell lines can all result in the increased p21 protein levels, cell cycle arrest and suppression of tumour cell colony formation.

<u>microRNA processing and maturation regulated by p53</u>

A recent study revealed an additional mechanism for p53 in the regulation of miRNAs. In recent times, p53 has also been associated with the regulation of miRNA processing and promoting the maturation of several miRNAs, which also plays a part in the role of p53 in tumour suppression. p53 provokes the post-transcriptional maturation of numerous miRNAs. Drosha has an essential role in processing pri-miRNA transcripts into pre-miRNAs. On DNA damage, p53 stimulates the Drosha-mediated processing of distinct miRNAs with presumed tumour suppressive functions, such as miR-16-1, miR-143, and miR-145. These miRNAs down-regulate key regulators of cell cycle progression and cell proliferation, including Cyclins E and D, CDK4 and CDK6 (as a target of miR-16-1 and miR-145) (Freeman & Espinosa 2013). For example, K-RAS is a target of miR-143. These miRNAs are decreased in various human cancers, and overexpression of these miRNAs reduces tumour cell proliferation. The interaction between p53 and Drosha occurs following doxorubicin treatment through the DEAD-

box RNA helicases p68 and p72, both of which are needed by Drosha to function for most miRNA maturation. p53 mutants, which are transcriptionally non-functional, hinder complex formation between Drosha and p68, resulting in decreased miRNA processing (Suzuki *et al.*, 2009). While p53 promotes the maturation of miRNAs, p53 also monitors the maturation of miRNAs. A loss in miRNA maturation is frequently seen in many human diseases and it can stimulate p53 signalling and provoke p53-mediated senescence. This miRNA dis-regulation may be a result of impaired miRNA processing. The ablation of Dicer and loss of mature miRNAs in embryonic fibroblasts activate p53 and induce senescence which could be rescued by deletion of p53 (Mudhasani *et al.*, 2008). Since p53-mediated senescence is an important mechanism by which p53 exerts its function in tumour suppression, loss of p53 function may greatly facilitate the tumorigenic potential of cells with reduced levels of mature miRNAs. These findings have further confirmed a direct link between p53 tumour suppressor and miRNA biogenesis apparatus, and also further emphasises the key role played by p53 in the regulation of miRNA expression and maturation, and therefore the functions of miRNAs. Additionally, transcription-independent modulation of miRNA biogenesis is implicated in a tumour suppressive program governed by p53 (Suzuki *et al.*, 2009), which could be a new mechanism by which p53 mutation lends itself to cancer.

Regulation of p53 and its pathway by microRNAs

Computational analyses estimate that over 30% of all genes and the majority of genetic pathways are subject to regulation by multiple miRNAs (Bentwich *et al.*, 2005; Lim *et al.*, 2005; Griffiths-Jones *et al.*, 2006). This raises a possibility that some specific miRNAs could regulate p53 and its pathway. To test this possibility, we screened for miRNAs which could potentially regulate p53 expression by performing an *in silico* search for putative binding sites of miRNAs in the 3'UTR of human p53 gene. The first indication that miRNAs could regulate the p53 pathway came about following on from a genetic screen for miRNAs that work with oncogenic RAS to provoke cellular transformation. Evidence has suggested that particular miRNAs are capable of regulating p53 and its pathway at differing levels. miRNAs can provoke direct repression of p53 expression, or they can stimulate repression of negative regulators of p53 (Takwi & Li 2009). It was demonstrated that p53 is subject to the negative

regulation by specific miRNAs. miR-372 and miR-373 have been shown to promote proliferation and tumourigenesis of testicular germ cells which contain the oncogene Ras and wild-type p53. This is brought about by prevention of p53-mediated CDK inhibition by these miRNAs, potentially through direct inhibition of LATS2 tumour suppressor (Voorhoeve *et al.*, 2007). In response to genotoxic stress, miR-504 negatively regulates human p53 protein expression by binding in its 3'UTR at two sites, impairing p53 transcriptional activity, p53-induced apoptosis and G1 cell cycle arrest, resulting in tumourigenesis. Furthermore, miR-504 overexpression promotes tumourigenicity of colon cancer cells *in vivo* (Hu *et al.*, 2010). miR-125b, which is expressed in the brain of zebrafish and humans, also negatively regulates p53 expression by targeting the 3'UTR of p53, which was confirmed by predictions made via computational software. This leads to hindered apoptosis in human neuroblastoma and lung fibroblasts, and its overexpression is often associated with megakaryoblastic leukaemia, myelodysplastic syndrome, and acute myeloid leukaemia. In contrast, the downregulation of miR-125b is observed in prostate, ovarian, neck, head, and breast cancers, as well as glioblastoma (Le *et al.*, 2009). These results demonstrate that miR-125b is an important negative regulator of p53 and p53-induced apoptosis during development and during the stress response. In addition to the direct negative regulation of p53 expression, there are miRNAs, e.g. miR-29, miR-34a, and miR-122, which positively regulate p53 by repressing negative regulators of p53. Recently, miR-29 was identified as another positive regulator of p53 (Park *et al.*, 2009). p53 upregulation is brought about by the miR-29 family members miR-29a, miR-29b, and miR-29c, which down-regulate the p53 negative regulators GTPase CDC42 and p85a, in order to provoke p53-mediated apoptosis. p85a, is a regulatory subunit of PI3 kinase (PI3K). The PI3K/AKT pathway can negatively regulate p53 activity through the direct phosphorylation and activation of MDM2 by AKT (Zhou *et al.*, 2001). Down-regulation of miR-29 miRNAs has been reported in various cancers, including leukemia, and lung and breast cancers. miR-34a, a transcription target of the p53 protein, was found to positively regulate p53 activity. miR-34a and miR-449a directly negatively regulate SIRT1, a negative regulator of p53 acetylation levels which deacetylates Lys382 of p53 (Luo *et al.*, 2001), to provoke p53-induced apoptosis (Fornari *et al.*, 2009; Park *et al.*, 2009; Yamakuchi *et al.*, 2008). E2F1 has been shown to upregulate the levels of miR-

34a and miR-449a, which in turn, decrease CDK6 levels, demonstrating the role of these miRNAs in a negative feedback system with E2F1. miR-449 is highly expressed in the trachea, testes, and lungs (Lizé *et al.*, 2010). Cyclin G1, which is highly expressed in numerous human cancers, is negatively regulated by miR-122. Cyclin G1 is transcriptionally regulated by p53. Cyclin G1 recruits PP2A phosphatase to form a complex, which then dephosphorylates and activates MDM2, which, in turn, inhibits p53 and induces its degradation. Thus, Cyclin G1 forms a negative feedback loop with p53 to negatively regulate p53. Cyclin G1 is overexpressed in several human cancers, including breast cancers. By directly repressing the expression of Cyclin G1, miR-122 increases p53 protein levels and activity and inhibits tumourigenesis in human hepatocellular carcinoma (HCC) (Fornari *et al.*, 2009).

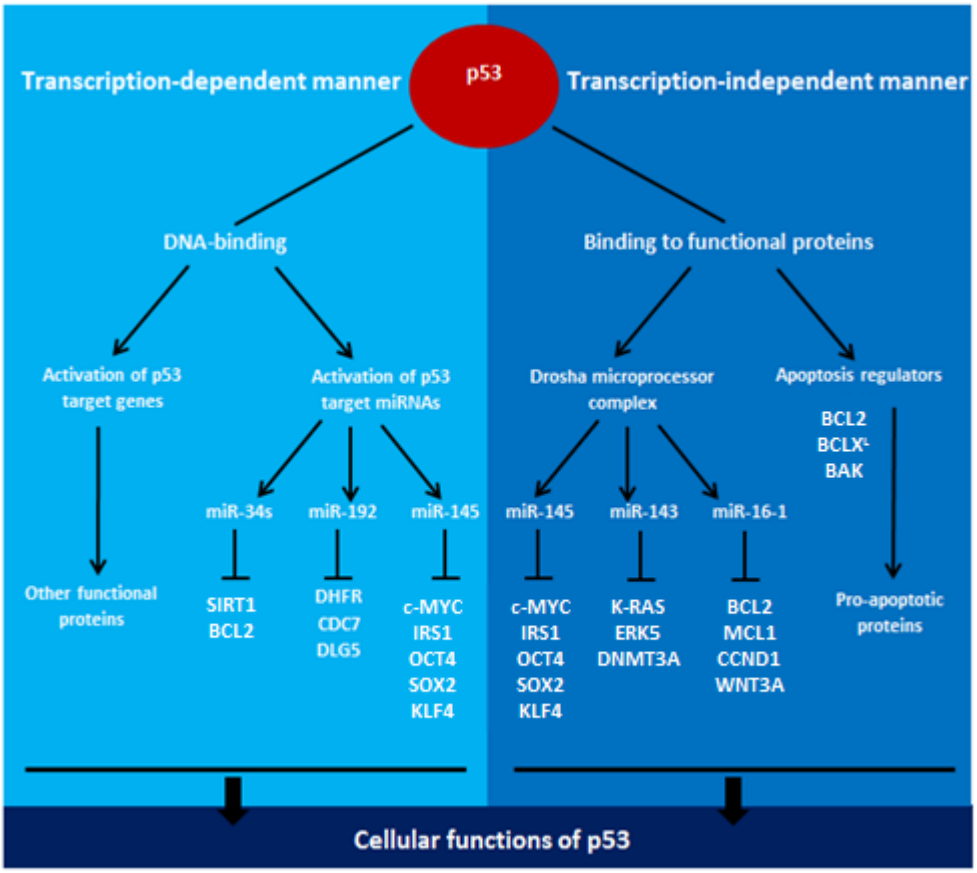


Figure 1.14 p53-microRNA network
p53, its regulators, as well as its target genes, comprise an intricate p53 network consisting of a vast array of genes and their products, which control various physiological responses to pressures of a cancer origin. Recent studies have shown that close interactions between miRNAs and p53 occur at multiple levels.
Adapted from Shi *et al.*, 2010.

Conclusion

Via the regulation of the transcription or maturation of distinct miRNAs by p53, miRNAs have the capacity as a novel set of p53 target genes to facilitate the function of p53 as a tumour suppressor. Furthermore, miRNAs could provide a new regulatory mechanism to closely regulate p53 activity and its protein levels. By identifying functionally important regulatory associations linking p53 and miRNAs, it will help characterising functionality of the p53 pathway in cancer (Feng *et al.*, 2011; Hermeking 2012).

The regulation of p53-dependent microRNA expression in response to genotoxic stress

Many p53-dependent miRNAs have been suggested to play a key role in cancer by regulating expression levels of oncogenes (He *et al.*, 2007; Suzuki *et al.*, 2009). Amongst those, two miRNAs, miR-16 and miR-26a are important effectors of p53 pathway in response to genotoxic stress.

Predicted p53-regulated microRNAs - miR-16

The miR-16 family members comprise a group of miRNAs that are related to the miR-15 gene family. Prediction and experimental validation of the miR-16 family members specifically localised them to vertebrates, including humans.

The first experimental evidence that miRNAs are involved in mammalian carcinogenesis was reported in chronic lymphocytic leukaemia (CLL), the most common form of adult leukemia in the Western world. In 2002 the miRNAs miR-15 and miR-16 were identified as the first cancer genes, which are also implicated in the progression of CLL (Calin *et al.*, 2002). These miRNAs make up the miR-15a/miR-16-1 cluster, which has been shown to be down-regulated and/or removed by chromosomal deletions in CLL cells. Analyses were conducted on $CD5^+$ B-lymphocytes, the cells from which CLL originates and whose accumulation is renowned in the progress of B-CLL, to reveal the gene cluster encoding miR-15 and miR-16. In B lymphocytes, the miR-15a/miR-16-1 cluster is highly expressed whereas the miR-15b/miR-16-2 cluster is detected as minimal levels in a normal human state (Aqeilan *et al.*, 2010).

miR-15a and miR-16-1 have been shown to express at lower levels in pituitary adenomas than in normal pituitary tissue. These data led to the hypothesis that miR-15a and miR-16-1 function as tumour suppressors and that their inactivation by allelic loss may contribute to tumourigenesis. Alterations in miR-16 expression have also been seen in brain, breast, colon, lung, ovarian, pancreatic, prostate, and stomach cancers. The miR-15a and miR-16-1 sequence could also be a target of inactivation by point mutations that contribute to the initiation and/or progression of cancer.

These miRNAs exert their roles as tumour suppressors by down-regulating BCL2, whose overexpression is often observed in CLL. The miR-15a/miR-16-1 cluster has also been shown to target Cyclin D1 and WNT3A, which promote several prostate

tumourigenic features, including survival, proliferation, and invasion. The mature sequence of the miR-16-2 gene is the same as miR-16-1, indicating similar functions (Yue & Tigyi 2010).

Mature miR-16 is derived at chromosome 13q14 from miR-16-1, and at chromosome 3q25 from miR-16-2, where miR-16 forms its cluster, with either miR-15a or miR-15b respectively (Guglielmelli *et al.*, 2011). Karyotype analysis revealed that over 50% of CLL patients have genetic changes in the 13q14.3 region of chromosome 13, and this is the most frequently observed abnormality in chromosomes associated with CLL. The same chromosomal region is also deleted in 60% of prostate cancers, 50% of mantle cell lymphomas, and 40% of multiple myelomas. Initially, the identification of cancer genes involved in the 13q14.3 deletions in CLL failed, despite the extensive effort of several research groups. A region of over 1Mb has been fully sequenced and characterised in detail and identified several genes, but none of them were found to be involved in the initiation or progression of CLL or other human tumours. Subsequent analysis of a 30kb deletion in one case of CLL associated with retinoblastoma and the chromosomal breakpoint translocation t(2:13)(q32;q14) in a differing patient led to identification of the critical region containing the tightly associated miRNAs miR-15a and miR-16-1.

In non-small cell lung cancer (NSCLC) cells, it was shown that miR-34a and miR-15a/miR-16-1 act together to provoke cell cycle arrest in an RB-dependent manner. This synergistic effect was abolished in cells where Cyclin E1, which is uniquely targeted by miR-15a/16, was silenced via RNA interference (Bandi & Vassella 2011). miRNAs of the miR-15/16/195/424/497 family were discovered to sensitise cisplatin-resistant cells to apoptosis by their targeting of CHK1 and WEE1 (Pouliot *et al.*, 2012).

A loss in miR-16 expression is seen in a vast number of cancer cells, and therefore it would be an ideal candidate to target for intervention via therapy (Liu *et al.*, 2008).

Predicted p53-regulated microRNAs - miR-26

Nearly 100 cancer-derived cell lines were analysed for varied sequences in 15 miRNAs implicated in tumourigenesis by their known target transcripts or their localisation to sites frequently linked to chromosomal instability. One of these miRNAs is miR-26. Various microarray expression profiles showed that miR-26 expression is disordered in a number of human tumours. miR-26a-1, miR-26a-2 and miR-26b are the only 3

subtypes of the hsa-miR-26 family, and are located in chromosomes 3, 12 and 2, respectively. The mature miRNA of miR-26a-1 and miR-26a-2 possesses the same sequence, with the exception of 2 different nucleotides in mature miR-26b.

Just like miR-16, miR-26 is specifically found in vertebrates, including humans. miRNAs play crucial roles in numerous biological processes via their target genes. It is known that miR-26 plays a significant role in the growth, development and cell differentiation of different tissues. It has been show that in various cancers miR-26 is dis-regulated, but its main functions are still not known (Lu *et al.*, 2011; Gao & Liu 2011; Iorio *et al.*, 2005). In several tumour types, such as bladder and breast cancers, miR-26 is down-regulated and it may display traits of a tumour suppressor during cancer progression in the same tumours (Gao & Liu 2011). miR-26 expression is also decreased in oral squamous cell carcinoma, anaplastic carcinomas, Burkitt lymphoma HCC, and rhabdomyosarcoma.

With regards to miR-26a-1, compared to the rest of the miR-26 family, it is the most abundant member in humans. In tumours, a reduced level of miR-26a-1 is observed (Ji *et al.*, 2009). The location of miR-26a-1 is at a tumour-specific chromosomal aberration, within an allelic deletion seen in various epithelial cancers (Diederichs & Haber 2006). Silencing of miR-26a can lead to hepatocellular carcinoma (Ji *et al.*, 2009). In human hepatocellular carcinoma cells, overexpression of miR-26a induces G1 phase cell cycle arrest, through down-regulating Cyclins D2 and E2, as well as a decrease in cells of the S stage (Kota *et al.*, 2009). Down-regulation of miR-26a is a regular occurrence in nasopharyngeal carcinoma. Overexpressed miR-26a in nasopharyngeal cells directly represses oncogenic enhancer of zeste homolog 2 (EZH2) expression, leading to cell cycle and cell growth inhibition, and therefore hindering tumourigenesis. In addition, miR-26a directly down-regulates an oncogenic protein metadherin (MTDH) to provoke apoptosis in, breast cancer cells (Zhang *et al.*, 2011). As a result, the expression of both $p14^{ARF}$ and $p21^{CIP1}$ CDK inhibitors are increased and the cyclin-dependent kinases CDK4 and CDK6 expression is suppressed, as is the expression of Cyclins D3 and E2, and c-MYC (Lu *et al.*, 2011). Overexpressed miR-26a has been shown to hinder human breast carcinogenesis, and it initiates apoptosis through pathways activated by caspase 8 and 9. A study on miRNA expression profiles in c-MYC driven tumourigenesis reported that the expression of miR-26 decreased in

Burkitt lymphoma (BL), an aggressive variant of non-Hodgkin's B-cell lymphoma. miR-26a was consistently repressed by c-MYC in multiple tumours, indicating that this miRNA may have a strong tumour-suppressor function in c-MYC induced lymphomas. The results showed that a significant degression of EZH2 in the gene expression profile was induced by miR-26a over-expression, in both human BL-derived and murine lymphoma cell lines. c-MYC may thus contribute to the up-regulation of EZH2 via the down-regulation of its targeting miRNA. The suppression of the miR-26a-mediated attenuation of EZH2 expression by c-MYC was shown to play a critical role in lymphomagenesis. A positive feedback loop comprising c-MYC and EZH2 was involved in the formation of the malignant lymphoma phenotype (Sander *et al.*, 2008).

miR-26b targets carboxy-terminal domain small phosphatases (CTDSP2s) to repress their expression, and therefore provoke neuronal differentiation. CTDSP2s form part of a complex implicated in the regulation of activating genes accountable for controlling neuronal stem cell differentiation. miR-26b expression is increased during neurogenesis, implicating miR-26b in a negative feedback loop with CTDSP2 in neural stem cells, where miR-26b inhibition occurs at the level of a precursor (Dill *et al.*, 2012). It has to be noted that miR-26b not always plays a tumour suppressive role. In several tumours miR-26b also directly targets and controls tumour suppressor PTEN expression. The latter, when mutated, triggers the PI3K/AKT survival pathway in glioma. This pathway is hyperactive in several cancers, resulting in reduced apoptosis, increased cell survival and propagation, and subsequent tumourigenesis. Down-regulation of miR-26b expression in conjunction with miR-128 upregulation results in pituitary tumour growth suppression via stabilisation of PTEN. miR-128 decreases expression of oncogenic protein BMI1, which is a repressor of PTEN (Palumbo *et al.*, 2012).

Relating to therapeutic intervention, adenovirus-associated delivery mechanisms have already been used to administer miR-26a to cancer cells, in order to induce apoptosis and therefore prevent cancer progression (Di Leva *et al.*, 2012). miR-26 expression is not only disordered in tumourigenesis but also alterable in non-tumour diseases. Primary billiary cirrhosis (PBC) caused by chronic cholestasis is often accompanied by autoimmune diseases such as rheumatoid arthritis and scleroderma. Padgett *et al* observed that a total of 35 independent miRNAs in the miRNA expression profile are

disordered and that miR-26a is one of the down-regulated miRNAs. The predicted targets of these alternative miRNAs are known to affect cell proliferation, apoptosis, inflammation, oxidative stress and metabolism associated with the development of PBC. The roles that miR-26 plays in non-tumour diseases have yet to be clarified and warrants further studies. miR-26 expression has been observed in neural tissue, where it accumulates in considerable amounts during the development of the embryo. Reduced oxygen levels trigger its response, where in hypoxic surroundings, its overexpression hinders the cell cycle and cell proliferation. The function of miR-26 in myogenesis is the clearest case in the studies of miRNA in normal tissue growth and development. Overexpression of miR-26a in murine myogenic C2C12 cells induced creatine kinase activity, an enzyme that markedly increased during myogenesis. MyoD and myogenin mRNA expression levels were also up-regulated, and EZH2 was identified as a potential target of miR-26a. miR-26 was found to play a role in normal tissue growth and development and to have an impact upon cell proliferation and differentiation; however, the mechanism remains to be clarified (Wong & Tellam 2008).

Other predicted targets of miR-26 are WEE1 and CHK1 protein kinases. The checkpoint kinases ATR, CHK1 and WEE1 are key regulators of DNA damage surveillance pathways. Genotoxic stress is sensed by the cell via DNA damage responsive kinases ATR and ATM. Upon activation, these kinases transfer the signal to their downstream effectors, kinases CHK1 and CHK2, respectively. WEE1 has a major cell-cycle function in control of the G2/M transition by phosphorylating and thus inactivating cell cycle dependent kinase CDC2 (CDK1). WEE1 is overexpressed in various cancer types, including glioblastoma and breast cancer where p53 function is compromised (De Witt Hamer *et al.*, 2011). Thus, it is tempting to speculate that overexpression of miR-26 in these tumours by p53 may result in mitotic catastrophe and cell death (Fig. 1.15).

Predicted cascade of events

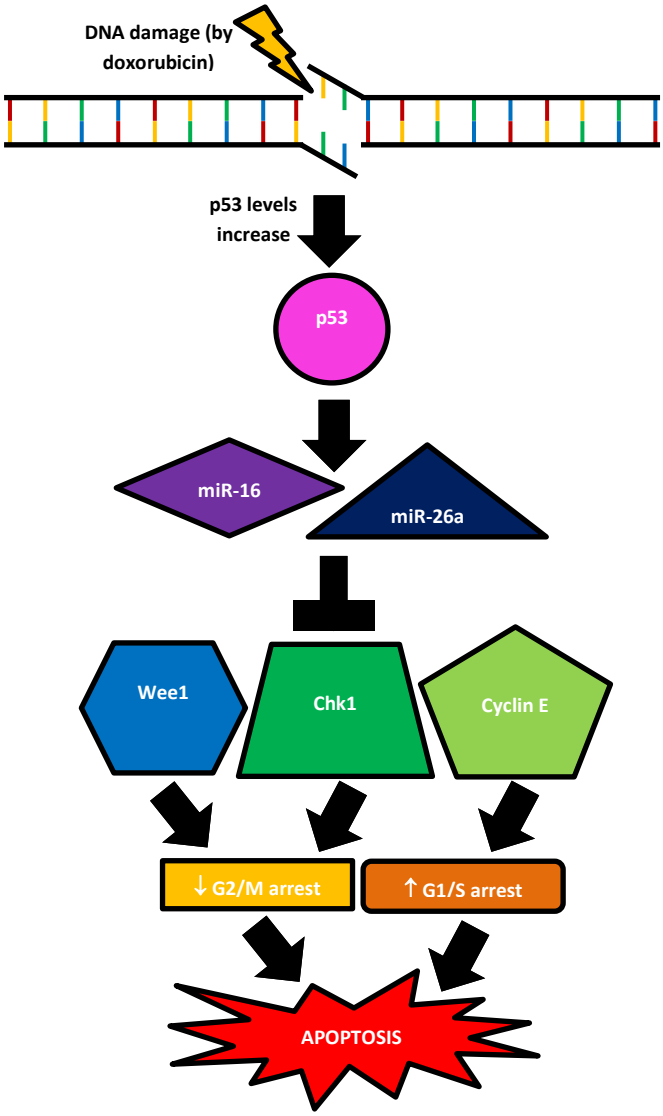


Figure 1.15 Predicted cascade of events

Exposure to DNA damaging agents, including the chemotherapeutic agent doxorubicin, affects the integrity of the genome by generating double-strand breaks in DNA, leading to potential loss of genetic information. These detected stress signals are then conveyed to the p53 protein, and leads to an increased p53 half-life resulting in p53 accumulating in cells. We wanted to determine if miR-16 and miR-26a expression was p53-dependent. We also wanted to determine if miR-16 and miR-26a target Cyclin E, CHK1, and WEE1 for down-regulation. Reduced CHK1 and WEE1 levels would lead to decreased G2/M arrest, and reduced Cyclin E levels would result in increased G1/S arrest. These events would eventually bring about apoptosis.

MATERIAL AND METHODS

PART 1: miR-16 and miR-26a in cancer

MICRORNA EXPRESSION PROFILING OF MIR-16 AND MIR-26A

(See Fig. 3.1)

Various cancer tissue specimens (breast, hepatocellular, ovarian, and prostate) belonging to cancer patients were used for this investigation.

RNA extraction

Tissue samples kept at -80°C were place on dry ice, and then cut using a cryostat microtome at -30°C. TRIzol® Reagent was added to tissues (as previously described).

Agarose gel electrophoresis

(As previously described)

cDNA synthesis

A TaqMan MicroRNA Assays Human Panel-Early Access Kit was used. The reverse transcriptase (RT) master mix was prepared in an eppendorf on ice, consisting of 0.15µl 100mM dNTPs, 1µl MultiScribe™ Reverse Transcriptase (50U/µl), 1.5µl 10x Reverse Transcription Buffer, 0.19µl RNase Inhibitor, (20U/µl), 4.2µl nuclease-free water, 3µl primer, and 5µl RNA. The reaction was mixed gently, and centrifuged briefly, then kept on ice until the RT reaction plate was being prepared.

The RT reaction plate was prepared. For each 15µl RT reaction, RT master mix was combined with total RNA in the ratio of 7µl: 5µl. This was mixed gently, and centrifuged briefly. The RT Primer plate was thawed on ice. 12 µl of RT master mix containing total RNA was dispensed into each well of the RT Primer plate. 3µl of RT primer from the appropriate wells of the RT Primer Plates was transferred into the wells of the RT reaction plate, bringing the total reaction volume to 15µl. The plate was sealed using MicroAmp® adhesive film, mixed gently, and briefly centrifuged. The plate was incubated on ice for 5 mins.

The RT reaction was run in a thermal cycler as follows: “hold” at 16°C for 30 mins, then “hold” at 42°C for 30mins, “hold” at 85°C for 5 mins, and kept at 4°C infinitely.

Reagent	**Supplier**
TaqMan MicroRNA Assays Human Panel-Early Access Kit	Applied Biosystems

Q-PCR

For a 20µl PCR reaction, 10µl TaqMan 2x Universal PCR Master Mix (No AmpErase UNG) was combined with 6.7µl nuclease-free water in an eppendorf, mixed gently and centrifuged briefly. 2µl of each appropriate TaqMan MicroRNA Assay Mix (10x) was transferred from the TaqMan Plates into the eppendorf. 1.3µl of the RT product from the RT reaction plate was transferred into the eppendorf. The eppendorf was mixed gently and centrifuged briefly. The PCR reaction plate was prepared by dispensing 20µl of the complete PCR master mix into each well. The plate was sealed, mixed gently, then centrifuged briefly.

The PCR reaction plate was run using an ABI PRISM 7900 Real Time PCR System, using the following thermal cycling conditions: "hold" for 10 mins at 95°C, and then 40 cycles of "denaturing" for 15 secs at 95°C, and "annealing/elongation" for 60 secs at 60°C.

The data was analysed by viewing the amplification plots, and setting the baseline and threshold values.

Reagent	**Supplier**
TaqMan MicroRNA Assays Human Panel-Early Access Kit	Applied Biosystems

PART 2: Mechanisms of p53-dependent control for miR-16 and miR-26a

MIR-16 AND MIR-26A Q-PCR

(See Fig. 3.2, 3.3, and 3.4)

In order to confirm microarray expression data suggesting that miR-16-2 and miR-26a expression are regulated by p53.

Maintenance of human cell lines

All reagents were used at room temperature. Dulbeccos's Modified Eagle's medium (DMEM) was supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS) and penicillin-streptomycin (Pen/Strep) (100U/ml and 100µg/ml, respectively), as well as 2mM L-Glutamine.

To passage, growth media was aspirated off adhered cell populations by pipette. Cells were washed once in 2ml PBS, which was then aspirated off. For cells in a 10cm plate, 2ml Trypsin-EDTA was added and the cells were incubated at room temperature for 5 mins until cells had detached from the plate. 6ml DMEM was added to neutralise Trypsin-EDTA, and transferred to a 15ml centrifuge tube. The cells were centrifuged for 5 mins at 1200rpm, the supernatant aspirated, and the cell pellet was re-suspended in 2ml DMEM for splitting into 2 new plates (1ml cells/DMEM per plate). For a 10cm plate, cells were seeded into 10ml DMEM. Newly-plated cells were gently rocked from side-to-side in the plate to ensure an even dispersal of cells in the plate. All cell lines were maintained in a humidified 5% CO_2 atmosphere at 37°C and passaged upon reaching 80% confluency.

U2OS (human osteosarcoma origin) and U2OS pLKO p53 (p53 knockdown by specific small hairpin (sh)RNA, puromycin used for antibiotic selection at 0.5µg/ml) cell lines were cultured in DMEM.

Reagent	Supplier
Dulbecco's Modified Eagle medium (DMEM) (high glucose, GlutaMAX™, pyruvate)	Gibco
Foetal Bovine Serum (FBS)	Gibco
L-Glutamine	Gibco
Penicillin-streptomycin	Gibco
Phosphate-buffered saline (PBS)	Gibco
Trypsin-EDTA (0.05%, Phenol red)	Gibco
Puromycin	Gibco

Long-term storage of human cell lines

Cells were frozen for storage when 80% confluent in culture plates. Cells in 10cm plates were washed and trypsinised before being collected by centrifugation (see previous method). Cells were resuspended in 4.5ml DMEM (as before) supplemented with 10% v/v DMSO. 1.5ml aliquots were transferred to 3 x 2ml cryotubes for freezing (1 cryotube to be revived per 10cm plate). Cryotubes were kept at -80°C overnight, then transferred to liquid nitrogen storage racks after 24 hrs.

Reagent	Supplier
Dulbecco's Modified Eagle medium (DMEM) (high glucose, GlutaMAX™, pyruvate)	Gibco
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich

Cell treatment with DNA damaging agent doxorubicin

0.5µM doxorubicin solution was added to DMEM of plated cells for 0, 12, and 24 hrs, and the plate was gently rocked from side-to-side to ensure an even dispersal of doxorubicin. Cell lines were maintained in a humidified 5% CO_2 atmosphere at 37°C. Non-treated cells were used as a control.

Reagent	Supplier
Doxorubicin hydrochloride	Sigma-Aldrich

Collection of cells

Cells were washed (doxorubicin treatment stopped with this step) and trypsinised before being collected by centrifugation (as before). The cell pellet was frozen at -80°C until required.

Cell lysis

Cell pellets were solubilised by addition of 50µl 1x SDS-PAGE loading buffer by pipette (50mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 100mM DTT, and 0.1% bromophenol blue). Samples were denatured at 95°C for 5 mins in a heat-block, and then placed on ice. Samples were then spun at 13,000rpm for 5 mins at 4°C. The supernatant was loaded onto a 10% polyacrylamide gel by pipette.

Reagent	**Supplier**
Bromophenol blue	Sigma-Aldrich
Dithiothreitol (DTT)	Fisher Scientific
Glycerol	Sigma-Aldrich
Hydrochloric acid (HCl)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Tris base	Sigma-Aldrich
Ultrapure water	Milli-Q

Handcasting polyacrylamide gels

1 inner glass plate and 1 x 1.5mm outer glass plate were washed well with distilled water, and allowed to air-dry following a wash in absolute ethanol. This glass pair was assembled in a glass cassette sandwich in a multi-casting chamber of the Mini-PROTEAN polyacrylamide gel electrophoresis (PAGE) system (Bio-Rad).

Firstly 15ml resolving gel (10% acrylamide, 0.4M Tris-HCl pH 8.8, 0.1% SDS, 4ml ultrapure water, 0.05% APS, and 15µl TEMED) was made and left to set for 30 mins, once overlaid with distilled water. Water was removed, and the 5ml stacking gel (5% acrylamide, 0.1M Tris-HCl pH 6.8, 0.1% SDS, 3.5ml ultrapure water, 0.1% APS, and 15µl TEMED) was made and overlaid on top of the resolving gel once set. A 1.5mm comb was gently added into the top of the stacking gel, which was left to set for 30 mins.

Reagent	Supplier
Acrylamide	Sigma-Aldrich
Ammonium persulphate (APS)	Sigma-Aldrich
Ethanol	Fisher Scientific
Hydrochloric acid (HCl)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris base	Sigma-Aldrich
Ultrapure water	Milli-Q

SDS-PAGE

The handcast gel was placed in the electrophoresis cell, and 500ml 1x SDS-running buffer (0.1% SDS, 25mM Tris, and 0.2M glycine) added. Samples were loaded by pipette, alongside 4µl protein ladder in the first gel lane. Electrophoresis was carried out for 1 hr, initially at 120V, and then increased to 160V once samples had entered the resolving gel. Electrophoresis was stopped just before the dye front had run out of the gel.

Reagent	Supplier
Glycine	Sigma-Aldrich
Protein Ladder (EZ-Run Prestained Rec)	Fisher Scientific
SDS (laemmli) sample buffer	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Tris base	Sigma-Aldrich
Ultrapure water	Milli-Q

Western blotting

Using a tank wet transfer system, resolved proteins were electrophoretically transferred from the polyacrylamide gel and immobilised onto a nitrocellulose membrane in a Mini Trans-Blot cell. The gel and membrane sandwich were held together between two fibre pads and blotting filter paper sheets and placed into the tank within a gel holder cassette. The cassette was submerged under 500ml 1x transfer

buffer (25mM Tris, 192mM glycine, and 10% methanol) within the tank, and an ice block kept the tank contents cool. The transfer was carried out at 100V for 60 mins.

Reagent	Supplier
Glycine	Sigma-Aldrich
Methanol	Fisher Scientific
Tris base	Sigma-Aldrich
Ultrapure water	Milli-Q

Protein detection

Prior to immunological detection, blocking unbound membrane sites was carried out. Nitrocellulose membranes were carefully transferred to a small container, and incubated in 10ml 5% bovine serum albumin (BSA) dissolved in TBST (0.1% Tween-20 in 1x Tris-buffered saline (TBS)) for 60 mins gently rocking at room temperature.

Reagent	Supplier
Bovine serum albumin (BSA)	Fisher Scientific
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The blocked membrane was then incubated in 10ml Ab-6 primary antibody at 1:1000 dilution in 5% BSA/TBST buffer and 100x 2% sodium azide (NaN_3) overnight rocking gently at 4°C.

Reagent	Supplier
Ab-6 (antibody against p53)	Calbiochem
Bovine serum albumin (BSA)	Fisher Scientific
Sodium azide (NaN_3)	Sigma-Aldrich
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The membrane was washed 3x 15 mins in TBST at room temperature rocking to remove unbound antibody.

Reagent	Supplier
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The membrane was incubated in 10ml horseradish peroxidase (HRP)-conjugated secondary antibody (Goat anti-mouse IgG-HRP) diluted 1: 10,000 in 5% BSA/TBST buffer for 50 mins gently rocking at room temperature.

Reagent	Supplier
Bovine serum albumin (BSA)	Fisher Scientific
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The membrane was washed 3x 5 mins rocking vigorously to remove unbound secondary antibody.

Reagent	Supplier
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The signal was developed based on the chemiluminescence. The membrane was exposed to an Immobilon Western Chemiluminescent HRP Substrate for 3 mins.

Reagent	Supplier
Immobilon Western Chemiluminescent HRP Substrate	Millipore

The chemiluminescent signal from the membrane was detected by X-ray film development. To gather information from the membrane, it was necessary to perform multiple exposures to produce data.

Reagent	Supplier
X-ray imaging film	Kodak

The membrane was stripped of all antibodies using Restore Western Blot Stripping Buffer (Thermo Scientific). The membrane was washed 1x 5 mins in TBST at room temperature rocking, to get rid of Chemiluminescent HRP Substrate. The membrane was stripped for 1x 15 mins in the Stripping Buffer at room temperature rocking. The membrane was washed 3x 15 mins in TBST at room temperature rocking, to get rid of Stripping Buffer.

Reagent	Supplier
Restore Western Blot Stripping Buffer	Thermo Scientific
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

The membrane was blocked (as previously described), with addition of 100x 2% sodium azide (NaN_3) to the blocking solution. The membrane was washed 3x 15 mins in TBST at room temperature rocking. The membrane was normalised with Ku70 primary antibody (as previously described).

Reagent	Supplier
Bovine serum albumin (BSA)	Fisher Scientific
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Ku70 (primary antibody for normalisation)	Abcam
Sodium azide (NaN_3)	Sigma-Aldrich
Tris-buffered saline (TBS)	Fisher Scientific
Tween-20	Sigma-Aldrich
Ultrapure water	Milli-Q

Preparation of RNA extracts

To prepare RNA for Q-PCR, harvested cells washed in 30µl 1x PBS were homogenised with 1ml (per 10cm plate of cells) Tri reagent in a 15ml tube, incubated at room temperature for 5 mins, then centrifuged at 10,000rpm for 10 mins at 4°C. 100µl chloroform was added to the supernatant that had been transferred to an eppendorf, incubated at room temperature for 15 mins, and centrifuged at 10,000rpm for 15 mins at 4°C. RNA, partitioned to the aqueous phase, was then precipitated with 500µl isopropanol, incubated at room temperature for 10 mins, and centrifuged at 10,000rpm for 10 mins at 4°C. 1ml 75% ethanol was added to the RNA pellet, centrifuged at 10,000rpm for 5 mins, and air-dried. The RNA pellet was solubilised in 30µl RNase-free water in a 65°C heat block, and stored at -80°C until required.

Reagent	Supplier
Chloroform	Fisher Scientific
DEPC-treated water	Ambion
Ethanol	Fisher Scientific
Isopropanol	Fisher Scientific
Phosphate-buffered saline (PBS)	Gibco
Tri reagent	Invitrogen

Agarose gel electrophoresis

RNA was analysed by agarose gel electrophoresis to assess the yield. RNA was combined with 1x loading buffer (10ml 6x loading buffer: 25mg bromophenol blue, 3ml glycerol, and 25mg xylene cyanol) in a 5:1 ratio. A 2% agarose gel was made by dissolving agarose in 1x TAE buffer (10x TAE: 48.4g Tris, 11.4ml acetic acid, and 3.7g EDTA) supplemented with ethidium bromide (0.5µg/ml). Once set, samples were loaded onto the gel, and the first gel lane was loaded with a DNA ladder. Samples were resolved by electrophoresis in TAE buffer, which was carried out at 100V for 10 mins. Resolved RNA was analysed by UV transillumination (302nm) and images captured using a GeneGenius gel imaging system.

Reagent	**Supplier**
Acetic acid	Fisher Scientific
Agarose	Fisher Scientific
Bromophenol blue	Sigma-Aldrich
DNA ladder	New England Biolabs (NEB)
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Tris base	Sigma-Aldrich
Xylene cyanol	Sigma-Aldrich

cDNA synthesis

Total RNA was used for first strand cDNA synthesis with the miScript II RT kit (miScript PCR starter kit). 1µg RNA was retrotranscribed in a 20µl reaction containing 4µl 1x miScript buffer, 2µl 1x nucleics mix (Qiagen), 20U RNase inhibitor, 2µl miScript reverse transcriptase mix (Qiagen), and RNase-free water up to 20µl in an eppendorf. RNA was denatured and first strand synthesis was carried out at 37°C for 60 mins in a regulated incubator. The miScript reverse transcriptase mix was inactivated by heating at 95°C for 5 mins in a heat-block, and then stored at -20°C until required.

Reagent	**Supplier**
miScript PCR starter kit	Qiagen

Oligonucleotide design

Oligonucleotides for Q-PCR were for the primary (pri) and mature sequences of miR-16-2 and miR-26a, and were designed to be 18-24 nucleotides in length with a GC: AT ratio of approximately 50%, thus ensuring a practical annealing temperature of around 50°C. miR-16-1 was used as a negative control.

Primers were initially validated at 350nM and 700nM using control cDNA (undiluted and 1/10 diluted) to assess the miR-16-2 or miR-26a products amplified. This was done by melt curve and agarose gel electrophoresis analysis, which was also used to assess annealing temperatures suitable for the primers. However the basic PCR reaction consisting of one cycle of activation at 95°C for 10 mins, followed by 40 cycles of denaturation at 95°C for 15 secs, annealing at 55° for 15 secs, and elongation at 72°C for 15 secs.

Primer	**Forward**	**Reverse**	**Supplier**
Primary (pri) miR-16-2	5'-GCACGTAAATATTGGCGTAGTGAA -3'	5'-AAAATAGTTGCTGTATCCCTGTCACA -3'	Invitrogen
Primary (pri) miR-26a-1	5'-CGTGGCCTCGTTCAAGTAATC -3'	5'-AACCAAGAATAGGCCCATTGG -3'	Invitrogen
Mature miR-16-2	5'-TTTCTCTATCGATAGGTACCTTTTTGGAATAAAAGCATTG -3'	5'-CCGGAATGCCAAGCTTTCAAAGTTTCGTTCTTTCAG-3'	Invitrogen
Mature miR-26a-1	5'-TTTCTCTATCGATAGGTACCGCACACATTCAAAAAGCTGC -3'	5'-CTTAGATCGCAGATCTGTGCAGGTCCCAATGGG-3'	Invitrogen

Q-PCR

Samples prepared for Q-PCR were run in triplicate in a Rotor-Gene 6000 (Corbett) Q-PCR cycler for 1 hr. Reactions were each 20µl in volume (10µl SensiMix SYBR Green

mix, 1µl 500nM miR-16-2 or miR-26a primer, 8µl water, and 1µl 1/10 diluted cDNA). Control reactions did not contain cDNA.

Reagent	**Supplier**
DEPC-treated water	Ambion
Mature miR-16-2 or miR-26a primers	Invitrogen
Primary (pri) miR-16-2 or miR-26a primers	Invitrogen
SensiMix SYBR No-ROX kit	Bioline

MIR-16 AND MIR-26a LUCIFERASE ASSAY

(See Fig. 3.5, 3.6, 3.7, 3.8, and 3.9)

As p53 was shown to affect expression of several miRNAs, at both the levels of transcription and post-transcription, we assessed whether p53 regulates miR-16-2 and miR-26a expression directly by binding their promoters and activating their transcription.

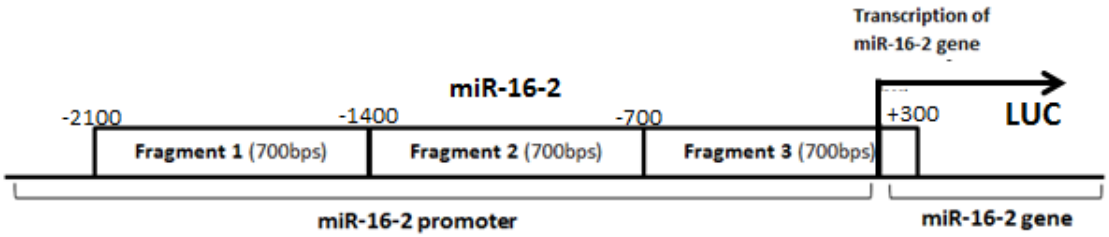
Cloning

A basic cloning procedure was followed involving amplification of insert DNA by polymerase chain reaction (PCR), restriction digestion of purified PCR products and destination vector, and then ligation of the two under appropriate conditions as outlined below. DNA was prepared from bacterial cultures by mini-prep (QIAGEN). The specific details of each stage are described below.

Oligonucleotide design

Oligonucleotides for PCR-based cloning were designed encompassing miR-16-2 and miR-26a-1 promoter regions with predicted p53REs/binding sites, and to be 18-24 nucleotides in length with a GC:AT ratio of approximately 50%, thus ensuring a practical annealing temperature of around 50°C. To incorporate appropriate restriction enzyme sites for cloning (miR-16-2 fragment 1: 5' - KpnI, 3' - NheI, miR-16-2 fragment 2: 5' - NheI, 3' - BglII, miR-16-2 fragment 3: 5' - BglII, 3' - HindIII, miR-16-2 whole promoter piece: 5' – KpnI, 3' - HindIII, miR-26a fragment 1: 5' - KpnI, 3' - BglII, miR-26a fragment 2: 5' - KpnI, 3' - BglII, and miR-26a whole promoter piece: 5' - KpnI, 3' - BglII), additional bases were added to the 5' end of the primer sequences with extra bases added in to ensure the maintenance of the correct reading frame upon insertion into the destination vector, as necessary. A GC clamp (4 bases), was added 5' to the restriction site sequence to allow for efficient digestion.

A



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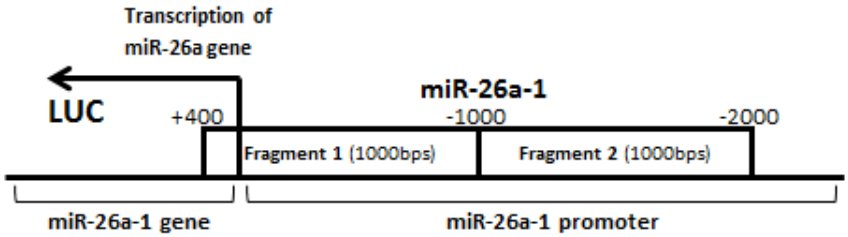


Figure 2.1 Schemes of miR-16-2 and miR-26a-1 promoter fragments for cloning
Oligonucleotides for PCR-based cloning were designed encompassing **(A)** miR-16-2 and **(B)** miR-26a-1 promoter regions with predicted p53REs/binding sites.

Primer	Forward	Reverse	Supplier
miR-16-2 (promoter fragment 1)	5'-GGTACCTTTTTGGAATAAAAGCATTG-3'	5'-GCTAGCCTTTCCCTTAACCTTAAAAG-3'	Invitrogen
miR-16-2 (promoter fragment 2)	5'-GCTAGCTGTTAAAGTAGATTTGCAATTG-3'	5'-AGATCTCAACATCCTTAAATGTCTTT-3'	Invitrogen
miR-16-2 (promoter fragment 3)	5'-AGATCTGAAATCTTCTTCGAAGCCATG-3'	5'-AAGCTTTCAAAGTTTCGTTCTTTCAG-3'	Invitrogen
miR-16-2 (whole promoter-fragments 1,2 and 3)	5'-TTTCTCTATCGATAGGTACCTTTTTGGAATAAAAGCATTG-3'	5'-CCGGAATGCCAAGCTTTCAAAGTTTCGTTCTTTCAG-3'	Invitrogen
miR-26a-1 (promoter fragment 1)	5'-GGTACCGCACACATTCAAAAAGCTGC-3'	5'-AGATCTTGAATTTTAAAGAGTTTGAT-3'	Invitrogen
miR-26a-1 (promoter fragment 2)	5'-GGTACCGCATGTTTACTGAGCCTCAG-3'	5'-AGATCTGTGCAGGTCCCAATGGG-3'	Invitrogen
miR-26a-1 (whole promoter-fragments 1 and 2)	5'-TTTCTCTATCGATAGGTACCGCACACATTCAAAAAGCTGC-3'	5'-CTTAGATCGCAGATCTGTGCAGGTCCCAATGGG-3'	Invitrogen

Polymerase chain reaction

To obtain insert DNA for cloning, PCR amplification was carried out using PfuTurbo® DNA polymerase. A standard reaction typically contained 500ng template DNA, 6pM forward primer, 6pM reverse primer, 1x Cloned Pfu DNA polymerase reaction buffer, 4.5mM dNTPs, 1U Pfu Turbo DNA polymerase, and the appropriate volume of water to complete the reaction volume of 20µl in a PCR tube. Annealing temperatures and elongation times suitable for each set of primers and template, respectively, were employed as appropriate, however the basic PCR reaction consisted of one cycle of activation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for the appropriate extension time (1 min per kb), followed by one cycle of extension at 72°C for 10 mins.

Reagent	**Supplier**
Cloned Pfu DNA polymerase reaction buffer (10×)	Stratagene
Deoxyribonucleotides (dNTPs)	Promega
Pfu Turbo DNA polymerase	Stratagene
miR-16-2 or miR-26a primers	Invitrogen
Ultrapure water	Milli-Q

DNA ligation (Blunt-end cloning)

Using a blunt-end cloning protocol, ligation reactions were carried out in a volume of 20µl in a PCR tube. Typically, a vector: insert molar ratio of 1:2 was employed, in a standard reaction mix of 50ng pJET1.2/blunt cloning vector DNA, 100ng insert (miR-16-2 or miR-26a) DNA, 1U T4 DNA ligase diluted in 10µl of 2x ligation buffer, and the rest of the reaction volume made up with nuclease-free water. Reactions were incubated overnight at room temperature.

Reagent	**Supplier**
CloneJET PCR Cloning Kit	Thermo-Scientific

For miR-16-2 and miR-26a (whole promoters), sub-cloning into the pJET 1.2/blunt cloning plasmid was not carried out. Instead they were cloned straight into the pGL3

luciferase reporter vector, upstream of a luciferase gene, using the In-Fusion® HD cloning kit.

Bacterial transformation

For cloning, 10µl of ligation mix was added to 100µl of TOP10 chemically competent cells in an eppendorf. The mix was incubated on ice for 15 mins, heat shocked at 42°C for 2 mins in a heat block to induce plasmid DNA uptake, and then return to the ice for a further 2 mins. Under sterile conditions, 100µl of S.O.C media was added to the cells and the transformation mix was incubated for 30 mins at 37°C and shaking at 200rpm. Following this incubation, under sterile conditions, these cells were spread onto LB agar plates containing ampicillin at a concentration of 100µg/ml agar. These plates were inverted and incubated overnight for 16 hrs at 37°C in a regulated incubator. The following morning plates were checked for bacterial colonies.

Reagent	**Supplier**
Ampicillin	Sigma-Aldrich
Luria broth (LB) agar	Fisher Scientific
S.O.C medium	Invitrogen
TOP10 competent cells	Invitrogen
Ultrapure water	Milli-Q

Isolation of plasmid DNA by DNA miniprep

Individual bacterial colonies were picked off LB agar plates, inoculated into 3ml LB containing 3µl 1000x ampicillin and incubated overnight for 16 hrs at 37°C, 200rpm for clone screening. Following this incubation, cells were collected by centrifugation (2000rpm for 10 mins) and DNA was isolated using the QIAprep Spin Miniprep kit. DNA was eluted in 50µl of sterile water.

Reagent	**Supplier**
Ampicillin	Sigma-Aldrich
LB broth	Fisher Scientific
QIAprep spin miniprep kit	Qiagen
Ultrapure water	Milli-Q

Restriction enzyme digest

1µg pJET plasmid DNA was digested with 1U of restriction endonuclease (see table below) in the suitable buffer at 37°C for 3 hrs in a regulated incubator. Digested reactions were resolved on an agarose gel (1%).

DNA insert	**Restriction endonucleases (and buffer)**	**Supplier**
miR-16-2 (promoter fragment 1)	BglII (NEBuffer 3)	New England Biolabs (NEB)
miR-16-2 (promoter fragment 2)	BglII (NEBuffer 3)	New England Biolabs (NEB)
miR-16-2 (promoter fragment 3)	BglII (NEBuffer 3)	New England Biolabs (NEB)
miR-16-2 (whole promoter-fragments 1, 2, and 3)	KpnI and HindIII (NEBuffer 2)	New England Biolabs (NEB)
miR-26a-1 (promoter fragment 1)	BglII (NEBuffer 3)	New England Biolabs (NEB)
miR-26a-1 (promoter fragment 2)	BglII (NEBuffer 3)	New England Biolabs (NEB)
miR-26a-1 (whole promoter-fragments 1 and 2)	KpnI and BglII (NEBuffer 2)	New England Biolabs (NEB)

DNA Sanger sequencing

Automated DNA sequencing was employed to verify the presence of the appropriate insert and the correct reading frame. This was carried out by the Protein Nucleic Acid Chemistry Laboratory (PNACL, Leicester) using their high-throughput Applied Biosystems 3730 Genetic Analyser. 0.5µg pJET plasmid DNA template in a 10µl volume (for a 3-10 kb template), and 1pmol/µl pJET forward and reverse primers in a 10µl volume each were supplied to PNACL.

Primer	Forward	Reverse	Supplier
pJET	5'-CGACTCACTATAGGGAGAGCGGC-3'	5'-AAGAACATCGATTTTCCATGGCAG-3'	Thermo-Scientific

Restriction enzyme digest

pJET constructs containing insert (miR-16-2 or miR-26a) DNA, and pGL3-Basic destination vector were digested with BglII restriction enzyme using NEBuffer 3 at 37°C overnight in a regulated incubator.

Reagent	Supplier
NEBuffer 3	New England Biolabs (NEB)
BglII	New England Biolabs (NEB)
Ultrapure water	Milli-Q
pGL3-Basic luciferase reporter vector	Promega

Dephosphorylation of pGL3 vector

Following digestion, pGL3 was dephosphorylated to prevent self-ligation of this vector. The 5' phosphates of the linearised vector were removed by incubating the entire vector reaction volume with 1U/1µl Shrimp alkaline phosphatase (SAP) for 1 hr at 37°C in a regulated incubator, and then SAP was inactivated at 65°C for 15 mins in a heat block.

Reagent	Supplier
Shrimp alkaline phosphatase (SAP)	Thermo-Scientific

Purification for cloning

The total reaction volumes of digested insert (miR-16-2 or miR-26a) DNA and digested, dephosphorylated pGL3 were separated by agarose gel and then purified for cloning using the QIAquick gel extraction kit. Purified DNA was eluted in 20µl of sterile water.

Reagent	Supplier
Isopropanol	Fisher Scientific
QIAquick gel extraction kit	Qiagen
Ultrapure water	Milli-Q

DNA ligation

Ligation reactions were carried out using a T4 DNA ligase. Typically, a standard reaction mix was 125ng pGL3 DNA, 150ng insert (miR-16-2 or miR-26a) DNA and 5U T4 DNA ligase diluted in 1x T4 DNA Ligase Reaction Buffer, and the rest of the volume made up with water to 10µl in a PCR tube. Reactions were incubated overnight at room temperature.

Reagent	Supplier
T4 DNA Ligase	New England Biolabs (NEB)
T4 DNA Ligase Reaction Buffer (10x)	New England Biolabs (NEB)
Ultrapure water	Milli-Q

miR-16-2 and miR-26a (whole promoter-all fragments) ligation into the pGL3 luciferase reporter vector was carried out using the In-Fusion® HD cloning kit. Ligation reactions were carried out using an In-Fusion HD Enzyme Premix. Typically, a standard reaction mix was 100ng pGL3 DNA, 200ng insert (miR-16-2 or miR-26a) DNA and 2µl 5x In-Fusion HD Enzyme Premix diluted in water. 10µl reactions were incubated for 15 mins at 50°C in a heat-block.

Reagent	Supplier
In-Fusion HD Cloning Kit	Clontech
Ultrapure water	Milli-Q

Bacterial transformation

(As previously described)

Isolation of plasmid DNA by DNA miniprep

(As previously described)

Restriction enzyme digest

1µg pGL3 plasmid DNA was digested with 1U of BglII restriction enzyme using NEBuffer 3 at 37°C for 3 hrs in a regulated incubator.

DNA Sanger sequencing

(As previously described)

Primer	Forward	Reverse	Supplier
pGL3 "RVprimer3"	5'-CTAGCAAAATAGGCTGTCCC-3'	5'-GACGATAGTCATGCCCCGCG-3'	Invitrogen

Isolation of plasmid DNA by DNA maxiprep

For isolation of large quantities of ultrapure transfection grade plasmid DNA, DNA plasmid maxipreps were used.

1µl of plasmid DNA was added to 100µl of TOP10 competent cells for bacterial transformation as previously described.

Starter cultures were made as previously described, except a bacterial colony was inoculating into 100ml LB in a conical flask. Cells were harvested by centrifugation at 7000rpm for 15 mins at 4°C. Plasmid DNA was isolated using a QIAGEN plasmid maxi kit according to the manufacturer's instructions. Isolated plasmid DNA was resuspended in 100µl 10mM Tris-HCl (pH 8) in an eppendorf.

Reagent	Supplier
Ampicillin	Sigma-Aldrich
Hydrochloric acid (HCl)	Fisher Scientific
LB broth	Fisher Scientific
Plasmid maxi kit	Qiagen
Tris base	Sigma-Aldrich
Ultrapure water	Milli-Q

Site-directed mutagenesis

A deletion mutant that lacks a putative p53RE in the miR-26a promoter was generated for use as a negative control.

Oligonucleotide design

Primers for site-directed mutagenesis were approximately 40 nucleotides in length. The forward primer consisted of a region approximately 15 bases 5' of the mutation site, a SacII restriction site, and a region of 15 bases 3' of the mutation site. The mutation site is a deletion of a specific region which is 170bps in the miR-26a-1 fragment 1 promoter piece, where p53 is predicted to bind. The mutation site is 430bps from the start of miR-26a-1 fragment 1. The reverse primer is complimentary to the forward primer.

Primer	**Forward**	**Reverse**	**Supplier**
miR-26a-1 (promoter fragment 1 mutant)	5'-GGCACCTGCTGAGGGTCCGCGGTGCAAGAAGGGGCTTC-3'	5'-GAAGCCCCTTCTTGCACCGCGGACCCTCAGCAGGTGCC-3'	Invitrogen

p53 Response Element Sequence	From (base)	To (base)
CAGGACCTGTGTGGGCACGG	432	451
ATATTGCCCAGCAGGTCTGG	494	513
CCCAGCAGGTCTGGGATTGC	500	519
GCAGGTCTGGGATTGCTCCG	504	523
CTAGGAAGGTCTGGGGTTGA	560	579
AAGCACAGGCCCAGCAAAGC	579	598
TGGTATGTTTCAGGGGATGT	660	679
CAGGCCAGGCCAGGGGGTAG	794	813

Table 2.1 p53 response elements (REs) present in miR-26a-1 fragment 1
Site-directed mutagenesis was used to delete a specific region in the miR-26a-1 fragment 1 promoter piece, where p53 is predicted to bind. This generated a miR-26a-1 mutant to use as a control in the luciferase assay.

Polymerase chain reaction (PCR)

To induce nucleotide changes in plasmid DNA, 5ng template DNA (miR-26a fragment 1/pGL3 construct) was amplified by PCR in 2x 25µl reaction mix containing either 125pM forward or reverse mutagenesis primers, 5mM dNTPs, and 1.25U Pfu Turbo DNA polymerase in 1x Pfu buffer, made up to the required volume with water in PCR tubes. The PCR amplification cycle consisted of activation at 95°C for 30 secs, followed by 3 cycles of denaturation at 95°C for 35 secs, annealing at 50°C for 1 min and an elongation step of 68°C for 12 mins. The two PCR reactions were combined and PCR was run for a further 9 cycles.

DpnI digest

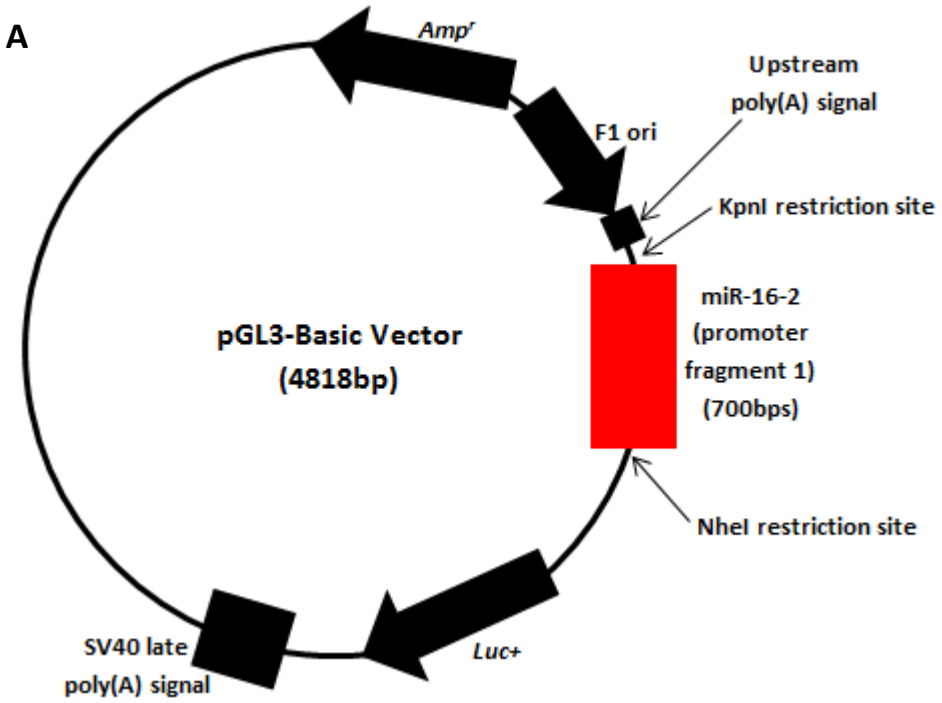
To eliminate parental plasmid DNA, the PCR mix was digested with DpnI enzyme. Typically, 2.5U 0.1µl DpnI was added to the reaction in an eppendorf, and incubated at 37°C for 1 hr in a heat-block.

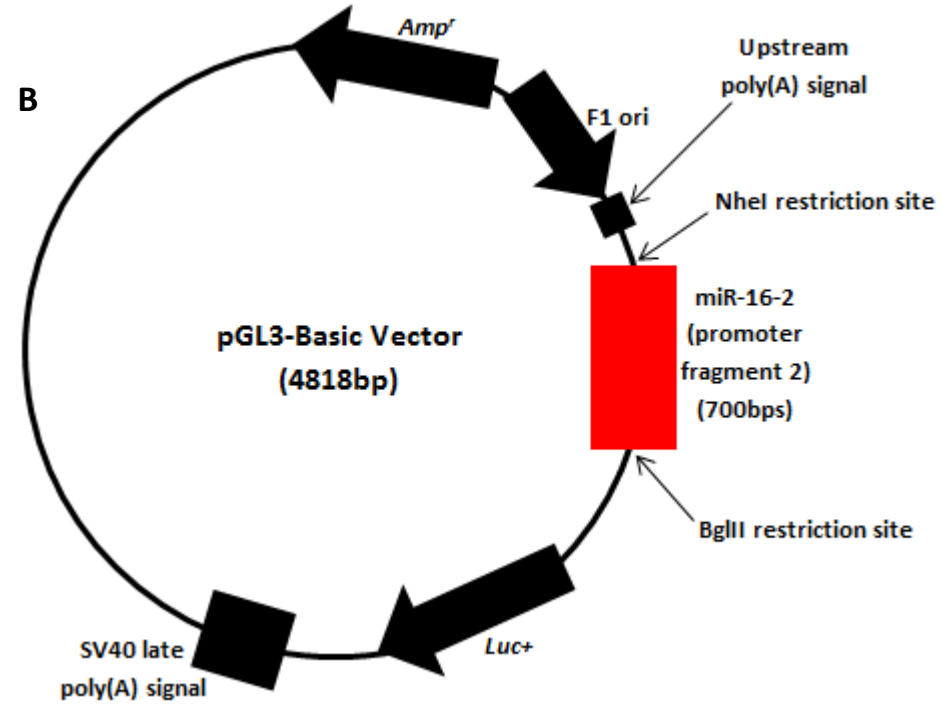
Reagent	**Supplier**
DpnI	New England Biolabs (NEB)

DNA Sanger sequencing

(As previously described)

Primer	**Forward**	**Reverse**	**Supplier**
pGL3 "RVprimer3"	5´-CTAGCAAAATAGGCTGTCCC-3´	5´-GACGATAGTCATGCCCCGCG-3´	Invitrogen


A
Amp^r
Upstream
poly(A) signal
F1 ori
KpnI restriction site
pGL3-Basic Vector
(4818bp)
miR-16-2
(promoter
fragment 1)
(700bps)
NheI restriction site
SV40 late
poly(A) signal
Luc+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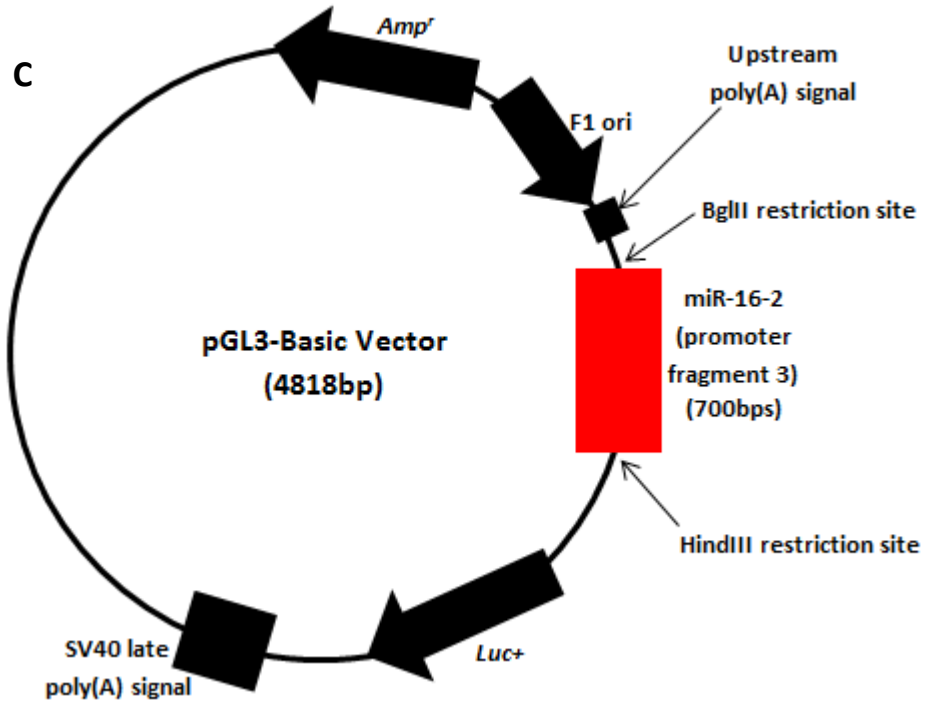
Amp^r
Upstream
poly(A) signal
F1 ori
NheI restriction site
pGL3-Basic Vector
(4818bp)
miR-16-2
(promoter
fragment 2)
(700bps)
BglII restriction site
SV40 late
poly(A) signal
Luc+

C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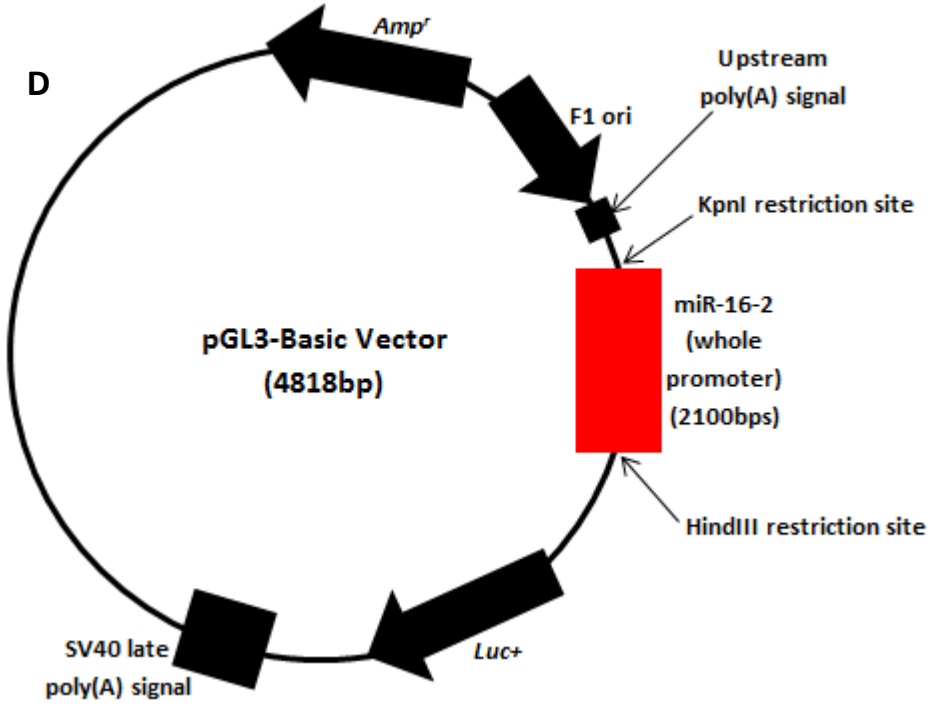
fragment 3)
(700bps)
HindIII restriction site
SV40 late
poly(A) signal
Luc+

D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miR-16-2
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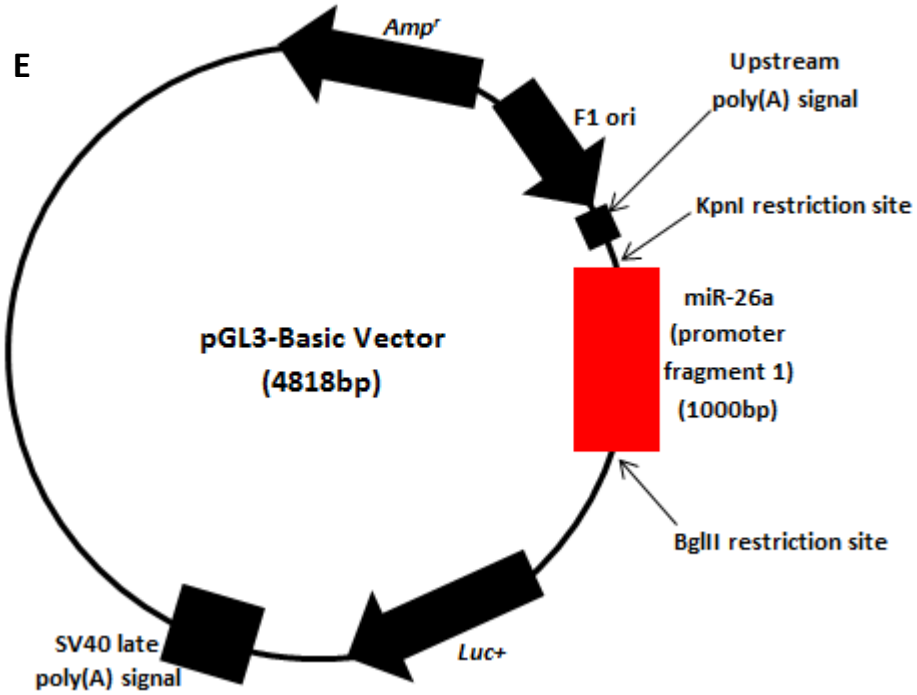
promoter)
(2100bps)
HindIII restriction site
SV40 late
poly(A) signal
Luc+

E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miR-26a
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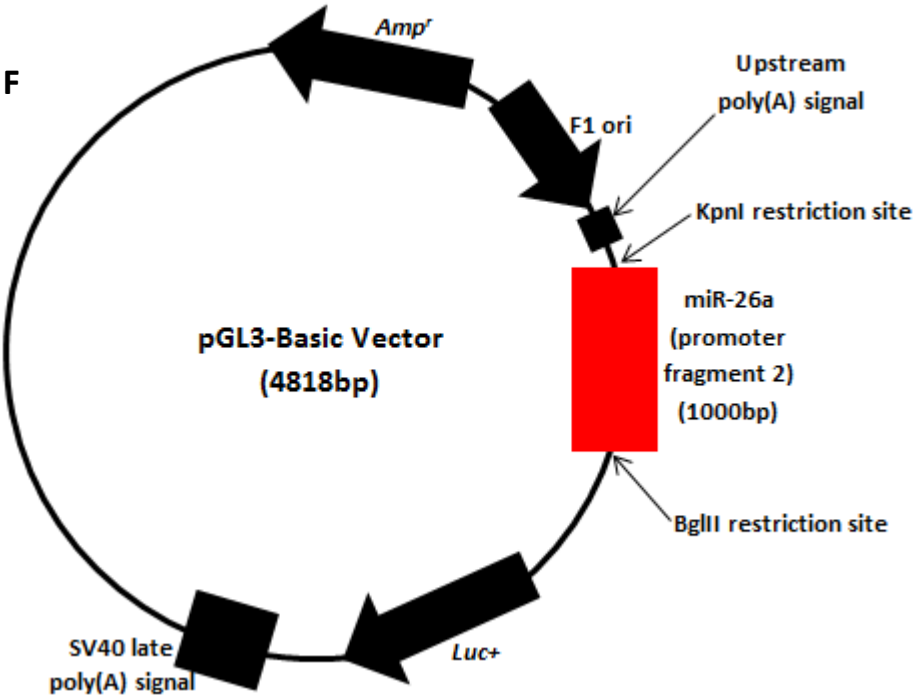
fragment 1)
(1000bp)
BglII restriction site
SV40 late
poly(A) signal
Luc+

F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KpnI restriction site
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miR-26a
(promoter
fragment 2)
(1000bp)
BglII restriction site
SV40 late
poly(A) signal
Luc+

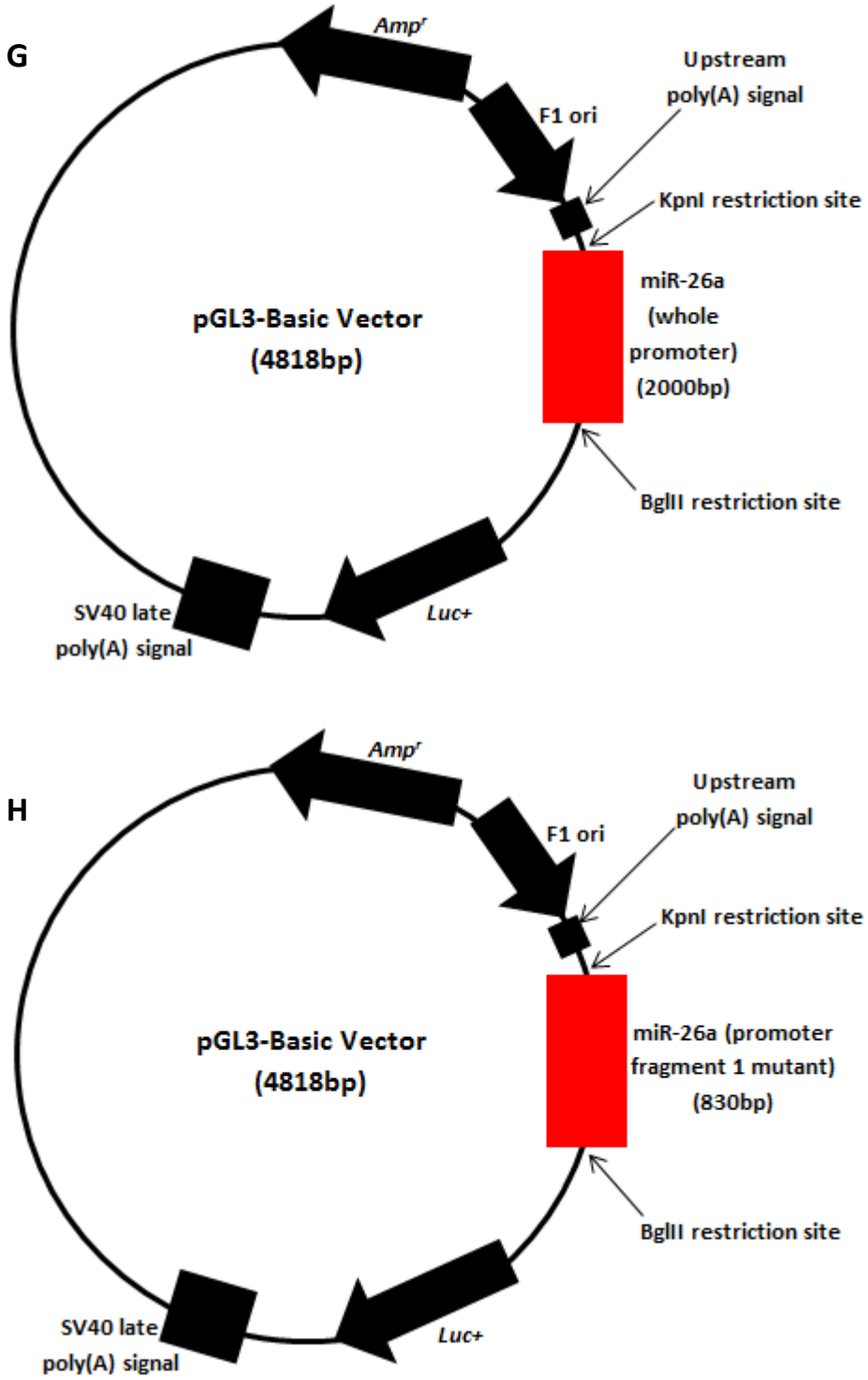


Figure 2.2 Schemes of miR-16-2 and miR-26a-1 promoter luciferase constructs for luciferase assay

Completed miR-16-2 **(A)**, **(B)**, **(C)**, **(D)** and miR-26a-1 **(E)**, **(F)**, **(G)**, **(H)** promoter luciferase constructs for use in luciferase assay.

Transfection

Transient transfection of cultured mammalian cells to induce expression of appropriate recombinant genes was achieved using Lipofectamine 2000 with U2OS and U2OS pLKO p53 cells.

Cells were seeded at a density of 10^5 in 24-well culture plates 24 hrs prior to transfection.

1.5µg plasmid DNA (0.5µg β-galactosidase, 0.5µg miRNA luciferase reporter, 0.25µg p53 (wild-type/K4R/R175H/Δ40/Δ133 mutants) and 0.25µg control pcDNA3) was added to 50µl Opti-MEM in a sterile tube, and 3.75µl Lipofectamine 2000 added to 50µl Opti-MEM in a separate sterile tube. The tube contents were incubated for 5 mins, after which the Lipofectamine/Opti-MEM mix was added to the DNA/Opti-MEM mix. This mix was incubated for 20 mins, during which DMEM in the 24-well culture plate was aspirated off and replaced with fresh DMEM. The DNA/Lipofectamine/Opti-MEM mix was added gently and drop-wise to the plated cells in DMEM. The plate was gently rocked from side-to-side to ensure an even dispersal of the DNA/Lipofectamine/Opti-MEM mix. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Control reactions that did not have p53 had p53 substituted with 0.25µg extra control pcDNA3.

Following a 15 hr incubation period (5% CO_2, 37°C), plated DMEM was aspirated off and replaced with fresh DMEM.

Reagent	**Supplier**
Dulbecco's Modified Eagle medium (DMEM) (high glucose, GlutaMAX™, pyruvate)	Gibco
Lipofectamine 2000 reagent	Invitrogen
Opti-MEM reduced serum medium	Gibco

Cell treatment with DNA damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Preparation of cell lysates

DMEM was aspirated off (doxorubicin treatment stopped with this step) and all cells were washed in 0.5ml 1x PBS (per 24-well dish). PBS was aspirated off and cells were treated with 200µl Biovision 1x lysis buffer (per 24-well dish) for 60 mins, rocking vigorously at room temperature. Cells were then frozen at -80°C at least overnight, until required.

Reagent	**Supplier**
Luciferase assay kit (200 assays)	Biovision
Phosphate-buffered saline (PBS)	Gibco

Preparation for luminometer

To make the β-galactosidase substrate, 10ml β-galactosidase solution (1mM $MgCl_2$, 10mM KCl, 60mM Na_2HPO_4, and 40mM NaH_2PO_4) was combined with 20mg dissolved o-nitrophenyl-β-d-galactopyranoside (ONPG; β-galactosidase substrate), and 35µl β-mercaptoethanol in a 15ml tube.

In order to be able to normalise results, the absorbance of the reaction had to be measured. 100µl β-galactosidase substrate was added to a 96-well transparent plate (per well), alongside 80µl whole cell lysate from the 24-well reaction plate (per well). The plate was incubated at 37°C for 15 mins. ONPG added to the lysate will turn yellow at a rate and intensity proportional to the expression of β-galactosidase. To this effect you can analyse how much expression occurs of your original gene based on how much β-galactosidase gets created under the same conditions.

In order to read the luminescence of the reaction, 20µl whole cell lysate was added to a 96-well opaque plate (per well).

Using the luminometer spectrometer

Soon after, the luminometer (PerkinElmer) was used to read the absorbance of the reactions at 450nm, from the 96-well transparent plate. This was followed by reading the luminescence. 100µl substrate A (Biovision) was pipetted into the 96-well opaque plate (per well). Within 10 mins, 100µl substrate B (Biovision) was injected into the same plate (per well), and the luminescence read.

Reagent	Supplier
Luciferase assay kit (200 assays)	Biovision
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich
O-nitrophenyl-β-D-galactopyranoside (ONPG)	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich
Sodium phosphate monobasic (NaH_2PO_4)	Sigma-Aldrich
Ultrapure water	Milli-Q
β-mercaptoethanol	Fisher Scientific

Western blotting

(As previously described) using cell lysate samples.

Antibody	Supplier
Ab-6 (antibody against p53 N-terminus)	Calbiochem
Ab-1 (antibody against p53 C-terminus)	Calbiochem
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Ku70 (primary antibody for normalisation)	Abcam

MIR-16 AND MIR-26a CHIP ASSAY

(See Fig. 3.10, 3.11, 3.12, and 3.13)

To investigate whether endogenous p53 affects transcription of miR-16 and miR-26a by binding to its promoter, Chromatin Immunoprecipitation (ChIP) assay using a p53-specific antibody was carried out. In the miR-26a-1 promoter, DNA regions with putative p53REs were 3000bps upstream of the transcription start site, and 500bps, 1500bps, and 2000bps downstream of this start site. In the miR-16-2 promoter, DNA regions with putative p53REs were 4000bps and 700bps downstream of the transcription start site. miR-16-1 was used as a control. Since p53 was shown to interact with numerous RNA processing proteins, such as the Drosha complex, we predicted that p53 could recruit these enzymes to promoters of certain miRNA genes (miR-26a-1) to help in the processing of these miRNAs coupled with transcription. To address this question, we also carried out ChIP analysis using antibodies against Drosha and p68 helicase, which is known for its interaction with Drosha.

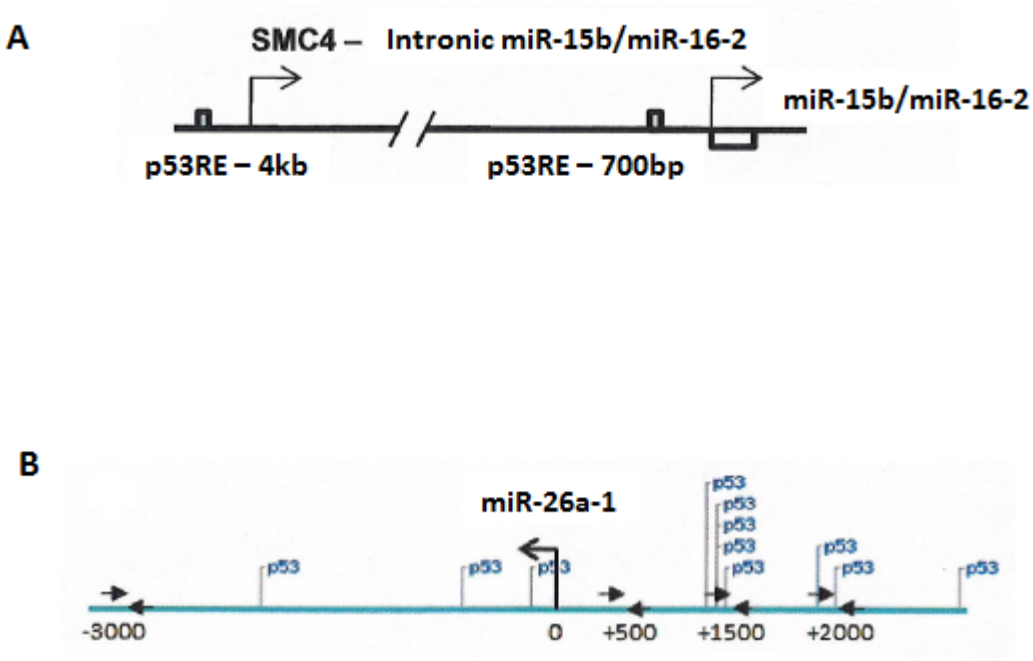


Figure 2.3 Schemes of miR-16-2 and miR-26a-1 promoters for chromatin immunoprecipitation (ChIP) assay
miR-16-2 **(A)** and miR-26a-1 **(B)** promoters with predicted p53 (response elements) REs.

Crosslinking of DNA-binding proteins

U2OS and U2OS pLKO p53 cells were cultured on 10cm culture plates up to 90-100% confluency and non-treated or treated with 0.5µM doxorubicin for 0, 3, 6, 12, 14, and 24 hrs (as previously described).

DMEM was aspirated off plates (doxorubicin treatment stopped with this step), and cells were washed with 2ml 1x PBS (per plate). PBS was aspirated off and 10ml 1x PBS was added (per plate), as well as 670µl 16% formaldehyde (to give a final concentration of 1% formaldehyde). The plate was put on a rocking platform for 13 mins at room temperature, gently rocking. The reaction was stopped by adding 560µl 2.5M glycine (to give a final concentration of 125mM glycine per plate). The plate was put on a rocking platform as previously described. A scraper was used to remove cells from the plate, cells collected in a 15ml tube by pipette, and centrifuged at 1200rpm for 5 mins at room temperature. The solution was aspirated off and the cells were washed 2x with 2ml 1x PBS (per plate – PBS added then cells centrifuged then PBS aspirated off). Cell pellets were frozen at -80C until required.

Reagent	**Supplier**
Formaldehyde	Sigma Aldrich
Glycine	Sigma Aldrich
Phosphate buffered saline (PBS)	Gibco

Cell lysis and sample preparation for immunoprecipitation

The cell pellet was resuspended in 4ml Lysis Buffer 1 in a 15ml tube, and incubated for 30 mins at 4°C, rotating on a drum. Cells were spun down at 2000rpm for 10 mins at 4°C and the supernatant was aspirated off.

The cell pellet was resuspended in 4ml Lysis Buffer 2 and incubated for 15 mins at 4°C, rotating. Cells were spun down at 1000rpm for 15 mins at 4°C and the supernatant was aspirated off.

The cell pellet was resuspended in 1.2ml of Immunoprecipitation (IP) Buffer (200mM sodium chloride (NaCl) with 0.5% SDS). For every 10cm plate of cells, 1.2ml IP Buffer 3 was used. Samples were split into 4 eppendorfs (300µl samples) and cells were sonicated for 30 mins at maximum power in a bath of ice and water (30 mins run split into 3x 10 mins (30 secs on and 30 secs off). The cell samples were transferred to a

15ml tube and spun down for 10 mins at 13,000rpm at 4°C. The supernatant was retained, and the pellet discarded. The supernatant was split into 2 eppendorfs, each with 600µl, and 1 was used for immunoprecipitation (IP) and the other kept at -80°C. The supernatant was diluted in a 1:1 ratio with 600µl IP buffer without SDS, and kept for IP. Before proceeding, a 50µl aliquot was kept for input.

Immunoprecipitation of target protein

Pre-clearing was carried out to reduce non-specific binding to either Protein A or Protein G (blocked with salmon sperm DNA) agarose beads. 15µl packed beads (per $3x10^6$ cells) in an eppendorf were washed with 1ml IP Buffer 1 (buffer added, beads spun down at 2000rpm for 3 mins at 4°C, and buffer aspirated off).

Beads were incubated with the supernatant for 1.5 hrs at 4°C with rotation, spun down as before, supernatant collected, and IP continued. The beads were incubated with a 1:1 ratio of 5% BSA (to a final concentration of 2.5% BSA) at 4°C rotating and overnight. The supernatant was incubated with 1µg antibody against the protein of interest, and rotated overnight at 4°C.

The next day, the beads/BSA mix were incubated with the supernatant/antibody mix, and rotated at 4°C for 3 hrs. The beads were spun down, supernatant aspirated off, then beads washed with 1ml IP buffer with 0.02% SDS.

Beads were resuspended in 0.7ml IP buffer (with 200mM NaCl and 0.02% SDS), and transferred to Spin-X centrifuge tube filter columns. Beads were rotated for 5 mins at 4°C, then spun down. Beads were washed 1x with IP buffer (200mM NaCl and 0.05% SDS), then 2x with IP-500 buffer (500mM NaCl), 2x with lithium chloride (LiCl) buffer, and 2x with Tris-EDTA (TE) buffer (pH 8) with no rotation but inverted 6x in the tubes. After spinning down, the column was transferred to a new eppendorf.

Beads were incubated with 100µl Elution buffer (buffer warmed to 65°C in advance) for 30 mins at 65°C in a water bath, and then spun down for 5 mins at 13,000rpm at 4°C, and the supernatant kept. 150µl Elution Buffer was added to the 50µl input (input from post-sonication).

Reagent	Supplier
Ab-6 (antibody against p53)	Calbiochem
p53 (mono-methyl K372) antibody	Abcam
Drosha antibody	Abcam
p68 antibody	Santa Cruz Biotechnology
Bovine serum albumin (BSA)	Fermentas
Protein A or G agarose beads/Salmon sperm DNA	Millipore

Reverse of DNA-protein cross-link and DNA purification

5M NaCl was added to samples to a final concentration of 200mM (4µl for IP sample, and 8µl for input sample), and incubated overnight at 65°C. A 1:1 ratio of TE buffer was added to samples, as well as 0.1µg/µl RNase A for 1 hr at 45°C in a water bath. 20µg Proteinase K was added to samples and samples incubated at 55°C in a water bath for 3 hrs.

DNA was purified from samples using the QIAquick PCR purification kit (Qiagen). 200µl input was used for PCR purification. DNA was eluted in 50µl sterile water and run on an agarose gel (1.8% gel).

Reagent	Supplier
Sodium chloride (NaCl)	Fisher Scientific
Proteinase K	Sigma Aldrich
QIAquick PCR purification kit	Qiagen
RNase A	Sigma Aldrich
Ultrapure water	Milli-Q

Lysis Buffer 1	Lysis Buffer 2	IP Buffer	IP500 Buffer	LiCl Buffer	Elution Buffer	TE Buffer
10mM Tris-Cl (pH 8)	10mM Tris-Cl (pH 8)	20mM Tris-Cl (pH 8)	20mM Tris-Cl (pH 8)	10mM Tris-Cl (pH 8)	10mM Tris-Cl (pH 8)	10mM Tris-Cl (pH 8)
0.25% Triton X-100	200mM NaCl	200mM NaCl	500mM NaCl	250mM LiCl	0.5mM EDTA	0.5mM EDTA
10mM EDTA	10mM EDTA	0.5% Triton X-100	0.5% Triton X-100	1% Triton X-100	1% SDS	
0.5mM EGTA	0.5mM EGTA	0.5% NP40	0.5% NP40	0.5% NP40		
1mM PMSF	1mM PMSF	0.05% DOC	0.05% DOC	0.1% DOC		
		0.05% SDS	1mM PMSF	0.5mM EDTA		
		1mM PMSF		1mM PMSF		

Q-PCR

The same Q-PCR protocol was used as for "MIR-16 AND MIR-26A Q-PCR", but IP samples were used in place of cDNA samples.

Western blotting

(As previously described)

Reagent	Supplier
Ab-6 (antibody against p53)	Calbiochem
p53 (mono-methyl K372) antibody	Abcam
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Goat anti-rabbit IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC

MIR-16 AND MIR-26a FACS ANALYSIS

(See Fig. 3.14, 3.15, and 3.16)

By use of FACs analysis we analysed the effect of miR-26a and miR-16 on cell cycle arrest and apoptosis of cells non-treated or treated with the DNA damaging agent, doxorubicin.

Transfection

U2OS and U2OS pLKO p53 cells were seeded at a density of 5 x 10^5 in 6-well culture plates (per well) 24 hrs prior to transfection (as previously described).

3nM/15nM miR-16/miR-15/miR-26a oligonucleotides, or 10nM/50nM inhibitors, and 18.75µl Lipofectamine 2000 were mixed in 500µl Opti-MEM, and added drop-wise to cells in fresh DMEM. Pre-miR™ negative control was used as a control.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with the DNA damaging agent doxorubicin

Cells were treated or non-treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Cell preparation

DMEM/doxorubicin mix was aspirated off plates (doxorubicin treatment stopped with this step), and cells were washed with 1x PBS. PBS was aspirated off and Accutase™ Enzyme Cell Detachment Medium was used to detach cells from the plate (10ml Medium per 75cm^2 surface area). A cell count was carried out using a haemocytometer, as well as a viability analysis with Trypan Blue (Dead cells take up the blue dye). Cells were centrifuged at 1200rpm for 5 mins in a 15ml tube, and resuspended in an appropriate volume of 1x eFluor® NC Flow Cytometry Staining Buffer so that the final cell concentration was 2x10^7/ml.

Reagent	**Supplier**
Accutase™ Enzyme Cell Detachment Medium	Affymetrix
eFluor® NC Flow Cytometry Staining Buffer (5x)	Affymetrix
Phosphate-buffered saline (PBS)	Gibco
Trypan Blue	Sigma Aldrich

Staining cell surface antigens

Cells were aliquoted as 50µl in eppendorfs. 10µg/ml primary antibody was added so that the final staining volume was 100µl (50µl of cell sample + 50µl of antibody). Cells were incubated for 30 mins in the dark at 4°C, transferred to 15ml tubes, and then washed in 2x 2ml Flow Cytometry Staining Buffer (per 15ml tube). Cells were pelleted by centrifugation at 2000rpm at 4°C for 5 mins. The supernatant was discarded between washes, and cells were resuspended in 2ml Flow Cytometry Staining Buffer.

Reagent	**Supplier**
Ab-6 (antibody against p53)	Calbiochem
eFluor® NC Flow Cytometry Staining Buffer (5x)	Affymetrix

Staining intracellular antigens (for cytoplasmic proteins)

After the last wash, the supernatant was discarded and cells were fixed by adding 100µl Intracellular (IC) Fixation Buffer (per tube). The mix was incubated in the dark at room temperature for 20 mins. 2ml 1x Permeabilisation Buffer was added to the sample. Samples were centrifuged at 2000rpm at room temperature for 5 mins, and the supernatant was discarded. This was carried out twice. Cells were resuspended in 100µl 1x Permeabilisation Buffer (per tube). 10µg/ml fluorochrome-labelled antibody Phycoerythrin (PE) conjugated to Ab-6) for detection of intracellular antigens was added to cells and incubated in the dark at room temperature for 20 mins. 2ml 1x Permeabilisation Buffer was added to each tube. Samples were centrifuged as before, and the supernatant was discarded. 2ml Flow Cytometry Staining Buffer was added to each tube. Samples were centrifuged, and the supernatant was discarded. Stained cells were resuspended in 2ml Flow Cytometry Staining Buffer and data was acquired on a flow cytometer.

Reagent	Supplier
Ab-6 (antibody against p53)	Calbiochem
eFluor® NC Flow Cytometry Staining Buffer (5x)	Affymetrix
Intracellular (IC) Fixation Buffer	Affymetrix
Permeabilisation Buffer	Affymetrix
Phycoerythrin (PE)	Sigma Aldrich

Staining dead cells with propidium iodide

After staining cells for surface antigens, cells were washed 2x with Flow Cytometry Staining Buffer, and then resuspended in 2ml of the same buffer. For every 100µl cells, 5µl propidium iodide was added, and incubated for 15 mins at room temperature before cells were analysed on the flow cytometer.

Reagent	Supplier
eFluor® NC Flow Cytometry Staining Buffer (5x)	Affymetrix
Propidium iodide	Sigma Aldrich

Use of Flourescence-activated cell sorting (FACS)

The flow cytometer was used for the measurement and sorting of fluorescently-labelled cells. A laser excites the fluorescent dyes bound to cells. A detector measures the strength of fluorescent light for each cell, which is displayed as a histogram.

PART 3: Targets of miR-16 and miR-26a

CHK1 AND WEE1 WESTERN BLOTTING

(See Fig. 3.17 and 3.18)

To validate the published targets of miR-16 and miR-26a, we carried out western blotting following transfection with the corresponding miRNAs.

Transfection

U2OS, U2OS pLKO p53, and H1299 cells were seeded at a density of 10^5 in 24-well culture plates (per well) 24 hrs prior to transfection (as previously described).

3nM/15nM miR-15/miR-16/miR-26a oligonucleotides, or 10nM/50nM inhibitors, and 3.75µl Lipofectamine 2000 were mixed in 100µl Opti-MEM, and added drop-wise to cells in fresh DMEM. Pre-miR™ negative control was used as a control.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with DNA damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Preparation of cell lysates

(As previously described in "MIR-16 AND MIR-26a LUCIFERASE ASSAY" section)

Western blotting

(As previously described)

Antibody	**Supplier**
β-tubulin (primary antibody for normalisation)	Cell Signalling Technology
CHK1 monoclonal (primary antibody)	Cell Signalling Technology
WEE1 (primary antibody)	Biovision
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Goat anti-rabbit IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC

WEE1 Q-PCR

(See Fig. 3.19)

Transfection

H1299 cells were seeded at a density of 5 x 10^5 in 6-well culture plates (per well) 24 hrs prior to transfection (as previously described).

25nM/75nM miR-16/miR-26a oligonucleotides and 18.75µl Lipofectamine 2000 were mixed in 500µl Opti-MEM, and added drop-wise to cells in fresh DMEM. Pre-miR™ negative control was used as a control.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with DNA-damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Collection of cells

Cells were washed (doxorubicin treatment stopped with this step) and trypsinised before being collected by centrifugation (as before). The cell pellet was frozen at -80°C until required.

Preparation of RNA extracts

(As previously described)

Agarose gel electrophoresis

(As previously described)

cDNA synthesis

Total RNA was used for first strand cDNA synthesis with Ready-To-Go You-Prime First-Strand Beads. Briefly, 1µg of total RNA was combined with DEPC-treated water up to 30µl in an eppendorf, heated at 65°C for 10 mins in a heat-block, then chilled on ice for 2 mins. The RNA solution was then combined with first-strand reaction mix beads (buffer, dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAguard™, and RNase/DNase-free BSA). 5µg Oligo(dT) primer and DEPC-treated water mix was added to the reaction to reach a total volume of 33µl, and allowed to sit at room temperature for 1 min. The reaction was mixed gently by vortex and centrifuged briefly, and incubated at 37°C for 60 mins in a regulated incubator.

Reagent	Supplier
DEPC-treated water	Ambion
Oligo (dT) primer	Invitrogen
Ready-to-go you-prime first-strand beads	GE Healthcare Life Sciences

Oligonucleotide design

Oligonucleotides for Q-PCR were designed encompassing the full-length of the Wee1 3'UTR, and they were 18-24 nucleotides in length with a GC: AT ratio of approximately 50%, thus ensuring a practical annealing temperature of around 50°C (as previously described).

Primer	Forward	Reverse	Supplier
WEE1 3'UTR	5'-GCATCAGCTAAACTTCCACC-3'	5'-GTATGCTCACGCAGTGCTTG-3'	Invitrogen

Q-PCR

(As previously described)

CHK1 AND WEE1 LUCIFERASE ASSAY

(See Fig. 3.20 and 3.21)

One of the most common experimental approaches to test the efficacy of targeting for specific miRNAs is to evaluate stability of the luciferase gene fused to the 3'UTR of the gene of interest. To engage this approach we constructed a plasmid that carries the 3'UTR of WEE1 or CHK1 attached to the luciferase gene, whose transcription is driven by a minimal SV40 promoter.

Cloning - oligonucleotide design

Oligonucleotides for PCR-based cloning were designed encompassing the predicted binding sites (at www.microrna.org) for miR-16 in the CHK1 3'UTR (494bps) and for miR-16 and miR-26a in the WEE1 3'UTR (1299bps), and to be ~20 nucleotides in length (see below). To incorporate XbaI restriction enzyme sites for cloning, additional bases were added to the 5' end of the primer sequences with extra bases added in. A GC clamp (4 bases), was added 5' to the restriction site sequence to allow for efficient digestion.

Primer	Forward	Reverse	Supplier
CHK1 3'UTR	5'-ACCGATCTAGAAGCCAGAAGATTTGGCTTCC-3'	5'-TTCGGTCTAGAGGCTTCGCTTCACAGACTGA-3'	Invitrogen
WEE1 3'UTR	5'-CCAAGTCTAGAGAACCGCTCTGTCAGCCTTA-3'	5'-AAAGATCTAGAAGAAAACAGGCATCACAAGGA-3'	Invitrogen

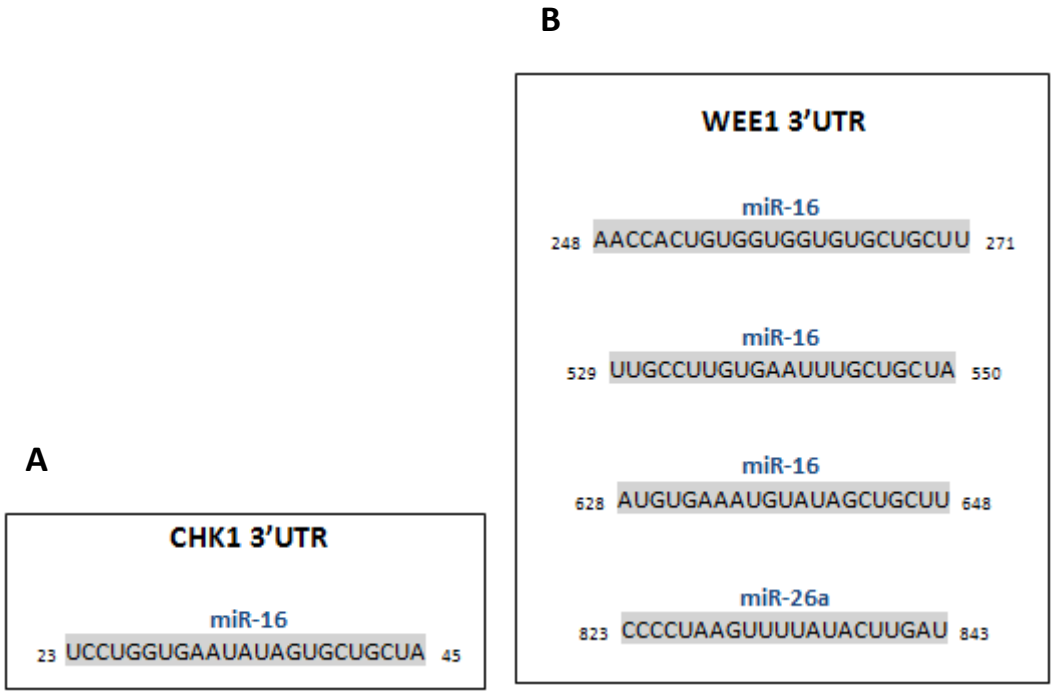


Figure 2.4 Predicted miR-16 and miR-26a binding sites in CHK1 and WEE1 3'UTRs
CHK1 3'UTR **(A)** and WEE1 3'UTR **(B)** with predicted miR-16 and miR-26a binding sites.

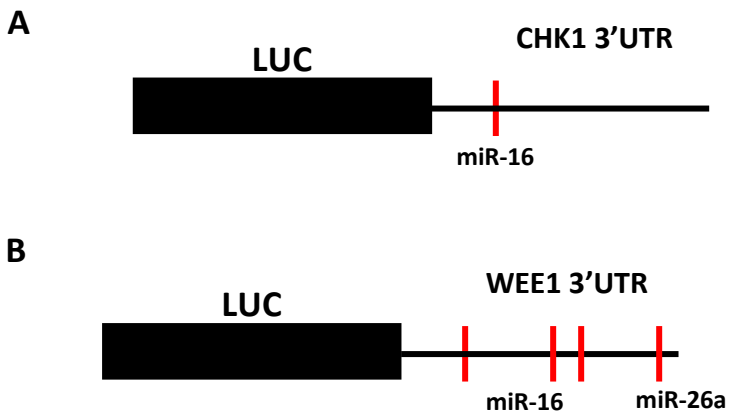


Figure 2.5 Schemes of CHK1 and WEE1 3'UTRs in luciferase constructs
CHK1 3'UTR **(A)** and WEE1 3'UTR **(B)** with predicted miR-16 and miR-26a binding sites were cloned separately into a luciferase plasmid downstream of a luciferase gene.

Polymerase chain reaction

To obtain insert DNA for cloning, PCR amplification was carried out using PfuTurbo® DNA polymerase (as previously described). A standard reaction typically contained template cDNA (not DNA).

Reagent	Supplier
CHK1 or WEE1 primers	Invitrogen

Restriction enzyme digest

Amplified CHK1 and WEE1 3'UTRs, and pGL3-Control destination vector were digested with XbaI restriction enzyme using NEBuffer 4 at 37°C overnight in a regulated incubator.

Reagent	Supplier
NEBuffer 4	New England Biolabs (NEB)
XbaI	New England Biolabs (NEB)
pGL3-Control luciferase reporter vector	Promega

De-phosphorylation of pGL3 vector

(As previously described)

Purification for cloning

(As previously described)

DNA ligation

Ligation reactions were carried out using a T4 DNA ligase as previously described.

Bacterial transformation

(As previously described)

Isolation of plasmid DNA by DNA miniprep

(As previously described)

Restriction enzyme digest

1µg pGL3 plasmid DNA was digested with 1U XbaI restriction enzyme using NEBuffer 4 at 37°C for 3 hrs in a regulated incubator.

DNA Sanger sequencing

(As previously described)

Primer	Forward	Reverse	Supplier
pGL3 "RVprimer3"	5'-CTAGCAAAATAGGCTGTCCC-3'	5'-GACGATAGTCATGCCCCGCG-3'	Invitrogen

Isolation of plasmid DNA by DNA maxiprep

(As previously described)

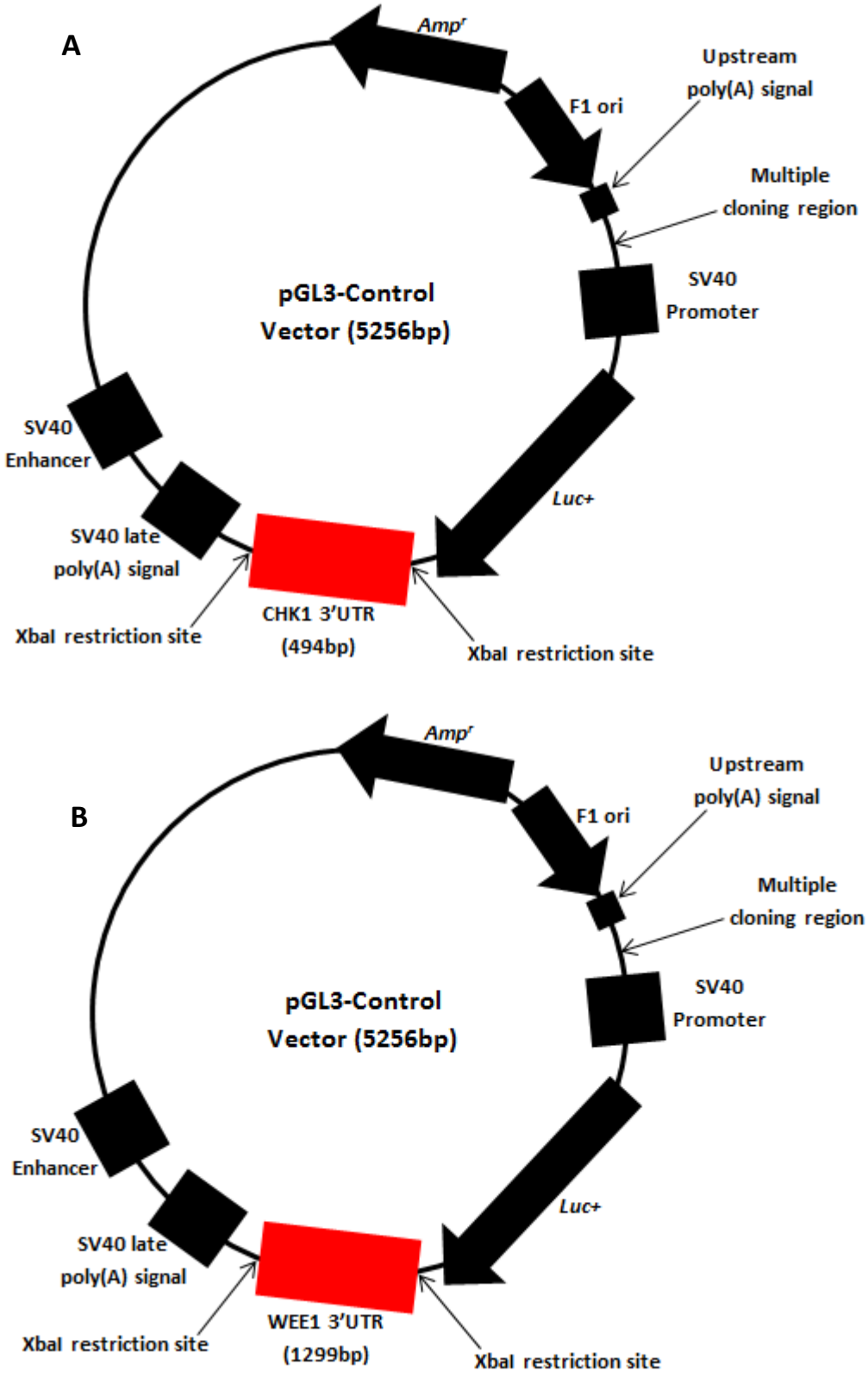


Figure 2.6 Schemes of CHK1 and WEE1 3'UTRs luciferase constructs for luciferase assay

Complete CHK1 3'UTR **(A)** and WEE1 3'UTR **(B)** luciferase constructs for luciferase assay.

Transfection

H1299 cells were seeded at a density of 10^5 in 24-well culture plates (per well) 24 hrs prior to transfection (as previously described).

1.5µg plasmid DNA (0.5µg β-galactosidase, 0.5µg CHK1/WEE1 3'UTR luciferase reporter, and 0.5µg control pcDNA3), 15nM miR-16/miR-26a oligonucleotides and 3.75µl Lipofectamine 2000 were mixed in 100µl Opti-MEM, and added drop-wise to cells in fresh DMEM. 15nM pre-miR™ negative control was used as a control.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with DNA damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Preparation of cell lysates

(As previously described in "MIR-16 AND MIR-26a LUCIFERASE ASSAY" section)

Preparation for luminometer

(As previously described)

Using the luminometer spectrometer

(As previously described)

CYCLIN E WESTERN BLOTTING

(See Fig. 3.22 and 3.23)

We wanted to characterise the effect of miR-16 on expression of Cyclin E in more detail.

Transfection

H1299 and U2OS cells were seeded at a density of 10^5 in 24-well culture plates (per well) 24 hrs prior to transfection (as previously described).

3nM/15nM miR-16 oligonucleotides, or 10nM/50nM inhibitors, and 3.75µl Lipofectamine 2000 were mixed in 100µl Opti-MEM, and added drop-wise to cells in fresh DMEM. Pre-miR™ negative control was used as a control.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with DNA damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 or 24 hrs (as previously described).

Preparation of cell lysates

(As previously described in "MIR-16 AND MIR-26a LUCIFERASE ASSAY" section)

Western blotting

(As previously described)

Antibody	**Supplier**
β-tubulin (primary antibody for normalisation)	Cell Signalling Technology
Cyclin E monoclonal (primary antibody)	Santa Cruz Biotechnology
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Goat anti-rabbit IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Ku70 (primary antibody for normalisation)	Abcam

p53, SET9, AND E2F1 WESTERN BLOTTING

(See Fig. 3.24)

We wanted to characterise the effect of SET9 on expression of p53.

Transfection

U2OS (control), U2OS pLKO p53, and U2OS (SET9 knockdown) cells were seeded at a density of 10^5 in 24-well culture plates (per well) 24 hrs prior to doxorubicin treatment (as previously described).

Cell treatment with DNA damaging agent doxorubicin

Cells were non-treated or treated with 0.5µM doxorubicin for 14 hrs (as previously described).

Preparation of cell lysates

(As previously described in "MIR-16 AND MIR-26a LUCIFERASE ASSAY" section)

Western blotting

(As previously described)

Antibody	**Supplier**
β-tubulin (primary antibody for normalisation)	Cell Signalling Technology
E2F1 (primary antibody)	Santa Cruz Biotechnology
Ab-6 (antibody against p53)	Calbiochem
SET9 (primary antibody)	Santa Cruz Biotechnology
Goat anti-mouse IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC
Goat anti-rabbit IgG-HRP (secondary antibody)	ImmunoChemistry Technologies, LLC

CYCLIN E LUCIFERASE ASSAY

(Fig. 3.25 and 3.26)

SET9 may be an important indirect regulator of Cyclin E expression. To test this possibility, we decided to compare the levels of transcriptional activation driven by the Cyclin E promoter by using the corresponding luciferase plasmids.

The Cyclin E promoter luciferase constructs used contained either wild-type or mutant (negative control) E2F1 response elements. E2F1 and Cyclin E are involved in an autoregulatory loop that ultimately affects the progression of cells through the G_1 phase of the cell cycle.

Transfection

H1299, H1299 SET9 KD (SET9 knocked-down with a specific small hairpin shRNA), U2OS, or U2OS SET9 KD cells were seeded at a density of 10^5 in 24-well culture plates (per well) 24 hrs prior to transfection (as previously described).

1.5µg plasmid DNA (0.5µg β-galactosidase, 0.5µg Cyclin E promoter luciferase reporter, and 0.5µg control pcDNA3) and 3.75µl Lipofectamine 2000 were mixed in 100µl Opti-MEM, and added drop-wise to cells in fresh DMEM. Control reactions did not have Cyclin E, which was substituted with 0.5µg extra control pcDNA3.

Following a 15 hr incubation period, DMEM was replaced with fresh DMEM.

Cell treatment with doxorubicin

Cells were treated with or without 0.5µM doxorubicin for 14 hrs (as previously described).

Preparation of cell lysates

(As previously described in "MIR-16 AND MIR-26a LUCIFERASE ASSAY" section)

Preparation for luminometer

(As previously described)

Using the luminometer spectrometer

(As previously described)

RESULTS

The p53 pathway and miRNAs

Recent studies have shown that interactions between miRNAs and p53 occur at multiple levels. Initial findings showed a difference in expression of several miRNAs in $HCT116^{p53+}$ colon cancer cells versus $HCT116^{p53-}$. Previous experiments in the lab utilised microarray expression analysis of miRNAs followed by Q-PCR to identify miRNAs dependent on p53 and DNA damage. Based on these preliminary data, two miRNAs have been identified whose expression was dependent on p53, which are miR-16 and miR-26a.

miR-16

In 2002 the miRNAs miR-15 and miR-16 were identified as the first cancer genes, which are implicated in the progression of CLL, which is the most common type of adult leukaemia. Alterations in miR-16 expression have been seen in brain, breast, colon, lung, ovarian, pancreatic, prostate, and stomach cancers. There are two copies of miR-16, miR-16-1 and miR-16-2, located on chromosome 13 and 4, respectively. Originally, miR-16 was shown to exert its role as a tumour suppressor by down-regulating BCL2, which is overexpressed in CLL. In B lymphocytes, the miR-15a/miR-16-1 cluster is highly expressed whereas the miR-15b/miR-16-2 cluster is detected as minimal levels in a normal human state. A loss in miR-16 expression is seen in a vast number of cancer cells, and therefore it would be an ideal candidate to target for intervention via therapy.

miR-26a

miR-26 is found in vertebrates, including humans. A panel of 91 cancer-derived cell lines was analysed for sequence variations in 15 miRNAs involved in tumourigenesis by virtue of their known target transcripts or their localisation to sites of frequent chromosomal instability. One of these miRNAs was miR-26. In several tumour types, such as bladder and breast cancers, miR-26 is down-regulated and it may display traits of a tumour suppressor during cancer progression in the same tumours. With regards to miR-26a-1, compared to the rest of the miR-26 family, it is the most abundant member in humans. In tumours a reduced level of miR-26a-1 is observed. The location of miR-26a-1 is at a tumour-specific chromosomal aberration, within an allelic deletion seen in various epithelial cancers. EZH2 is down-regulated by miR-26a, as

demonstrated by overexpressed miR-26a in Burkitt lymphoma and nasopharyngeal carcinoma. Relating to therapeutic intervention, adenovirus-associated delivery mechanisms have already been used to administer miR-26a to cancer cells, in order to provoke apoptosis and therefore prevent cancer progression.

Thus, there is a large wealth of evidence suggesting that miR-16 and miR-26a may be important targets of p53. This work is focused on preliminary elucidation of regulatory mechanisms by which p53 controls expression of miR-16 and miR-26a and characterisation of their gene targets involved in the p53 network.

PART 1: miR-16 and miR-26a in cancer

We carried out Q-PCR to predict the survival probability of cancer patients based on miR-16 and miR-26a miRNA signatures (Fig. 3.1). Breast, hepatocellular, ovarian, and prostate cancer tissue specimens belonging to cancer patients were used for this study. miR-16 and miR-26a expression was analysed by Q-PCR with a TaqMan MicroRNA Assays Human Panel-Early Access Kit.

Generally, high miR-16 and high miR-26a expression are associated with increased breast, prostate, ovarian, and hepatocellular cancer survival (Fig. 3.1A-F). With breast cancer progression survival is increased with high miR-16 and high miR-26a expression following 30 months from initial cancer onset (Fig.3.1A and B). Generally, breast cancer survival declines with time (Fig.3.1A and B), as does ovarian and hepatocellular cancer survival (Fig. 3.1E and F respectively). With ovarian and hepatocellular cancer progression survival is increased with high miR-16 and high miR-26a expression following 5 and 15 months respectively from initial cancer onset (Fig. 3.1E and F respectively). With prostate cancer progression survival is increased with high miR-16 and miR-26a expression, which remains steady over time (Fig.3.1C and D).

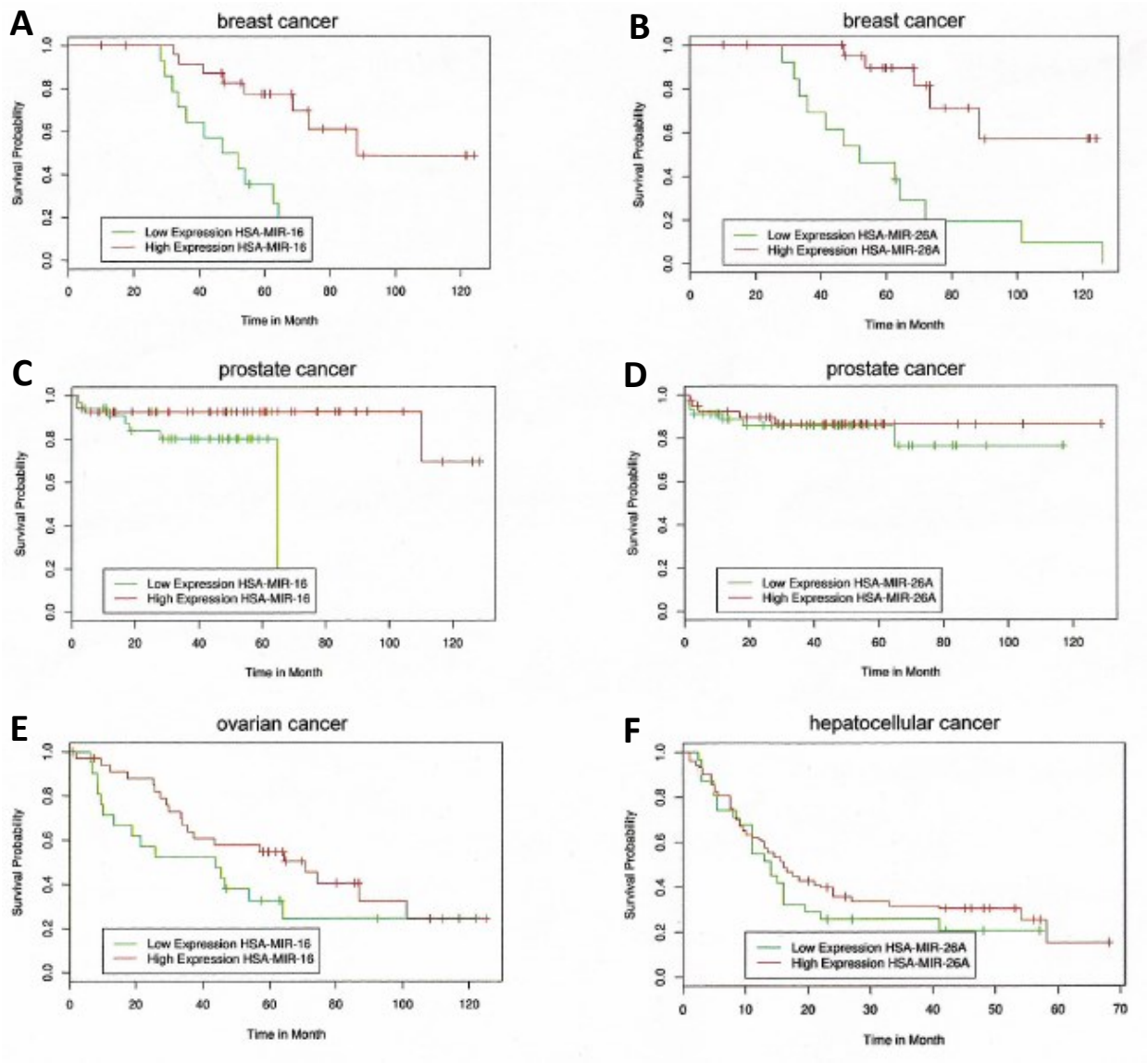


Figure 3.1 Survival probability of cancer patients based on miR-16 and miR-26a microRNA signatures

Breast, hepatocellular, ovarian, and prostate cancer tissue specimens belonging to cancer patients were used for this study. Extracted RNA was used for cDNA synthesis, and then miR-16 and miR-26a expression was analysed by Q-PCR with a TaqMan MicroRNA Assays Human Panel-Early Access Kit.

PART 2: Mechanisms of p53-dependent control for miR-16 and miR-26a

Expression patterns of miRNAs differ significantly between different cancer cell lines. To analyse p53-dependent expression of miRNAs we decided to generate isogenic cell lines that differ only in the levels of p53 expression. $HCT116^{p53+}$ and $HCT116^{p53-}$ constructed in the Vogelstein lab (Vogelstein *et al.*, 1989) have a significant disadvantage, as they constitutively express beta-catenin, which is known to interact with p53 and alter its transcription programme. To solve this problem, we have constructed a $p53^{-}$ isogenic cell line from the $p53^{+}$ human osteosarcoma cell line, U2OS, which has a normal p53 response. In these cells (U2OS pLKO p53), expression of p53 was down-regulated by shRNA.

As can be seen from this figure, lentiviral infection of U2OS cells with shRNA-p53 produced a stable knockdown in these cells. Importantly, even after DNA damage with doxorubicin, when the p53 protein is stabilised (Fig. 3.2, compare bar 1 and 2), there was no detectable signal for p53 in U2OS pLKO p53 cells as judged by western blotting analysis using a p53-specific antibody (Fig. 3.2, bar 3 and 4). Note, that the total protein levels were comparable as evident from the Ku70 signal used as a loading control (Fig. 3.2, bottom panel).

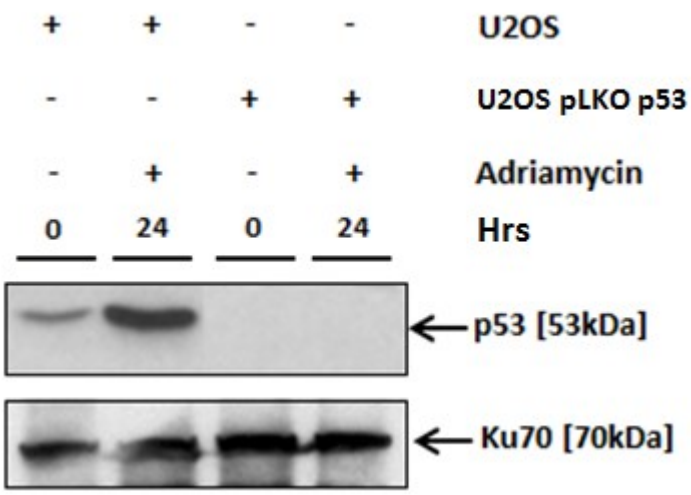


Figure 3.2 Knockdown of endogenous p53 protein in U2OS cells
Immunoblotting was carried out to confirm the knockdown of endogenous p53 in U2OS cells. Whole cell extracts were prepared and samples were separated by SDS-PAGE. Immunoblots were carried out with the p53-specific antibody Ab-6 and samples were normalised against tubulin. U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with Adriamycin (doxorubicin) to induce DNA damage for 0 and 24 hours. Cells not treated with Adriamycin were used as controls.

p53-regulated microRNA expression

Once we established a system with isogenic cell lines that differ only in p53 expression, next step was to confirm the microarray expression data suggesting that expression of miR-16-2 and miR-26a are regulated by p53. To do this, we employed Q-PCR-based analysis using primers specific for these miRNAs (Fig. 3.3).

Our results clearly showed that expression levels of both miR-16 and miR-26a was dependent on p53 even in the absence of DNA damage (Fig. 3.3, compare bar 1 and 3, panel A and B). However, upon DNA damage conferred by treatment of cells with doxorubicin, there was a moderate but consistent increase in the endogenous levels of miR-16 and mir-26a expression (1.3-fold and 1.6-fold for miR-16 and miR-26a respectively) as evident from Fig. 3.3 (compare bar 1 and 2 in panels A and B). It should be noted, that the levels of p53 protein increased dramatically after 24 hours of treatment as shown in Fig. 3.2. Also, there was a slight increase in the levels of miR-16 and miR-26a expression after DNA damage even in the absence of p53 (Fig. 3.3, bar 3 and 4, panels A and B), suggesting p53-independent regulatory mechanisms of miR-16 and miR-26a. Additionally, these results suggest that other stress-response transcription factors, for example E2F1, may control expression of these genes.

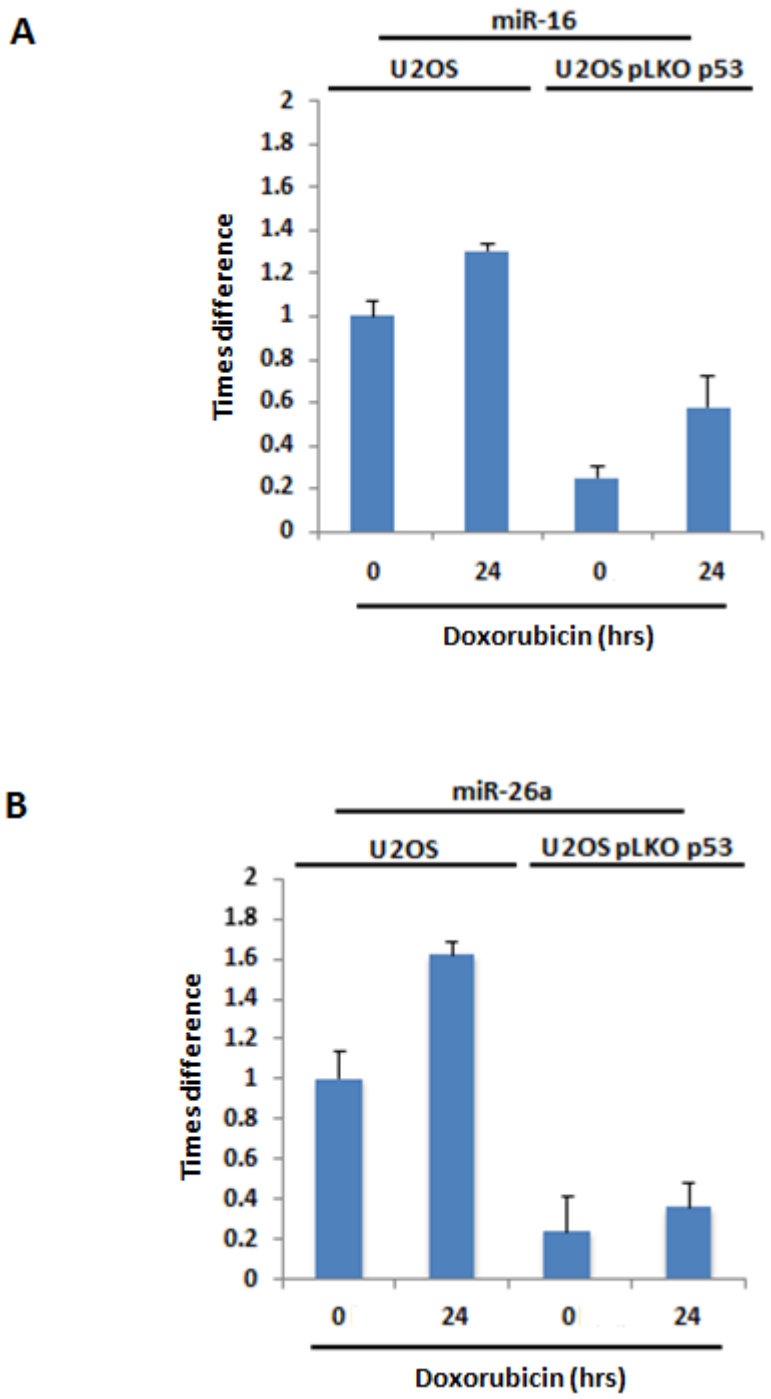


Figure 3.3 Effect of p53 on endogenous miR-16 and miR-26a expression using Q-PCR

RNA from U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells treated with doxorubicin to induce DNA damage for 0 and 24 hours was used in cDNA synthesis. Mature miR-16 **(A)** and miR-26a **(B)** expression was measured by Q-PCR with SYBR Green dye. Samples were normalised against U6. Cells not treated with doxorubicin were used as controls. $p = 0.05$

p53 was recently shown to affect expression of miRNAs both at the level of transcription and post-transcriptional maturation (Suzuki *et al.*, 2009). We decided to take a closer look at what level this regulation occurs. To this end, we designed primers that discriminate between pri-miRNA and processed mature miRNAs and performed Q-PCR using U2OS cells with wild-type and knockdown expression of p53 (U2OS pLKO p53). In addition, both types of cells were non-treated or treated with doxorubicin for 12 and 24 hours to induce DNA damage (Fig. 3.4).

As evident from this figure, only miR-26a showed p53-dependent response on the level of transcription at 12 and 24 hours after doxorubicin treatment (Fig. 3.4, upper panel). miR-16-2 showed a transient decrease in transcription at 12 and 24 hours after DNA damage in the absence of p53. However, both miRNAs demonstrated a significant decrease in the levels of mature miRNA expression in the absence of p53 (Fig. 3.4, bottom panel). In addition, there was a direct correlation between the time of DNA damage treatment and mature miRNA production.

Thus, these data suggest that p53 can control expression of miRNAs on several levels; miR-16 is regulated at a transcriptional level and miR-26a at post-transcriptional levels. Also, DNA damage facilitates expression of miR-16-2 and miR-26a-1.

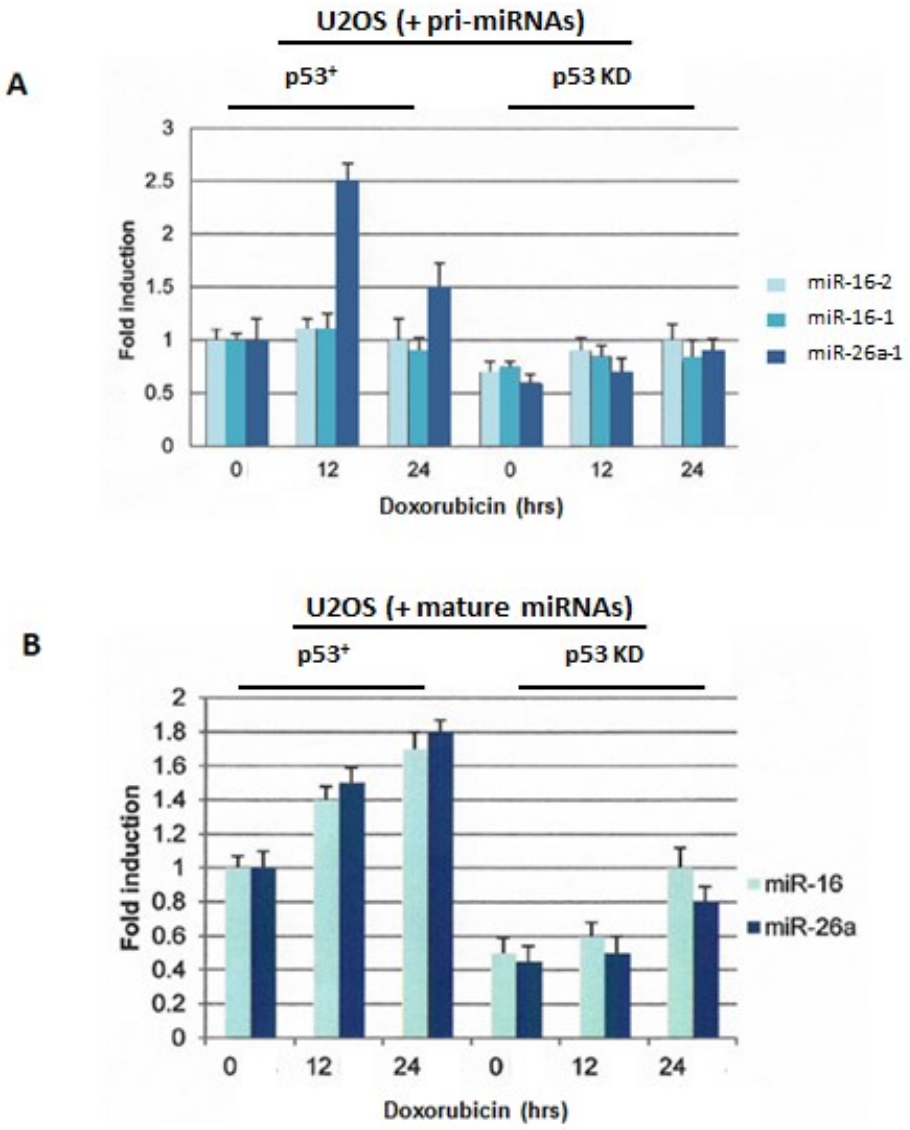


Figure 3.4 Effect of p53 on expression of primary and mature miR-16 and miR-26a Transcription levels of primary (pri) miR-16-2, miR-26a-1, and miR-16-1 (control for miR-16-2) are shown in different colours **(A)**. U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with doxorubicin to induce DNA damage for 0 (control), 12, and 24 hours followed by Q-PCR. Samples were normalised against GAPDH. Similarly, expression levels of mature miR-16 and miR-26a were measured by Q-PCR. U2OS and U2OS pLKO p53 cells were treated with doxorubicin to induce DNA damage for 0, 12, and 24 hours **(B)** Note, that since the mature products of miR-16-1 and miR-16-2 are identical it was impossible to distinguish these two non-coding RNAs. $p = 0.05$

Transcriptional regulation of miRNAs by p53

Since p53 was shown to affect expression of several miRNAs both on the transcriptional and post-transcriptional levels, we decided to assess whether p53 regulates the expression of miR-16-2 and miR-26a directly by binding their promoters and activating their transcription. To address this question, we decided to use a luciferase assay.

Thus, we cloned different fragments of upstream sequences of miR-26a and miR-16-2 genes into a pGL3-Basic luciferase vector (Fig 3.5). Specifically, the upstream sequence of miR-26a gene was divided into two fragments (Fig 3.5). This was done because the bioinformatics software programme Consite (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) predicted existence of several p53REs scattered along those sequences. Therefore, we wanted to test the functionality of these p53RE sites separately. Similarly, the upstream sequences of miR-16-2 gene were divided into three fragments and also cloned into pGL3 vector (Fig 3.5). Putative p53REs was predicted to reside in the vicinity of the TATA box region of the miR-15b/miR-16-2 locus. The results of successful cloning procedures are shown in Fig. 3.5A, B, C, D and E.

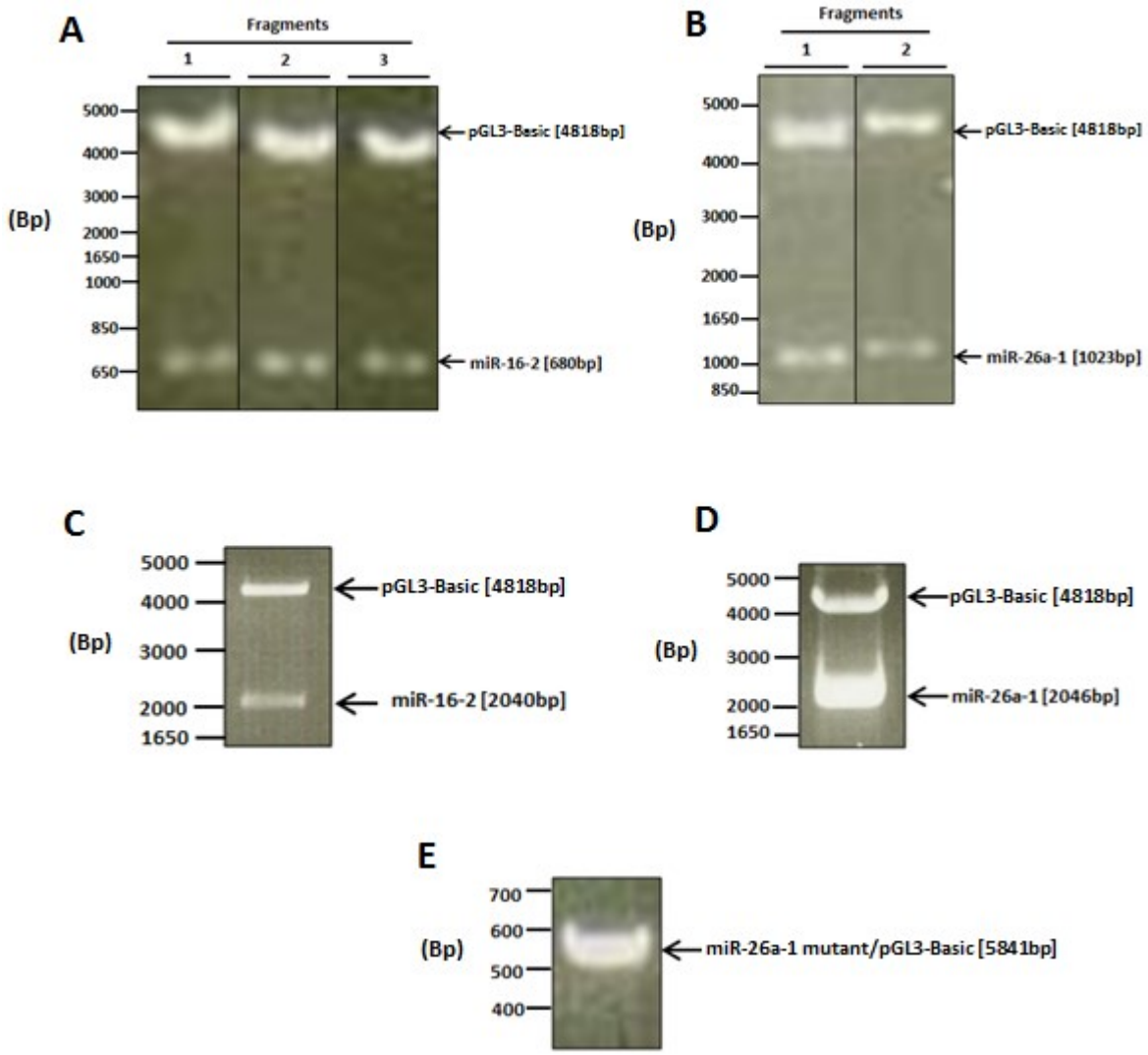


Figure 3.5 Generation of miR-16-2 and miR-26a-1 promoter luciferase constructs
The miR-16-2 promoter fragments **(A)**, and full-length promoter **(C)**, and miR-26a-1 promoter fragments **(B)** and full-length promoter **(D)** were cloned separately into a pGL3-Basic luciferase reporter plasmid. A mutant construct was generated by site-directed mutagenesis from pGL3/miR-26a-1 promoter fragment 1 **(E)** to use as a control for miR-26a-1. Miniprep plasmid DNA was prepared for each construct and screened for positive clones by restriction digest with
mutant) and DNA sequencing.

Western blot analysis was carried out to confirm the expression of ectopic p53 wild-type and p53 mutant isoforms K4R (lysine residue acetylation and methylation sites 320, 372, 373 and 382 replaced with arginine residues), R175H (arginine residue 175 in the DNA-binding domain replaced with a histidine residue), transactivation-deficient mutant Δ40 (deletion of the first 40 amino acids), or DNA binding-deficient mutant Δ133 (deletion of the first 133 amino acids) transfected in U2OS pLKO p53 (p53 knockdown) cells used for luciferase assays. Cells were treated with doxorubicin for 14 hours to induce DNA damage. Samples were normalised against Ku70 (Fig. 3.6D), and cells not transfected with p53 were used as a control.

p53 wild-type, K4R, and R175H mutants were positively detected with a size of 53kDa (Fig.3.6A). Truncated mutants p53Δ40 and p53Δ133 were confirmed at 39kDa (Fig. 3.6B), and at 29kDa (Fig. 3.6C) respectively.

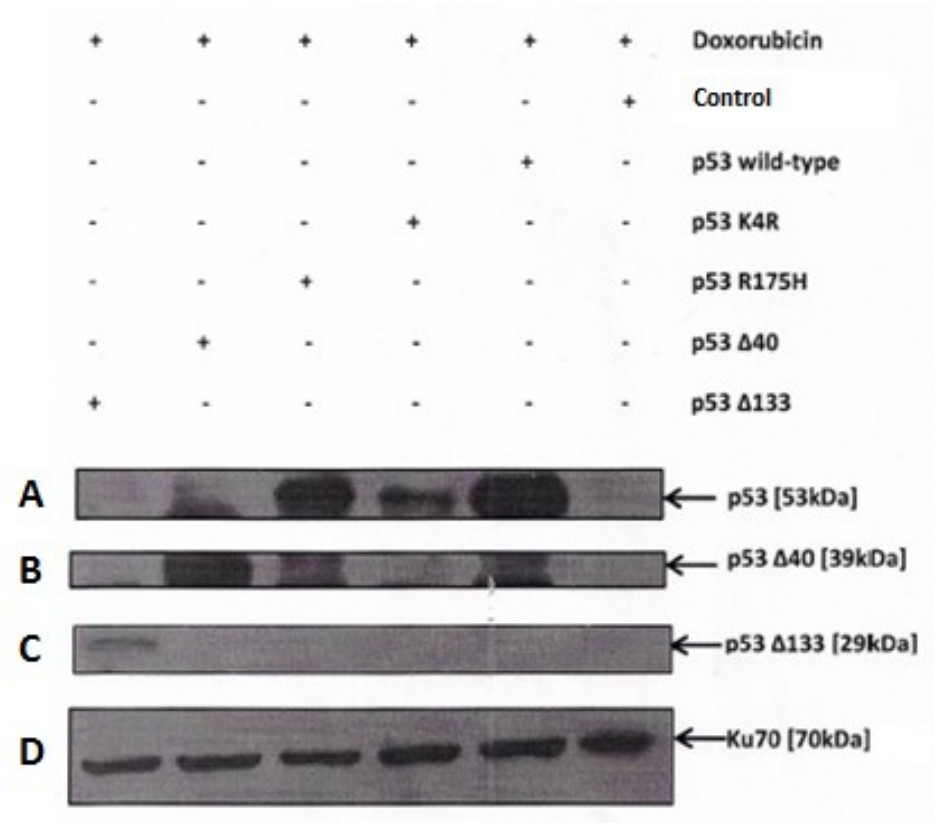


Figure 3.6 Expression of ectopic p53 in U2OS (p53 knockdown) cells
Immunoblotting was carried out to confirm the expression of ectopic p53 wild-type and p53 mutant isoforms K4R (lysine residue acetylation and methylation sites 320, 372, 373 and 382 replaced with arginine residues), R175H (arginine residue 175 in the DNA-binding domain replaced with a histidine residue), transactivation-deficient mutant Δ40 (deletion of the first 40 amino acids), or DNA binding-deficient mutant Δ133 (deletion of the first 133 amino acids) transfected in U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells used for luciferase assays. Cells were treated with doxorubicin for 14 hours to induce DNA damage. Whole cell extracts were prepared and samples were separated by SDS-PAGE. Immunoblots were carried out with the p53-specific antibodies Ab-1 and Ab-6 and samples were normalised against Ku70. Cells not transfected with p53 were used as a control.

The purpose of this experiment was to assess whether these promoters were indeed dependent on p53. The resulting luciferase constructs encompassing the promoters of miR-16-2 and miR-26a genes, with or without p53-expressing vector, were transfected into U2OS pLKO p53 cells, which harbours knockdown of the TP53 gene. The choice of this cell line was dictated by the concern that endogenous p53 could interfere with the ectopic one. It is well established that p53 undergoes various post-transcriptional modifications that activate its functions. Thus, transfected cells were also treated with doxorubicin for 14 hours to induce DNA damage (Fig. 3.7).

Expression levels of luciferase constructs that contained fragments of miR-16-2 upstream sequences were significantly higher than the negative control (empty pGL3-Basic vector) or a vector that contained the promoter of miR-16-1 gene, which does not contain p53REs in its sequence (Fig. 3.7, panel A, compare bar 1 and 6 with bar 2,3, and 4). Surprisingly, the full-length upstream sequence (2100bp) of miR-16-2 promoter severely attenuated luciferase expression (Fig. 3.7, panel A, compare bar 2, 3, 4 with bar 5). This phenomenon needs to be further investigated.

Unexpectedly, U2OS pLKO p53 cells lacking ectopic p53, showed a very similar trend of luciferase activity compared to U2OS cells expressing p53. In fact, the levels of luciferase activity for constructs bearing fragments of miR-16-2 promoter in p53 knockdown cells were slightly higher than those in $p53^{+}$ cells (Fig. 3.7).

This effect cannot be accounted for by high background level of activity for miR-16-2 luciferase constructs since the full-length construct showed a very low level of activation in these cells. Neither could it be attributed to the low efficiency of transfection with ectopic p53 plasmid, because the results of western blotting analysis of transfected U2OS pLKO p53 cells with the plasmid expressing p53 confirmed that the p53 protein was successfully expressed in those cells (Fig. 3.6).

Transfection of miR-16-2 reporter constructs into U2OS pLKO p53 cells without p53 yielded –approximately the same levels of luciferase activity as miR-26a reporter constructs, except relating to the full-length miR-26a promoter. The full-length miR-26a promoter displayed 2-fold higher activity than individual miR-26a fragments (Fig. 3.7 compare bars 7, 8 and 9). The mutant miR-26a promoter construct had much lower luciferase activity, indicating that the p53REs may be utilised by other transcription

factors, thus supporting a p53-independent regulatory mechanism for miR-26a, as well as miR-16.

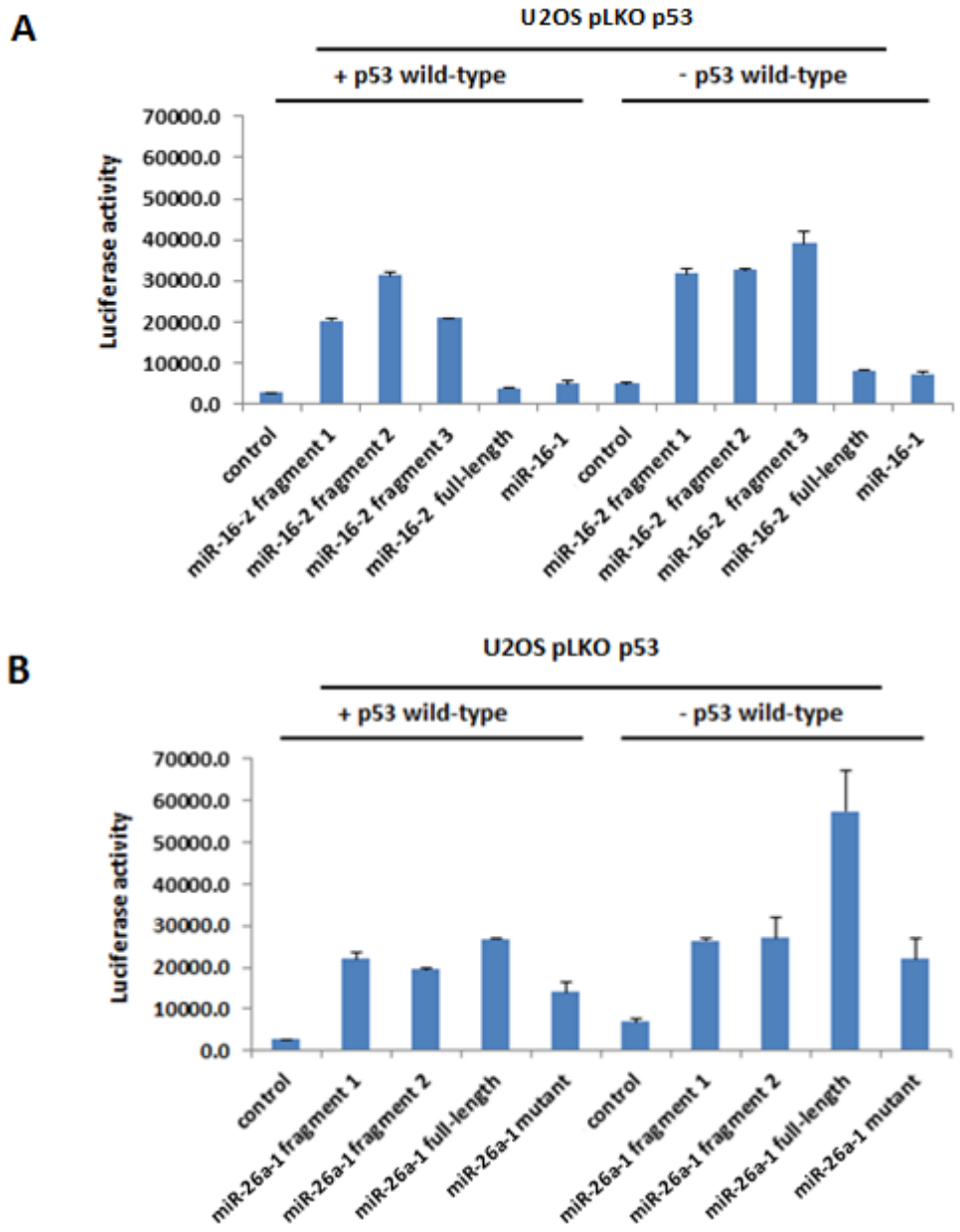


Figure 3.7 Effect of p53 on ectopic miR-16-2 and miR-26a-1 promoter activities using luciferase assay

miR-16-2 **(A)** and miR-26a-1 **(B)** promoter luciferase constructs and ectopic p53 wild-type were transfected using Lipofectamine 2000 reagent into U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells. Cells were treated with doxorubicin for 14 hours to induce DNA damage. pGL2 luciferase reporter plasmid and cells not transfected with p53 wild-type were used as controls. p = 0.05

The p53 protein is known to undergo regulation in response to genotoxic stress. This is achieved mostly on post-translational level through various post-translational modifications. Therefore, we wanted to examine the effect of post-translational modifications on the ability of p53 to transactivate both miR-16- and miR-26a-containing luciferase reporters (Fig. 3.8). Since the full-length miR-16-2 promoter did not display any appreciable luciferase activity, we decided to focus on individual fragments.

To further explore transcriptional effects of p53 mutants, in addition to p53 R175H (arginine residue 175 mutated to histidine) and p53 K4R (lysine residues subject to acetylation, methylation, neddylation, and ubiquitination mutated to arginine) mutants, we employed two other deletion mutants of p53 that lacked either the first amino-terminally situated transactivation domain (p53Δ40), or the whole amino-terminus (p53Δ133). Importantly, these deletion mutants are present in the cells of various forms of breast cancer and therefore have clinical relevance (Soussi 2007).

The rationale behind this experiment was that we sought to distinguish the requirements of different domains of p53 for transactivation. To preserve the transcriptional environment of endogenous p53, we used U2OS cells with knocked down expression of p53 (U2OS pLKO p53) (Fig. 3.8).

R175H p53 mutant showed the highest level of transactivation for each miR-16-2 promoter fragment (Fig. 3.8A). Generally, the Δ133 p53 mutant showed lower levels compared to the levels induced by wild-type p53, suggesting that the absence of the first 133 amino acids in p53 could negatively affect the association with endogenous transcription factor(s) that bind to the p53RE in the miR-16-2 promoter. Wild-type p53 was also able to activate transcription of miR-16-2 fragments, albeit to a lesser extent compared to p53 K4R mutant and p53 R175H mutant. None of the p53 proteins were able to transactivate miR-16-1 promoter construct, suggesting that these effects were specific.

Next, we focused on studying the effect of p53 mutants on promoter regions of the miR-26a-1 gene, since it showed transcriptional dependence on p53 in our previous experiments. Both fragments of miR-26a promoter showed dependency on various p53 tested, and in both cases wild-type p53 showed the weakest transactivation

potential. The level of p53-dependent response varied between the p53 mutants (Figure 3.8B).

When cells were transfected with wild-type p53 or p53 mutants, together with miR-26a fragment reporter constructs (Fig. 3.8B), generally Δ40 mutant of p53 showed the highest level of transactivation for each miR-26a promoter fragment (Fig. 3.8B). Transactivation of both miR-26a fragments was carried out most weakly by p53 K4R mutant. It is plausible that because p53 K4R mutations affect acetylation, which is critical for p53 stability, the overall stability of this p53 mutant was lower and thus transactivation was not as efficient as by p53 Δ40 mutant or wild-type p53.

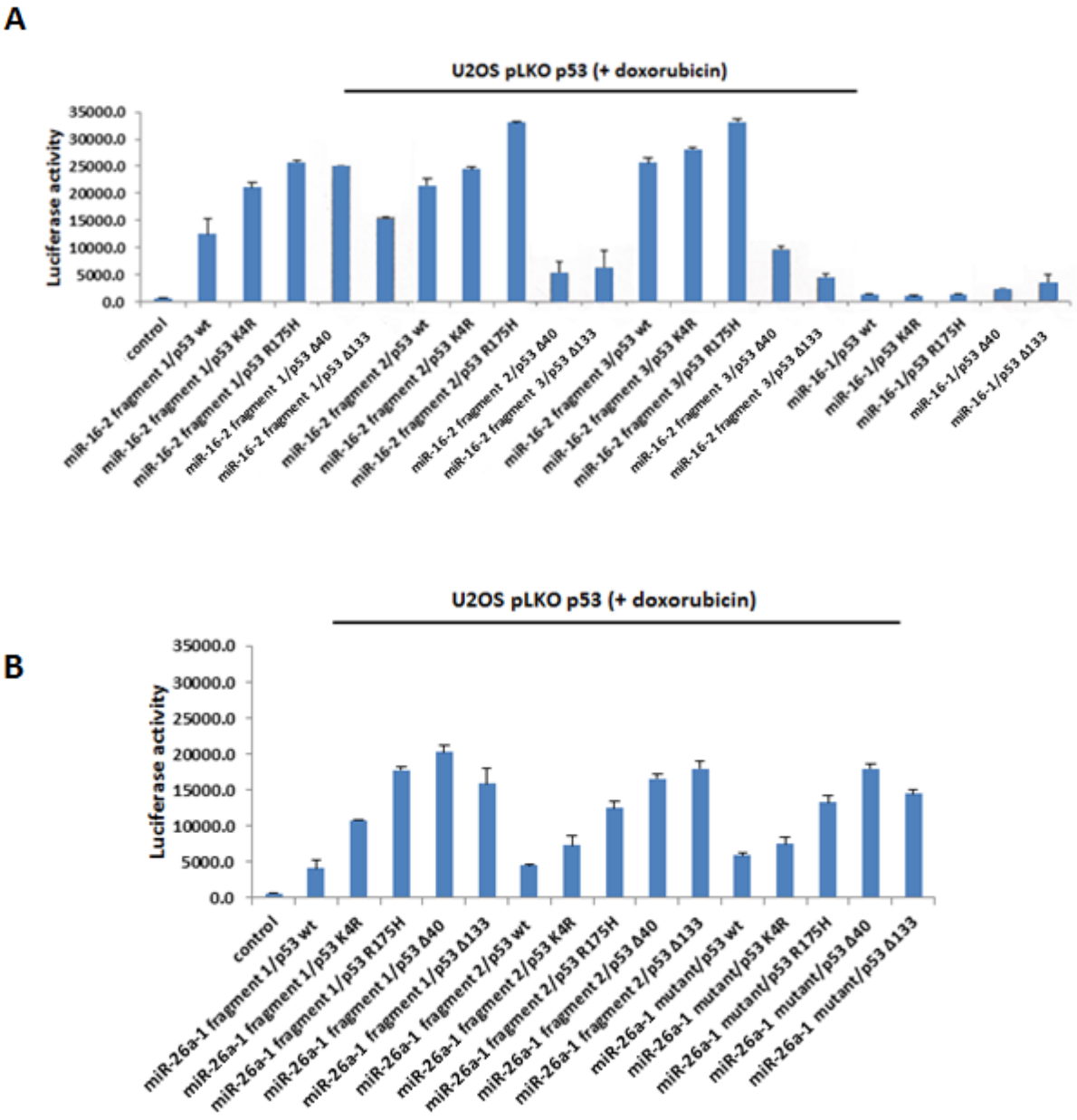


Figure 3.8 Effect of p53 mutants on promoter activities of miR-16-2 and miR-26a-1

miR-16-2 **(A)** and miR-26a-1 **(B)** promoter luciferase constructs were transfected into U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells. These cells were also transfected with ectopic p53 wild-type or p53 mutant isoforms K4R (lysine residue acetylation and methylation sites 320, 372, 373 and 382 replaced with arginine residues), R175H (arginine residue 175 in the DNA-binding domain replaced with a histidine residue), transactivation-deficient mutant Δ40 (deletion of the first 40 amino acids), or DNA binding-deficient mutant Δ133 (deletion of the first 133 amino acids). Cells were treated with doxorubicin for 14 hours to induce DNA damage. pGL2 luciferase reporter plasmid was used as a control. p = 0.05

Luciferase constructs encompassing the promoters of miR-16-2 and miR-26a genes were transfected into U2OS, which expresses a wild-type copy of the TP53 gene. It is well established that p53 undergoes various post-transcriptional modifications upon DNA damage that activate its functions. Thus, transfected cells were either treated or not treated with doxorubicin to induce DNA damage for 14 hours (Fig. 3.9 left and right panels, respectively).

Expression levels of luciferase constructs that contained miR-16-2 fragments were significantly higher than the negative control (empty pGL3-Basic vector) or the control luciferase construct containing miR-16-1 promoter, which does not contain p53REs in its sequence (3.9A). Surprisingly, the full-length miR-16-2 promoter failed to activate luciferase expression (Fig. 3.9A). Presumably, the full-length miR-16-2 promoter construct may contain a repressor motif that is broken down in the individual fragments. However, this phenomenon needs further investigation. When the transfected U2OS cells were treated with doxorubicin, luciferase activity of the miR-16-2 fragment 2-containing construct increased 1.5 fold in comparison with that in untreated cells (Fig. 3.9A), indicating that DNA damage positively affected transcription initiation from this fragment. A similar trend was noted with the other two miR-16-2 fragments.

Interestingly, when U2OS cells transfected with luciferase vectors that contained the miR-26a fragments were analysed, the highest luciferase activity was observed in the case of the full-length miR-26a-containing construct (Fig. 3.9B), which is in contrast to the situation with miR-16-2. This difference in activation of full-length miR-26a compared to individual miR-26a fragments was further amplified in cells treated with DNA damage (Fig. 3.9B left and right panels). Taken together, these results suggest that the promoter of miR-26a responds to DNA damage stress.

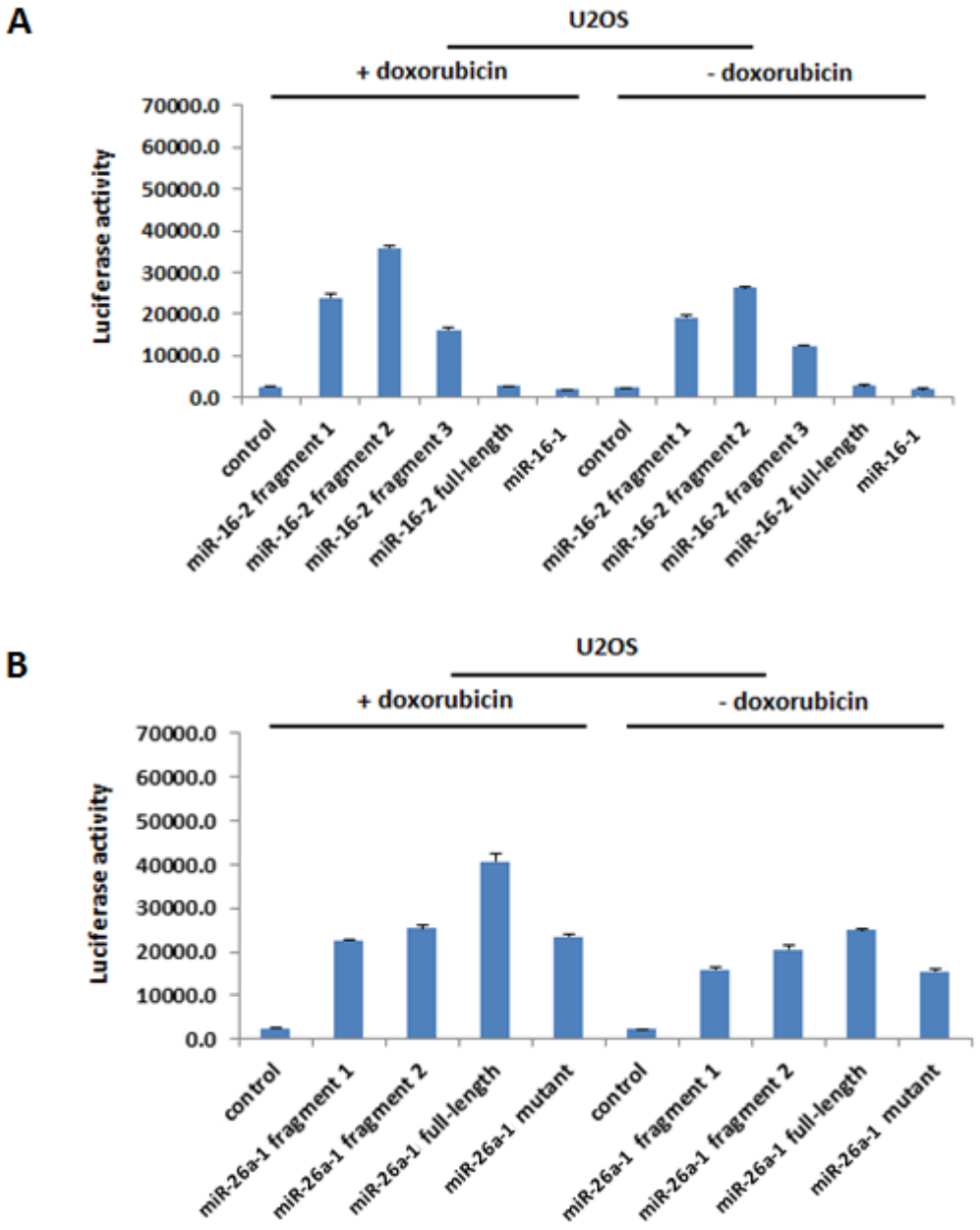


Figure 3.9 Effect of genotoxic stress on promoter activities of miR-16-2 and miR-26a-1

miR-16-2 **(A)** and miR-26a-1 **(B)** promoter luciferase constructs were transfected using Lipofectamine 2000 reagent into U2OS ($p53^{+}$) cells, treated with or without doxorubicin for 14 hours to induce DNA damage. pGL2 luciferase reporter plasmid was used as a control. $p = 0.05$

To examine whether endogenous p53 affects transcription of miR-26a by binding to its promoter, ChIP assay using p53-specific antibody was carried out (Fig. 3.10). Initially, we analysed the distribution of p53 binding sites in the promoter of miR-26a gene, using a bioinformatics approach with the Consite program (Fig. 2.3B).

Our results on luciferase assays indicated that the second fragment of the miR-26a promoter contained the most p53REs (region from +1000bp to +2000bp shown in Fig. 2.3B). To verify these data several sets of primers encompassing putative p53REs both in the promoter and in the downstream region were designed. Endogenous p53 before and after 24 hours treatment with doxorubicin were immunoprecipitated from U2OS cells. Cells with knocked-down expression of p53 (U2OS pLKO p53) were used as negative control (Fig. 3.10).

Our ChIP results suggest that maximal p53 binding activity was observed in the region of 1500-2000bp upstream of the start site. There was very low binding observed downstream of the start site (-3000bp), indicating that most of the p53REs are located in the upstream promoter region.

Since p53 was reported to interact with several RNA processing proteins, including the Drosha complex, we hypothesized that p53 may recruit these enzymes to promoters of certain miRNA genes to facilitate the processing of these miRNAs coupled with transcription. To address this question, we performed ChIP analysis using antibodies against Drosha and p68 helicase, which is known to interact with Drosha (Fig. 3.10B and C).

U2OS cells expressing wild-type p53 and cells with knocked-down expression of p53 (U2OS pLKO p53) were used in this experiment to assess whether recruitment of these RNA processing proteins to miR-26a was p53-dependent. Interestingly, we observed 4-fold increase of Drosha binding to the miR-26a promoter (+1500 region) in U2OS ($p53^+$) cells treated with doxorubicin for 24 hours, compared with Drosha binding in untreated U2OS ($p53^+$) cells, and with U2OS (knockdown p53) cells, where very low binding of Drosha was observed (Fig. 3.10B). Collectively, these results suggest that Drosha is likely recruited to the miR-26a promoter in a p53-dependent manner.

Surprisingly, binding of p68 to the same region of miR-26a promoter although increased after DNA damage, did not depend on p53, because similar increases in

binding were observed both in U2OS ($p53^{+}$) and U2OS (knockdown p53) cells (Fig. 3.10C).

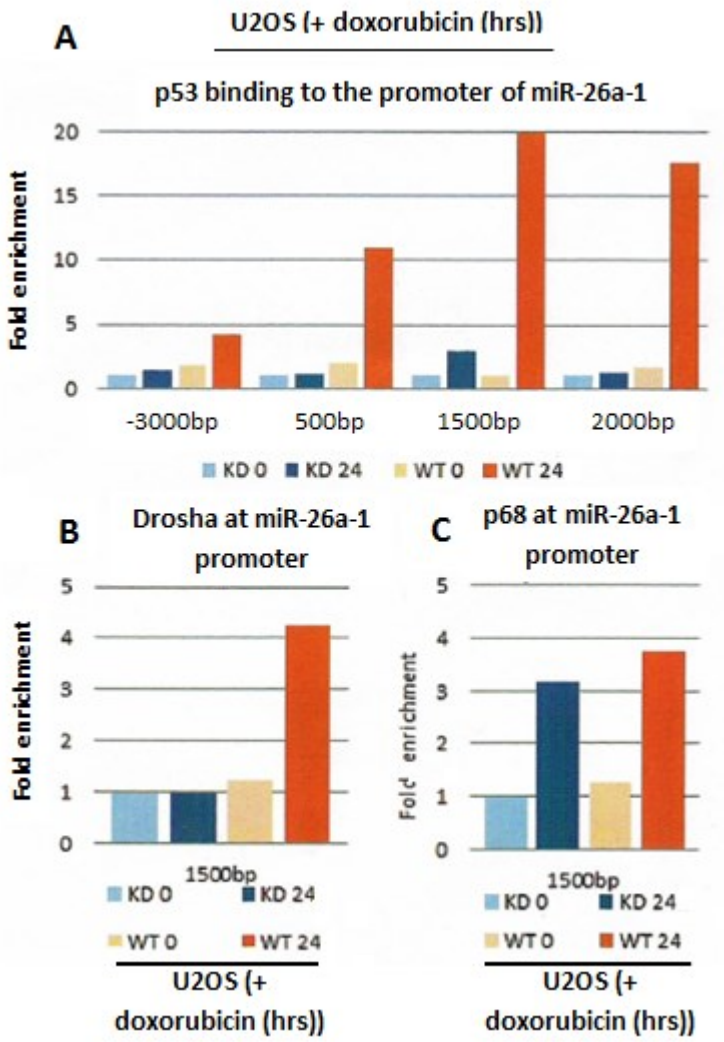


Figure 3.10 ChIP assay of p53, Drosha and p68 on the miR-26a promoter
U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with doxorubicin to induce DNA damage for 0 (control) and 24 hours followed by ChIP assay. **(A)** p53 binding in the upstream and downstream regions of the miR-26a-1 locus. **(B)** Binding of the Drosha protein to the miR-26a-1 locus. **(C)** Binding of p68 to the miR-26a-1 locus.

We decided to correlate spatially the regions of maximal binding for p53, Drosha and p68 to examine their possible co-dependence in DNA binding (Fig. 3.11).

p53 showed the highest occupancy of the miR-26a promoter in the region between +1500bp and +2000bp in U2OS ($p53^+$) cells treated with doxorubicin for 24 hours (Fig. 3.11A).

The binding profile of p68 was different from the p53 one. p68 displayed the highest binding activity in the region of +500bp in U2OS ($p53^+$) cells after 24 hours doxorubicin. Moreover, a lack of p53 in U2OS (knockdown p53) cells did not significantly affect the binding of p68. These results suggested that although p68 is recruited to the promoter region of miR-26a, this likely occurs in a p53-independent, but DNA damage-dependent fashion (Fig. 3.11B).

In contrast to p68, the binding profile of Drosha to miR-26a clearly showed dependence on p53. However, the highest occupancy of Drosha was observed in the region between +500-1500bp, which is more proximal to the start site compared to the peak of p53 binding that maps to the region of +1500-2000bp (Fig. 3.11C). Collectively, our results suggest that Drosha is recruited to the promoter of miR-26a-1 gene in a DNA damage- and p53-dependent manner.

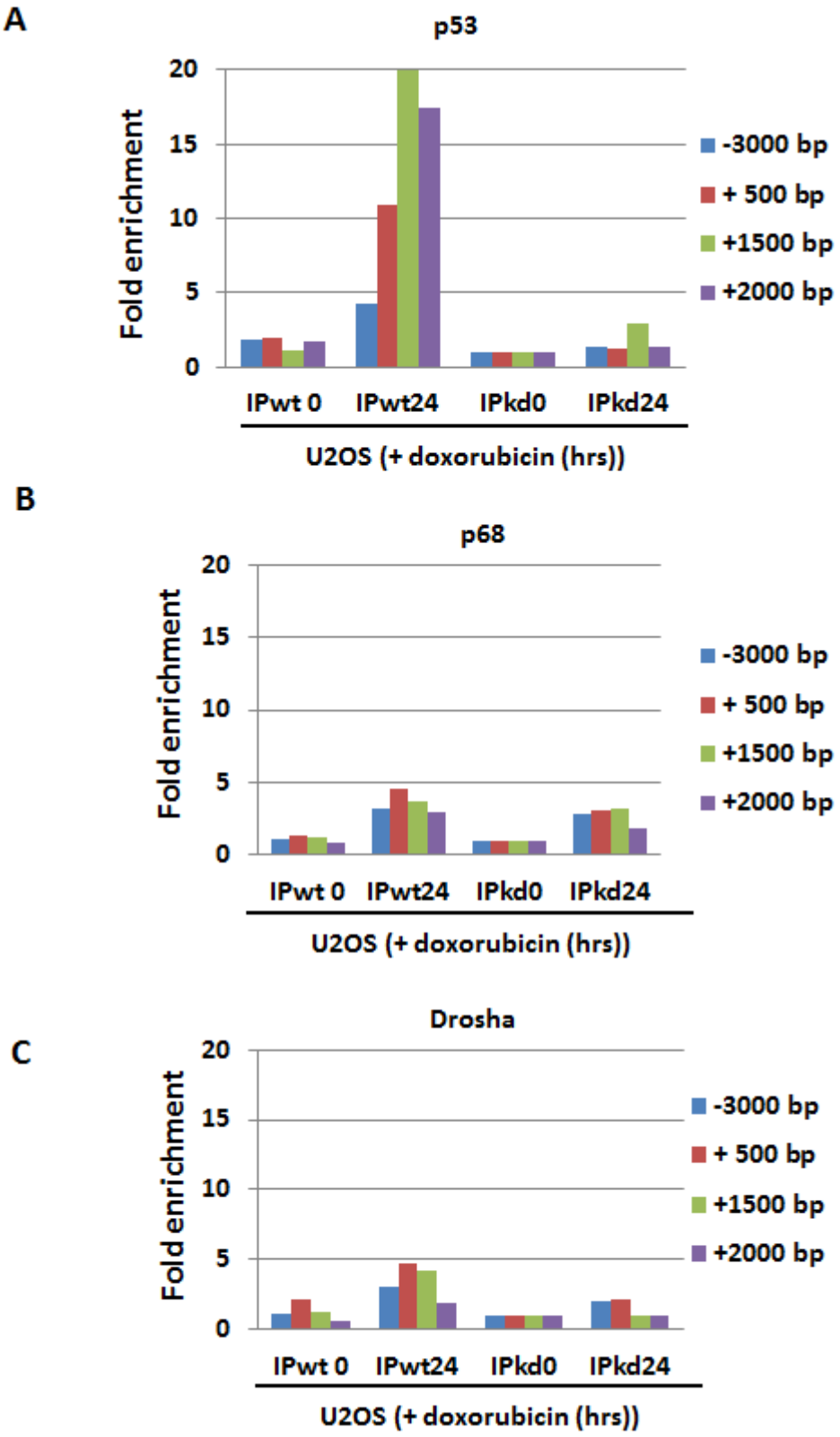


Figure 3.11 ChIP assay of p53, Drosha and p68 on the miR-26a-1 promoter
(A) p53 distribution along the miR-26a-1 locus measured by ChIP assay in U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells treated with doxorubicin to induce DNA damage for 0 (control) and 24 hours. **(B)** Distribution of the p68 binding sites along the miR-26a-1 locus measured by ChIP assay. **(C)** Distribution of the Drosha binding sites along the miR-26a-1 locus measured by ChIP assay.

Results of our luciferase reporter assay experiments indicate that p53 likely affected miR-16-2 transcription in a non-direct way. Thus, we employed ChIP assay to test whether p53 binds to the promoter of miR-16-2 gene (Fig. 3.12).

To determine whether endogenous p53 affects transcription of miR-16 by binding to its promoter, ChIP assay using a p53-specific antibody was carried out (Fig. 3.12). Primarily, we analysed the distribution of p53 binding sites in the promoter of miR-16 gene, using a bioinformatics approach with the Consite program (Fig. 2.3A).

U2OS ($p53^{+}$) cells were treated with doxorubicin to induce DNA damage for 0 (control), 3, 6, and 12 hours followed by ChIP assay. The miR-15b/miR-16-2 cluster resides in intron 5 of the SMC4 gene (Ofir *et al.*, 2011). p53 binding in the downstream region (SMC4 promoter) of the miR-15b/miR-16-2 locus, and p53 binding in the downstream region (-700bp) of the miR-15b/miR-16-2 locus was analysed.

Generally, increased DNA damage by doxorubicin increased binding of p53 to the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Increasing DNA damage increases p53 binding at the SMC4 promoter more significantly than at the -700bp region in the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Even in the absence of DNA damage, p53 still bound at its RE in the downstream region (-700bp) of the miR-15b/miR-16-2 locus to the same degree as treatment with 3 hours doxorubicin (Fig. 3.12B). In the absence of DNA damage, p53 bound to a similar degree in both the downstream regions (SMC4 promoter and -700bp) of the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Therefore, p53 binding to miR-16-2 promoter is not DNA damage-dependent, but DNA damage can increase this binding.

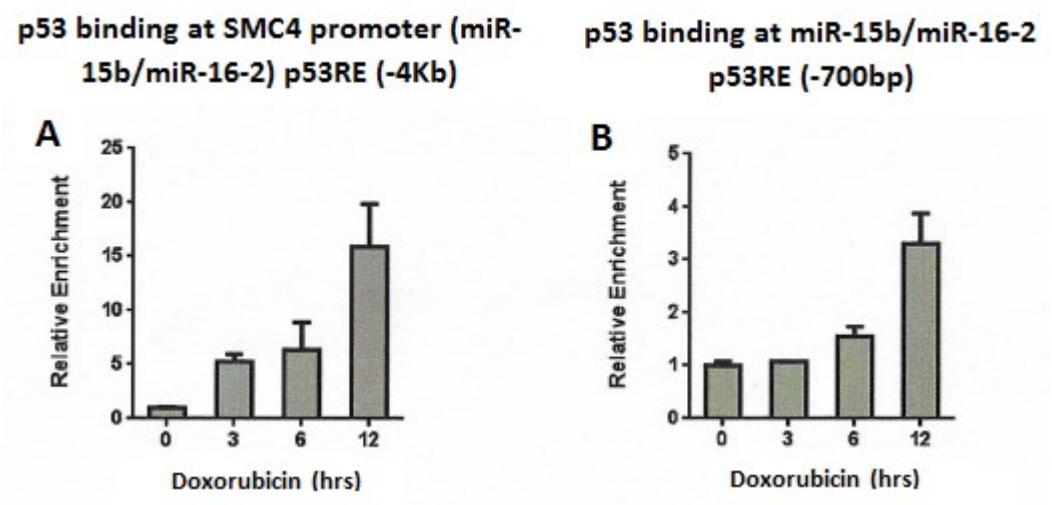


Figure 3.12 ChIP assay of p53 on the miR-15b/miR-16-2 promoter

U2OS ($p53^{+}$) cells were treated with doxorubicin to induce DNA damage for 0 (control), 3, 6, and 12 hours followed by ChIP assay. **(A)** p53 binding in the downstream region (SMC4 promoter) of the miR-15b/miR-16-2 locus. **(B)** p53 binding in the downstream region (-700bp) of the miR-15b/miR-16-2 locus. $p = 0.05$

Since DNA binding of p53 is affected by post-translational modifications, such as acetylation and methylation, we also tested whether methylated p53 was able to bind the miR-16-2 promoter (Fig. 3.13). Methylation of p53 on K372 by SET9 lysine methyltransferase was shown to enhance its subsequent acetylation (Ivanov *et al.*, 2007). Our ChIP results suggest that indeed p53 bound miR-16-2 promoter (Fig. 3.12 and 3.13). DNA damage enhanced the occupancy of the promoter by p53. Importantly, this binding was specific, because no appreciable binding of p53 to the promoter of miR-16-1 was observed (Fig. 3.13E). In addition, bound p53 was methylated by SET9 upon DNA damage induced by doxorubicin (Fig. 3.13C). Thus, our data suggest that p53 binds the promoter of miR-16-2 in response to DNA damage.

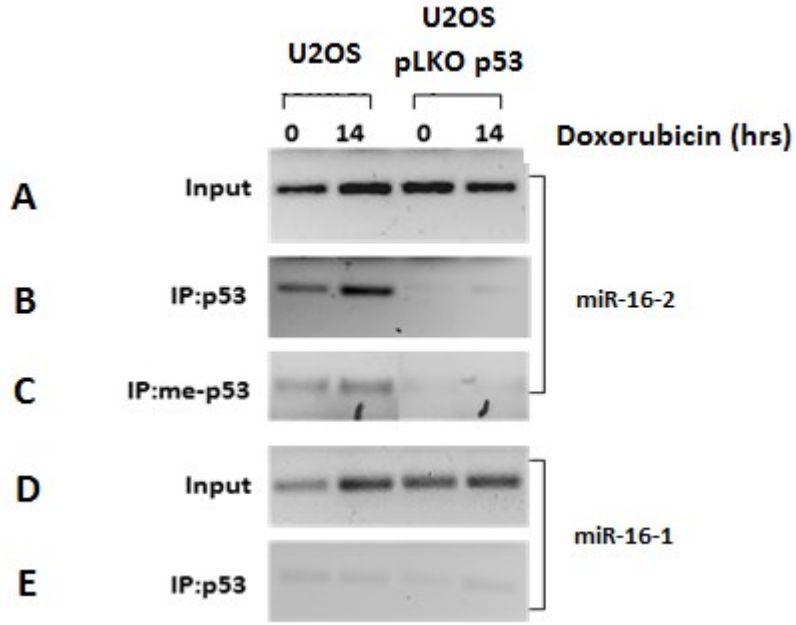


Figure 3.13 ChIP assay of p53 and methylated p53 on the miR-16-1 and miR-16-2 promoters

U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with doxorubicin to induce DNA damage for 0 (control) and 14 hours followed by ChIP assay. **(A) (B)** and **(C):** Binding of p53 and K372-methylated p53 to the miR-16-2 promoter in U2OS cells. **(D)** and **(E)** Binding of p53 to the miR-16-1 promoter in U2OS cells. Input materials were used as controls.

p53-regulated miRNAs and apoptosis

p53 exerts its functions of tumour suppressor as a transcription factor that promotes expression of genes, whose products induce cell cycle arrest and/or apoptosis in response to genotoxic stress.

The effect of p53 on apoptosis was determined in U2OS ($p53^{+}$) and U2OS knockdown p53 (U2OS pLKO p53) cells. Cells were treated with doxorubicin to induce DNA damage for 0 (control) and 14 hours (Fig. 3.14). Cells were analysed by FACS, and U2OS cells were scored for apoptosis based on PI staining.

In U2OS cells not treated with doxorubicin, cells arrested in the G1 phase and then stopped in the G2/M phase (Fig. 3.14A). A similar trend was seen in U2OS (knockdown p53) cells not treated with doxorubicin, but slightly more cells arrested in G2/M phase (Fig. 3.14B). In U2OS cells treated with doxorubicin, similar populations of cells arrested in both the G1 phase and in the G2/M phase (Fig. 3.14C). In U2OS (knockdown p53) cells treated with doxorubicin, cells were only arrested in the G2/M phase, with a minimal population in G1 phase (Fig. 3.14D).

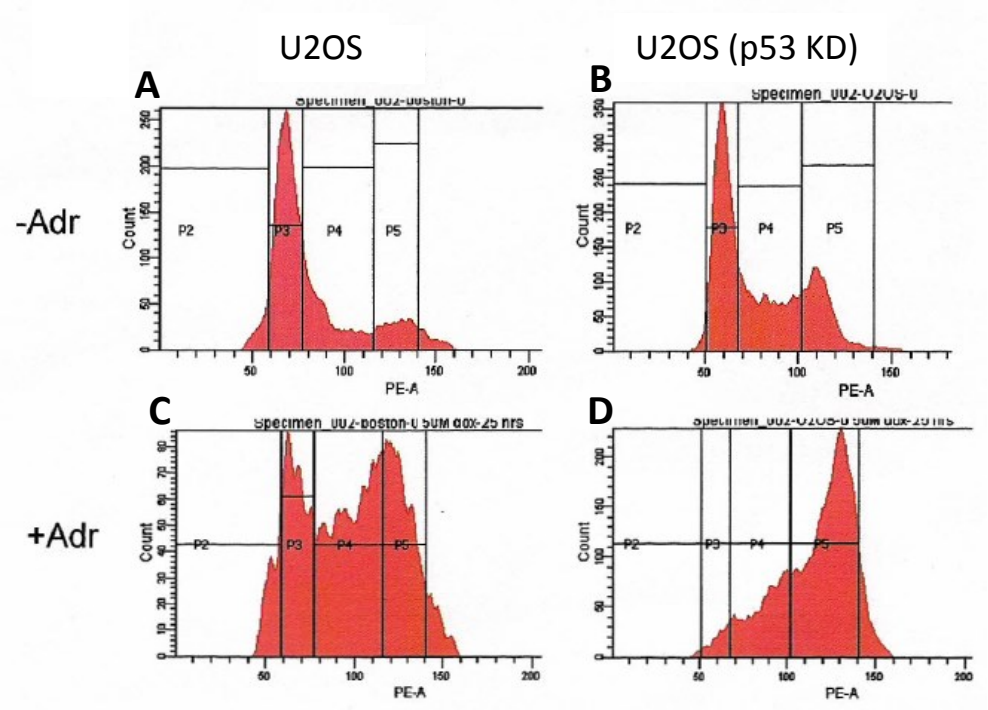


Figure 3.14 Effect of p53 on apoptosis in U2OS (p53^{+}) and (knockdown p53) cells

U2OS (p53^{+}) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with Adriamycin (doxorubicin) to induce DNA damage for 0 (control) and 14 hours **(A)**, **(B)**, **(C)**, and **(D)**. Cells were stained with propidium iodide (PI), harvested and analysed by FACS. U2OS cells were scored for apoptosis based on PI staining.

Our experimental data indicate that miR-16 and miR-26a are regulated by p53 and thus may contribute to p53-dependent physiological response to genotoxic stress. Using FACs analysis we examined the effect of miR-16 and miR-26a on cell cycle arrest and apoptosis of cells non-treated or treated with DNA damaging doxorubicin (Fig. 3.15).

In the absence of DNA damage, overexpression of miR-26a increased the population of cells in the G1 phase and decreased cells in G2/M phase, compared to control cells transfected with control scrambled oligonucleotide. On the contrary, overexpressed miR-16 decreased the number of G1 cells and increased G2/M cells, compared to control cells (Fig. 3.15A and B).

U2OS cells used for the experiment transiently arrest in the G1 phase and then stop in the G2/M phase. Thus, if miR-16 and miR-26a affect cell cycle progression by slowing cells in the G1 phase, then overexpression of these oligonucleotides in DNA damage-treated cells should reduce the population of G2/M phase cells.

In fact, doxorubicin-treated cells transfected with miR-16 and miR-26a showed decreased G2/M distribution compared to control cells. Accordingly, the number of cells arrested in G1 increased (Fig. 3.15A and B).

In the presence and absence of DNA damage, cells transfected with miR-16 and miR-26a show increased apoptosis compared to control cells, which is more significant in DNA-damaged cells (Fig. 3.15B). In this way, DNA damage sensitises cells to undergo apoptosis. miRNAs of the miR-15/16/195/424/497 family were discovered to sensitise cisplatin-resistant cells to apoptosis by their targeting of CHK1 and WEE1 kinases, which are implicated at G1/S and G2/M cell cycle checkpoints, respectively (Pouliot *et al.*, 2012).

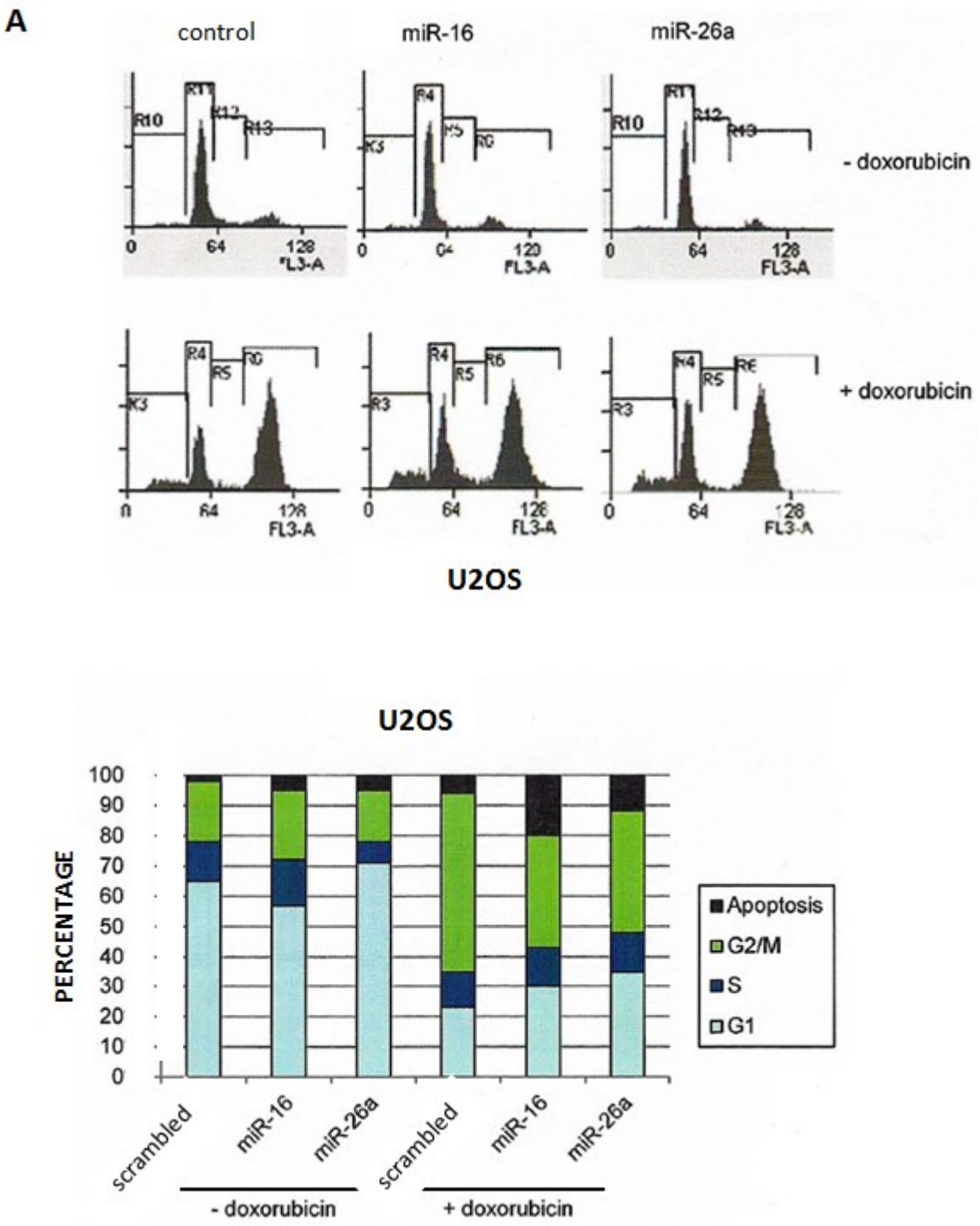


Figure 3.15 Effect of p53-dependent miR-16 and miR-26 on apoptosis in U2OS (p53+) and (p53-) cells

U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were transfected with the indicated microRNAs and treated with doxorubicin to induce DNA damage for 0 (control) and 14 hours. Cells were stained with Propidium iodide (PI), harvested, and analysed by FACS. U2OS cells were scored for apoptosis based on PI staining **(A)**. **(B)** U2OS cells transfected with the corresponding precursors of miR-16 and miR-26 (and scrambled control), and treated with doxorubicin to induce DNA damage, were scored for apoptosis based on PI staining. Cells were analysed by FACs. Different colours denote cell state.

We wanted to determine if miR-16 and miR-26a induction of apoptosis was a p53-dependent effect (Fig. 3.16). As miR-15 is part of a gene cluster with miR-16 (miR-15a/miR-16-1 cluster or miR-15b/miR-16-2 cluster), we decided to analyse miR-15 as well (Aqeilan *et al.*, 2010).

To this end, U2OS and U2OS pLKO p53 cells were transfected with precursors of miR-15, miR-16, and miR-26a as well as their inhibitors (antagomirs). Following transfection, these cells were either treated or not treated with doxorubicin (Fig. 3.16). Ectopic expression of either precursor of miR-15 or its inhibitor did not significantly affect the levels of DNA damage-induced apoptosis in p53-positive cells. Interestingly, inhibition of miR-16 in p53-positive cells treated with doxorubicin caused a significant increase of apoptosis, whereas overexpression of miR-16 did not have this effect. Overexpression of miR-26a facilitated p53-dependent apoptosis upon DNA damage.

Collectively, these results suggest that miR-26a cooperates with p53 to induce apoptosis, and miR-16 enhances p53-mediated cell cycle arrest in the G1 phase.

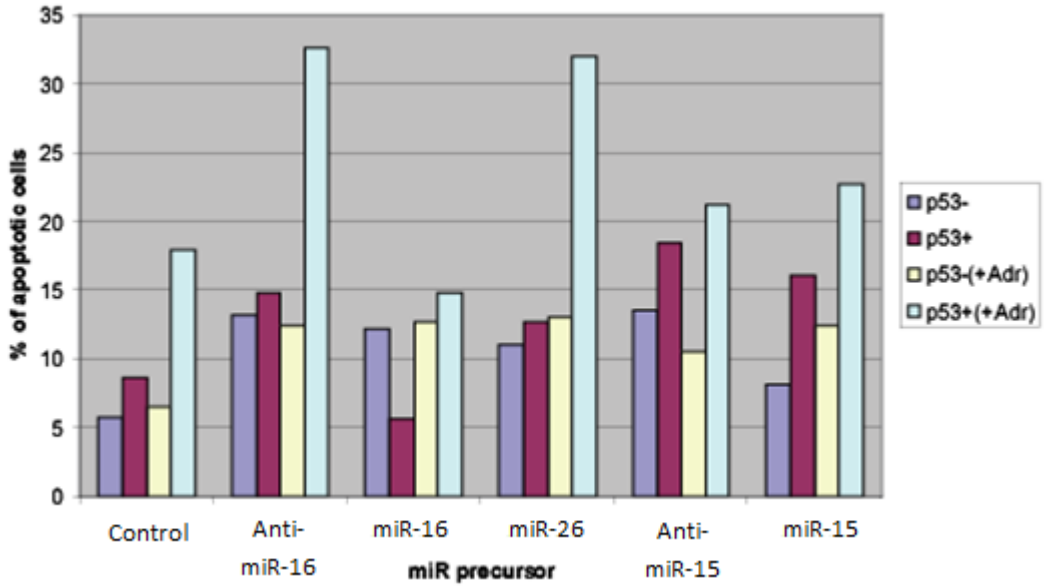


Figure 3.16 Effect of p53-dependent miR-15, miR-16 and miR-26a on apoptosis in U2OS (p53+) and (p53-) cells

U2OS ($p53^+$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were transfected with the corresponding precursors and antagonists of miR-15, miR-16 and miR-26a, and treated with doxorubicin to induce DNA damage for 14 hours. Cells were stained with Propidium iodide (PI), harvested, and analysed by FACS. Cells were scored for apoptosis based on PI staining. Different colours denote $p53^+$/knockdown cells with/without DNA damage.

PART 3: Targets of miR-16 and miR-26a

miR-16 and miR-26a regulation of CHK1 and WEE1

miRNAs in the miR-15/16/195/424/497 family were found to sensitise cisplatin-resistant cells to apoptosis by targeting the WEE1 and CHK1 genes for down-regulation. WEE1 and CHK1 are already recognised as important targets for cancer therapy. Therefore, we decided to investigate whether miR-16 and miR-26a indeed target these genes in H1299 ($p53^-$) and U2OS ($p53^+$) cells.

First, we tested whether miR-16 is able to affect the expression of CHK1 in H1299 cells (Fig. 3.17A). Since CHK1 is an important element of DNA damage signalling pathway, cells were treated with doxorubicin to induce genotoxic stress (Fig. 3.17A). miR-16 oligonucleotide was transfected in two different concentrations of 3nM and 15nM. To evaluate the specificity of targeting for these miRNAs, miR-16 inhibitor was also tested in concentrations of 10nM and 50nM. Transfection with miR-16 oligonucleotide showed a clear reduction of CHK1 levels compared to control cells. In addition, overexpression of miR-16 inhibitor in concentrations of 50nM resulted in restoration of CHK1 expression. Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.17A).

A bioinformatics approach using software programme Targetscan (www.targetscan.com) has identified WEE1 as a potential target of miR-16 and miR-26a. Thus, we sought to verify this prediction experimentally.

First, we tested whether miR-16 and miR-26a affect the expression of WEE1 in H1299 cells (Fig. 3.17B). Similar to the CHK1 western blot (Fig. 3.17A) cells were treated with doxorubicin to induce genotoxic stress, and miR-16 and miR-26a oligonucleotides were transfected into cells (Fig. 3.17B). miR-16 and miR-26a were shown to clearly reduce WEE1 expression compared to control cells, with this reduction being more distinct for miR-16. Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.17B).

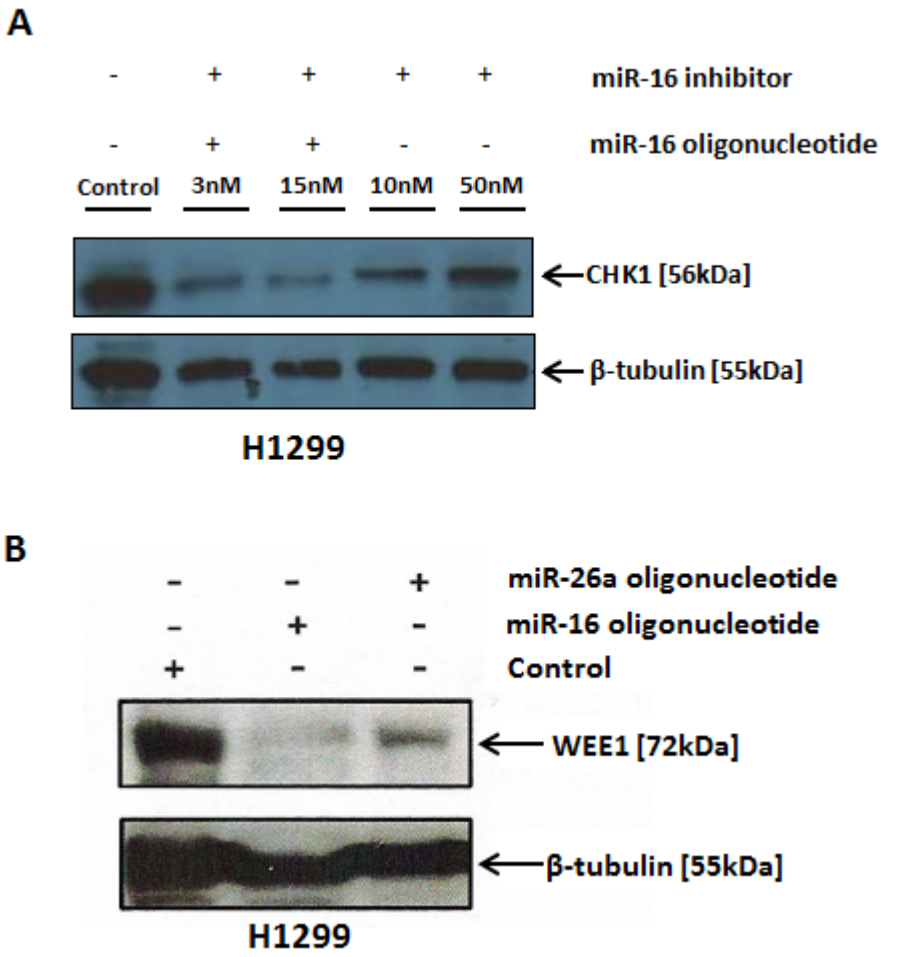


Figure 3.17 Effect of miR-16 and miR-26a on expression of cell cycle-dependent genes CHK1 and WEE1

H1299 cells were transfected with either control scrambled RNA (control), or miR-16, or miR-26a followed by the treatment with doxorubicin for 14 hours. Cells were then harvested and analysed by Western blotting against CHK1 and WEE1. β-tubulin was used as a loading control.

Additionally, we wanted to test whether p53 has an effect on the expression of CHK1 and WEE1. This was carried out in U2OS cells by Western blotting analysis (Fig. 3.18). U2OS ($p53^{+}$) and U2OS (knockdown p53) cells were treated with and without doxorubicin to induce genotoxic stress (Fig. 3.18).

In the presence of p53, there was a clear reduction of CHK1 and WEE1 expression compared to control (knockdown p53) cells, further amplified on DNA damage (Fig. 3.18). In addition, on DNA damage alone, there was also a clear reduction of CHK1 and WEE1 levels compared to control non-treated cells, in knockdown p53 cells (Fig. 3.18). Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.18).

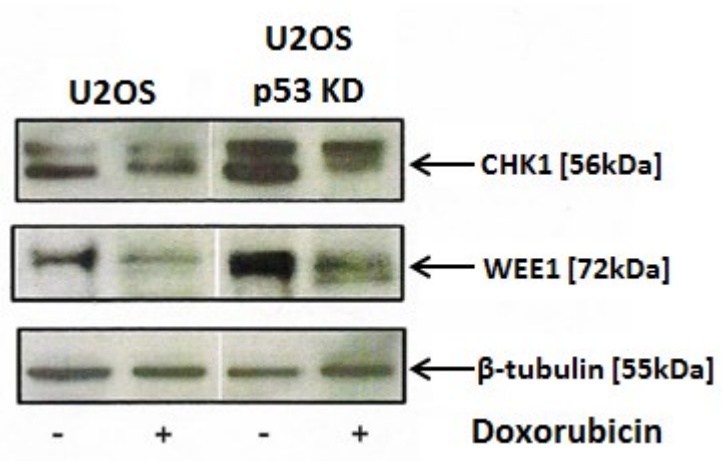


Figure 3.18 Effect of p53 on expression of cell cycle-dependent genes CHK1 and WEE1

U2OS ($p53^{+}$) and U2OS pLKO p53 (p53 knockdown with small hairpin shRNA) cells were treated with or without doxorubicin for 14 hours. Cells were then harvested and analysed by Western blotting against CHK1 and WEE1. β-tubulin was used as a loading control.

H1299 cells were transfected with miR-16 and miR-26a oligonucleotides and transcriptional levels of WEE1 were evaluated by Q-PCR. Cells were transfected with the corresponding oligonucleotides in two concentrations of 25nM and 75nM. The RNA level of U6 was used for normalisation (Fig. 3.19).

These results indicate that miR-16 and miR-26a did reduce transcription of the WEE1 gene, compared to controls (Fig. 3.19). Relating to a lower concentration (25nM) of the tested miRNAs, miR-16 significantly reduced WEE1 expression, compared to the control, and miR-26a which was linked to a minor WEE1 expression reduction (Fig. 3.19).

With a higher concentration (75nM) of the tested miRNAs, miR-16 also reduced WEE1 levels compared to the control, but not to as great an extent as with a lower concentration of miR-16 (Fig. 3.19).

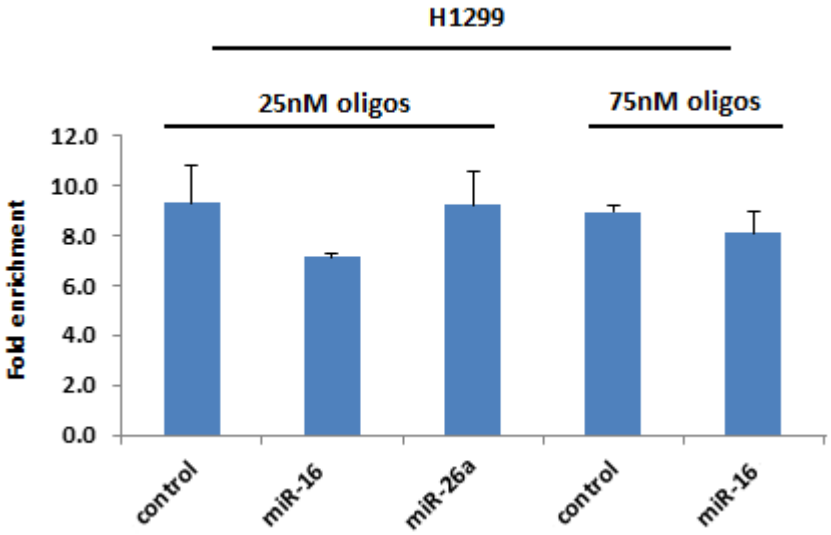


Figure 3.19 Effect of miR-16 and miR-26a on WEE1 expression
H1299 cells were transfected with miR-16 and miR-26a oligonucleotides, and scrambled control (25nM or 75nM) using Lipofectamine 2000, and treated with doxorubicin for 14 hours. Transcriptional levels of WEE1 were evaluated by Q-PCR with SYBR Green dye. U6 was used for normalisation. $p = 0.05$

One of the most common experimental approaches to test the efficiency of targeting for specific miRNAs is to evaluate stability of the luciferase gene fused to the 3'UTR of the gene of interest that is presumed to be targeted by miRNA.

To employ this approach we have constructed a plasmid that carries the 3'UTR of WEE1 or CHK1 attached to the luciferase gene, whose transcription is driven by a minimal SV40 promoter.

The results of this cloning are shown in Fig. 3.20. The full-length 3'UTR of WEE1 or CHK1 were cloned separately into a pGL3-Control luciferase reporter plasmid. The identity of each 3'UTR sequence was verified by restriction digest with XbaI and DNA Sanger sequencing.

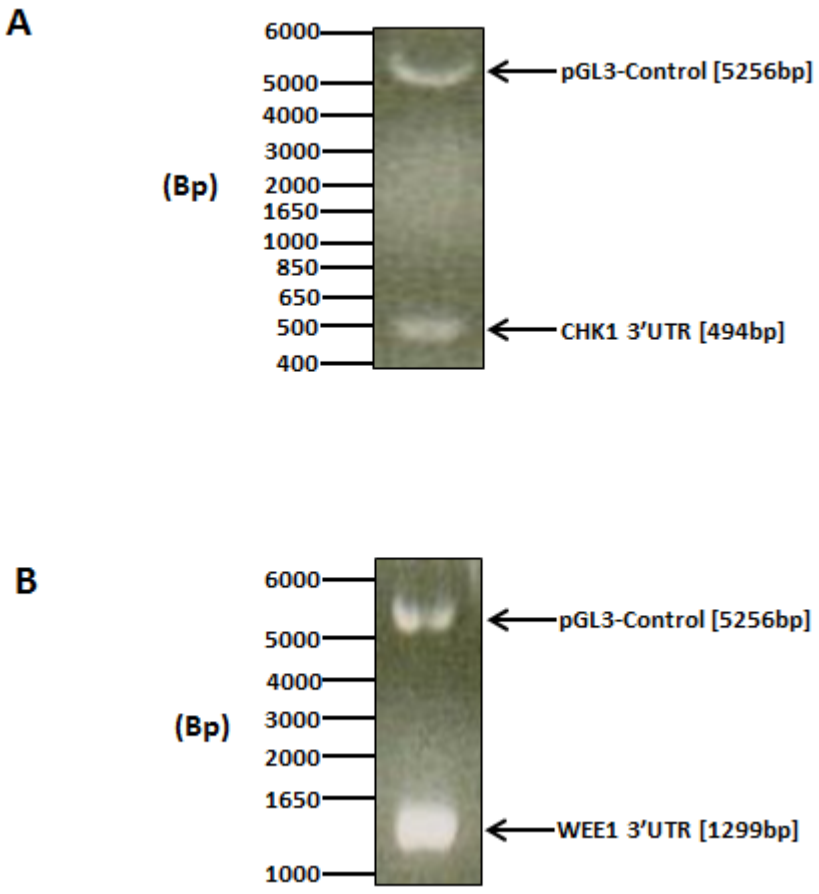


Figure 3.20 Generation of CHK1 and WEE1 3'UTR luciferase constructs
The CHK1 3'UTR **(A)**, and WEE1 3'UTR **(B)** were cloned separately into a pGL3-Control luciferase reporter plasmid. Miniprep plasmid DNA was prepared for each construct and screened for positive clones by restriction digest with XbaI and DNA sequencing.

The CHK1 and WEE1 3'UTR luciferase constructs were transfected into H1299 cells in the presence or absence of miR-16 and miR-26a oligonucleotides. Scrambled oligonucleotide was used as a negative control (Fig. 3.21).

miR-16 and miR-26a together had a greater effect on reducing WEE1 expression, compared to when miR-16 and miR-26a were transfected into cells separately, and compared to the control (Fig. 3.21). When miR-16 and miR-26a were transfected into cells separately, miR-16 had a greater effect on reducing WEE1 expression compared to miR-26a, which had a minimal effect (Fig. 3.21).

Additionally, miR-16 and miR-26a together had a significant effect on reducing CHK1 expression, compared to the control (Fig. 3.21).

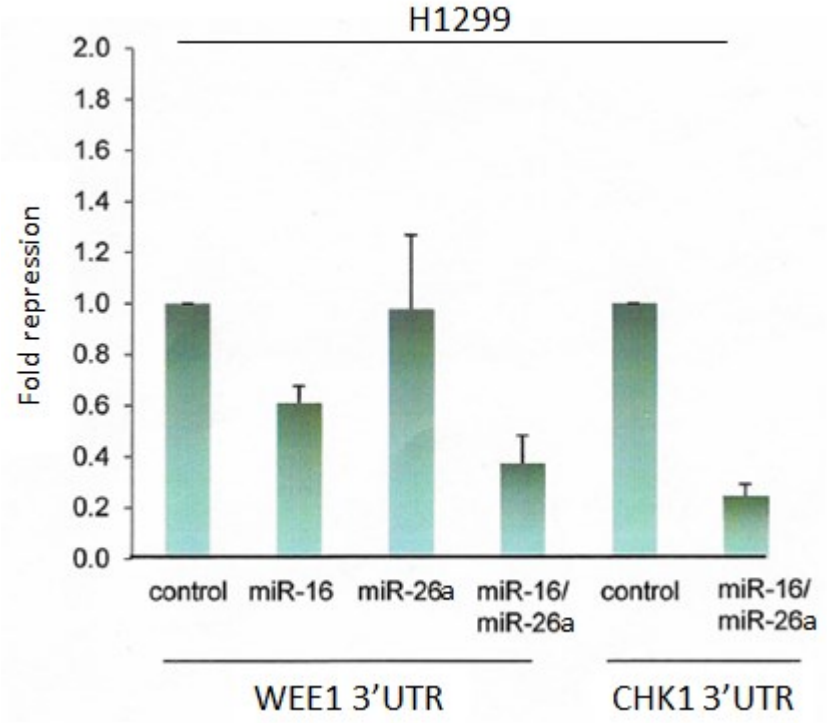


Figure 3.21 Effect of miR-16 and miR-26a on CHK1 and WEE1 expression using luciferase assay

CHK1 3'UTR and WEE1 3'UTR luciferase constructs, and miR-16/miR-26a oligonucleotides (or scrambled control) were transfected using Lipofectamine 2000 reagent into H1299 cells. Cells were treated with doxorubicin for 14 hours to induce DNA damage. $p = 0.05$

miR-16 and miR-26a regulated Cyclin E and SET9

To validate Cyclin E as a published target of miR-16, we employed Western blotting analysis after transfection with the corresponding miRNA.

To characterise the effect of miR-16 on expression of Cyclin E in more detail we transfected

H1299 cells with two different concentrations of miR-16 oligonucleotide (3nM and 15nM), subjected cells to DNA damage, followed by Western blotting against Cyclin E. Increasing concentrations of miR-16 inhibitors (10nM and 50nM) were also used to show the specificity of this miRNA in respect to Cyclin E (Fig. 3.22).

15nM miR-16 completely eliminated Cyclin E expression (Fig. 3.22). Importantly, 10nM miR-16 inhibitor restored Cyclin E expression compared to the scrambled control. Overall, our data confirm that miR-16 targets Cyclin E and mediates down-regulation of its respective mRNA.

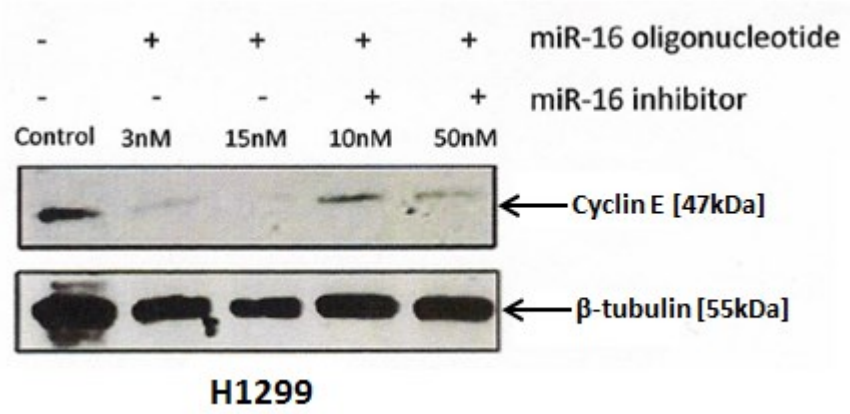


Figure 3.22 Effect of miR-16 on expression of cell cycle-dependent gene Cyclin E
H1299 cells were transfected with either scrambled RNA (control), miR-16 (3nM or 15nM), or miR-16 inhibitor (10nM or 50nM) followed by the treatment with doxorubicin for 14 hours. Cells were then harvested and analysed by Western blotting against Cyclin E. β-tubulin was used as a loading control.

We also wanted to determine the effect p53 has on miR-16 regulation of Cyclin E expression (Fig. 3.23). We transfected U2OS ($p53^{+}$) cells with a mixture of miR-15 and miR-16 oligonucleotides, followed by Western blotting analysis for Cyclin E. In addition, these cells were non-treated or treated with doxorubicin for 24 hours to induce DNA damage (Fig. 3.23).

miR-15/miR-16 effectively decreased Cyclin E expression, compared to cells transfected with scramble control (Fig. 3.23). This effect was specific because the level of Ku70 was not affected by these miRNAs (Fig. 3.23).

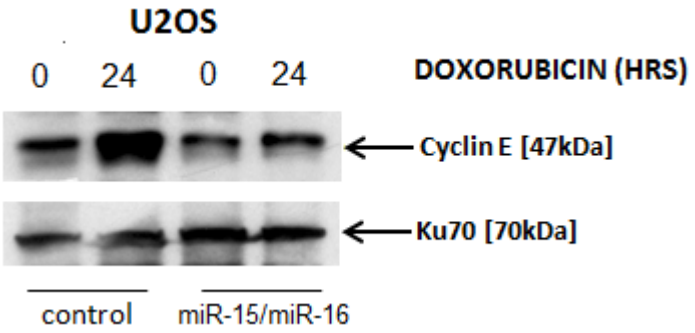


Figure 3.23 Effect of miR-15/miR-16 on expression of cell cycle-dependent gene Cyclin E
U2OS cells were transfected with either scrambled RNA (control) or miR-15/miR-16 oligonucleotides, followed by the treatment with doxorubicin for 0 or 24 hours. Cells were then harvested and analysed by Western blotting against Cyclin E. Ku70 was used as a loading control.

SET9 methyltransferase has been shown to methylate histones as well as non-histone proteins including p53. After DNA damage, methylation of p53 by SET9 at Lys372 in its C-terminus leads to p53 stabilisation, containing it in the nucleus, and transcriptional activation (Chuikov *et al.*, 2004).

In U2OS ($p53^+$ and $SET9^+$) cells, p53 levels are higher compared to in knockdown SET9 cells (Fig. 3.24). Additionally, DNA damage clearly increases p53 levels.

One of the targets of miR-16 is Cyclin E (Fig. 3.22 and 3.23), which is positively regulated by E2F1, as part of cell cycle regulation. The latter is regulated by SET9. It has also been shown that E2F1 positively regulates miR-16 expression, which, in turn, down-regulates E2F1 and Cyclin E expression. To add extra layer of complexity, p53 is known to repress E2F1 expression, but on the contrary, E2F1 activates expression of p53. Therefore, these mutual regulatory mechanisms form a complex circuitry that needs to be deciphered.

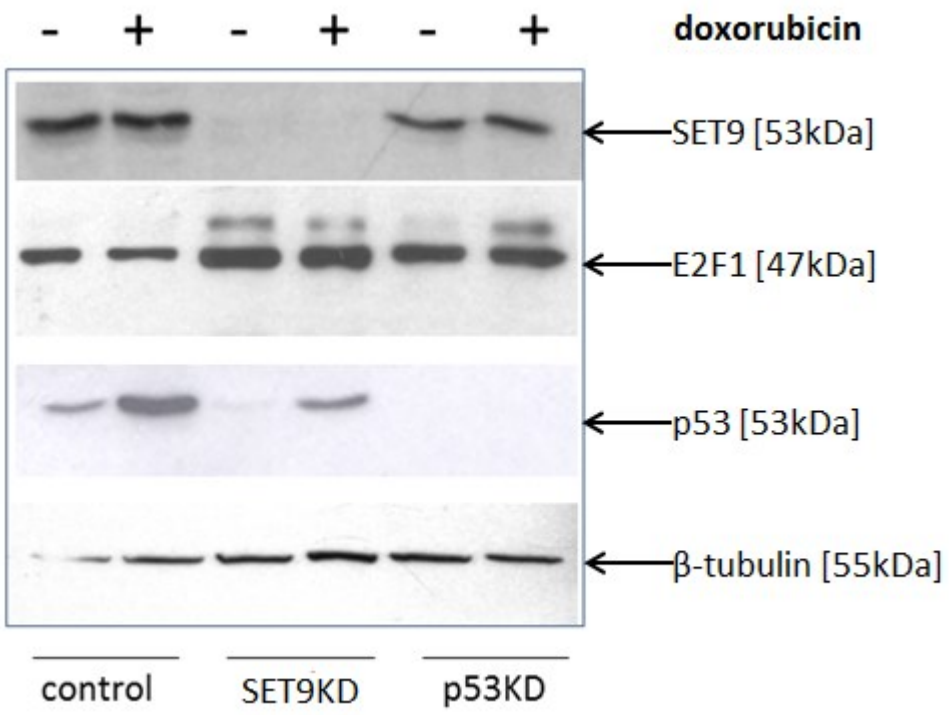


Figure 3.24 Effect of SET9 and p53 on E2F1 expression
U2OS (SET9 knockdown) and U2OS pLKO p53 cells were treated or non-treated with doxorubicin for 14 hours to induce DNA damage. U2OS cells were used as a control. Cells were harvested and analysed by Western blotting against SET9, E2F1, and p53. β-tubulin was used as a loading control.

Our results already demonstrate that Cyclin E is regulated by p53-dependent miR-16 (Fig. 3.22 and 3.23). Previous data from our lab suggest that transcriptional activity of p53 is dependent on lysine-specific methyltransferase SET9 (Ivanov *et al.*, 2007). SET9 methylates p53 and renders it transcriptionally active. Methylated p53 is also found in the chromatin-bound fraction of the miR-16 promoter. Therefore, SET9 may be an important indirect regulator of Cyclin E expression.

To test this possibility, we decided to compare the levels of transcriptional activation driven by the Cyclin E promoter as a function of SET9. To achieve this, we used luciferase reporter plasmids that contained within the Cyclin E promoter either wild-type or mutant E2F1REs. Notably, E2F1 also responds to DNA damage by modulating transcription of its target genes.

The Cyclin E promoter was cloned into a pGL2 luciferase reporter plasmid to study the effects of SET9 on the expression of Cyclin E. The activity of the luciferase Cyclin E promoter construct was analysed in different cell lines.

Importantly, this luciferase assay was performed in cells treated with doxorubicin to induce DNA damage, as it is known from the literature that E2F1 is activated in response to DNA damage (Fig. 3.25A). We also tested the level of activation of Cyclin E reporter in cells without doxorubicin treatment (Fig. 3.25B).

First, we analysed the effect of SET9 on Cyclin E transcription in H1299 ($SET9^{+}$) and (SET9 knockdown) cells. To generate the latter cell line, SET9 expression was stably repressed by specific shRNA. On DNA damage, activation of Cyclin E promoter in H1299 cells expressing SET9 was significantly higher (6-fold) than that in matching SET9 knockdown cells (Fig. 3.25A). In the absence of DNA damage, activation of Cyclin E promoter in H1299 cells expressing SET9 was also higher than that in matching SET9 knockdown cells, but by only 3-fold (Fig. 3.25B).

As a negative control, pGL2 luciferase plasmid without any promoter was used. To validate the specificity of the effects observed, a construct bearing Cyclin E promoter with mutations in E2F1REs was used. Mutant Cyclin E clearly showed less induction compared to the level of activity of wild-type Cyclin E (Fig. 3.25A and B).

Results of this experiment demonstrate that SET9-dependent methylation is required for activation of Cyclin E even in the absence of DNA damage.

To confirm the presence of SET9 in cells, cells treated with doxorubicin were harvested and analysed by Western blotting against SET9 (Fig. 3.24).

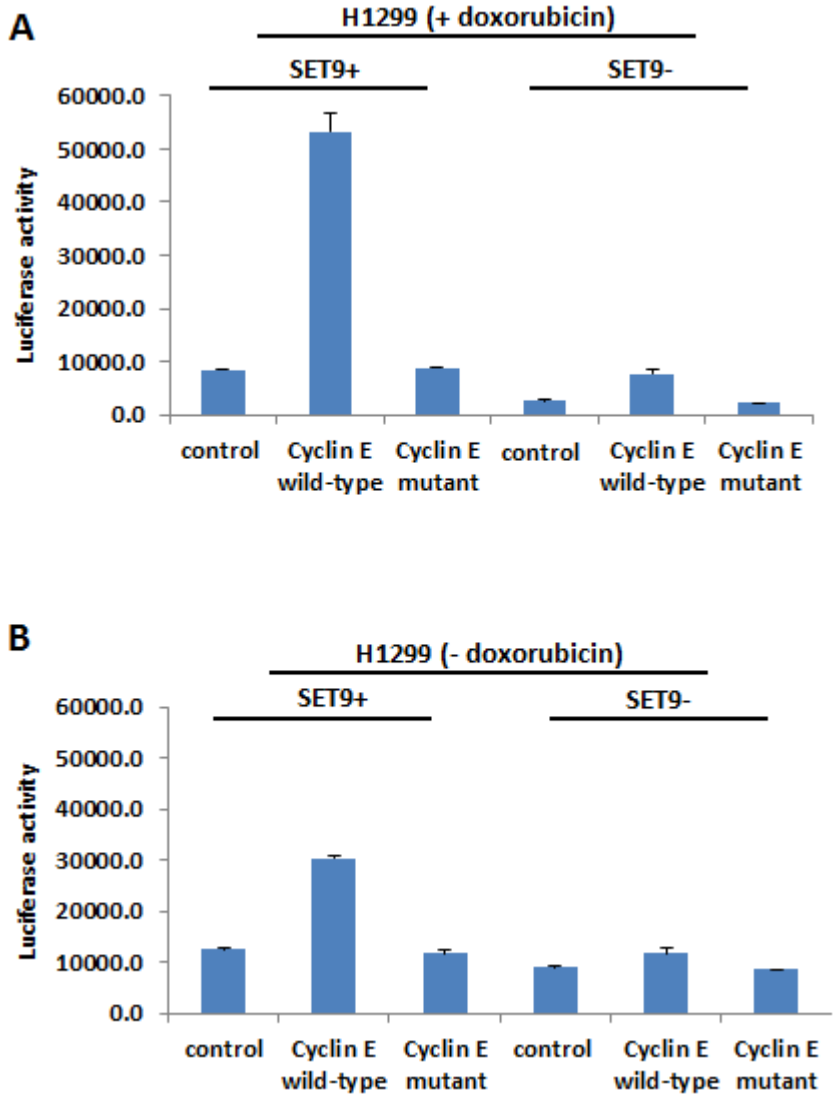


Figure 3.25 Effect of SET9 on ectopic Cyclin E expression using luciferase assay
Cyclin E promoter luciferase constructs were transfected using Lipofectamine 2000 reagent into H1299 (SET9+) and H1299 (SET9-) (SET9 knockdown with small hairpin shRNA) cells. Cells were treated **(A)** or non-treated **(B)** with doxorubicin for 14 hours to induce DNA damage. pGL2 luciferase reporter plasmid and Cyclin E mutant promoter luciferase construct were used as controls. $p = 0.05$

To follow, we repeated luciferase assay, but this time in U2OS ($p53^+$) cells, to determine the effect of p53 on SET9 regulation of Cyclin E. Again, cells were either treated or non-treated with doxorubicin to induce DNA damage, and hence activation and stabilisation of p53 (Fig. 3.26A and B).

Similar to in H1299 cells (Fig. 3.25A), SET9 was important for activation of Cyclin E in U2OS cells treated with doxorubicin (Fig. 3.26A). However, the difference in the activity between U2OS ($SET9^+$) and U2OS (SET9 knockdown) cells (Fig. 3.26A) was not as dramatic as in H1299 cells (Fig. 3.25A) - (1.5-fold versus 7-fold difference in Cyclin E activation, respectively). This may be due to the presence of active p53 in U2OS cells (Fig. 3.26A), which inhibits E2F1 upon DNA damage.

When the luciferase assay was repeated in U2OS cells without DNA damage (Fig. 3.26B), the level of activation of wild-type Cyclin E was lower compared to in DNA damage-treated cells (Fig. 3.26A). However, the difference in Cyclin E activation between U2OS $SET9^+$ and SET9 knockdown cells was greater in non-treated cells (3-fold) (Fig. 3.26B) compared to in U2OS cells after DNA damage (1.5-fold) (Fig. 3.26A).

Collectively, our data suggest that SET9 is an important factor that regulates transactivation of Cyclin E.

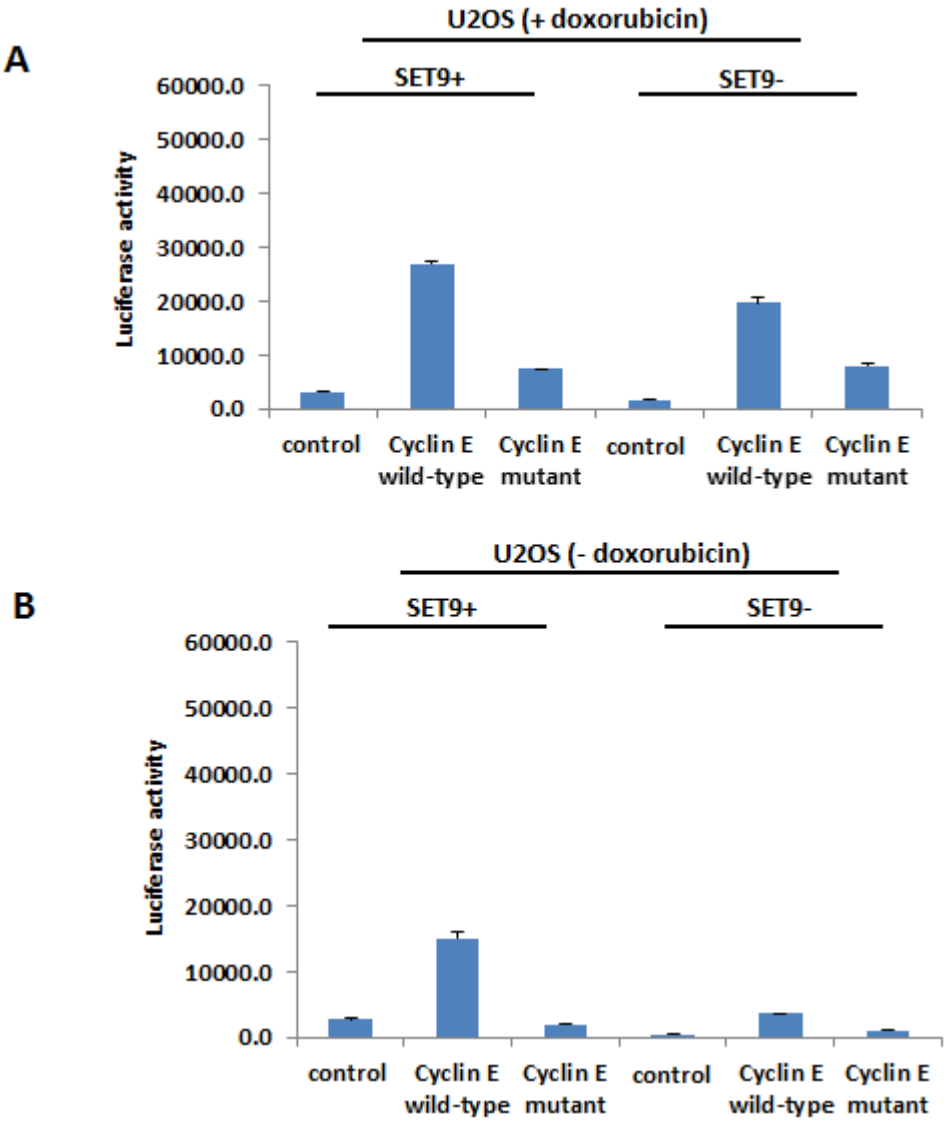
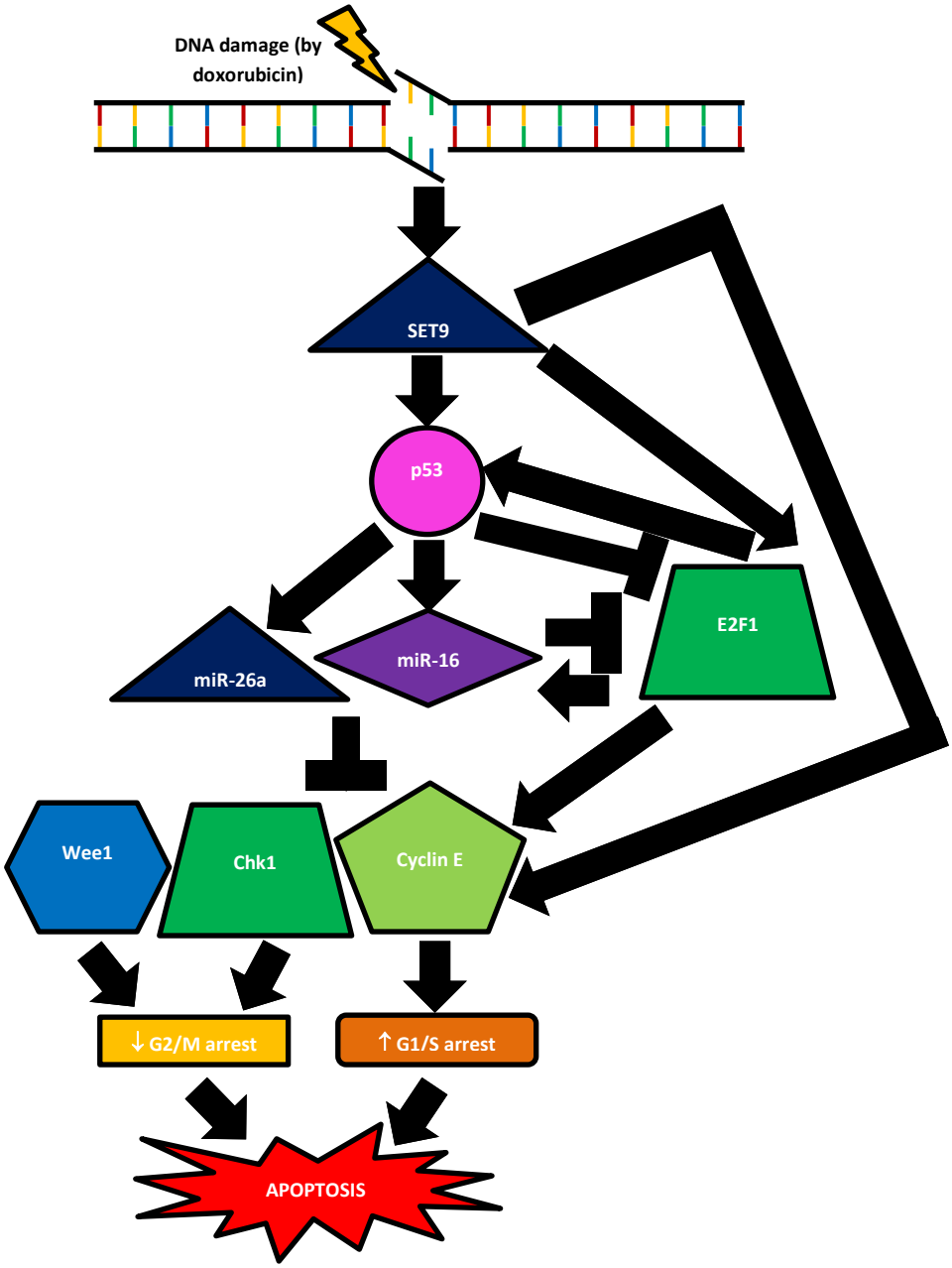


Figure 3.26 Effect of SET9 on ectopic Cyclin E expression using luciferase assay
Cyclin E promoter luciferase constructs were transfected using Lipofectamine 2000 reagent into U2OS (SET9+) and U2OS (SET9-) (SET9 knockdown with small hairpin shRNA) cells. Cells were treated **(A)** or non-treated **(B)** with doxorubicin for 14 hours to induce DNA damage. pGL2 luciferase reporter plasmid and Cyclin E mutant promoter luciferase constructs were used as controls. $p = 0.05$

Overall cascade of events

Our results showed that in response to DNA damage, miR-16 and miR-26a expression levels are controlled by p53-dependent and p53-independent mechanisms, potentially involving other stress-response transcription factors such as E2F1. Our data also confirms that miR-16 and miR-26a directly target Cyclin E, CHK1, and WEE1 for down-regulation. Additionally, SET9 directly controls Cyclin E expression. Reduced CHK1 and WEE1 levels leads to decreased G2/M arrest, and reduced Cyclin E levels results in increased G1/S arrest. This eventually brings about apoptosis.



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DISCUSSION

The p53 pathway and miRNAs

In recent times, it has been shown that miRNAs play a key role in the p53 network. p53 has been demonstrated to regulate the miRNA gene transcription, which in turn, helps in cell cycle arrest and apoptosis. The cross-regulation of p53 and miRNAs in human disease, specifically cancer, adds another complex level to cellular responses. The functionality of the p53 pathway in cancer may be elucidated by identification of regulatory associations between p53 and miRNAs, as well as the downstream targets of the latter (Schetter *et al.*, 2010; Feng *et al.*, 2011).

Amongst the p53-dependent miRNAs that have already been suggested to play a key role in cancer by regulating expression levels of oncogenes (He *et al.*, 2007; Suzuki *et al.*, 2009), two miRNAs, miR-16 and miR-26a, have been identified as key effectors of the p53 pathway in response to genotoxic stress.

Preliminary findings presented differences in expression of various miRNAs in $HCT116^{p53+}$ colon cancer cells versus $HCT116^{p53-}$ (Vogelstein *et al.*, 1989). Previous experiments in the lab involved the use of microarray expression analysis of miRNAs, followed by Q-PCR to identify miRNAs dependent on p53 and DNA damage. Based on this initial data, two miRNAs were identified whose expression was p53-dependent. These miRNAs are miR-16 and miR-26a.

miR-16

In 2002 miR-15 and miR-16 were identified as the first cancer genes, which are also implicated in CLL progression (Calin *et al.*, 2002). Altered miR-16 expression has also been observed in stomach, prostate, pancreatic, ovarian, lung, colon, breast, and brain cancers. miR-16 exerts its tumour suppressor role by down-regulation of BCL2, which is overexpressed in CLL (Yue & Tigyi 2010). As loss in miR-16 expression is seen in many cancers, it would be an ideal therapeutic target (Liu *et al.*, 2008).

miR-26a

It has been shown that overexpressed miR-26a in nasopharyngeal cells directly represses oncogenic EZH2 expression, resulting in cell cycle and cell growth inhibition, and prevention of tumourigenesis. miR-26a-induced EZH2 repression by c-MYC was shown to have a key role in lymphomagenesis (Wong & Tellam 2008). miR-26a also directly down-regulates the oncogenic protein metadherin (MTDH) to bring about

apoptosis in breast cancer cells (Zhang *et al.*, 2011). Relating to therapeutic intervention, delivery mechanisms via adenovirus have already been used to administer miR-26a to cancer cells, in order to induce apoptosis, and therefore stop cancer from progressing (Di Leva *et al.*, 2012).

PART 1: miR-16 and miR-26a in cancer

We carried out Q-PCR to predict the survival probability of cancer patients based on miR-16 and miR-26a microRNA signatures (Fig. 3.1). Breast, hepatocellular, ovarian, and prostate cancer tissue specimens belonging to cancer patients were used for this study. miR-16 and miR-26a expression was analysed by Q-PCR with a TaqMan MicroRNA Assays Human Panel-Early Access Kit. This method of miRNA profiling is highly sensitive and provides log-linear amplification over at least six orders of magnitude.

Generally, high miR-16 and high miR-26a expression are associated with increased breast, prostate, ovarian, and hepatocellular cancer survival (Fig. 3.1A-F). With breast cancer progression survival is increased with high miR-16 and high miR-26a expression following 30 months from initial cancer onset (Fig.3.1A and B). Generally, breast cancer survival declines with time (Fig.3.1A and B), as does ovarian and hepatocellular cancer survival (Fig. 3.1E and F respectively). With ovarian and hepatocellular cancer progression survival is increased with high miR-16 and high miR-26a expression following 5 and 15 months respectively from initial cancer onset (Fig. 3.1E and F respectively). With prostate cancer progression survival is increased with high miR-16 and miR-26a expression, which remains steady over time (Fig.3.1C and D).

The first reports indicating a potential role of miRNAs in cancer was of a miR-15a/miR-16-1 deletion at chromosome position 13q14, which is commonly found in CLL, as well as mantle cell lymphoma and prostate cancer. The majority of miRNAs are down-regulated in cancer, as shown by the first signatures gained of miRNA expression in tumours (Lu *et al.*, 2005). For example, let-7 was shown to exhibit tumour suppressive features, as shown by many of the let-7 family members mapped to chromosomal regions often deleted in tumours and absent in several cancers, such as lung, breast, and cervical.

miRNA signatures may have important diagnostic and prognostic roles as biomarkers in cancer, and thus providing key information for prognosis and therapy efficacy (Waldman & Terzic 2008). miRNA expression profiling has been used to identify the origin of tumours with an undetermined origin, and also to differentiate between various tumour subtypes (Wittmann & Jäck 2010). A miRNA expression signature differentiating between cancerous tissues and normal tissues has been established,

profiling numerous miRNAs which show a changed expression in various tumours (Calin & Croce 2006). Thus, frequently de-regulated pathways in cancer may have these miRNAs as downstream targets. As miRNA deregulation is classically seen in cancer, it can be predicted that these miRNAs would be essential therapeutic targets.

PART 2: Mechanisms of p53-dependent control for miR-16 and miR-26a

Expression patterns of miRNAs differ significantly between different cancer cell lines. To analyse p53-dependent expression of miRNAs we decided to generate isogenic cell lines that differ only in the levels of p53 expression. A well-known pair of colon cancer cell lines, HCT116^{p53+} and HCT116^{p53-} constructed in the Vogelstein lab has a significant disadvantage, as they constitutively express beta-catenin, which is known to interact with p53 and alter its transcription programme. To solve this problem, we have constructed a p53^{-} isogenic cell line from the p53^{+} human osteosarcoma cell line, U2OS, which has a normal p53 response. In these cells, expression of p53 was down-regulated by shRNA. This stable knockdown approach is a convenient alternative to genetic knockouts, which are quite laborious and costly. However, the success of such stably knockdown cell line relies on the efficiency of a particular small hairpin RNA.

The most significant challenge relating to shRNA-based therapeutics is delivery. ShRNA is usually delivered via a vector. Also, there might be off-target effects and the shRNA may silence other unintended genes. In the development of new and successful shRNA-based therapeutics, these challenges must be considered.

As can be seen from this figure, lentiviral infection of U2OS cells with shRNA-p53 produced a stable knockdown in these cells. Lentiviral infection provides a very high efficiency of delivering the cloned material as many copies of the virus can infect the same cell. In addition, lentiviruses are able to infect a broad spectrum of cells, including senescent cells, although with a lower efficiency. Importantly, even after DNA damage with doxorubicin, when the p53 protein is stabilised (Fig. 3.2, compare bar 1 and 2), there was no detectable signal for p53 in U2OS pLKO p53 cells as judged by western blotting analysis using a p53-specific antibody Ab-1, which is specific to the N-terminus of p53 protein (Fig. 3.2, bar 3 and 4). Note, that the total protein levels were comparable as evident from the Ku70 signal used as a loading control (Fig. 3.1, bottom panel). The main advantages of using western blotting to detect proteins are its vast specificity and sensitivity.

The p53 protein responds to several stress signals, such as DNA damage. These detected signals are portrayed to p53 via numerous enzymes that provoke post-translational modifications to p53. This results in an increased p53 half-life leading to p53 protein accumulation in cells (Appella & Anderson 2001). The first stress type to

be discovered to activate p53 was DNA damage (Efeyan & Serrano 2007). DNA damage can affect the integrity of the genome by generating double-strand breaks and introducing mutations in DNA, leading to possible rearrangements in the genome or a loss in genetic information (Khanna & Jackson 2001).

Doxorubicin (Adriamycin is one of its brand names) is a key chemotherapeutic agent used in treatment of solid epithelial and mesenchymal tumours, in addition to leukaemia. Several mechanisms have been put forward to be implicated in doxorubicin-induced cytotoxicity, including altered p53 expression, death receptor activation, DNA intercalation, DNA strand breakage by inhibition of topoisomerase II, and oxidative stress (Dunkern *et al.*, 2003). Doxorubicin has been shown to elevate wild-type p53 levels in human cells (Nelson & Kastan 1994).

p53-regulated microRNA expression

Once we established a system with isogenic cell lines that differ only in p53 expression, next step was to confirm the microarray expression data suggesting that expression of miR-16-2 and miR-26a are regulated by p53. To do this, we employed Q-PCR-based analysis using primers specific for these miRNAs (Fig. 3.3).

Our results clearly showed that expression levels of both miR-16 and miR-26a was dependent on p53 even in the absence of DNA damage (Fig. 3.3, compare bar 1 and 3, panel A and B). However, upon DNA damage conferred by treatment of cells with doxorubicin, there was a moderate but consistent increase in the endogenous levels of miR-16 and mir-26a expression (1.2 fold and 1.4 fold for miR-16 and miR-26a respectively) as evident from Fig. 3.3 (compare bar 1 and 2 in panels A and B). It should be noted, that the levels of p53 protein increased dramatically after 24 hours of treatment as shown in Fig. 3.2. Also, there was a slight increase in the levels of miR-16 and miR-26a expression after DNA damage even in the absence of p53 (Fig. 3.3, bar 3 and 4, panels A and B), suggesting p53-independent regulatory mechanisms of miR-16 and miR-26a. Additionally, these results suggest that other stress-response transcription factors, for example E2F1, control expression of these genes. E2F1 binds and stimulates the DNA-binding, transactivation and apoptotic functions of p53. Specifically, E2F1 binds residues 347–370 of p53 and therefore enhances the nuclear retention of Ser315-phosphorylated p53 (Fogal *et al.*, 2005). Alternatively, p53 may exercise its control not directly, but on the post-transcriptional level, as Suzuki *et al.*

has already shown that p53 enhances the post-transcriptional maturation of numerous miRNAs with growth-suppressive functions, including miR-16-1, miR-143 and miR-145, in response to DNA damage, or through binding to other transcriptional factors, such as E2F1.

p53 was recently shown to affect expression of microRNAs both at the level of transcription and post-transcriptional maturation (Suzuki *et al.*, 2009). We decided to take a closer look at what level this regulation occurs. To this end, we designed primers that discriminate between pri-miRNA and processed mature miRNAs and performed Q-PCR using U2OS cells with wild-type and knockdown expression (U2OS pLKO p53) of p53. In addition, both types of cells were non-treated or treated with doxorubicin for 12 and 24 hours to induce DNA damage (Fig. 3.4).

As evident from this figure, only miR-26a showed p53-dependent response on the level of transcription at 12 and 24 hours after doxorubicin treatment (Fig. 3.4, upper panel). miR-16-2 showed a transient decrease in transcription at 12 and 24 hours after DNA damage in the absence of p53. However, both miRNAs demonstrated a significant decrease in the levels of mature miRNA expression in the absence of p53 (Fig. 3.4, bottom panel). In addition, there was a direct correlation between the time of DNA damage treatment and mature miRNA production.

Thus, these data suggest that p53 can control expression of miRNAs on several levels; miR-16 is regulated at a transcriptional level and miR-26a at post-transcriptional levels. Also, DNA damage facilitates expression of miR-16-2 and miR-26a-1.

Recently it was demonstrated that p53 induces the transcriptional expression of those miRNAs which exhibit tumour suppressive functions, by binding to p53REs in the promoters of these miRNAs. As a result, RNA products of these genes aid in the p53 function of a tumour suppressor (He *et al.*, 2007). In 2007, numerous papers showed the miR-34 family members, of homologous miRNAs miR-34a, miR-34b, and miR-34c, as the first verified miRNAs directly targeted by p53 (Chang *et al.*, 2007; Corney *et al.*, 2007; He *et al.*, 2007; Raver-Shapira *et al.*, 2007; Tazawa *et al.*, 2007). In the presence of genotoxic stress, expression of *miR-34a* provokes p53-dependent apoptosis, G1 cell cycle arrest and senescence (Fig. 1.13).

p53 has also been linked with the regulation of miRNA processing and promoting maturation of many miRNAs, which play an essential part in the role of p53 as a

tumour suppressor. p53 brings about the post-transcriptional maturation of several miRNAs. Drosha has a key role in processing pri-miRNA transcripts to pre-miRNAs. On DNA damage, p53 provokes the Drosha-mediated processing of distinct miRNAs with presumed tumour suppressive functions, including miR-16-1, miR-143, and miR-145. These miRNAs down-regulate essential regulators of cell cycle progression and cell proliferation, such as Cyclins E and D, CDK4 and CDK6 (as a target of miR-16-1 and miR-145) (Freeman & Espinosa 2013). p53 and Drosha interactions occur after doxorubicin treatment via the DEAD-box RNA helicases p68 and p72, which are both required by Drosha to function for miRNA maturation. Transcriptionally non-functional p53 mutants hinder complex formation between Drosha and p68, leading to reduced miRNA processing (Suzuki *et al.*, 2009).

Transcriptional regulation of miRNAs by p53

Since p53 was shown to affect expression of several miRNAs both on the transcriptional and post-transcriptional levels, we decided to assess whether p53 regulates the expression of miR-16-2 and miR-26a directly by binding their promoters and activating their transcription. To address this question, we decided to use a luciferase assay.

This type of assay is based on detection of the reporter luciferase plasmid luminescence. If the promoter or its fragment is bound by a specific transcription factor, then transcription initiation occurs and the luciferase gene starts expressing. This assay helps to determine whether a transcription factor binds the respective response element that controls transcription of the luciferase gene.

Thus, we cloned different fragments of upstream sequences of miR-26a and miR-16-2 genes into a pGL3-Basic luciferase vector (Fig 3.5). Specifically, the upstream sequence of miR-26a gene was divided into two fragments (Fig 3.5). This was done because the bioinformatics software programme Consite (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) predicted existence of several p53 REs scattered along those sequences. Therefore, we wanted to test the functionality of these p53RE sites separately. Similarly, the upstream sequences of miR-16-2 gene were divided into three fragments and also cloned into pGL3 vector (Fig 3.5). Putative p53REs was predicted to reside in the vicinity of the TATA box region of the miR-15b/miR-16-2 locus. The results of successful cloning procedures are shown in Fig. 3.5A, B, C, D and E.

Western blot analysis was carried out to confirm the expression of ectopic p53 wild-type and p53 mutant isoforms K4R (lysine residue acetylation and methylation sites 320, 372, 373 and 382 replaced with arginine residues), R175H (arginine residue 175 in the DNA-binding domain replaced with a histidine residue), transactivation-deficient mutant Δ40 (deletion of the first 40 amino acids), or DNA binding-deficient mutant Δ133 (deletion of the first 133 amino acids) transfected in U2OS pLKO p53 (p53 knockdown) cells used for luciferase assays. Cells were treated with doxorubicin for 14 hours to induce DNA damage. Samples were normalised against Ku70 (Fig. 3.6D), and cells not transfected with p53 were used as a control.

p53 wild-type, K4R, and R175H mutants were positively detected with a size of 53kDa (Fig.3.6A). Truncated mutants p53 Δ40 and p53 Δ133 were confirmed at 39kDa (Fig. 3.6B), and at 29kDa (Fig. 3.6C) respectively.

The direct inactivation of the *p53* gene occurs in over 50% of all human tumours which exhibit non-functional DNA mutations in the *p53* gene (Sigal & Rotter 2000). Most of the remaining malignancies exhibit mutations upstream or downstream of the p53 regulatory network, leading to a deactivated p53 pathway (Olivier *et al.*, 2010). Here, the activity of p53 can be attenuated in many ways. For example, several DNA tumour viruses encode proteins that inactivate p53. These include SV40 large T-antigen, adenovirus E1B55kDa protein, and the E6 oncoprotein of human papilloma virus (HPV) types 16 and 18, which bind to p53 and destabilise the protein or inactivate its functionality (Levine 2009; Lin *et al.*, 1994).

95% of p53 mutants originating from tumours have a missense mutation, usually a point mutation (most often found in arginine amino acids 102-292) within the genomic region which encodes its DNA-binding domain (Cho *et al.*, 1994). Of all the mutations that occur in the DNA-binding domain, around a third occur in the six “hotspot” residues (Figure 1.6) (Ho 2005). Due to this, p53 mutants have changed or abolished affinity for their target genes, leading to a binding deficit and thus a deficiency in the sequence-specific transactivation ability of p53 (Ozaki & Nakagawara 2010), and they may also portray oncogenic characteristics (Sigal & Rotter 2000). Wild-type p53 under normal, unstressed conditions is a very short-lived protein, whereas missense mutations result in the production of a full-length altered p53 protein with an elongated half-life (Strano *et al.*, 2007). Many stable mutant forms of p53 exert a

dominant-negative effect on the remaining wild-type p53 allele, which abrogate the ability of wild-type p53 to inhibit cellular transformation, especially when the mutant protein is expressed in excess of its wild-type counterpart (Brosh & Rotter 2009; Oren & Rotter 2010). These observations have led to the ‘‘gain of- function’’ hypothesis, which indicates that p53 mutation is not just equivalent to losing wild-type p53 functionality; in fact, a strong selection for maintained expression of mutant p53 proteins conveys a positive role for certain p53 mutants in tumourigenesis (Freed-Pastor & Prives 2012).

The purpose of this experiment was to assess whether these promoters were indeed dependent on p53. The resulting luciferase constructs encompassing the promoters of miR-16-2 and miR-26a genes, with or without p53-expressing vector, were transfected into U2OS pLKO p53 cells, which harbours knockdown of the TP53 gene. The choice of this cell line was dictated by the concern that endogenous p53 could interfere with the ectopic one. It is well established that p53 undergoes various post-transcriptional modifications, including phosphorylation, methylation and acetylation, which activate its functions and increase its protein stability (Barlev *et al.*, 2001). Thus, transfected cells were also treated with doxorubicin to induce DNA damage (Fig. 3.7).

Expression levels of luciferase constructs that contained fragments of miR-16-2 upstream sequences were significantly higher than the negative control (empty pGL3-Basic vector) or a vector that contained the promoter of miR-16-1 gene, which does not contain p53REs in its sequence (Fig. 3.7, panel A, compare bar 1 and 6 with bar 2,3, and 4). Surprisingly, the full-length upstream sequence (2100bp) of miR-16-2 promoter severely attenuated luciferase expression (Fig. 3.7, panel A, compare bar 2, 3, 4 with bar 5). This phenomenon needs to be further investigated.

It is possible that the full-length promoter construct may contain a repressor motif that lost its integrity in the individual fragments. Another possibility is that this regulatory region may form a repression loop, which is removed by an interaction with a distal enhancer. For example, in bacteria the *lac* operon is repressed by DNA looping mediated by the lac repressor protein (Becker *et al.*, 2013). Due to the structural properties of the DNA binding and tetramerisation domains of p53, the latter may form DNA loops by forming a tetramer from a pair of dimers bound to remote p53RE (Okorokov *et al.*, 2006). However, this phenomenon needs further exploration.

Unexpectedly, U2OS pLKO p53 cells lacking ectopic p53, showed very similar trends of luciferase activity compared to the cells expressing p53. In fact, the levels of luciferase activity for constructs bearing fragments of miR-16-2 promoter in p53 knockdown cells were slightly higher than those in $p53^{+}$ cells (Fig. 3.7).

This effect cannot be accounted for by high background level of activity for miR-16-2 luciferase constructs since the full-length construct showed very low level of activation in these cells. Neither could it be attributed to the low efficiency of transfection with p53 plasmid, because the results of western blotting analysis of transfected U2OS pLKO p53 cells with plasmid expressing p53, confirmed that the p53 protein was successfully expressed in those cells (Fig. 3.6).

A plausible explanation for this is that endogenous auxiliary transcription factors that mediate transcription of this construct may be repressed by excessive amount of ectopic p53. For example, overexpression of wild-type p53 can repress transcription by hypoxia-inducible factor-1 (HIF-1) transcription factor (Blagosklonny *et al.*, 2001).

Transfection of miR-16-2 reporter constructs into U2OS pLKO p53 cells without p53 yielded approximately the same levels of luciferase activity as miR-26a reporter constructs, except the one containing the full-length miR-26a promoter. Full-length miR-26a promoter displayed 2-fold higher activity than individual miR-26a fragments (Fig. 3.7 compare bar 7, 8 and 9). The mutant miR-26a promoter construct had much lower luciferase activity, indicating that p53REs may be utilised by other transcription factors, for example, E2F1 or NF-Y, which have overlapping consensus sites with p53 (Benatti *et al.*, 2008; Choi & Rho 2002). Therefore this supports a p53-independent regulatory mechanism for miR-26a and miR-16.

The p53 protein is known to undergo regulation in response to genotoxic stress. This is achieved mostly on post-translational level through various post-translational modifications. Therefore, we wanted to examine the effect of post-translational modifications on the ability of p53 to transactivate both miR-16- and miR-26a-containing luciferase reporters (Fig. 3.8). Since the full-length miR-16-2 promoter did not display any appreciable luciferase activity, we decided to focus on individual fragments.

Of all the modifications to p53, N-terminal phosphorylation is the most studied, as well as being the best described, and it has been shown to be key in p53 stabilisation. The

half-life of p53 is augmented approximately 7-fold to 200 minutes following DNA damage, resulting in p53 accumulating in cells (Appella & Anderson 2001). In response to several stresses, including DNA damage, all threonines and serines in the N-terminal of p53 in its first 89 residues are either phosphorylated or dephosphorylated. After DNA damage, p53 phosphorylation takes place through several kinases, including ATM, ATR, CHK1, CHK2, CK1, CK2, ERK, JNK, and p38.

p53 acetylation increases its stability, ability to bind DNA, and its transcriptional activity. p53 was the first non-histone protein to be subject to acetylation and deacetylation, provoked by cellular stress, such as DNA damage. Newer p53 modifications that were discovered include methylation, sumoylation, and neddylation, which all modify lysine residues. It is thought that these lysine residues fine-tune p53 reacting on stress. On DNA damage, methylated p53 is required for its successive acetylation, resulting in p53 protein stabilisation (Ivanov *et al.*, 2007). Methylation of p53 by SET9 at Lys372 in its C-terminus results in p53 stabilisation, retaining it in the nucleus, and leading to its transcriptional activation (Chuikov *et al.*, 2004). Sumoylation induces and represses p53 transcriptional activity. Neddylation occurs on Lys370, Lys372, and Lys373 p53 residues, which also are all ubiquitinated (Xirodimas *et al.*, 2004).

To investigate whether p53 directly binds to its regulatory elements in the promoters of miR-16-2 and miR-26a-1 genes, one of the most common DNA binding mutants of p53 (R175H – arginine residue at position 175 mutated to histidine) was tested using the luciferase assay in U2OS pLKO p53 cells, as well as p53 K4R mutant (Fig. 3.8).

Post-translational modifications which are affected in the p53 K4R mutant are acetylation (K320R/K373R/K373R/K382R), methylation (K372R/K382R), neddylation (K372R/K373R), and ubiquitination (K372R/K373R/K382R) (Maclaine & Hupp 2009). These specific mutations occur in the C-terminal regulatory domain of p53, and could result in decreased p53 stability (due to reduced acetylation and methylation), DNA binding ability (due to reduced acetylation and methylation), transcriptional activity (due to reduced acetylation and methylation) (Ivanov *et al.*, 2007), or protein-protein interactions (due to reduced methylation) (Chuikov *et al.*, 2004). Enhanced p53 transcriptional activity (due to reduced neddylation) (Xirodimas *et al.*, 2004) and p53

stability (due to reduced ubiquitination) could also be a consequence (Waning *et al.*, 2010).

To further explore transcriptional effects of p53 mutants, in addition to p53 R175H and p53 K4R mutants, we employed two other deletion mutants of p53 that lacked either the first amino-terminally situated transactivation domain (p53Δ40), or the whole amino-terminus (p53Δ133). Importantly, these deletion mutants are present in the cells of various forms of breast cancer and therefore have clinical relevance (Soussi 2007). Activation levels of miR-16-2 and miR-26a luciferase reporters in U2OS pLKO p53 cells with ectopic wild-type p53 acted as a control.

Several isoforms of p53 exist, including wild-type p53, p53-β, p53-γ, Δ40p53, Δ40p53-β, Δ40p53-γ, Δ133p53, Δ133p53-β, Δ133p53-γ, and Δp53 (Fig. 1.5). Three mRNA splice variants that can be transcribed from the human *p53* gene are p53, p53i9, and Δ40p53. Δ40p53 with an N-terminal truncation is still able to activate gene expression, because of its partial transactivation domain. Δ40p53 inhibits the transcriptional activity of p53, p53-mediated apoptosis, changes the cellular location of p53, and prevents its MDM2-mediated degradation. Many reports demonstrated the expression of the p53 homologues in several cancer types (Bourdon *et al.*, 2011).

The rationale behind this experiment was that we sought to distinguish the requirements of different domains of p53 for transactivation. To preserve the transcriptional environment of endogenous p53, we used U2OS cells with knocked down expression of p53 (U2OS pLKO p53) (Fig. 3.8).

R175H p53 mutant showed the highest level of transactivation for each miR-16-2 promoter fragment (Fig. 3.8A). Generally, the Δ133 p53 mutant showed lower levels compared to the levels induced by wild-type p53, suggesting that the absence of the first 133 amino acids in p53 could negatively affect the association with endogenous transcription factor(s) that bind to the p53RE in the miR-16-2 promoter. Wild-type p53 was also able to activate transcription of miR-16-2 fragments, albeit to a lesser extent compared to p53 K4R mutant and p53 R175H mutant. None of the p53 proteins were able to transactivate miR-16-1 promoter construct, suggesting that these effects were specific.

Next, we focused on studying the effect of p53 mutants on promoter regions of the miR-26a-1 gene, since it showed transcriptional dependence on p53 in our previous

experiments. Both fragments of miR-26a showed dependency on various p53 tested, and in both cases wild-type p53 showed the weakest transactivation potential. The level of p53-dependent response varied between the mutants.

When cells were transfected with wild-type p53 or p53 mutants, together with miR-26a fragment reporter constructs (Fig. 3.8B), generally Δ40 mutant of p53 showed the highest level of transactivation for each miR-26a promoter fragment (Fig. 3.8B). Transactivation of both miR-26a fragments was carried out most weakly by p53 K4R mutant. It is plausible that because p53 K4R mutations affect acetylation, which is critical for p53 stability, the overall stability of this p53 mutant was lower and thus transactivation was not as efficient as by p53 Δ40 mutant or wild-type p53.

The data raises the possibility that the p53RE situated in the promoter of miR-26a-1 gene may also be dependent on the presence of other p53 family members, such as TP73 and TP63. U2OS cells express all of these three isoforms. The p53-related genes, *p63* and *p73*, were first identified in 1997 (Kaghad *et al.*, 1997; Yang *et al.*, 1998). The two proteins share structural and functional homologies with p53, especially in the DNA binding domain. These proteins share similarities in their domain architecture with p53. These traits enable p63 and p73 to form homo- and hetero-oligomers, bind to p53 DNA-binding sites, resulting in the transactivation of p53-responsive genes, and thus induce cell cycle arrest, senescence, or apoptosis, in response to DNA damage (Fig. 1.4) (De Laurenzi & Melino 2000). Full-length isoforms TAp63 and TAp73 bind to p53REs and induce the expression of p53 target genes through their transactivation domain (Melino *et al.*, 2003; Benard *et al.*, 2003). N-terminally truncated isoforms ΔNp63 and ΔNp73 specifically bind to p53REs and directly activate specific target genes (Dohn *et al.*, 2001; Wu *et al.*, 2003; Liu *et al.*, 2004). On stress, p63 and p73 work with p53 to regulate tumourigenesis (Yang & McKeon 2000).

Alternatively, these mutations confer a dramatic change into the structure of p53 (Freed-Pastor & Prives 2012). Moreover, recent data indicate that this gain-of-function mutant is able to aggregate with other transcriptional factors and thus change the transcription (Gaiddon *et al.*, 2001). If mutants bind a repressor and sequester it from the promoter of miR-26a, then this would explain our results.

Luciferase constructs encompassing the promoters of miR-16-2 and miR-26a genes were transfected into U2OS, which expresses a wild-type copy of the TP53 gene. It is

well established that p53 undergoes various post-transcriptional modifications upon DNA damage that activate its functions. Thus, transfected cells were either treated or not treated with doxorubicin to induce DNA damage for 14 hours (Fig. 3.9 left and right panels, respectively).

Expression levels of luciferase constructs that contained miR-16-2 fragments were significantly higher than the negative control (empty pGL3-Basic vector) or the control luciferase construct containing miR-16-1 promoter, which does not contain p53REs in its sequence (3.9A). Surprisingly, the full-length miR-16-2 promoter failed to activate luciferase expression (Fig. 3.9A). Presumably, the full-length miR-16-2 promoter construct may contain a repressor motif that is broken down in the individual fragments. However, this phenomenon needs further investigation. When the transfected U2OS cells were treated with doxorubicin, luciferase activity of the miR-16-2 fragment 2-containing construct increased 1.5 fold in comparison with that in untreated cells (Fig. 3.9A), indicating that DNA damage positively affected transcription initiation from this fragment. A similar trend was noted with the other two miR-16-2 fragments.

Interestingly, when U2OS cells transfected with luciferase vectors that contained the miR-26a fragments were analysed, the highest luciferase activity was observed in the case of the full-length miR-26a-containing construct (Fig. 3.9B), which is in contrast to the situation with miR-16-2. This difference in activation of full-length miR-26a compared to individual miR-26a fragments was further amplified in cells treated with DNA damage (Fig. 3.9B left and right panels). Taken together, these results propose that the miR-26a promoter responds to DNA damage stress and this transcriptional response is p53-dependent. However, at present, we were not able to pin-point the exact location of p53RE in the promoter of miR-26a-1 gene. It is possible that there is an additional enhancer element, which controls transcription of this gene. Further investigations are required to elucidate this question.

To examine whether endogenous p53 affects transcription of miR-26a by binding to its promoter, ChIP assay using p53-specific antibody was carried out (Fig. 3.10). Compared with earlier methods devised to study transcription factor-DNA interactions, the advantage of the ChIP assay is that the interaction of a transcription factor with its target genes is captured in the native context of chromatin in living cells.

The biogenesis of miRNAs is an intricate procedure (Fig. 1.11). pri-miRNA is cleaved at the hairpin stem by the RNAse III endonuclease Drosha to form a smaller hairpin precursor pre-miRNA, 60-100 base pairs in size (Lee *et al.*, 2003). In humans, this microprocessor complex comprises Drosha associated with DGCR8, a cofactor which is believed to aid Drosha in recognising its substrate (Han *et al.*, 2004). DGCR8 interacts with pri-miRNAs at the base of its stem structure, whilst Drosha provokes its cleavage (Bohnsack *et al.*, 2004).

ChIP assay was carried out to examine whether endogenous p68 and Drosha affects transcription of miR-26a, as p53 has been shown to interact with the Drosha complex and its components. Drosha has already been shown to be recruited to miR-23a-1 promoter (Ballarino *et al.*, 2009), miR-199a promoter, and miR-214 promoter (Newman & Hammond 2010).

Initially, we analysed the distribution of p53 binding sites in the promoter of miR-26a gene, using a bioinformatics approach with the Consite program (Fig. 2.3B).

Our results on luciferase assays indicated that the second fragment of the miR-26a promoter contained the most p53REs (region from +1000bp to +2000bp shown in Fig. 2.3B). To verify these data several sets of primers encompassing putative p53REs both in the promoter and in the downstream region were designed. Endogenous p53 before and after 24 hours treatment with doxorubicin were immunoprecipitated from U2OS cells. Cells with knocked-down expression of p53 (U2OS pLKO p53) were used as negative control (Fig. 3.10).

Our ChIP results suggest that maximal p53 binding activity was observed in the region of 1500-2000bp upstream of the start site. There was very low binding observed downstream of the start site (-3000bp), indicating that most of the p53REs are located in the upstream promoter region.

Since p53 was reported to interact with several RNA processing proteins, including the Drosha complex, we hypothesized that p53 may recruit these enzymes to promoters of certain miRNA genes to facilitate the processing of these miRNAs coupled with transcription. To address this question, we performed ChIP analysis using antibodies against Drosha and p68 helicase, which is known to interact with Drosha (Fig. 3.10B and C).

U2OS cells expressing wild-type p53 and cells with knocked-down expression of p53 (U2OS pLKO p53) were used in this experiment to assess whether recruitment of these RNA processing proteins to miR-26a was p53-dependent. Interestingly, we observed 4-fold increase of Drosha binding to the miR-26a promoter (+1500 region) in U2OS ($p53^+$) cells treated with doxorubicin for 24 hours, compared with Drosha binding in untreated U2OS ($p53^+$) cells, and with U2OS (knockdown p53) cells, where very low binding of Drosha was observed (Fig. 3.10B). Collectively, these results suggest that Drosha is likely recruited to the miR-26a promoter in a p53-dependent manner.

Surprisingly, binding of p68 to the same region of miR-26a promoter although increased after DNA damage, but did not depend on p53, because similar increases in binding were observed both in U2OS ($p53^+$) and U2OS (knockdown p53) cells (Fig. 3.10C).

We decided to correlate spatially the regions of maximal binding for p53, Drosha and p68 to examine their possible co-dependence in DNA binding (Fig. 3.11).

p53 showed the highest occupancy of the miR-26a promoter in the region between +1500bp and +2000bp in U2OS ($p53^+$) cells treated with doxorubicin for 24 hours (Fig. 3.11A).

The binding profile of p68 was different from the p53 one. p68 displayed the highest binding activity in the region of +500bp in U2OS ($p53^+$) cells after 24 hours doxorubicin. Moreover, a lack of p53 in U2OS (knockdown p53) cells did not significantly affect the binding of p68. These results suggest that although p68 is recruited to the promoter region of miR-26a, this likely occurs in a p53-independent, but DNA damage-dependent fashion (Fig. 3.11B).

In contrast to p68, the binding profile of Drosha to miR-26a clearly showed dependence on p53. However, the highest occupancy of Drosha was observed in the region between +500-1500bp, which is more proximal to the start site compared to the peak of p53 binding that maps to the region of +1500-2000bp (Fig. 3.11C). Collectively, our results suggest that Drosha is recruited to the promoter of miR-26a-1 gene in a DNA damage- and p53-dependent manner.

Results of our luciferase reporter assay experiments indicate that p53 likely affected miR-16-2 transcription in a non-direct way. Thus, we employed ChIP assay to test whether p53 binds to the promoter of miR-16-2 gene (Fig. 3.12).

To determine whether endogenous p53 affects transcription of miR-16 by binding to its promoter, ChIP assay using a p53-specific antibody was carried out (Fig. 3.12). Primarily, we analysed the distribution of p53 binding sites in the promoter of miR-16 gene, using a bioinformatics approach with the Consite program (Fig. 2.3A).

U2OS ($p53^{+}$) cells were treated with doxorubicin to induce DNA damage for 0 (control), 3, 6, and 12 hours followed by ChIP assay. The miR-15b/miR-16-2 cluster resides in intron 5 of the SMC4 gene (Ofir *et al.*, 2011). p53 binding in the downstream region (SMC4 promoter) of the miR-15b/miR-16-2 locus, and p53 binding in the downstream region (-700bp) of the miR-15b/miR-16-2 locus was analysed.

Generally, increased DNA damage by doxorubicin increased binding of p53 to the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Increasing DNA damage increases p53 binding at the SMC4 promoter more significantly than at the -700bp region in the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Even in the absence of DNA damage, p53 still bound at its RE in the downstream region (-700bp) of the miR-15b/miR-16-2 locus to the same degree as treatment with 3 hours doxorubicin (Fig. 3.12B). In the absence of DNA damage, p53 bound to a similar degree in both the downstream regions (SMC4 promoter and -700bp) of the miR-15b/miR-16-2 locus (Fig. 3.12A and B). Therefore, p53 binding to miR-16-2 promoter is not DNA damage-dependent, but DNA damage can increase this binding.

Since DNA binding of p53 is affected by post-translational modifications, such as acetylation and methylation, we also tested whether methylated p53 was able to bind the miR-16-2 promoter (Fig. 3.13). Methylation of p53 on K372 by SET9 lysine methyltransferase was shown to enhance its subsequent acetylation (Ivanov *et al.*, 2007). Our ChIP results suggest that indeed p53 bound miR-16-2 promoter (Fig. 3.12 and 3.13). DNA damage enhanced the occupancy of the promoter by p53. Importantly, this binding was specific, because no appreciable binding of p53 to the promoter of miR-16-1 was observed (Fig. 3.13E). In addition, bound p53 was methylated by SET9 upon DNA damage induced by doxorubicin (Fig. 3.13C). Thus, our data suggest that p53 binds the promoter of miR-16-2 in response to DNA damage.

p53-regulated miRNAs and apoptosis

p53 exerts its functions of tumour suppressor as a transcription factor that promotes expression of genes, whose products induce cell cycle arrest and/or apoptosis in response to genotoxic stress.

The effect of p53 on apoptosis was determined in U2OS ($p53^{+}$) and U2OS knockdown p53 (U2OS pLKO p53) cells. Cells were treated with doxorubicin to induce DNA damage for 0 (control) and 14 hours (Fig. 3.14). Cells were analysed by FACS, and U2OS cells were scored for apoptosis based on PI staining. The crucial advantage of using flow cytometry is the one-by-one measuring principle: each and every particle is analysed individually. This allows accurate counting, measurement of cell properties and classification. Rare particles are also detected.

In U2OS cells not treated with doxorubicin, cells arrested in the G1 phase and then stopped in the G2/M phase (Fig. 3.14A). A similar trend was seen in U2OS (knockdown p53) cells not treated with doxorubicin, but slightly more cells arrested in G2/M phase (Fig. 3.14B). In U2OS cells treated with doxorubicin, similar populations of cells arrested in both the G1 phase and in the G2/M phase (Fig. 3.14C). In U2OS (knockdown p53) cells treated with doxorubicin, cells were only arrested in the G2/M phase, with a minimal population in G1 phase (Fig. 3.14D).

Activated p53 functions via the regulation of the transcription of its target genes. These target gene protein products are the last executors to provoke reversible cell cycle arrest, senescence, or apoptosis, all occurrences involved in tumour suppression (Fig. 1.9). When high levels of genotoxic stress persist and cannot be repaired, then the p53-dependent apoptotic or senescence response is followed. The function of p53 in this way helps to protect against neoplasia. Via the extrinsic apoptotic pathway, p53 activates DR5, the death domain-containing receptor for TRAIL, in response to DNA damage (Wu *et al.*, 1997) and, as a result, provokes cell death via caspase 8. Cell cycle arrest permits DNA to be repaired to allow for cell survival (Pellegata *et al.*, 1996). Following DNA damage, p53 halts cell cycle progression at G1/S and G2/M phases (Ceribelli *et al.*, 2006; Kastan & Kuerbitz 1993). Cell cycle arrest genes regulated by p53, whose protein products are involved in G2/M transition following DNA damage, include $p21^{WAF1}$, *GADD45*, and *14-3-3σ* (Luk *et al.*, 2012). It is still debatable whether

p53 is always present at specific promoters, including $p21^{WAF1}$, or if it solely binds on genotoxic stress (Barlev *et al.*, 2001; Espinosa & Emerson 2001).

p53 exerts its functions of tumour suppressor as a transcription factor that promotes expression of genes, whose products induce cell cycle arrest and/or apoptosis in response to genotoxic stress. Our experimental data indicate that miR-16 and miR-26a are regulated by p53 and thus may contribute to p53-dependent physiological response to genotoxic stress.

Using FACs analysis we examined the effect of miR-16 and miR-26a on cell cycle arrest and apoptosis of cells non-treated or treated with DNA damaging doxorubicin (Fig. 3.15).

In the absence of DNA damage, overexpression of miR-26a increased the population of cells in the G1 phase and decreased cells in G2/M phase, compared to control cells transfected with control scrambled oligonucleotide. On the contrary, overexpressed miR-16 decreased the number of G1 cells and increased G2/M cells, compared to control cells (Fig. 3.15A and B).

U2OS cells used for the experiment transiently arrest in the G1 phase and then stop in the G2/M phase. Thus, if miR-16 and miR-26a affect cell cycle progression by slowing cells in the G1 phase, then overexpression of these oligonucleotides in DNA damage-treated cells should reduce the population of G2/M phase cells.

In fact, doxorubicin-treated cells transfected with miR-16 and miR-26a showed decreased G2/M distribution compared to control cells. Accordingly, the number of cells arrested in G1 increased (Fig. 3.15A and B).

In the presence and absence of DNA damage, cells transfected with miR-16 and miR-26a show increased apoptosis compared to control cells, which is more significant in DNA-damaged cells (Fig. 3.15B). In this way, DNA damage sensitises cells to undergo apoptosis. miRNAs of the miR-15/16/195/424/497 family were discovered to sensitise cisplatin-resistant cells to apoptosis by their targeting of CHK1 and WEE1 kinases, which are implicated at G1/S and G2/M cell cycle checkpoints, respectively (Pouliot *et al.*, 2012).

We wanted to determine if miR-16 and miR-26a induction of apoptosis was a p53-dependent effect (Fig. 3.16). As miR-15 is part of a gene cluster with miR-16 (miR-

15a/miR-16-1 cluster or miR-15b/miR-16-2 cluster), we decided to analyse miR-15 as well (Aqeilan *et al.*, 2010).

To this end, U2OS and U2OS pLKO p53 cells were transfected with precursors of miR-15, miR-16, and miR-26a as well as their inhibitors (antagomirs). Following transfection, these cells were either treated or not treated with doxorubicin (Fig. 3.16). Ectopic expression of either precursor of miR-15 or its inhibitor did not significantly affect the levels of DNA damage-induced apoptosis in p53-positive cells. Interestingly, inhibition of miR-16 in p53-positive cells treated with doxorubicin caused a significant increase of apoptosis, whereas overexpression of miR-16 did not have this effect. Overexpression of miR-26a facilitated p53-dependent apoptosis upon DNA damage.

Collectively, these results suggest that miR-26a cooperates with p53 to induce apoptosis, and miR-16 enhances p53-mediated cell cycle arrest in the G1 phase.

PART 3: Targets of miR-16 and miR-26a

miR-16 exerts its main role as a tumour suppressor by down-regulation of BCL2. However, a significant volume of evidence has accumulated in the literature strongly suggesting that miR-16 targets genes involved in cell cycle progression.

The miR-15a/miR-16-1 cluster targets Cyclin D1 and WNT3A, which promote many prostate tumourigenic features, such as survival, proliferation, and invasion (Yue & Tigyi 2010). In non-small cell lung cancer (NSCLC) cells, miR-34a and miR-15a/miR-16-1 work together to induce cell cycle arrest in an RB-dependent manner. This synergistic effect was diminished in cells where Cyclin E1, a known target of miR-15a/miR-16-1, was silenced by means of RNA interference (Bandi & Vassella 2011). miRNAs of the miR-15/16/195/424/497 family sensitise cisplatin-resistant cells to apoptosis by targeting CHK1 and WEE1 (Pouliot *et al.*, 2012).

In human hepatocellular carcinoma cells, overexpression of miR-26a induces G1 phase cell cycle arrest, by down-regulation of Cyclins D2 and E2, and decreasing cells in S stage of the cell cycle (Kota *et al.*, 2009). Overexpressed miR-26a in nasopharyngeal cells represses oncogenic EZH2 expression, resulting in cell cycle and cell growth inhibition, and therefore preventing tumourigenesis. Suppression of miR-26a-mediated attenuation of EZH2 expression by c-MYC plays a critical role in lymphomagenesis (Sander *et al.*, 2008). miR-26a directly down-regulates oncogenic MTDH to provoke apoptosis in breast cancer cells (Zhang *et al.*, 2011). As a consequence, the expression of $p14^{ARF}$ and $p21^{CIP1}$ CDK inhibitors is augmented and the expression of cyclin-dependent kinases CDK4 and CDK6 is suppressed, as is Cyclins D3 and E2, and c-MYC expression (Lu *et al.*, 2011). Other predicted targets of miR-26 are WEE1 and CHK1 protein kinases.

To validate the published targets of miR-16 and miR-26a we employed several approaches, including western blotting after transfection with the corresponding miRNAs and luciferase reporter assay where the luciferase gene is fused to the 3'UTR region of target gene.

miR-16 and miR-26a regulation of CHK1 and WEE1

miRNAs of the miR-15/16/195/424/497 family sensitise cisplatin-resistant cells to apoptosis by targeting CHK1 and WEE1 (Pouliot *et al.*, 2012).

miR-26 also targets WEE1 and CHK1. The checkpoint protein kinases ATR, CHK1 and WEE1 are essential regulators of DNA damage surveillance pathways. Genotoxic stress is recognised by the cell through DNA damage-responsive ATR and ATM kinase. On activation, the kinases transfer the signal to their downstream effectors, CHK1 and CHK2, respectively. WEE1 has a key role in control of the G2/M transition of the cell cycle, by phosphorylation and inactivation of CDC2 (CDK1). WEE1 is overexpressed in several cancers, such as glioblastoma and breast cancer, where p53 functionality is compromised (De Witt Hamer *et al.*, 2011). One could predict that overexpression of miR-26 in these tumours by p53 may lead to mitotic catastrophe and cell death (Fig. 1.15).

WEE1 and CHK1 are already recognised as important targets for cancer therapy. Thus, these miRNAs are of clinical relevance and are documented to play roles in apoptotic response to DNA damage insult by genotoxic drugs. Therefore, we decided to investigate whether miR-16 and miR-26a indeed target these genes in H1299 ($p53^-$) and U2OS ($p53^+$) cells.

First, we tested whether miR-16 is able to affect the expression of CHK1 in H1299 cells (Fig. 3.17A). Since CHK1 is an important element of DNA damage signalling pathway, cells were treated with doxorubicin to induce genotoxic stress (Fig. 3.17A). miR-16 oligonucleotide was transfected in two different concentrations of 3nM and 15nM. To evaluate the specificity of targeting for these miRNAs, miR-16 inhibitor was also tested in concentrations of 10nM and 50nM. Transfection with miR-16 oligonucleotide showed a clear reduction of CHK1 levels compared to control cells. In addition, overexpression of miR-16 inhibitor in concentrations of 50nM resulted in restoration of CHK1 expression. Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.17A).

A bioinformatics approach using software programme Targetscan (www.targetscan.com) has identified WEE1 as a potential target of miR-26a and miR-16. Thus, we sought to verify this prediction experimentally.

First, we tested whether miR-16 and miR-26a affect the expression of WEE1 in H1299 cells (Fig. 3.17B). Similar to the CHK1 western blot (Fig. 3.17A) cells were treated with doxorubicin to induce genotoxic stress (Fig. 3.17B). miR-16 and miR-26a oligonucleotides were also transfected into cells.

miR-16 and miR-26a were shown to clearly reduce WEE1 expression compared to control cells, with this reduction being more distinct for miR-16. Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.17B).

Additionally, we wanted to test whether p53 is able to affect the expression of CHK1 and WEE1. This was carried out in U2OS cells by Western blotting analysis (Fig. 3.18). U2OS ($p53^{+}$) and U2OS (knockdown p53) cells were treated with and without doxorubicin to induce genotoxic stress (Fig. 3.18).

In the presence of p53, there was a clear reduction of CHK1 and WEE1 levels compared to control (knockdown p53) cells, further amplified on DNA damage (Fig. 3.18). In addition, on DNA damage, there was a clear reduction of CHK1 and WEE1 levels compared to control non-treated cells, in knockdown p53 cells (Fig. 3.18). Importantly, the levels of tubulin were not affected by these treatments (Fig. 3.18).

H1299 cells were transfected with miR-16 and miR-26a oligonucleotides and transcriptional levels of WEE1 were evaluated by Q-PCR. Cells were transfected with the corresponding oligonucleotides in two concentrations of 25nM and 75nM. The RNA level of U6 was used for normalisation (Fig. 3.19).

These results indicate that miR-16 and miR-26a did reduce transcription of the WEE1 gene, compared to controls (Fig. 3.19). Relating to a lower concentration (25nM) of the tested miRNAs, miR-16 significantly reduced WEE1 expression, compared to the control, and miR-26a which was linked to a minor WEE1 expression reduction (Fig. 3.19).

With a higher concentration (75nM) of the tested miRNAs, miR-16 also reduced WEE1 levels compared to the control, but not to as great an extent as with a lower concentration of miR-16 (Fig. 3.19). Very high background concentrations of oligonucleotides could have a more significant suppression effect.

One of the most common experimental approaches to test the efficiency of targeting for specific miRNAs is to evaluate stability of the luciferase gene fused to the 3'UTR of the gene of interest that is presumed to be targeted by miRNA.

To employ this approach we have constructed a plasmid that carries the 3'UTR of WEE1 or CHK1 attached to the luciferase gene, whose transcription is driven by a minimal SV40 promoter. In this way, via their 5' seed region, specific miRNAs would

bind their target 3'UTR in this luciferase construct and decrease luminescence emitted by the luciferase gene.

The results of cloning are shown in Fig. 3.20. The full-length 3'UTR of WEE1 or CHK1 of 494 nucleotides were cloned separately into a pGL3-Control luciferase reporter plasmid. The identity of each 3'UTR sequence was verified by restriction digest with XbaI and DNA Sanger sequencing.

The CHK1 and WEE1 3'UTR luciferase constructs were transfected into H1299 cells in the presence or absence of miR-16 and miR-26a oligonucleotides. Scrambled oligonucleotide was used as a negative control (Fig. 3.21).

miR-16 and miR-26a together had a greater effect on reducing WEE1 levels, compared to when miR-16 and miR-26a were transfected into cells separately, and compared to the control (Fig. 3.21). When miR-16 and miR-26a were transfected into cells separately, miR-16 had a greater effect on reducing WEE1 levels compared to miR-26a, which had a minimal effect (Fig. 3.21).

Additionally, miR-16 and miR-26a together had a significant effect on reducing CHK1 levels, compared to the control (Fig. 3.21).

<u>miR-16 and miR-26a regulated Cyclin E and SET9</u>

Cyclins E1 (also known as Cyclin E) drives cell entry into S phase from G1 phase (Geng *et al.*, 2003). Strong Cyclin E expression is often seen in advanced stage tumours, deeply invasive tumours, and tumours with lymph node metastasis. Cyclin E expression correlates with p53 expression. p53 expression is highest in Cyclin E positive tumours compared to in other tumours (Sakaguchi *et al.*, 2000).

To validate Cyclin E as a published target of miR-16, we employed Western blotting analysis after transfection with the corresponding miRNA.

To characterise the effect of miR-16 on expression of Cyclin E in more detail we transfected H1299 cells with two different concentrations of miR-16 oligonucleotide (3nM and 15nM), subjected cells to DNA damage, followed by Western blotting against Cyclin E. Increasing concentrations of miR-16 inhibitors (10nM and 50nM) were also used to show the specificity of this miRNA in respect to Cyclin E (Fig. 3.22).

15nM miR-16 completely eliminated Cyclin E expression (Fig. 3.22). 10nM miR-16 inhibitor restored Cyclin E expression compared to the scrambled control. Overall, our

data confirm that miR-16 targets Cyclin E and mediates down-regulation of its respective mRNA.

We also wanted to determine the effect p53 has on miR-16 regulation of Cyclin E expression (Fig. 3.23). We transfected U2OS ($p53^+$) cells with a mixture of miR-15 and miR-16 oligonucleotides, followed by Western blotting analysis for Cyclin E. In addition, these cells were non-treated or treated with doxorubicin for 24 hours to induce DNA damage (Fig. 3.23).

miR-15/miR-16 effectively decreased Cyclin E expression, compared to cells transfected with scramble control (Fig. 3.23). This effect was specific because the level of Ku70 was not affected by these miRNAs (Fig. 3.23).

SET9 methyltransferase has been shown to methylate histones as well as non-histone proteins including p53. After DNA damage, methylation of p53 by SET9 at Lys372 in its C-terminus leads to p53 stabilisation, containing it in the nucleus, and transcriptional activation (Chuikov *et al.*, 2004).

In U2OS ($p53^+$ and $SET9^+$) cells, p53 levels are higher compared to in knockdown SET9 cells (Fig. 3.24). Additionally, DNA damage clearly increases p53 levels.

One of the targets of miR-16 is Cyclin E (Fig. 3.22 and 3.23), which is positively regulated by E2F1, as part of cell cycle regulation. The latter is regulated by SET9. It has also been shown that E2F1 positively regulates miR-16 expression, which, in turn, down-regulates E2F1 and Cyclin E expression. To add extra layer of complexity, p53 is known to repress E2F1 expression, but on the contrary, E2F1 activates expression of p53. Therefore, these mutual regulatory mechanisms form a complex circuitry that needs to be deciphered.

In normal cells, E2F1 expression levels are regulated during the cell cycle. E2F expression levels are high at the G1/S transition. E2F1 binds and provokes DNA-binding, transactivation and apoptotic functionality of p53. This p53 regulation by E2F1 is dependent on the cell cycle (Fogal *et al.*, 2005). E2F1 has been shown to up-regulate the levels of miR-34a and miR-449a, which in turn, decrease CDK6 levels, demonstrating the role of these miRNAs in a negative feedback system with E2F1. miR-449 is highly expressed in the trachea, testes, and lungs (Lizé *et al.*, 2010). Notably, E2F1 also responds to DNA damage by modulating transcription of its target genes.

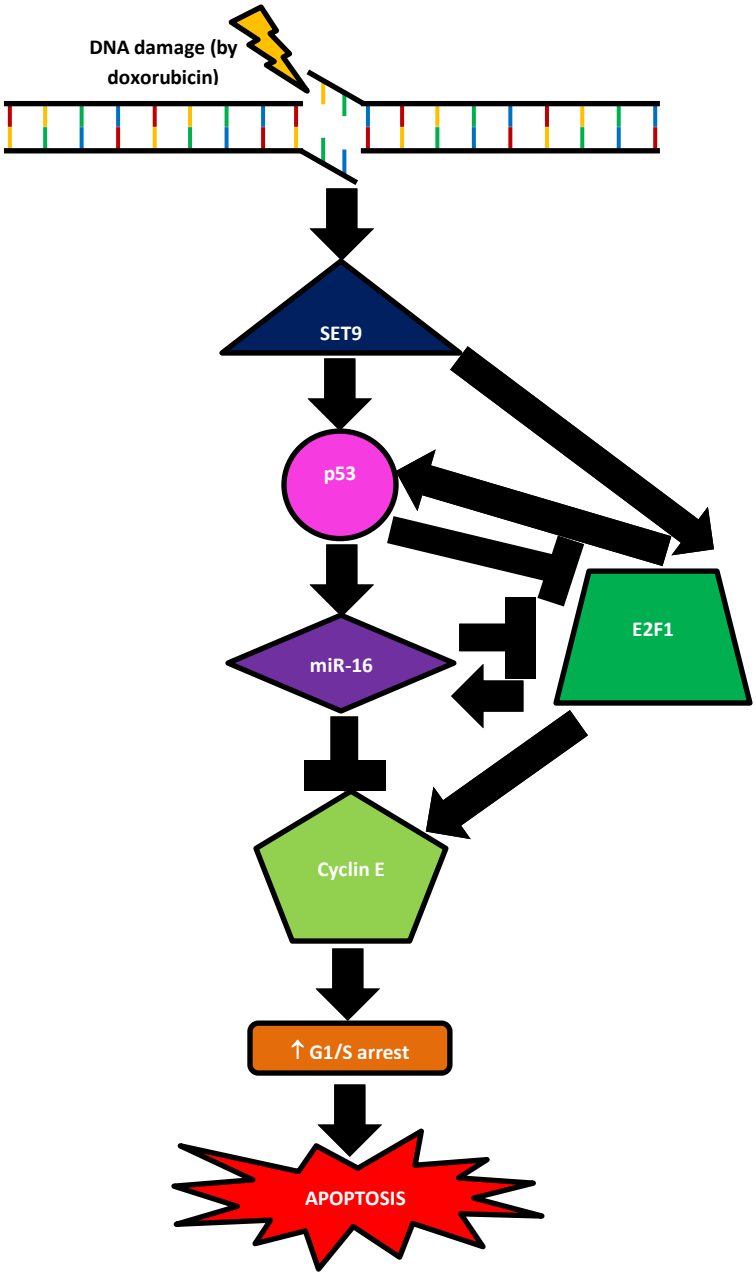

Figure 4.1 Scheme to illustrate the current SET9, p53, E2F1, miR-16, and Cyclin E network

One of the targets of miR-16 is Cyclin E, which is positively regulated by E2F1, as part of cell cycle regulation. The latter is regulated by SET9. It has also been shown that E2F1 positively regulates miR-16 expression, which, in turn, down-regulates E2F1 and Cyclin E expression. To add extra layer of complexity, p53 is known to repress E2F1 expression, but on the contrary, E2F1 activates expression of p53. We wanted to decipher whether, in response to DNA damage, SET9 directly controls Cyclin E expression.

Our results already demonstrate that Cyclin E is regulated by p53-dependent miR-16 (Fig. 3.22 and 3.23). Previous data from our lab suggest that transcriptional activity of p53 is dependent on lysine-specific methyltransferase SET9 (Ivanov *et al.*, 2007). SET9 methylates p53 and renders it transcriptionally active. Methylated p53 is also found in the chromatin-bound fraction of the miR-16 promoter. Therefore, SET9 may be an important indirect regulator of Cyclin E expression.

To test this possibility, we decided to compare the levels of transcriptional activation driven by the Cyclin E promoter as a function of SET9. To achieve this, we used luciferase reporter plasmids that contained within the Cyclin E promoter either wild-type or mutant E2F1REs.

Notably, E2F1 also responds to DNA damage by modulating transcription of its target genes.

The Cyclin E promoter was cloned into a pGL2 luciferase reporter plasmid to study the effects of SET9 on the expression of Cyclin E. The activity of the luciferase Cyclin E promoter construct was analysed in different cell lines.

Importantly, this luciferase assay was performed in cells treated with doxorubicin to induce DNA damage, as it is known from the literature that E2F1 is activated in response to DNA damage (Fig. 3.25A). We also tested the level of activation of Cyclin E reporter in cells without doxorubicin treatment (Fig. 3.25B). E2F1 protein has been shown to be a specific inducer of apoptosis, as well as p53 accumulation, relying on DNA damage. DNA damage results in specific induction of E2F1 accumulation, which is dependent on ATM kinase activity. The specificity of E2F1 induction echoes specificity in E2F1 phosphorylation by ATM and ATR kinase (Lin *et al.*, 2001).

First, we analysed the effect of SET9 on Cyclin E transcription in H1299 ($SET9^{+}$) and (SET9 knockdown) cells. To generate the latter cell line, SET9 expression was stably repressed by specific shRNA. On DNA damage, activation of Cyclin E promoter in H1299 cells expressing SET9 was significantly higher (6-fold) than that in matching SET9 knockdown cells (Fig. 3.25A). In the absence of DNA damage, activation of Cyclin E promoter in H1299 cells expressing SET9 was also higher than that in matching SET9 knockdown cells, but by only 3-fold (Fig. 3.25B).

As a negative control, pGL2 luciferase plasmid without any promoter was used. To validate the specificity of the effects observed, a construct bearing Cyclin E promoter

with mutations in E2F1REs was used. Mutant Cyclin E clearly showed less induction compared to the level of activity of wild-type Cyclin E (Fig. 3.25A and B).

Results of this experiment demonstrate that SET9-dependent methylation is required for activation of Cyclin E even in the absence of DNA damage.

One of the targets of miR-16 is Cyclin E, which is positively regulated by E2F1. The latter is regulated by SET9. It has also been shown that E2F1 positively regulates miR-16 expression, which, in turn, down-regulates E2F1 and Cyclin E expression. To add extra layer of complexity, p53 is known to repress E2F1 expression, but on the contrary, E2F1 activates expression of p53. Therefore, these mutual regulatory mechanisms form a complex circuitry that needs to be deciphered.

To confirm the presence of SET9 in cells, cells treated with doxorubicin were harvested and analysed by Western blotting against SET9 (Fig. 3.24).

As a next step in this direction, we repeated luciferase assay, but this time in U2OS ($p53^+$) cells, to determine the effect of p53 on SET9 regulation of Cyclin E. Again, cells were either treated or non-treated with doxorubicin to induce DNA damage, and hence activation and stabilisation of p53 (Fig. 3.26A and B).

Similar to the situation in H1299 cells (Fig. 3.25A), SET9 was important for activation of Cyclin E in U2OS cells treated with doxorubicin (Fig. 3.26A). However, the difference in the activity between U2OS ($SET9^+$) and U2OS (SET9 knockdown) cells (Fig. 3.26A) was not as dramatic as in the corresponding strains of H1299 cells (Fig. 3.25A) - (1.5-fold versus 7-fold difference in Cyclin E activation, respectively). This may be due to the presence of active p53 in U2OS cells (Fig. 3.26A), which inhibits E2F1 upon DNA damage.

When the luciferase assay was repeated in U2OS cells without DNA damage (Fig. 3.26B), the level of activation of wild-type Cyclin E was lower compared to in the DNA damage-treated cells (Fig. 3.26A). However, the difference in Cyclin E activation between U2OS $SET9^+$ and SET9 knockdown cells was greater in non-treated cells (3-fold) (Fig. 3.26B) compared to in U2OS cells after DNA damage (1.5-fold) (Fig. 3.26A).

Collectively, our data suggest that SET9 is an important factor that regulates transactivation of Cyclin E.

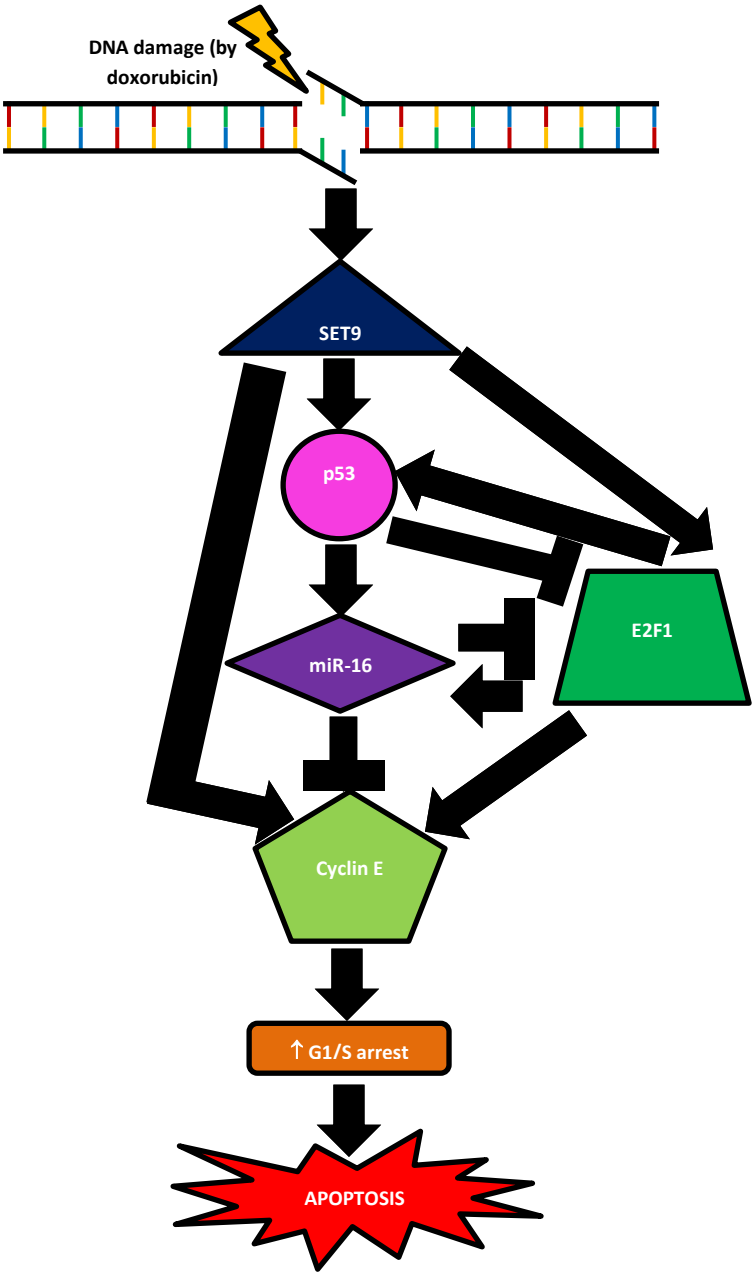

Figure 4.2 SET9 directly controls Cyclin E expression
Our results showed that in response to DNA damage SET9 directly controls Cyclin E expression. Reduced Cyclin E levels results in increased G1/S arrest. This eventually brings about apoptosis.

Overall cascade of events

Our results showed that in response to DNA damage, miR-16 and miR-26a expression levels are controlled by p53-dependent and p53-independent mechanisms, potentially involving other stress-response transcription factors such as E2F1. Our data also confirms that miR-16 and miR-26a directly target Cyclin E, CHK1, and WEE1 for down-regulation. Additionally, SET9 directly controls Cyclin E expression. Reduced CHK1 and WEE1 levels leads to decreased G2/M arrest, and reduced Cyclin E levels results in increased G1/S arrest. This eventually brings about apoptosis.

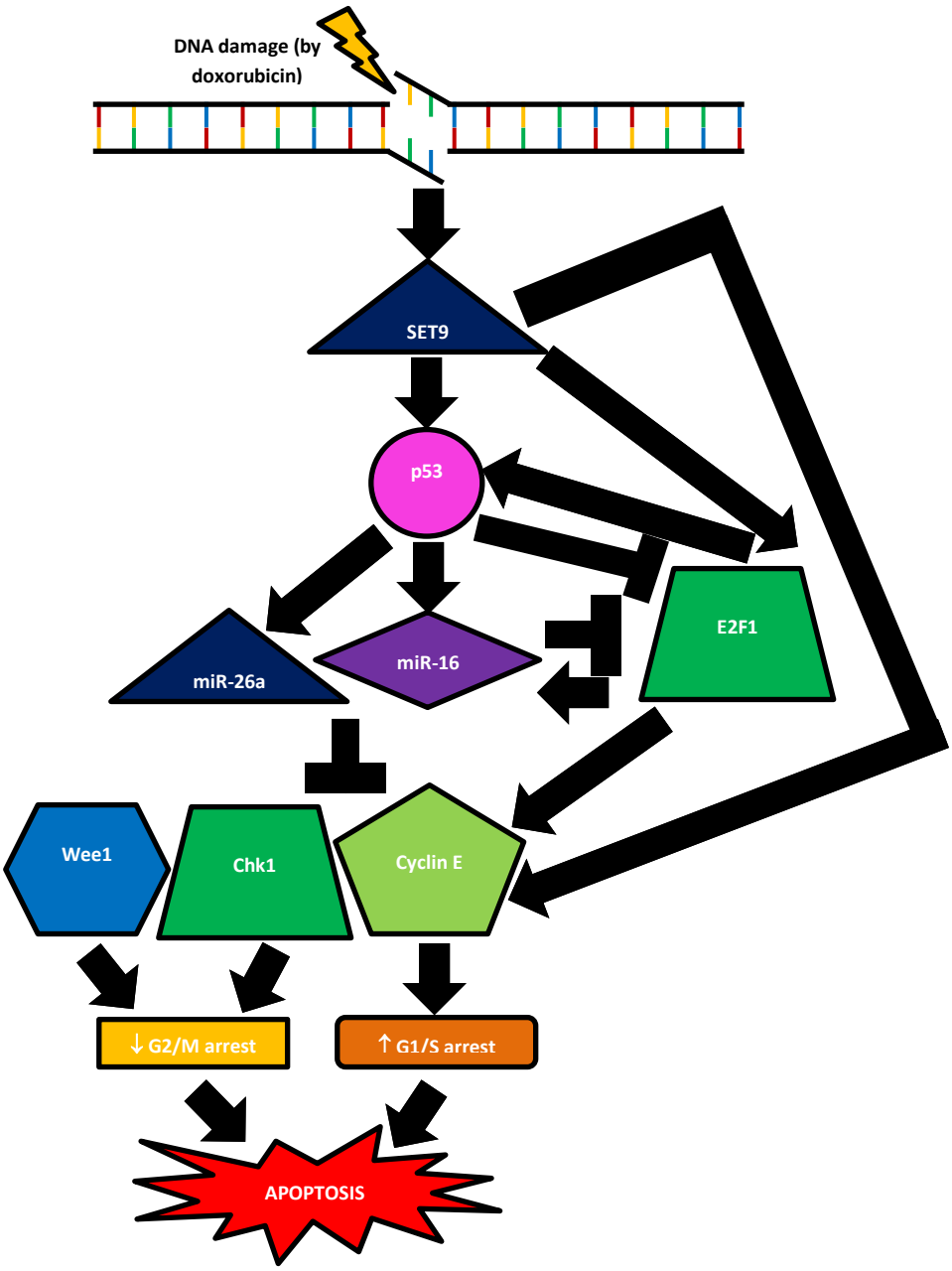

Figure 4.3 Overall cascade of events

Our results showed that in response to DNA damage, miR-16 and miR-26a expression levels are controlled by p53-dependent and p53-independent mechanisms, potentially involving other stress-response transcription factors such as E2F1. Our data also confirms that miR-16 and miR-26a directly target Cyclin E, CHK1, and WEE1 for down-regulation. Additionally, SET9 directly controls Cyclin E expression. Reduced CHK1 and WEE1 levels leads to decreased G2/M arrest, and reduced Cyclin E levels results in increased G1/S arrest. This eventually brings about apoptosis.

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