Regulation of wild-type and mutant p53 activity

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Abstract

The tumour suppressor p53 is extensively regulated by posttranslational modification, including modification by the small Ubiquitin-related modifier SUMO. The data presented here show that MDM2, previously described to promote Ubiquitin, Nedd8 and SUMO-1 modification of p53, can also enhance conjugation of endogenous SUMO-2/3 to p53. SUMOylation activity requires p53-MDM2 binding but does not depend on an intact RING finger. Both $p14^{ARF}$ and L11 can promote SUMO-2/3 conjugation of p53. However, unlike the previously described SUMO-1 conjugation of p53 by an MDM2-$p14^{ARF}$ complex, this activity does not depend on the ability of MDM2 to relocalise to the nucleolus. Strikingly, the SUMO consensus is not conserved in mouse p53, which is therefore not modified by SUMO-2/3. Ultimately, conjugation of SUMO-2/3 to p53 correlates with a reduction of both activation and repression of a subset of p53 target genes and guides the p53 response towards apoptosis rather than cell cycle arrest.

Roughly 30% of all cancers express a p53 protein containing a single amino acid exchange within the DNA binding domain. These mutant p53 proteins not only lose wild-type p53 function, but also gain new oncogenic properties that partially reflect the ability of mutant p53 to interact with and repress the p53-family transcription factors p63 and p73. Like wild-type p53, mutant p53 is also SUMOylated by MDM2, but SUMO-2/3 modification does not affect mutant p53's ability to interact with p63 and p73. p63 and p73 bind to the DNA binding domain of mutant p53, although these interactions do not require an aggregation domain that has been identified around isoleucine 254 within this region of p53. While the DNA binding domain of p73 is necessary for binding to mutant p53, the core domain of p63 is dispensable for the interaction with mutant p53. The p53-binding protein MDM2 binds TAp73α and ΔNp73α, but does not interact with TAp63α or ΔNp63α. Strikingly, addition of MDM2 to mutant p53-p73 complexes leads to the formation of a trimeric complex with MDM2, while addition of MDM2 to mutant p53-p63 complexes releases p63 from the inhibitory mutant p53 interaction.

Altogether, this study reveals ubiquitination-independent mechanisms, by which MDM2 influences both wild-type and mutant p53 activity.

Table of Contents

List of Figures

List of Tables

Accompanying Material

Part of this work has contributed to the publication that is enclosed at the back of the thesis:

Stindt MH, Carter S, Vigneron AM, Ryan KM, Vousden KH.
MDM2 promotes SUMO-2/3 modification of p53 to modulate transcriptional activity.
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Author's Declaration

I declare that I am the sole author of this thesis and the work presented here is entirely my own unless stated otherwise. This thesis does not include work that has been submitted for consideration for any other degree or qualification.

Abbreviations

2-P-Gly	2-Phosphoglycerate
3-P-Gly	3-Phosphoglycerate
6-PGL	6-Pospho-glucono-lactone
aa	Amino acids
Acetyl-CoA	Acetyl-Coenzyme A
AD	Acidic domain
AEC	ankyloblepharon-ectodermal dysplasia-clefting
AMP	Adenosine monophosphate
AMPK	AMP kinase
apaf	Apoptotic protease activating factor
APS	Ammonium persulfate
ARF	Alternative reading frame of $p16^{INK4A}$
ATM	Ataxia telangieactasia mutated
ATP	Adenonine 5'-triphosphate
ATR	Ataxia telangieactasia and Rad3 related
B2M	β_2-microglobulin
BAI	Brain-specific angiogenesis inhibitor
bp	Base pair
BPAG1	Bullous pemphigoid antigen
BRCA	Breast cancer susceptibility protein
CARP	Caspase associated RING proteins
Cdk	Cyclin-dependent kinase
CK2	Casein kinase 2
COP1	Coat protein
CRM1	Chromosome region maintenance 1
CSN	COP9 signalosome
Cy	Cytosol
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DRAM	damage-regulated autophagy modulator
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
DYRK2	Dual-specific tyrosine phosphorylation-regulated kinase 2
E6-AP	E6-associated protein
ECL	Enhance chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
F-1,6-P	Fructose-1,6-bisphospohate
F-2,6-P	Fructose-2,6-bisphosphate
F-6-P	Fructose-6-phospohate
FCS	Foetal calf serum
G-6-P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate-Dehyrodgenase
GA	Glutaraldehyde
GCN5	General control nonderepressible 5
GDF-15	Growth-differentiation factor-15
GFP	Green fluorescent protein
GLS2	Glutaminase 2
GLUT	Glucose transporter
GPX1	Glutathione peroxidase
GSH	reduced glutathione
HA	Haemagglutinin
HAUSP	Herpes-virus associated USP
HB-EGF	Heparin-binding epidermal growth factor
HDAC	Histone deacetylase
HECT	Homologous to E6-AP C-terminus
HGF	Hepatocyte growth factor
HIPK2	Homeodomain interacting protein kinase 2
HRP	Horseradish peroxidase
HSF	Heat shock factor
IHC	Immunohistochemistry
IKKα	Inhibitor of NF-κB kinase
IP	Immunoprecipitation
kD	kilo Dalton
LB	Lysogeny Broth
LDHB	Lactate Dehyrogenase B
MDM2	Mouse Double Minute 2
MDR-1	Multi drug resistance protein 1
MEM	Minimum essential medium
MIC-1	Macrophage inhibitory cytokine
miR	Micro RNA
MMP	Matrix metallo protease
mRNA	Messenger RNA
NAD	Nicotnamide adneine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator
NES	Nuclear export signal

NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NLS	Nuclear localisation signal
NP40	Nonidet P40
NQO1	NADH quinon oxidoreductase 1
NRD1	Nardisylin 1
Nu	Nucleus
PAGE	Poly-acrylamid gel electrophoresis
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate buffered saline
PCNA	Proliferating cellular antigen
PCR	Polymerase chain reaction
PHF6	PHD finger protein 6
PFA	Paraformaldehyde
PFK	Phosphofructokinase
PGM	Phosphoglucomutase
PI	Propidium iodide
PIAS	Protein Inhibitor of Activated STAT
PIG3	p53-inducible gene 3
PIR2	p73-induced RING2 protein
Pirh2	p53-induced RING H2 protein
Plk	Polo-like kinase
PKCζ	Protein kinase
PML	Promyelocytic leukaemia protein
PP	Pentosephosphate
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator
PRMT5	Protein arginine methyltransferase
PUMA	p53 upregulated modulator of apoptosis
Ras	Rat sarcoma protein
Rb	Retinoblastoma protein
RING	Really interesting new gene
RLU	Relative Luciferase units
RNA	Ribonucleic acid
RNF4	RING finger protein 4
ROS	Reactive oxygen species
RPLP0	Ribosomal protein, large, P0
SAM	Sterile α motif
SCO2	Synthesis of Cytochrome c oxidase
SDS	Sodium dodecyl sulfate
SENP	Sentrin-specific protease
SIM	SUMO interaction motif
Sip1	Smad-interacting protein 1

SREBP	Sterol regulatory Element binding protein
SUMO	Small ubiquitin-related modifier
SV	Simian virus
TAD	Transcription activation domain
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TCA	Tricarboxylic acid
TEAB	Triethyl ammonium bicarbonate buffer
TEMED	Tetramethylethylenediamine
TET	Tetramerisation domain
TID	Transcription inhibitory domain
TIGAR	Tp53 induced glycolysis and apoptosis regulator
TNF	Tumour necrosis factor
TopBP1	Topoisomerase βII binding protein
Tris	Tris(hydroxymethyl)aminomethane
UHFR2	Ubiquitin-like with PhD and RING finger
Ubc9	Ubiquitin-conjugating enzyme 9
UBL	Ubiquitin-like modifier
USP	Ubiquitin-specific protease
VHL	von Hippel-Lindau
wt	wild-type
WWP1	WW-domain containing protein
YFP	Yellow fluorescent protein
Zeb1	Zinc finger E-box binding homeobox 1

1 Introduction

1.1 Cancer: when cells grow out of control.

In a healthy individual, most cells are spatially confined to specific tissues and cell proliferation is strictly controlled. The term cancer describes a group of illnesses, where cells have begun to grow uncontrolled and invade tissue boundaries to form malignant tumours[1].

The degree of abnormal cellular behaviour can be classified into different severity grades. Hyperplasia describes the accumulation of excessively growing cells forming a histologically normal tissue organisation, which is considered a benign tumour. Dysplastic cells not only over-proliferate, but also adopt abnormal shape and tissue organisation, yet respect tissue boundaries. Neoplasms show a chaotic histology and penetrate surrounding tissue. Such malignant tumours, which can metastasise to distant organs, are commonly referred to as cancer.

Healthy cells can start to proliferate unconstrained and form tumours when incorporation of errors into the DNA leads to the alteration of genes that normally control important cell autonomous mechanisms such as DNA repair, cell division and cell movement. In order for a normal cell to be transformed to a tumour cell, mitogenic genes become activated or oncogenes are introduced via viral infections while surveillance and repair mechanisms are silenced.

Altogether, the cell physiology of tumour cells differs from that of normal cells in eight key aspects, the so-called hallmarks of cancer[2,3]:

First, normal cells only proliferate if stimulated by pro-growth factors, but tumour cells have acquired independence of external mitogenic signals. Many tumour cells display a different array of surface receptors, produce their own growth-factors or stimulate surrounding fibroblasts to secrete them[4]. Furthermore, signals from external growth factor receptors can be uncoupled if mitogenic pathways become constantly activated as in the case of Ras mutants constitutively driving signalling through the MAP-kinase pathway[5].

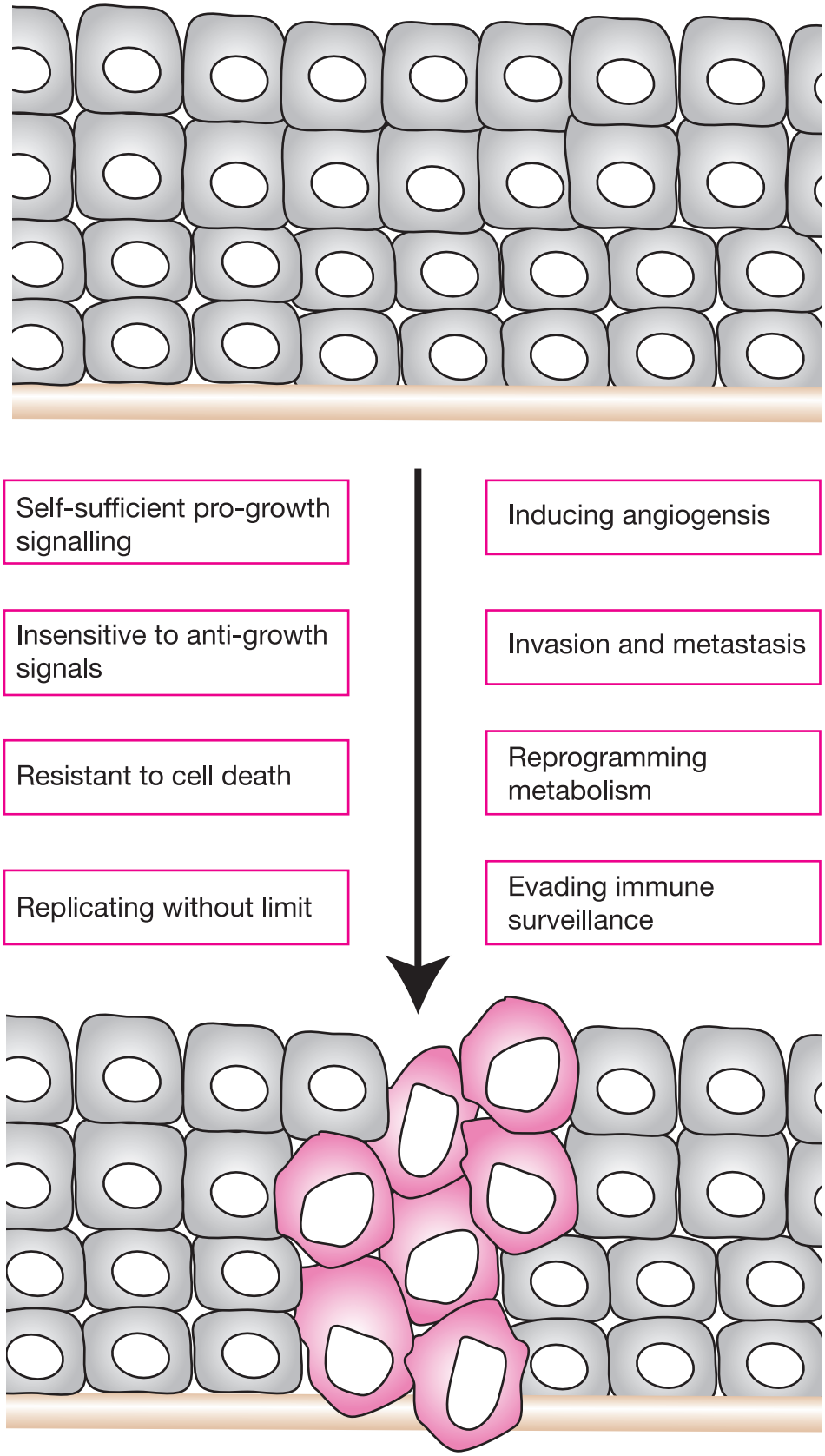

Figure 1-1: Eight Hallmarks of Cancer
Normal cells acquire self-sufficiency in pro-growth signalling, insensitivity to anti-growth and cell death signals, capabilities to replicate without limit, induce angiogenesis and invasion, reprogramme their metabolism and evade immune surveillance along the route of malignant transformation.

Second, tumour cells are insensitive to anti-growth signals that prevent cell cycle progression at the S-phase entry. Many exogenous anti-growth signals control the phosphorylation status of the Retinoblastoma protein (Rb) that blocks S-phase entry in its hypo-phosphorylated stage. It is therefore not surprising that tumour cells frequently harbour mutations in either Rb itself or in its phosphorylation pathway[6].

Normal cells are subject to multiple mechanisms of cell death including the intrinsic and extrinsic apoptosis pathways, necrosis and autophagy. The intrinsic apoptosis pathway can be triggered once internal faults such as DNA damage have been observed. Apoptosis thereby provides an effective barrier to cancer development. However, tumour cells have evaded this control mechanism enforced by tumour suppressors. For example, in order to avoid activation of intrinsic apoptosis, which is controlled by a balance or pro- and anti-apoptotic Bcl-2 family proteins, many cancers show increased levels of the anti-apoptotic Bcl-2[7].

Tumours can exceed the limited number of replications constraining healthy cell proliferation. Normal cells can divide about 70 times[8], but cancer cells divide much more often. As part of ageing, the telomere ends of chromosomes become shorter with every DNA replication, however many transformed cells activate the enzyme telomerase to actively rebuild the telomere ends[9].

Tumour cells, just like untransformed cells, need to be supplied with oxygen and nutrients by blood vessels. Usually, cells in a tissue are within a 100 µm diameter of a blood vessel. In order to stimulate formation of new blood vessels, tumour cells express high levels of pro-angiogenic factors, for example the vascular endothelial growth factor VEGF[10]. A local inflammatory response can furthermore promote angiogenesis[11].

Most cancer deaths are not caused by the primary tumour, but by metastasis to other organs[12]. During embryonic development, embryonic cells move around in order to form tissue layers and organs, but in an adult organism most non-hematopoietic cells are static in tissues and loss of cell-matrix interaction leads to a programmed form of cell death called anoikis[13]. However, tumour cells survive losing cell-cell and cell-matrix junctions without undergoing anoikis[14]. By altering the display

of cadherins and integrins on their cell surface, tumour cells convert to a mesenchymal morphology and become more motile[15]. Tumours do not only contain neoplastic cells and blood vessels, but also connective tissue. The stromal cells can be stimulated to express matrix-degrading enzymes that loosen up the surrounding fibres, facilitating metastasis of the neoplastic cells[16].

Furthermore, transformation coincides with reprogramming of the cell metabolism. Tumour cells produce most energy via glycolysis rather than mitochondrial oxidative phosphorylation, a phenomenon that was termed the "Warburg effect"[17]. Switching to glycolysis as major supply of ATP is possibly advantageous for tumour cells since many glycolysis intermediates can be used for anabolic pathways, for example synthesis of nucleotides and amino acids, which are required to build up a new cell[18]. Metabolic reprogramming also affects the level of reactive oxygen species[19], moderately high levels of which can drive cancer development[20]. The increased glucose uptake of tumour cells can be a useful tool for imaging tumours by positron emission tomography (PET) using radiolabelled glucose analogues[21].

Finally, tumour cells have escaped the surveillance of the immune system that should attack abnormal cells. Immuno-compromised mice develop more carcinogen-induced tumours than immuno-competent mice[22], suggesting that the immune system poses another barrier to tumour development in healthy organisms. Furthermore, the tumours formed in immuno-compromised mice fail to establish tumours when injected into immuno-competent animals[22].

Most of these cellular alterations are due to changes in the genetic information. DNA errors occur frequently during DNA replication and cell division, but tight control systems normally either trigger repair mechanisms or eliminate damaged cells in order to prevent faulty cells from multiplying. However, some mutations can escape the control system and if both proto-oncogenes are activated and tumour suppressor genes silenced, tumours can develop. Many tumours show not only single amino acid exchanges but also amplifications and deletions of entire chromosome pieces due to genomic instability after control checkpoints have been inactivated. Mutagens such as ultraviolet light, ionising radiation and tobacco smoke increase the rate of DNA errors and thereby raise the statistical chance of cell

transformation. Furthermore, the chromatin and the histones, which serve as scaffold structure, are subject to many modifications such as acetylation and methylation, specific patterns of which influence the accessibility of promoters and the rate at which genes are transcribed. Changes in these epigenetic marks are also frequently found in tumours and contribute to the genetic reprogramming[23].

1.2 The tumour suppressor p53

The p53 tumour suppressor is a key player in the cellular control system. If cells contain overactive oncogenes or DNA damage, p53 becomes activated and contributes to the repair of DNA damage and survival under low levels of stress and eliminates or terminally arrests damaged cells through the induction of programmes such as apoptosis and senescence under acute stress[24]. Through this genome-guarding function, p53 prevents the accumulation of malignant cells - demonstrated by the development of spontaneous sarcomas and lymphomas in p53-knockout mice[25]. Almost all tumour cells have escaped the control by p53 in some way, either by disruption upstream and downstream of p53, or by mutating p53 itself (in roughly half of all tumours)[26].

1.2.1 p53: guarding the genome

p53 is a member of the p53-family of transcription factors together with p63 and p73, which are discussed later (see section 1.5). p53 regulates the expression of many genes[27], however transcription-independent roles of p53 have also become apparent[28]. In a simplified model, transient or mild stress predominantly leads to activation of cell cycle arrest via activation of p53-target genes like the Cyclin-dependent kinase inhibitor p21 (also called CIP1 and WAF1)[29,30], whereas sustained or severe stress leads to apoptosis through the induction of p53-target-genes such as the BH3-domain proteins Bax[31] and PUMA[32]. A number of other roles for p53 are emerging, including regulation of angiogenesis and metabolism, contributing to a complex network of tumour-prevention. Strikingly, a p53 mutant, deficient in regulating both apoptosis and cell cycle arrest but maintaining the metabolic activities was recently shown to be sufficient to suppress tumour development[33].

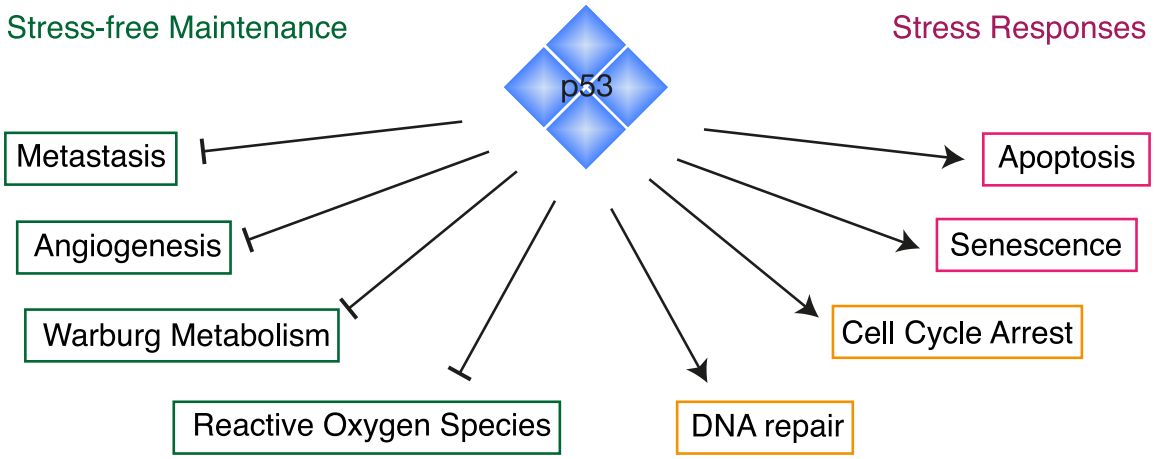

Figure 1-2: p53 responses depend on stress levels.
p53 counteracts tumour development with a range of preventative measures under stress-free circumstances (green), DNA repair and cell cycle arrest under low to medium stress levels (yellow) and induction of apoptosis or senescence under high stress levels (red).

1.2.2 p53 induces cell cycle arrest and DNA repair under low levels of stress.

If problems in the cells are at a resolvable level, p53 will not eliminate the cell but rather allow for repair of the damage. The transcription factor can induce and repress an array of genes leading to temporary arrests at the G_1 or G_2 cell cycle checkpoints in case nucleotide levels are low[34], the DNA template replicate contains errors or chromosomes are damaged. These temporary arrests allow for replenishment of nucleotide pools or completion of DNA repair and prevent multiplication of damaged cells.

The most prominent p53-target gene involved in the G_1-arrest is the Cyclin-dependent kinase (Cdk) inhibitor p21[29,30] that inhibits G_1/S specific kinases Cdk2, 3, 4 and 6[35], thereby preventing Retinoblastoma protein (Rb) hyper-phosphorylation and cell cycle progression into S-phase.

Furthermore, p53 induces two Cdk-inhibitors, which preferentially target the G_2/M-specific Cdk1/Cyclin B complex, leading to an arrest in G_2. While the 14-3-3σ protein sequesters the Cyclin-Cdk complex in the cytoplasm[36,37], Gadd45 is thought to dissociate Cdk1 (also called Cdc2) from its binding partner Cyclin B_1[38], with both mechanisms preventing progression into mitosis. In addition to transcriptionally activating Cdk-inhibitors, p53 transcriptionally represses Cyclin B_1[39], Cyclin B_2[40],

Cyclin A_2[41] and Cdk1[42], also contributing to the G_2-arrest. Moreover, p53 represses Topoisomerase II, which is not part of the Cyclin-Cdk complexes, but is crucial for chromosome segregation, and progression into mitosis[43].

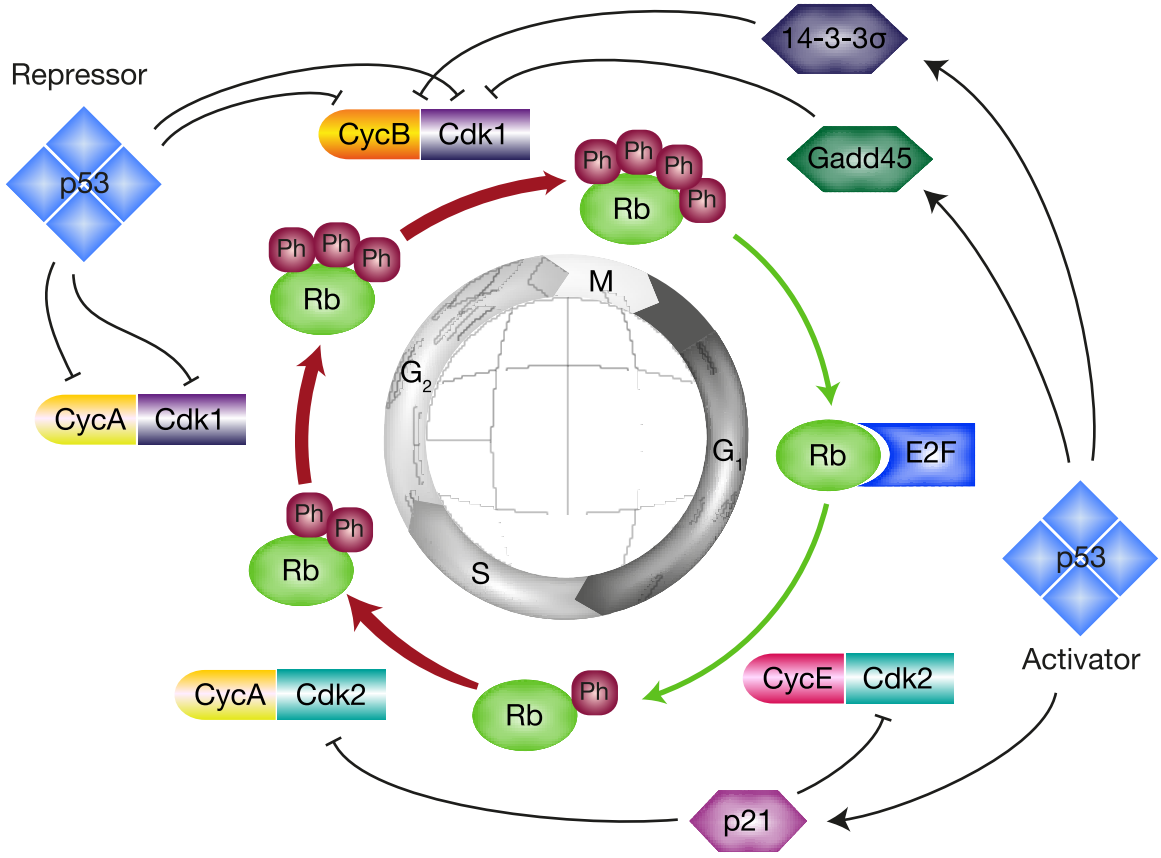

Figure 1-3: p53 activates cell cycle arrest.
p53's activities as transcriptional activator and repressor contribute to cell cycle arrest at G_1 and G_2 by preventing S-phase entry via the Cdk-inhibitor p21 and preventing G_2 exit via 14-3-3σ , Gadd45 Cdk1, Cyclin A and Cyclin B. Rb is unphosphorylated (green arrows) during G_1 and hyper-phosphorylated (red arrows) during S, G_2 and M-phase.

The p53 target Gadd45 not only dissociates the Cdk1-Cyclin B_1 complex but simultaneously activates DNA excision repair by binding to the Proliferating cellular antigen (PCNA)[44]. PCNA is a co-factor for DNA-polymerase δ, which is important for the re-synthesis of excised damaged DNA. Another p53 target gene also links cell cycle arrest to DNA repair: the Ribonucleotide reductase p53R2 is activated by p53 and produces nucleotides required for DNA repair[45]. p53 furthermore induces post-replicative mismatch repair by inducing the mismatch repair genes PMS2 and MLH1[46], which together form the endonuclease MutLα complex, introducing DNA strand breaks next to mismatched DNA in order to provide access for exonucleases

to remove the errors. Strikingly, even p53 itself was found to possess some 3' to 5' exonuclease activity[47].

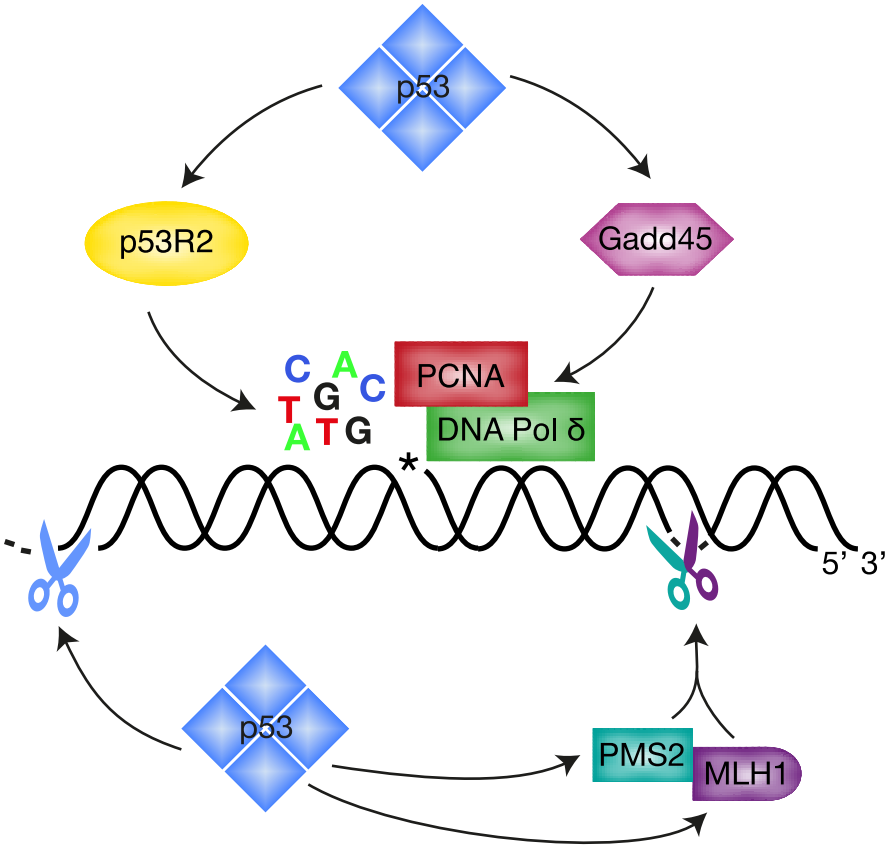

Figure 1-4: p53 activates DNA repair.
p53 induces DNA excision repair via PCNA and DNA Polymerase ∂ and contributes to nucleotide synthesis via p53R2. The p53 targets PMS2 and MLH1 form an endonuclease complex introducing DNA strand breaks at mismatch sites. p53 itself possesses some 3' to 5' exonuclease activity.

1.2.3 p53 induces apoptosis or pre-mature senescence under high levels of stress

p53 is a key regulator for eliminating cells on their road to transformation in response to oncogene activation and DNA damage, initiating cell death programmes, most famously apoptosis. Two pathways can initiate the apoptotic response: the extrinsic pathway, initiated by death factors binding to surface receptors such as Fas and the intrinsic pathway triggered by DNA damage or oncogene activation. Both apoptosis pathways converge in the activation of effector Caspases 3, 6 and 7, which cleave key cellular components such as lamins and actin, leading to the distinct apoptotic

phenotype of a condensed nucleus and blebbed membrane and finally to the formation of apoptotic bodies[48].

The extrinsic apoptosis pathway is initiated by death factors binding to death receptors on the cell surface, leading to activation of the initiator capsases 8 and 10, which then initatie the effector caspases. p53 can influence the extrinsic apoptosis pathway by up-regulating the expression of the Tumour necrosis factor (TNF) receptors Fas[49] and Killer/DR5[50].

The intrinsic apoptotic pathway is initiated by p53 when the state of a cell is beyond repair. p53 induces transcription of the pro-apoptotic Bcl-2 family member Bax[31], which locates to the outer mitochondrial membrane. Here Bax counteracts the anti-apoptotic Bcl-2 family members and triggers the collapse of the mitochondrial membrane potential and release of Cytochrome c into the cytoplasm. Then, Cytochrome c forms the Apoptosome together with Apaf1, inducing the initiator caspase 9, which cleaves and activates the effector caspases.

p53 not only induces the Bcl-2 family member Bax, but also the related BH3-only proteins PUMA[32] and Noxa[51], which contribute to the Cytochrome c release by binding anti-apoptotic Bcl-2 and activating Bax and Bak[52-54]. Moreover, p53 prevents the transcriptional activation of the anti-apoptotic Bcl-2 by inhibiting its transcription factor Brn3a[55].

Many more of p53's transcriptional targets, which do not contain any BH3 domain, contribute to triggering the apoptotic response. The p53-induced protein with death domain (PIDD)[56] plays a role in activating Caspases 2, 3 and 7[57]. Induction of the p53 target PERP stimulates apoptosis[58], while the role it plays in its location at the plasma-membrane remains unknown. A recently identified p53 target gene is the calcium-binding protein PDCD6 (also called ALG2), which promotes Cytochrome c release and accumulates in the nucleus during apoptosis[59]. Interestingly, this protein had previously been described to have a role in the cytoplasm during Fas-induced apoptosis[60].

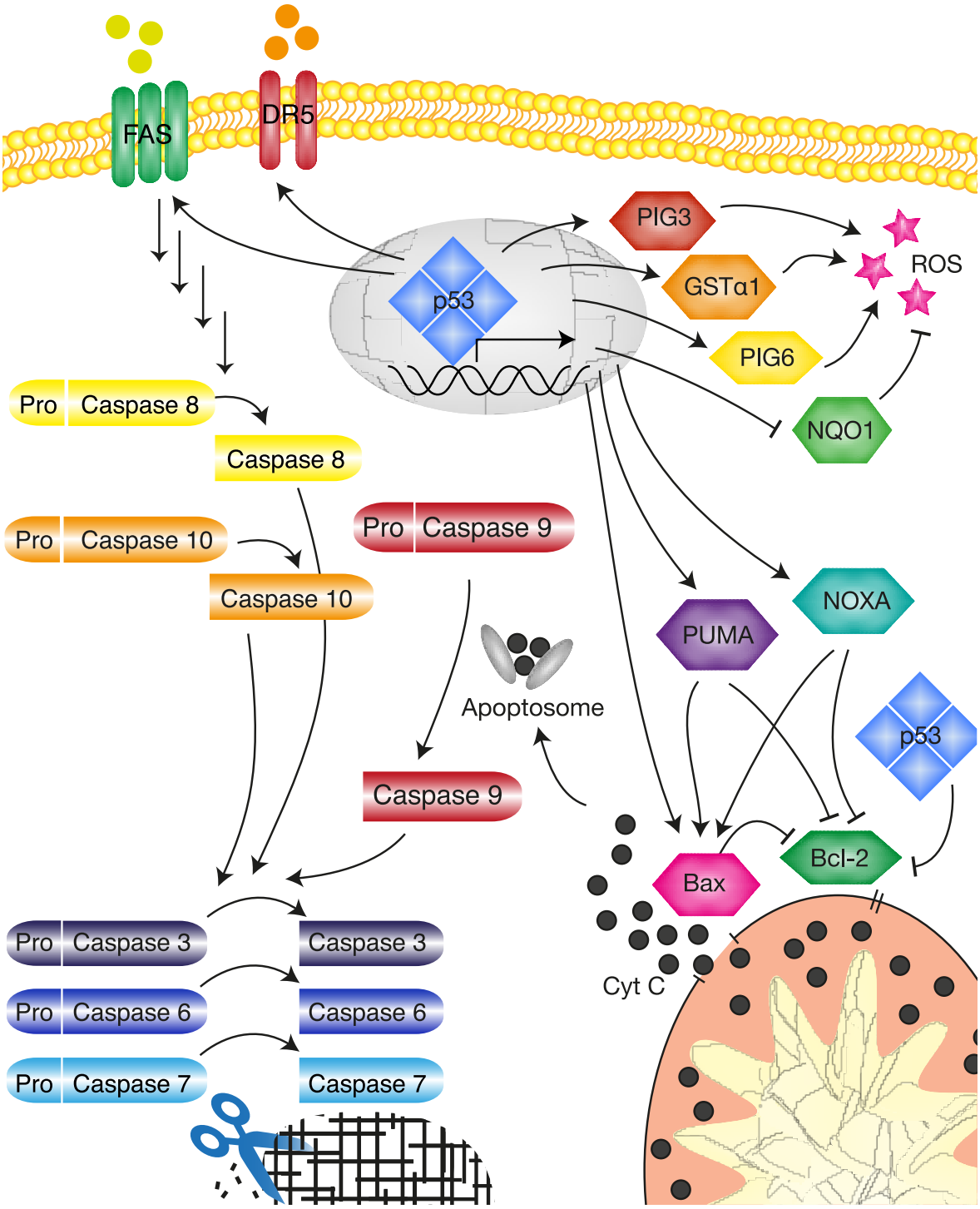

Figure 1-5: p53 activates the extrinsic and the intrinsic apoptotic pathways.
p53 induces the extrinsic (left side) and intrinsic (right side) apoptosis pathways, converging in the activation of effector caspases 3, 6 and 7, which cleave many cellular proteins, for example lamins, that provide the structure for the nucleus. Many p53 target genes furthermore contribute to apoptosis by producing reactive oxygen species (ROS).

Many reports suggest that reactive oxygen species play a vital role in the p53-induced apoptosis pathway[61,62]. In line with this observation, it was found that the p53 inducible gene 3 (PIG3) encodes an NADPH-dependent reductase[63], which generates reactive oxygen species, critical for the oxidation of the mitochondrial components as part of the disassembly of the apoptotic cell[64]. The p53-inducible

protein 6 (PIG6) encodes a proline oxidase, which also generates pro-apoptotic reactive oxygen species[65]. Finally, as part of the apoptotic response, p53 represses Nrf2-induced anti-oxidant genes such as the x-CT subunit of the Cysteine/Glutamate transporter, the Glutathione-S-transferase (GST-α1) and the NADPH Quinone oxidoreductase (NQO1), which is responsible for 2-electron reduction of Quinones[66].

Moreover, p53 contributes to the intrinsic apoptosis pathway directly at the mitochondria and in the cytoplasm independent of its transcriptional activity[28]. It was reported that p53 itself can translocate to the outer mitochondrial membrane and counteract the anti-apoptotic Bcl-2 family members Bcl-x_L and Bcl-2 there[67]. Furthermore, p53 can bind and activate the pro-apoptotic proteins Bak in the mitochondria[68] and Bax in the cytoplasm[69].

More recently, another form of cell death, called autophagy (self-eating) was also linked to the activity of p53. The transcription factor induces a lysosomal protein termed damage-regulated autophagy modulator (DRAM) that induces macro-autophagy[70]. While DRAM-induced autophagy was reported to contribute to p53's apoptotic response[71], autophagy can also promote cellular survival, for example by recycling non-essential cell components under conditions of starvation. Strikingly, p53-induced autophagy can also contribute to survival, as shown in Myc-driven lymphomas, where blocking p53-induced autophagy led to tumour regression[72,73]. Complicating the picture even further, p53 was shown to inhibit autophagy under non-stressed conditions[74], possibly via a cytoplasmic p53 protein pool[75]. Furthermore, p53 was reported to trigger cell death by necrosis when activated by oxidative stress. p53 was found associated with the Cyclophilin D complex, leading to opening of the mitochondrial transition pore and ultimately causing cell swelling and rupture[76].

Adding another layer of complexity, p53 not only transcriptionally regulates genes that are then translated into proteins, but also induces a number of micro-RNAs, such as microRNA34α, which regulates the expression of many anti-apoptotic genes by initiating degradation of their mRNA transcripts and contributes to the initiation of apoptosis[77]. One of the miR-34α regulated genes is the anti-apoptotic Bcl-2[78], which p53 also counteracts via multiple other transcription-dependent and independent

ways, either directly or via its target genes. The importance of miR-34α was recently demonstrated in a lung cancer model, where miR-34α treatment prevented tumour progression[79].

As well as physically eliminating damaged cells by initiating cell death, p53 can also trigger premature senescence, an irreversible cell cycle arrest that prevents damaged cells from multiplying. Two pathways converge to induce the permanent G_1 arrest after DNA damage, oncogene activation or telomere erosion: $p16^{INK4A}$ and the p53-target p21 inhibit Cyclin-dependent kinases to prevent hyper-phosphorylation of Rb, thereby inactivating the E2F transcription factors and cell cycle progression[80]. In contrast to transiently arrested cells, senescent cells undergo morphological changes, adopting a flattened cell shape and altering their chromatin with the appearance of senescence-associated heterochromatin foci[81].

1.2.4 p53 prevents angiogenesis, metastasis and Warburg metabolism.

p53's multiple mechanisms to prevent transformation of healthy cells do not end here. p53 is involved in preventing the acquisition of almost every single hallmark of cancer, posing a great risk for any cell with inactivated p53 and explaining why all cancer cells have lost p53 activity in some way.

Tumours need to be supplied with nutrients and oxygen via blood vessels, like normal cells and p53 contributes to suppressing the formation of new blood vessels by inducing anti-angiogenic factors. p53 promotes the expression of the matrix glycoprotein Thrombospondin 1[82] and a related brain-specific protein with Thrombospondin repeats, the angiogenesis inhibitor BAI-1[83], both of which inhibit neovascularisation[84]. Additionally, p53 induces a Collagen-4 prolyl hydroxylase, which cleaves Collagen and leads to the release of anti-angiogenic Collagen 4 and 18 fragments, inhibiting endothelial cell growth[85]. Moreover, p53 disrupts the signalling from areas of hypoxia to induce pro-angiogenic factors by suppressing expression of Hypoxia-inducible factor (HIF-1β) via induction of microRNA miR-107[86].

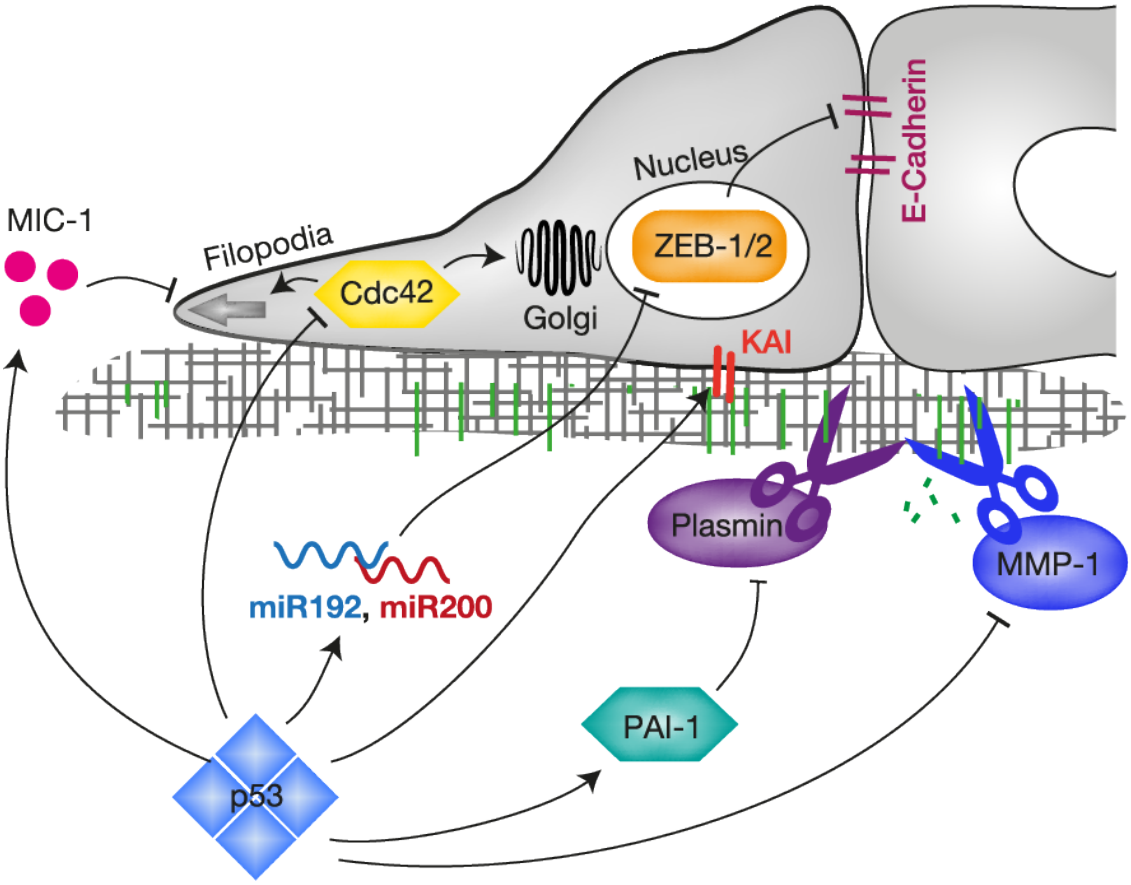

Figure 1-6: p53 inhibits the invasive phenotype.
p53 prevents epithelial-mesenchymal transition by repressing the ZEB-1/2 transcription factors, represses filopodia formation and cell polarisation (orientation of Golgi) via Cdc42 and prevents loosening of the extracellular matrix and collagen by repressing Plasmin and MMP-1. Furthermore the inhibitory cytokine MIC-1 prevents cell movement.

Overgrowing cells become neoplastic when they start to invade the surrounding tissue boundaries. p53 represses this invasive phenotype by a number of mechanisms: First, p53 prevents cells from moving by increasing the expression of KAI, a cell surface glycoprotein which contributes to cell-cell interaction and interaction of cells with the extracellular matrix[87]. Secondly, p53 inhibits the formation of filopodia induced by Cdc42 GTPase[88], which are a critical part of the invasive cell morphology. Cdc42 is also required for cell polarity[89] and p53's inhibitory effect prevents Golgi orientation in direction of movement, thereby perturbing directed cell migration.

Thirdly, p53 induces expression of the secreted TGFβ-family member Macrophage inhibitory cytokine MIC-1 (also known as growth differentiation factor GDF-15), which was shown to inhibit cell movement through transwell assays[90]. Furthermore, p53 reduces the expression of ZEB-1 (also known as δEF1) and ZEB-2 (also known as

Sip1) via up-regulation of the microRNAs miR-192 and miR-200[91,92]. The ZEB transcription factors have a crucial role in epithelial to mesenchymal transition, by suppressing E-Cadherin transcription[93,94]. Finally, p53 imposes a barrier to matrix remodelling by inducing the expression of plasminogen activator inhibitor PAI-1, which inhibits the activation of plasminogen and thereby prevents the proteolytic degradation of the extra-cellular matrix by Plasmin[95]. Additionally, p53 represses the expression of Matrix metalloproteinase 1 (MMP-1) that catalyses the break-down Collagen I, II and III[96], loosening the tissue and creating space for invading cells.

One of the recent additions to the hallmarks of cancer is the observation that tumour cells alter their metabolism to mainly rely on glycolysis and less on oxidative phosphorylation. It has become apparent that p53 antagonises these metabolic changes, the so-called Warburg-effect, via two mechanisms: On the one hand it stimulates mitochondrial respiration by activating expression of the Synthesis of Cytochrome C oxidase 2 (SCO2)[97]. This is a key regulator of the Cytochrome c oxidase complex, the last enzyme in the mitochondrial electron transfer chain, catalysing the oxygen-consuming step of the mitochondrial respiration. On the other hand it decreases glucose uptake by repressing the expression of glucose transporters GLUT1 and GLUT4[98] and down-regulating the GLUT3 glucose transporter via IKKα and NF-κB[99].

p53-induced ubiquitination and degradation of Phosphoglycerate mutase (PGM), which catalyses the eighth step of glycolysis, poses another barrier to completing glycolysis[100]. Moreover, p53 slows down the glycolytic flux by up-regulating the Fructose-2,6-bisphosphatase Tp53 induced glycolysis and apoptosis regulator (TIGAR)[101], lowering the levels of the fructose-2,6-bisphosphate that drives progression through glycolysis by activating the rate-limiting Phosphofructokinase.

Blocking progression through glycolysis drives diversion of the early intermediates through the pentose phosphate pathway. The NADPH produced in this enzymatic cascade is required to regenerate the reduced state of glutathione, once it has become oxidised by scavenging reactive oxygen species. Thereby, TIGAR not only suppresses the metabolic flow through glycolysis, but also increases the synthesis of antioxidant Glutathione, keeping intracellular reactive oxygen species low[101].

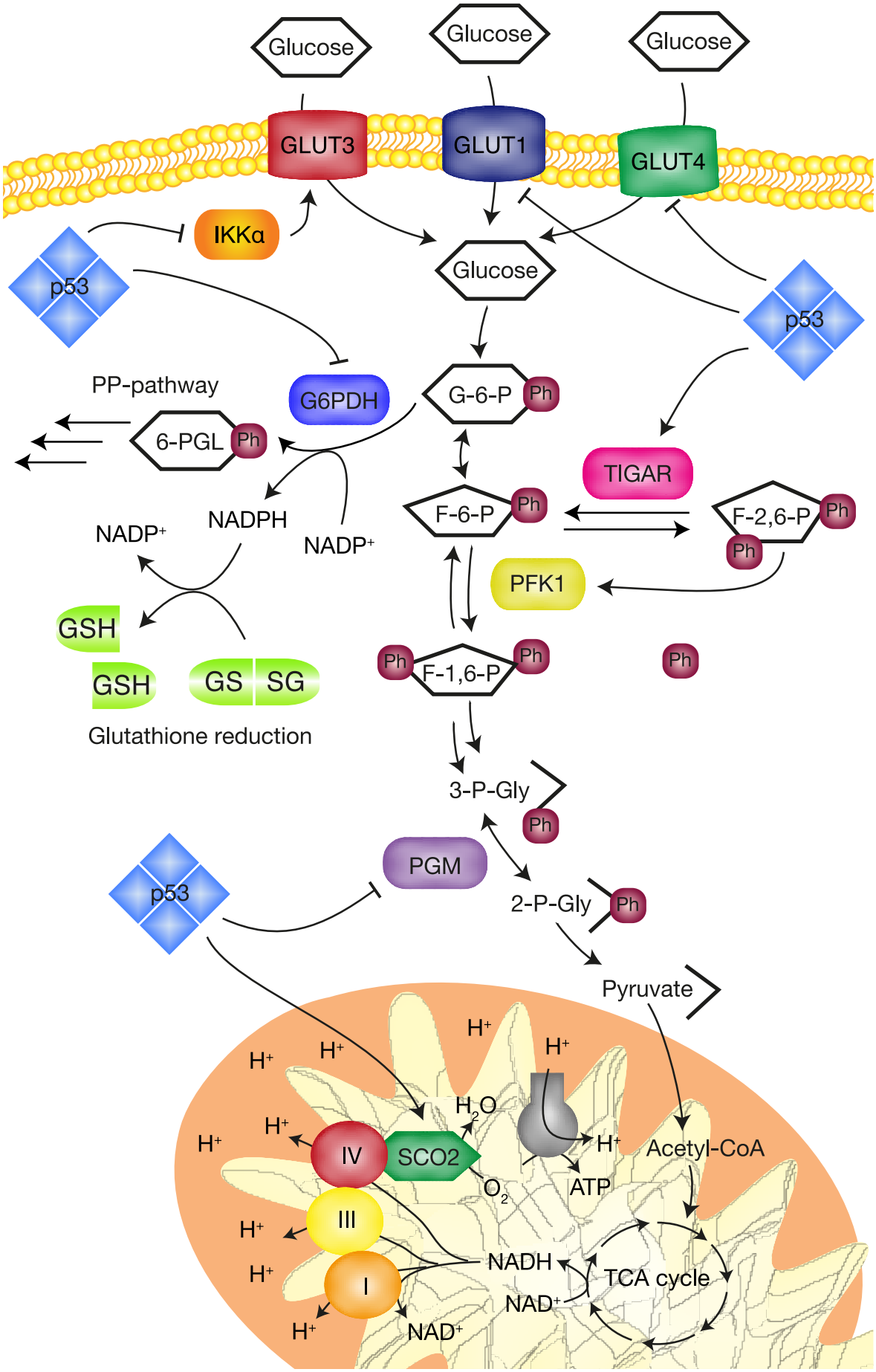

Figure 1-7: p53 inhibits glycolysis and promotes oxidative phosphorylation.
PP-pathway: Pentose phosphate pathway, TCA cycle: Tricarboxylic acid cycle

This is a critical measure in order to prevent genomic errors to be incorporated into the DNA in the first place. In line with preventing oxidative stress, p53 also induces the Glutaminase GLS2 that catalyses the conversion from glutamine to glutamate, which is required for the synthesis of Glutathione together with cysteine and glycine[102]. Furthermore, p53 induces expression of the antioxidant enzyme Glutathione peroxidase (GPX1), which scavenges hydrogen peroxide and organic hyperoxides[103] and of the mitochondrial matrix protein Aldehyde dehydrogenase (ALDH4), which also decreases the levels reactive oxygen species[104]. Moreover, a p53-induced family of cysteine sulfinyl reductases called Sestrins regenerate Peroxiredoxins, which are necessary for scavenging hydrogen peroxide[105].

Altogether, p53 can counteract transformation via a plethora of transcriptional target genes and mechanisms. On top of the repair or elimination of already damaged cells and its anti-angiogenic and anti-metastatic activity, p53 pre-empts genetic changes by keeping levels of intracellular reactive oxygen species low under physiological levels of stress.

Strikingly, it was recently reported, that p53 blocks the first rate-limiting first step of the pentose-phosphate pathway by inhibiting Glucose-6-phosphate dehydrogenase (G6PDH)[106]. This role is in opposition to the pentose-phosphate pathway-driving role of TIGAR[101]. However, since many intermediates of this pathway are required for anabolic processes, for example nucleotide synthesis[18], inhibiting this route counteracts biosynthesis and could suppress uncontrolled growth in line with p53's many other anti-oncogenic roles.

p53's bivalent role in metabolism, driving the pentose phosphate pathway through TIGAR while inhibiting it through suppression of G6PDH, is only one of the many apparent paradoxes in p53's many functions. p53 can induce and inhibit autophagy, it can keep ROS levels low and increase ROS levels, overall, p53's responses can range from promoting death to promoting survival. But these contradictory activities are never activated at the same time and the output depends on cellular circumstances and stress levels. Hence, p53's activity must be tightly controlled in order to activate an appropriate response in each case and there must be a network of signals that determines which panel of targets p53 will modulate. For example, the

activation of antioxidant genes under conditions of normal physiological stress levels, but the inactivation of antioxidant genes during the apoptotic response clearly require input about the intrinsic cellular situation being translated into p53's activity.

1.3 Regulation of p53 activity

p53 activity is mainly regulated at protein level by the Ubiquitin E3 ligase MDM2[107,108]. MDM2 itself is a p53 target gene, forming a negative feedback loop[109,110]. p53's transcriptional activity is furthermore fine-tuned by an array of posttranscriptional and posttranslational modifications as well as a vast number of interacting proteins.

1.3.1 Structure of p53.

The transcription factor p53 harbours two distinct N-terminal transactivation domains (TAD) TAD I: amino acids 1-40 and TAD II: amino acids 43-63[111], which interact with the basal transcription machinery, and an adjacent proline-rich domain (amino acids 63-91), required for the transactivation of some, but not all target genes[112].

Alignment of the p53 amino acid sequence across species reveals 5 areas of very high conservation, termed the conserved boxes. The conserved box I is located between amino acids 15 and 29 and required for interaction with the Ubiquitin ligase MDM2[113]. Interaction with MDM2 shields the transactivation domain and inhibits p53's transcriptional activity[114,115].

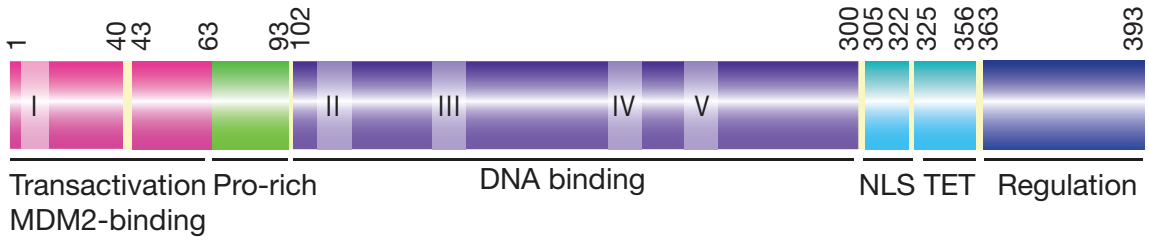

Figure 1-8: Structure of the p53 protein.
The domains of the p53 protein. Transactivation domains, Proline-rich domain, DNA binding domain, nuclear localisation signal (NLS), tetramerisation domain (TET), C-terminal regulatory domain. I-V indicates conserved boxes.

All other conserved boxes are located in the central DNA binding domain from amino acids 102 to 300. Structurally, the DNA binding domain forms an anti-parallel sandwich of β-sheets (Figure 1-9 A), while the DNA-binding surface is built up of a

loop-sheet-helix motif and two large loops interacting with the minor and major grooves of the DNA (Figure 1-9 B). The residues K120, S241, R248, R273, A276, C277, R280 and R283 are involved in contacting the DNA[116]. The DNA binding domain confers sequence-specific binding activity to promoters of p53 target genes harbouring a p53 response element containing 2 copies of the p53 consensus binding site 5'-PuPuPuC(A/T)(A/T)GPyPyPy - 3' separated by a spacer of up to 13 base pairs[117].

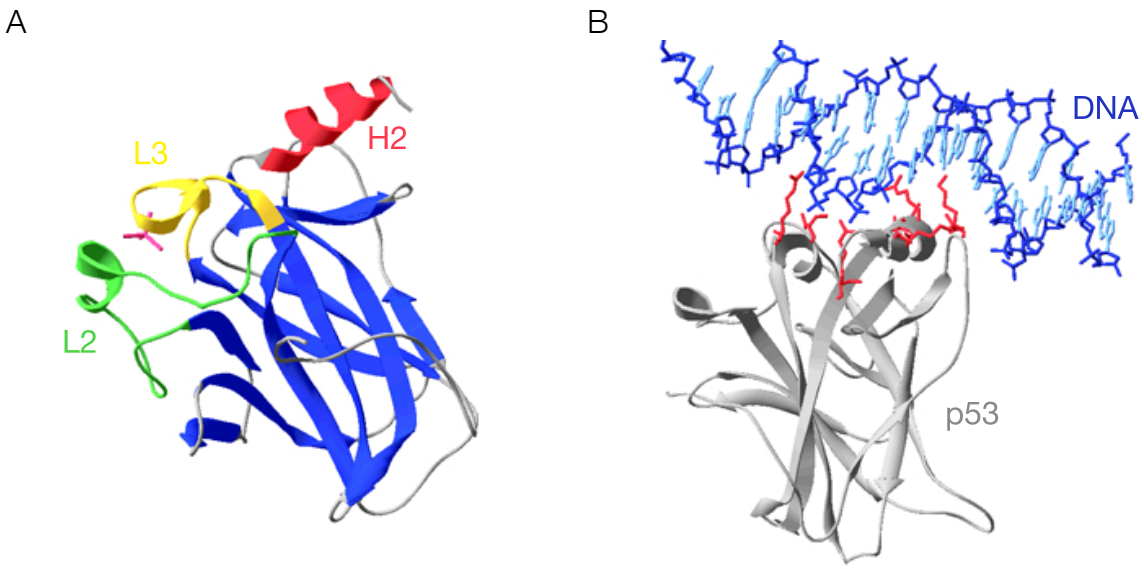

Figure 1-9: Structure of the DNA binding domain.
(A) The structure of the DNA binding domain consists of an anti-parallel sandwich of β-sheets (blue) with loops L2 (green), L3 (yellow) and helix H2 (red).
(B) The amino acids 120, 241, 248, 273, 276, 277, 280 and 283 (red) contact the DNA (blue).
Protein database (PDB) accession number 1TUP[116].

p53 contains nuclear localisation and nuclear export signals, which allow the protein to shuttle in and out of the nucleus. Three nuclear localisation signals for p53 have been described, the major bipartite NLS with two basic motifs between amino acid 305 and 322 and two less active sequences between amino acids 366 to 372 and 377 to 381[118]. p53 forms a tetrameric complex out of two homodimers via its oligomerisation domain in order to be an active transcription factor[119]. A nuclear export signal (amino acids 340 to 351) lies within the oligomerisation domain (amino acids 325 to 356) and it is thought that in its active state as tetramer, p53's nuclear export signal is masked[120].

p53's C-terminus adopts a flexible conformation and contains a regulatory domain (amino acids 363-393), which is subject to extensive posttranslational modification.

1.3.2 p53 isoforms

The p53 gene is located at chromosome 17p13.1 and spans 19200 base pairs across 11 exons. While the vast majority of research has focused on the full-length protein, 11 other p53 isoforms have been described more recently.

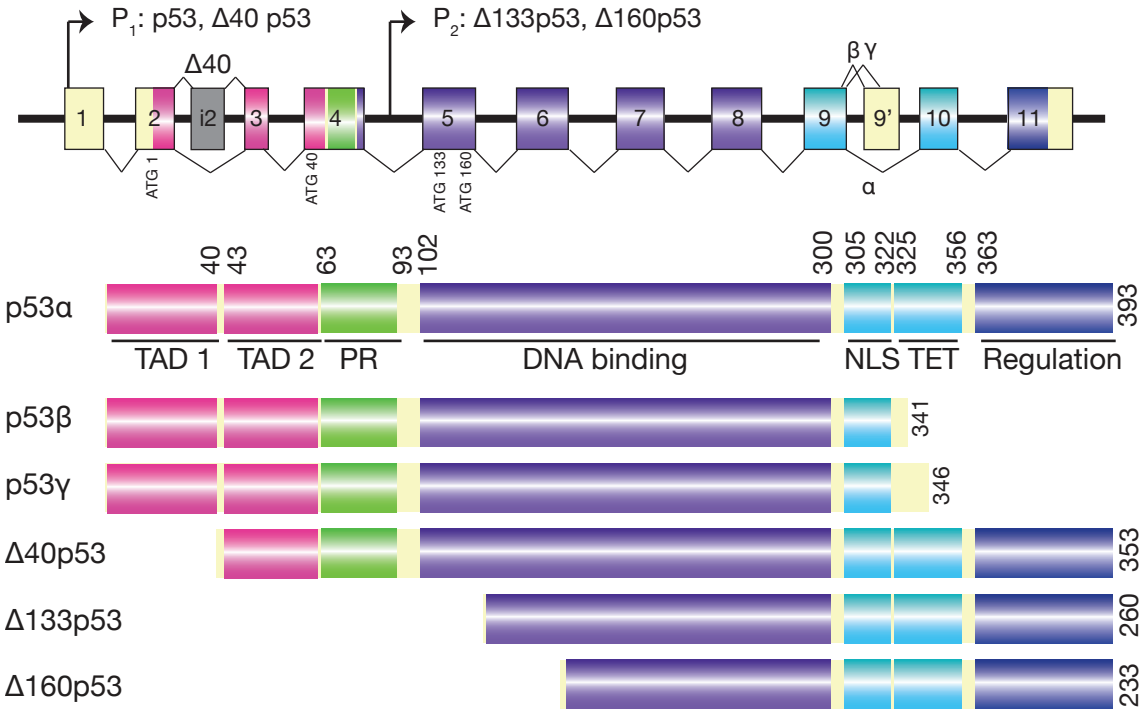

Figure 1-10: p53 Isoforms
Isoforms β and γ lack exons 10 and 11 after alternative splicing of intron 9. Δ40 p53 is initiated at an alternative ATG in exon 4. Δ133p53 and Δ160p53 are translated from an internal promoter in intron 4. Translation of Δ160p53 is initiated at a second ATG in exon 5.
TAD: transactivation domain, PR: proline rich domain, NLS: nuclear localisation signal, TET: Tetramerisation domain.

A second promoter is located in intron 4 of p53, and initiation of transcription here gives rise to an N-terminally deleted Δ133p53, which lacks the first 133 amino acids, including both transactivation domains and part of the DNA binding domain. As expected, this isoform cannot induce p53 target genes, by contrast it was described to inhibit wild-type p53 in a dominant negative fashion, in particular inhibiting p53-induced apoptosis[121]. Full-length p53 was reported to induce expression of the Δ133p53 isoform in response to stress by binding to its own internal promoter[122,123]. Recently, the Δ133p53 isoform was shown to promote angiogenesis and tumour progression and can therefore be classed as an oncogene rather than a tumour suppressor[124].

The Δ133p53 mRNA transcript also gives rise to another isoform. If translation is initiated at an alternative ATG at codon 160, an even shorter Δ160p53 protein, lacking the first 160 amino acids is produced[125]. Although its expression was observed in multiple cancer-derived cell lines, the function of this isoform has not yet been studied.

If p53 translation is initiated at an alternative ATG at codon 40 from the full-length transcript, the resulting protein lacks the first transactivation domain, but retains TADII[126]. Δ40p53 is also produced if intron 2 is alternatively spliced, which gives rise to a longer mRNA, containing multiple stop codons in the intron 2 sequence. Therefore a shorter protein starting at the later ATG 40 is produced[127]. The second transactivation domain seems to be sufficient to induce some p53 target genes[128], however it lacks the MDM2-binding N-terminus and is consequently not degraded by MDM2[127].

Intron 9 can be spliced by two alternative methods, giving rise to p53β and p53γ, which have prematurely terminated C-termini (with ten or fifteen additional amino acids, respectively) lacking the regulatory and the tetramerisation domains, but retaining the nuclear localisation signal[121]. While p53α has higher affinity towards the p21 and MDM2 promoters, p53β preferentially bind the p21 and Bax promoters[121].

Finally, these 3 C-terminal alternative splice events can occur at the same time as the 4 N-terminal deletions, giving rise to a total number of 12 isoforms, or possibly more, since three more C-terminal splice variants were reported recently[129].

Furthermore, the p53 gene contains a common single nucleotide polymorphism (SNP) in the proline-rich domain at codon 72, which can be a CCC, encoding a proline or a CGC, encoding an arginine residue. The ancestral form of p53 was found to be the proline variant[130]. While both variants are very common, the distribution changes according to ethnicity and the 72P variant was found to be more prevalent nearer the equator, suggesting that it might confer better UV light protection[131]. The SNP influences p53's transcriptional output: the prolyl-isomerase PIN1 binds to the proline rich domain and preferentially binds the R72 variant[132]. Furthermore, the P72 variant binds the anti-apoptotic iASPP better than R72[133], leading to R72 p53 being a

more potent inducer of apoptosis. Interestingly, p53 cancer mutants with the 72R variant bind p73 better than 72P, leading to a more sustained suppression of p73's apoptotic activity[134].

1.3.3 MDM2 keeps p53 levels in check

It is crucial to quickly accumulate and activate p53 in the event of stress, it is however equally critical to control p53's ability to inhibit proliferation and survival when cells are perfectly healthy. Therefore, under unstressed conditions, regulation of p53 protein turnover is controlled by a number of factors, one of the most important of which is the Ubiquitin-E3-ligase MDM2, which constantly ubiquitinates newly synthesised p53 protein, leading to its degradation[107,135,136]. The significance of this control is demonstrated by the MDM2-knockout mouse, which is embryonic lethal due to overactive p53, leading to widespread apoptosis. Importantly, the lethality can be rescued by simultaneous knockout of p53[137,138].

1.3.3.1 MDM2 ubiquitinates p53.

Multiple lysine residues in p53's C-terminus[139], DNA-binding[140], nuclear localisation and oligomerisation domains[141] are targeted for modification with the small (76 amino acids) protein Ubiquitin via a 3-enzyme cascade of a Ubiquitin-activating enzyme E1, a Ubiquitin-activating enzyme E2 and an E3 Ubiquitin-ligase. Despite the identification of multiple E3 ligases for p53, MDM2 is still the most prominent one.

The Ubiquitin-activating E1 enzyme catalyses the formation of an Ubiquitin-adenylate intermediate with Ubiquitin and ATP and consecutively transfers the activated Ubiquitin to a thiol-group of its own cysteine residue to form an E1-Ubiquitin thioester. While only one mammalian Ubiquitin E1 enzyme, UBE1, was known for a long time[142], more recently a second E1 UBE1L2 was identified[143]. The E2 enzyme accepts the activated Ubiquitin from the E1, also forming a thioester with one of its cysteine residues, resulting in a Ubiquitin-E2 complex. There are more than ten different Ubiquitin conjugating enzymes in mammals[144], however the predominantly used E2 in conjunction with MDM2 is Ubch5 B/C[145]. Finally, MDM2 serves as E3-enzyme, facilitating the formation of an isopeptide bond between the ε-group of the target lysine and the C-terminal glycine residue of Ubiquitin.

RING finger Ubiquitin ligases generally function as a dimer and the most efficient Ubiquitin-ligase for p53 is a heterodimer of MDM2 its closely related protein MDMX[146-148]. Although MDMX itself does not contain any catalytic activity, an MDMX protein containing a mutation, which prevents it from interacting with MDM2, is embryonic lethal due to overactive p53[149].

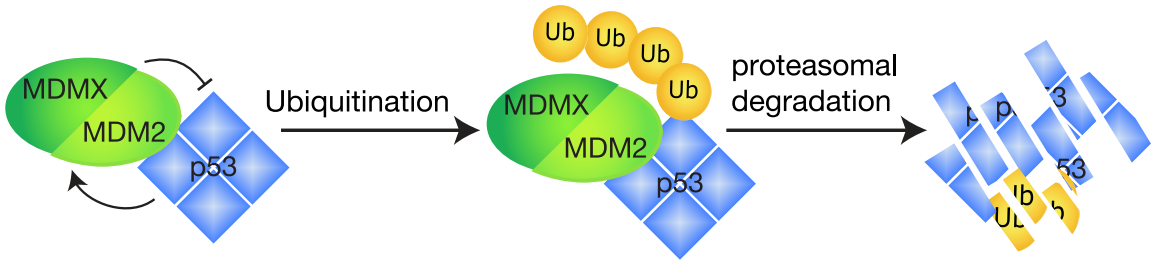

Figure 1-11: MDM2 negatively regulates p53.
The MDM2/MDMX heterodimer is a Ubiquitin-ligase for p53, inducing p53's proteasomal degradation. Furthermore, MDM2 shields the p53 transactivation domain, while p53 induces MDM2, forming a negative feedback loop.

All C-terminal lysine residues in p53 are targeted for ubiquitination and mutation of all six lysine residues to arginine (6KR) was reported to block degradation by MDM2[139], although the 6KR p53 protein could still be ubiquitinated by MDM2 in vitro. Knock-in mice with 6KR or 7KR p53 (the mouse sequence has an additional lysine in the C-terminus) did not display a more stable p53 protein[150,151], suggesting that ubiquitination of p53 is not limited to these C-terminal lysine residues. Indeed, a number of lysine residues in the DNA binding domain were also found to be ubiquitinated by MDM2[140] and Ubiquitin conjugation was mapped by mass spectrometry to lysines 101, 292, 305 and 319-321 in the DNA binding domain and lysine 357 in the tetramerisation domain[141].

Poly-Ubiquitin chains are built up by connecting Ubiquitin-molecules with a lysine residue of an already target-bound Ubiquitin by an isopeptide bond[152]. While mono-ubiquitination of p53 was linked to nuclear export[153], poly-ubiquitination by conjugation of four or more Ubiquitin-molecules linked via lysine 48 in Ubiquitin serve as a signal for degradation by the proteasome[154].

1.3.3.2 Structure of MDM2.

The E3 Ubiquitin ligase MDM2 (also called HDM2) belongs to the family of RING finger ligases, with the RING domain being crucial for its catalytic activity, directly contacting the Ubiquitin-conjugating E2 enzyme[155]. MDM2's atypical C2H2C4 RING finger domain is located in the C-terminus between the amino acids 440 and 497, with cysteines 438, 441, 461, 464, 475 and 477 and histidines 452 and 457 binding the zinc ion[156]. In line with this, mutation of the zinc-coordinating cysteine 464 perturbs the RING structure and completely abrogates MDM2's ability to ubiquitinate p53[157]. Consistent with the importance of an intact RING domain, the single amino acid substitution C462A MDM2 (equivalent to human C464A) knock-in mouse is embryonic lethal due to overactive p53[158]. The embryonic lethality can be rescued by simultaneous p53 knockout, just like the MDM2-knockout mouse[137,138].

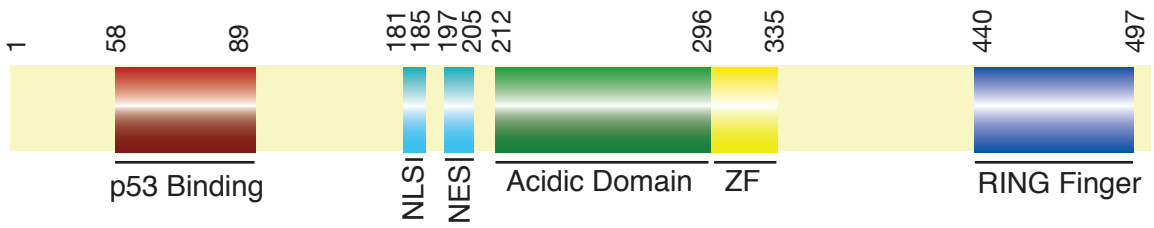

Figure 1-12: Structure of MDM2.
The MDM2 protein contains an N-terminal p53-Binding domain, a nuclear localisation signal (NLS) and nuclear export signal (NES), an acidic domain, a zinc finger (ZF) and a C-terminal RING finger.

MDM2 also contains a C4 zinc-finger further towards the N-terminus between amino acids 296 and 335 with cysteine residues 305, 308, 319 and 322 coordinating the zinc-ion[159]. Some cancer-associated MDM2 mutants harbour single amino acid exchanges of these zinc-coordinating cysteine residues[160,161]. Interestingly, the zinc finger mutant C305F MDM2 was shown to be able to ubiquitinate, but not degrade p53[162].

MDM2's N-terminus forms a deep hydrophobic binding pocket, which binds the α-helix formed by the conserved box I (amino acids 15-29) in the N-terminus of p53[163]. An MDM2 protein deleted for amino acids 58-89 is unable to bind p53[164]. Importantly, MDM2 not only regulates p53 by promoting its degradation, but also shields the transactivation domain thereby inhibiting the transactivation of p53's target genes[114,115].

MDM2 shuttles between nucleus and cytoplasm and contains a nuclear localisation signal (NLS) between amino acids 181 and 185[165] and a nuclear export signal (NES) between amino acids 197 and 205[166]. p53 also shuttles between nucleus and cytoplasm and MDM2 can promote the nuclear export of p53, which requires the export signal of p53, but not the NES of MDM2[167].

The central domain of MDM2 consists of an acidic domain between amino acids 212 and 296. Although not directly involved in the catalytic ubiquitination activity, this domain was shown to be crucial for efficient degradation of p53[168,169]. More recently, it has become apparent that the acidic domain serves as a second contact point with the core domain of p53[170-172]. The acidic domain is furthermore a platform for binding of multiple MDM2-interacting proteins, many of them inhibiting MDM2's Ubiquitin ligase activity, for example $p14^{ARF}$ and L11. MDM2's acidic domain was also found to bind to the tumour suppressor Retinoblastoma protein (Rb), resulting in Rb inactivation[173].

A short stretch of nine amino acids in the extreme C-terminus after the RING domain was recently reported to be essential for MDM2 dimerisation[174-176]. MDM2 is only a functional poly-Ubiquitin ligase, when present as a homodimer with itself or a heterodimer with its related protein MDMX (also called HDMX or MDM4) through their RING domains[146]. Although MDMX contains a very similar p53-binding domain to MDM2 and a RING finger, it harbours no Ubiquitin ligase activity. Nevertheless, MDMX knockout mice were found to be embryonic lethal and lethality could be rescued by simultaneous p53 knockout[177,178], just like the MDM2 knockout mouse. It then emerged, that MDMX negatively regulated p53 by promoting MDM2's Ubiquitin ligase activity[147,148,179]. The MDM2-MDMX heterodimer was found to show higher Ubiquitin ligase activity towards p53 and to be more stable than the MDM2 homodimer[180]. MDM2 is furthermore not only a Ubiquitin-ligase for p53, but also for itself[181] and MDMX[182,183].

1.3.3.3 Stress signals release p53 from MDM2's control.

Activation of p53 in response to stress is accompanied by a rapid stabilisation of the p53 protein, reflecting a block to MDM2's negative control. Various binding partners

and posttranslational modifications of both p53 and MDM2 were shown to regulate their interaction and the ability of MDM2 to target p53 for degradation.

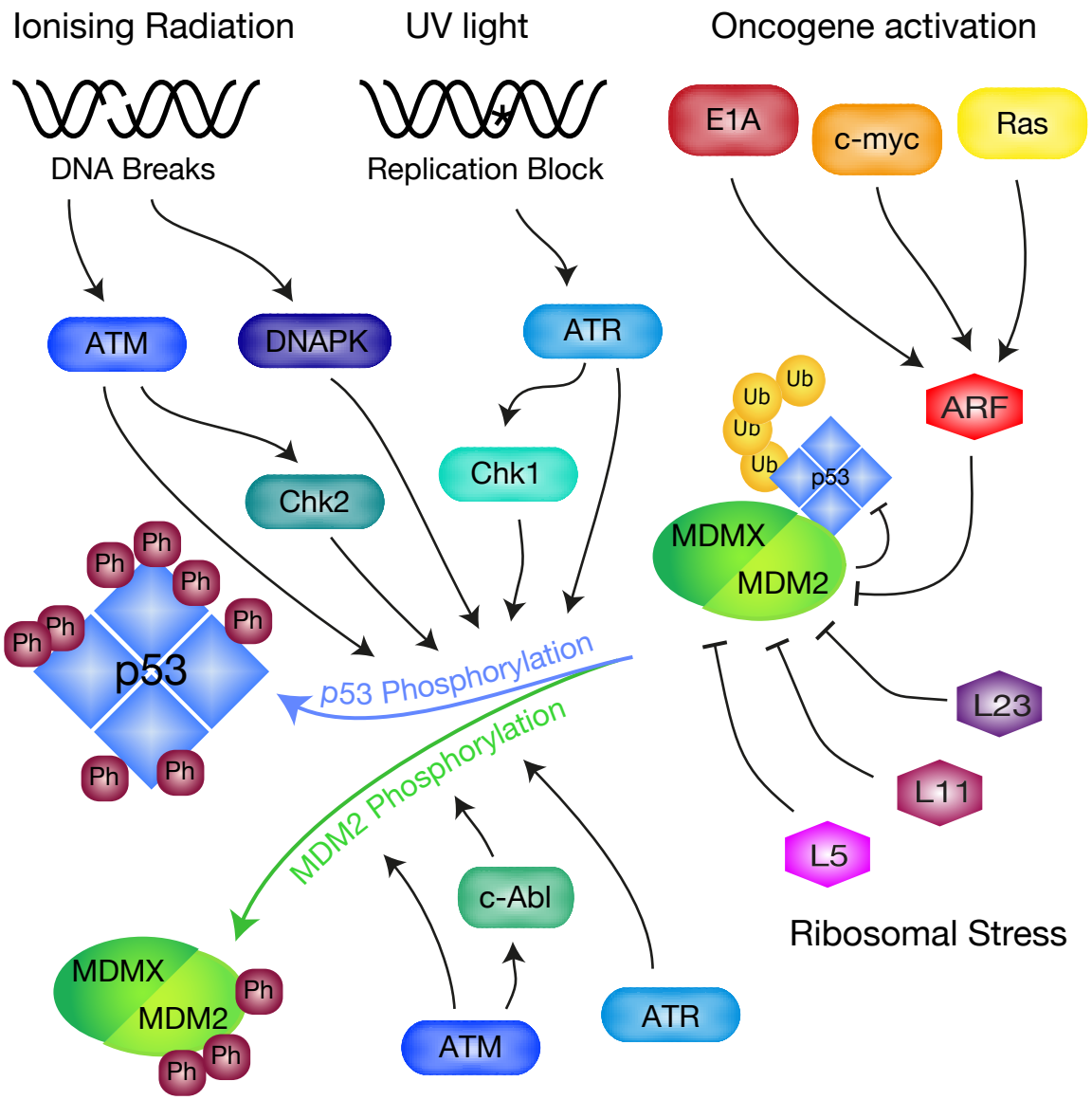

Figure 1-13: DNA damage, oncogene activation and ribosomal stress activate p53. Without stress, p53 is kept at low levels by its Ubiquitin ligase MDM2/MDMX. Ionising radiation and UV exposure cause DNA damage, which activate a number of kinases, phosphorylating both p53 and MDM2 and disrupting their inhibitory complex, leading to p53 stabilisation and activation. Oncogene activation and ribosomal stress inhibit MDM2's Ubiquitin E3 ligase activity without affecting p53-MDM2 binding.

MDM2 itself is a transcriptional target of p53[109,110], providing a positive feedback loop and MDM2 accumulation simultaneous with p53 stabilisation, resulting in quick restoration of low p53 levels once the p53-MDM2 interaction block is lifted.

Three major pathways activate p53 by releasing it from MDM2's negative control: DNA damage activates stress-induced kinases, which phosphorylate p53's N-

terminus[184] at residues critical for interaction with MDM2, resulting in a weakened interaction[185]. Oncogene activation results in expression of $p14^{ARF}$, which inhibits MDM2's ubiquitination activity[186] while not affecting p53-MDM2 binding[187]. Similarly, ribosomal stress releases ribosomal proteins such as L11 inhibiting MDM2's E3 ligase activity without impairing binding to p53[188].

A number of kinases are activated by DNA damage and target serine residues in p53's N-terminus for phosphorylation. The Ataxia telangiectasia mutated kinase (ATM) is activated in response to chromatin alterations, which are a consequence of DNA strand breaks induced by ionising radiation[189], and phosphorylates serine 15 and 37 in p53's N-terminus[190]. DNA strand breaks also stimulate the DNA-activated Protein Kinase (DNA-PK) targeting the same serine residues[191], later found to impair p53 binding to MDM2[185]. Serine 15 and 37 are also phosphorylated by the Ataxia telangiectasia and Rad3 related kinase (ATR)[192], which is induced by replication blockage and stalled replication forks induced by UV-irradiation.

Both ATM and ATR can activate further kinases involved in p53-phosphorylation: ATM activates Chk2 by phosphorylating it at threonine 68[193] and Chk2 then phosphorylates serine 20 of p53[194,195]. ATR activates the Chk1 kinase by phosphorylating it at serine 317 and serine 345[196,197], also leading to serine 20 phosphorylation of p53[198], contributing to p53's dissociation from MDM2. In addition, Chk1 phosphorylates threonine 18 of p53, if serine 15 has already been phosphorylated[199].

Interestingly, γ-irradiation not only stabilises the p53 protein, but was also found to increase ribosomal protein L26-mediated translation of p53 mRNA levels[200].

Along with p53, MDM2 is also subject to phosphorylation after DNA damage. However, MDM2 phosphorylation does not take place at the N-terminus, and does not affect its interaction with p53. Instead, ATR-mediated phosphorylation at serine 407[201] and ATM-mediated phosphorylation of serine 395[202,203] prevent MDM2-mediated nuclear export of p53. MDM2 phosphorylated at serine 395 was furthermore reported to bind to the p53 mRNA, promoting p53 protein synthesis in another mechanism to achieve accumulation of the p53 protein[204]. Two further serine

residues in MDM2's C-terminus serine 429 and 386 are also targeted by ATM and phosphorylation of these residues was proposed to disrupt dimerisation of the MDM2 RING domains, thereby contributing to p53 stabilisation by inhibiting MDM2-mediated poly-ubiquitination[205]. In response to ionising radiation, ATM furthermore activates the kinase c-Abl[206,207], which in turn phosphorylates MDM2's tyrosine 394, disrupting the p53-MDM2 complex[208].

But not only DNA damage poses a threat to healthy cells, activation of proto-oncogenes, an important step on the way to cancer development, also triggers p53 activation. The alternative reading frame of the INK4A locus gives rise to $p14^{ARF}$ [209], which was soon discovered to bind to the acidic domain of MDM2[210] and serve as inhibitor of its Ubiquitin ligase activity[186,211-213]. Binding of $p14^{ARF}$ exposes a cryptic nucleolar localisation signal in MDM2, leading to its nucleolar sequestration[214,215]. $p14^{ARF}$ expression is stimulated by many hyper-proliferative signals, including E1A[216], Myc[217] and Ras[218]. Lacking this crucial p53-activating mechanism, $p14^{ARF}$-knockout mice were found to be prone to spontaneous tumour development[219].

A similar mechanism activates p53 in response to ribosomal stress that can be induced by treatment with RNA polymerase II inhibitor Actinomycin D. By now, many small ribosomal proteins were reported to bind MDM2's acidic domain and inhibit its ubiquitination of p53: Namely, the ribosomal proteins involved in inhibiting MDM2 are the ribosomal large subunit proteins L11[188,220], L23 [221,222], L5[223,224] and the small subunit protein S7[225,226]. Activation of p53 by aberrant ribosome biogenesis was recently shown to be an important independent tumour suppressing mechanism: Tumours in a Myc-driven lymphoma mouse model formed more rapidly when MDM2 was substituted with an MDM2 mutant (MDM2 C305F), which could not bind to L11 or L5, but retained binding to $p14^{ARF}$ [227].

In line with p53's metabolic responsibilities, glucose starvation was found to activate p53 via the glucose-dependent Adenosine mono phosphate dependent kinase (AMPK)[228]. Under conditions of low glucose, the cell cycle should not proceed with cell division, but be halted until conditions have improved. Upon glucose starvation, AMPK induces phosphorylation of serine 15, once again contributing to the release of p53 from MDM2's negative control and activating the G_1/S checkpoint[229].

Similarly, cells stop dividing when oxygen is scarce. p53 protein levels were reported to accumulate under hypoxic conditions, possibly by down-regulation of MDM2[230]. Serine 15 of p53 was also found to be phosphorylated in response to hypoxia[231], which is probably mediated by ATR[232].

Clearly, p53 is activated by many different routes, often resulting in accumulation of p53 protein. However, importantly, the levels of p53 in unstressed cells are sufficient to induce the genes required for p53's functions in unstressed circumstances. Broadly speaking, protein levels can affect the selection of target genes activated by p53. In a simplified model low levels of p53 preferentially occupy high-affinity promoters, which are mostly genes involved in cell-cycle arrest, while high levels of p53 bind to lower affinity promoters, including the genes of the apoptotic response[233]. But while not directly affecting p53 protein levels, many other interacting proteins and posttranslational modifications modulate p53's transcriptional activity and determine the ultimate response activated by the transcription factor.

1.3.4 p53 co-factors

As a transcription factor, p53 interacts with many transcriptional cofactors to induce or repress its target genes. Particular p53-binding proteins can influence which of the many p53-regulated promoters is activated and thereby stimulate a selective p53 response.

Transcription factors influence the regulation of their target genes via recruiting transcriptional cofactors, which allow or prevent transcription of the target genes by facilitating or hindering the transcription machinery to gain access to the chromatin. One mechanism of chromatin remodelling is posttranslational modification of the Histones, around which the DNA is organised. Acetylation of lysine residues in Histones leads to the removal of the positive charge of the unmodified amino-group. Thus acetylated Histones are less positively charged. This reduces their affinity to the negatively charged DNA, which is organised around them, thereby allowing access for the transcription machinery[234]. p300 was the first Histone acetyltransferase discovered to be recruited to the p21 promoter and was found to acetylate p53-bound nucleosomes[235]. p53 also transactivates some of its genes in conjunction with a multi-subunit transcriptional activator complex called STAGA, containing the

Histone acetyltransferase GCN5, crucial for promoter activation. The STAGA complex interacts with p53 at its transactivation domains and is required for the activation of p21, Gadd45 and PUMA[236].

On the other hand, transcriptional repression by p53 can be mediated by binding of the co-repressor mSin3A, which recruits the Histone deacetylase HDAC1[237]. By removing the acetylation of Histones, a closed chromatin structure is achieved, which cannot be accessed by the transcription machinery. The pro-proliferation gene c-Myc and the anti-apoptotic gene Survivin are examples of p53-repressed genes by this mechanism[238,239]. p53 also represses target genes without directly binding to their promoter, but by forming a complex with other transcription factors. Many of the cell cycle genes repressed by p53 contain a CCAAT box, which is recognised by the transcription factor NF-Y. NF-Y interacts with p53 in the C-terminus and the complex of NF-Y and p53 is required for the recruitment of Histone deacetylases to the promoters of the G_2/M genes Cyclin B_1, Cyclin B_2, Cdk1 and Topoisomerase II[240].

Many other proteins can influence p53's transcriptional output: The ASPP family (Apoptosis stimulating proteins of p53) bind to p53's proline-rich domain and the core DNA binding domain. The ASPP1 and ASPP2 proteins were found to promote transactivation of Bax and PIG3, while not affecting expression of p21 and MDM2, promoting p53's apoptotic response[241]. Strikingly, another ASPP family member called iASPP binds to the same region in p53, but with the opposite outcome: In contrast to ASPP1 and ASPP2, iASPP inhibits rather than stimulates apoptosis[242].

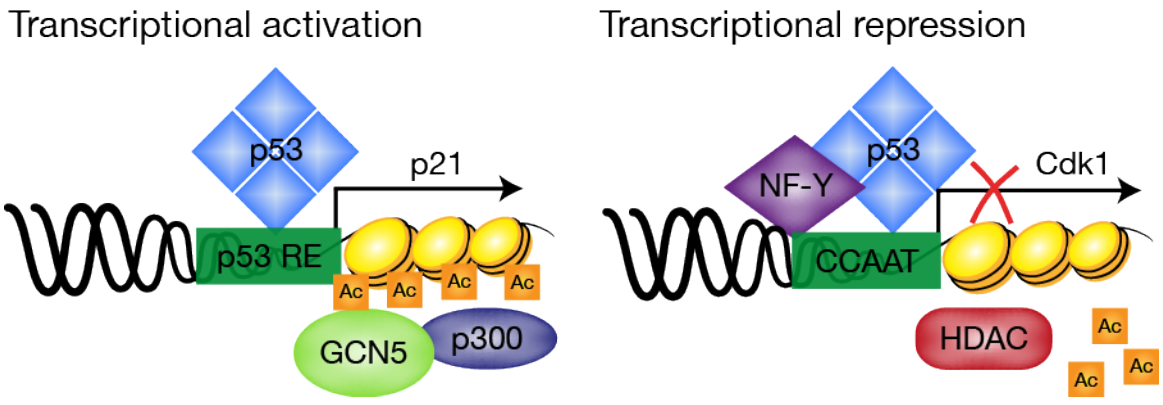

Figure 1-14: p53 activates and represses target genes.
p53 recruits Histone acetyltransferases (GCN5, p300) in order to activate target genes such as p21 and recruits Histone deacetylases (HDAC) to inhibit target genes such as Cdk1.

Moreover, the Haemapoietic zinc finger protein HZF interacts with p53's DNA binding domain and promotes the induction of cell cycle arrest[243]. p53 bound to HZF was found to preferentially activate expression of p21 and 14-3-3σ, while not inducing the promoters of Bax and Noxa.

An intriguing set of p53-binding transcription factors are the POU family members Brn3a and Brn3b. Interaction of Brn3a with p53 leads to activation of p21, but not Bax and Noxa[244], while interaction of Brn3b results in the opposite effect: activating Bax, but not p21[245].

Recruited by N-terminal serine and threonine phosphorylation of p53, the Prolyl-isomerase 1 (Pin1) induces conformational changes of the proline rich domain in p53[246], resulting in a change in its interaction partners: Pin1 generally stimulates transactivation by inducing p300-mediated acetylation of p53, which in turn is required for the transcription factor's sequence-specific DNA binding (see section 1.3.5.1). If p53 is phosphorylated at serine 46 in the transactivation domain, Pin1 furthermore leads to the dissociation of the inhibitory iASPP protein, promoting the induction of apoptosis[132]. This is by no means a complete of p53-interacting proteins and their effects, furthermore many indirect effects of p53-binding proteins are complicating the p53-signalling networks[247].

1.3.5 Posttranslational modifications modulate the p53 transcriptional response.

While p53-binding partners can modulate the outcome of the transcriptional response induced by p53, its activities are also modulated by a vast number of posttranslational modifications such as phosphorylation, acetylation, methylation, ubiquitination, neddylation and SUMOylation.

1.3.5.1 Phosphorylation

One of the first identified modifications on p53 was phosphorylation[248], which takes place on serine and threonine residues in all regions of the protein. Phosphate groups are conjugated to the hydroxyl-groups of polar amino acid residues by kinases in a condensation reaction. As phosphate groups are two to three times

negatively charged (depending on the pH), phosphorylation is often associated with conformational changes of its target proteins. Phosphatases remove phosphate groups in a hydrolysation reaction, allowing reversible and highly dynamic phosphorylation and dephosphorylation. Phosphorylation is often used to amplify minor effects via multistep kinase activation pathways and is thus involved in many cellular signalling cascades.

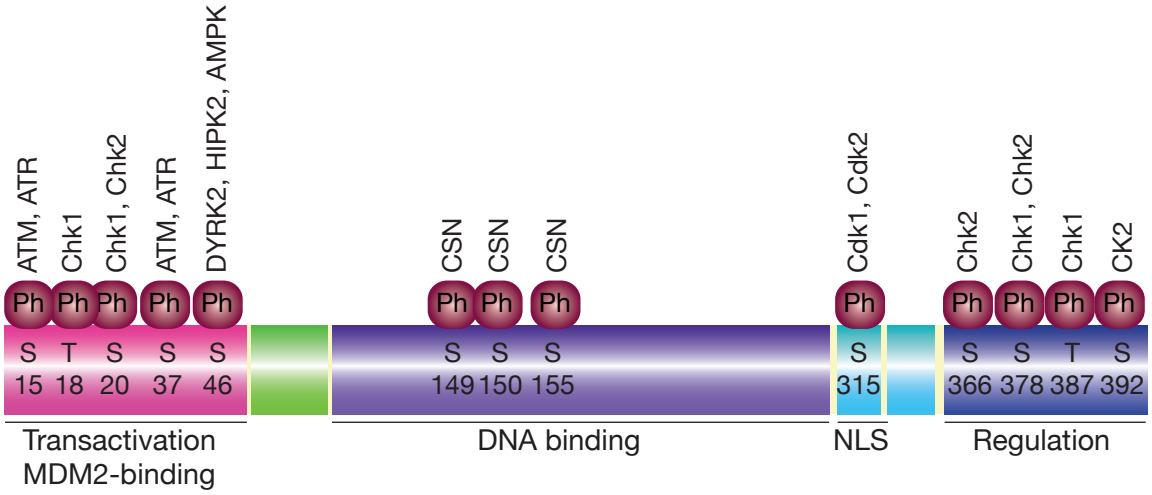

Figure 1-15: p53 is phosphorylated in the N-terminus, DNA binding domain and C-terminus.
Many different kinases target p53 for phosphorylation, including ATM, ATR, Chk1, Chk2, DYRK2, HIPK2, AMPK, CSN, Cdk1, Cdk2 and Casein Kinase 2 (CK2) affecting binding to MDM2, protein localisation, modulation of the transcriptional response and influencing modification of neighbouring residues. NLS: nuclear localisation domain.

In response to DNA damage and UV, phosphorylation takes place on p53's N-terminus on serine 15, threonine 18, serine 20 and serine 37, induced by the ATM, ATR, Chk1, Chk2 and DNA-PK kinases. These modifications result in decreased MDM2 binding and p53 stabilisation (see section 1.3.3.3). Other residues in p53 are also phosphorylated with consequences other than disrupting the p53-MDM2 interaction. Serine 46 in the second transactivation domain is phosphorylated by a number of different kinases and the serine 46-phosphorylated protein was reported to induce an apoptotic response, via the target gene p53AIP1[249]. The kinases involved in this modification are the Homeodomain protein kinase 2 (HIPK2)[250], which is controlled in a feedback loop via MDM2-mediated degradation[251], the dual-specific tyrosine phosphorylation-regulated kinase 2 (DYRK2), which translocates to the nucleus upon genotoxic stress[252] and AMP kinase (AMPK), phosphorylating p53 upon glucose deprivation[253].

Serine residue 315, which is phosphorylated by the Cyclin-dependent kinases Cdk1[248] and Cdk2[254], is located in the nuclear localisation signal of p53[255]. Indeed, serine 315 phosphorylation was reported to affect nuclear localisation of p53[120].

Serine 392 in the C-terminal regulatory domain of p53 is phosphorylated by Casein Kinase 2 (CK2) after treatment UV-light, but not γ-irradiation[256]. As part of the DNA-damage response this modification enhances p53 transcriptional activity, which is consistent with the observation that serine 392 phosphorylation stabilises the p53 tetramer in vitro[257].

The DNA-damage induced kinases Chk1 and Chk2 do not only phosphorylate p53's N-terminus, but also a number of serine and threonine residues the C-terminus. These modifications are thought to activate p53's transcriptional activity by inducing acetylation of nearby lysine residues[258].

Some serine and threonine residues in the DNA-binding domain (serine 149, threonine 150, threonine 155) are targeted for phosphorylation by the COP9 signalosome (CSN). These phosphorylation events occur under unstressed conditions and promote p53 degradation[259].

1.3.5.2 Acetylation

Acetyl-groups can be conjugated to either N-terminal amino groups or ε-amino groups on lysine residues throughout the protein.

A major role of acetylation is the regulation of Histone modification. The enzymes catalysing acetyl conjugation and deconjugation reactions are therefore called Histone acetyltransferases (HATs) and Histone deacetylases (HDACs), although they also target non-Histone proteins. The amino group of lysine residues carries a positive charge, which is removed by acetylation. This can alter protein conformations and influence DNA binding.

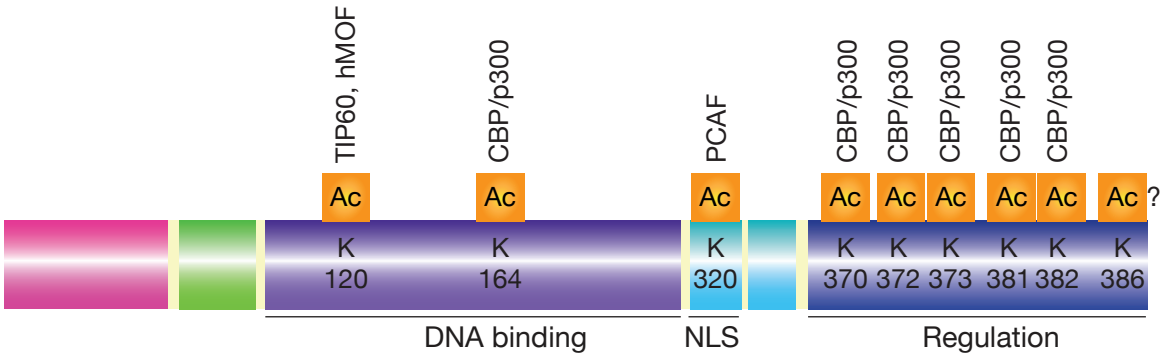

Figure 1-16: p53 is acetylated in the DNA binding domain and the C-terminus.
Different acetyltransferases contribute to p53's acetylation at multiple lysine residues in the DNA binding, nuclear localisation and C-terminal domains.

Acetylation of p53 predominantly takes place at lysine residues in the C-terminus and is generally associated with enhancing sequence-specific DNA-binding, which is probably due to a conformational change following the acetylation[260]. Moreover, acetylation has been reported to enhance p53 protein stability by blocking MDM2-mediated ubiquitination[261].

p53's main Histone acetyltransferase CBP/p300 was first identified as transcriptional co-activator[262] binding to p53's N-terminus[263] and phosphorylation of p53's serine 15 promoted this interaction[264]. CBP/p300 was later found to acetylate lysine residues 370, 372, 373, 381 and 382 in p53's C-terminus[260]. Acetylation of lysines 373 and 382 is induced upon DNA damage[265]. Acetylation of the C-terminal lysine residues induces overall sequence specific DNA binding of p53 and thereby transactivation of all its target genes. Although initially described as non-acetylated[260], mass-spectrometry analysis later found the sixth lysine residue in the C-terminus, lysine 386, to also be acetylated in COS cells[266].

The p300 and CBP associated factor (PCAF) acetylates p53 at lysine 320 in the nuclear localisation signal in response to DNA damage, when serine 33 and 37 are phosphorylated[267]. Lysine 320 acetylation also enhances sequence specific DNA binding of p53 and promotes activation of p21[268].

Interestingly, acetylation and ubiquitination target the same C-terminal lysine residues in p53 and it was reported that acetylation inhibits MDM2-mediated ubiquitination of p53[261]. On the other hand, the Ubiquitin-E3 ligase MDM2 reduces acetylation of p53

by displacing p300 bound to p53[265,269], degrading PCAF[270] and recruiting the deacetylating enzyme HDAC1[271].

p53's DNA binding domain is also subject to acetylation: Lysine 164 is targeted by CBP/p300, contributing to p21 activation[272] and lysine 120 is acetylated by the MYST family of Histone acetyltransferases, specifically Tip60[273] and hMOF[274], channelling p53's response towards the panel of apoptotic target genes such as PUMA and Bax.

While knock-in-studies with p53 proteins lacking the C-terminal lysine residues, did not reveal a major impact on p53 transactivation[150,151], a human protein lacking all 6 C-terminal lysines plus lysine 120 and 164 in the DNA binding domain failed to induce p21, PUMA, PIG3 and Bax, while still being able to transactivate MDM2[272]. Indeed, a knock-in mouse mutated at just the K120 and K164 acetylation sites in the DNA-binding domain (K117 and K161/162 in mouse), cannot induce cell cycle arrest, apoptosis or senescence, but retains ability to induce p53's metabolic target genes[33]. However, it is difficult to assess whether this ablation of p53's ability to transactivate most of its target genes is really due to the posttranslational modifications of the lysine residues mutated in this protein, since lysine 120 is known to interact with the major groove of the DNA and mutation of this single lysine residue already affects DNA binding[275].

1.3.5.3 Methylation

Methylation refers to the displacement of a hydrogen atom with a methyl (CH_3-) group. In proteins, the guanidinium group of arginine residues can be mono- or di-methylated by protein arginine methyltransferases and the ε-amino group of lysine residues can be mono-, di- or tri-methylated by lysine methyl transferases.

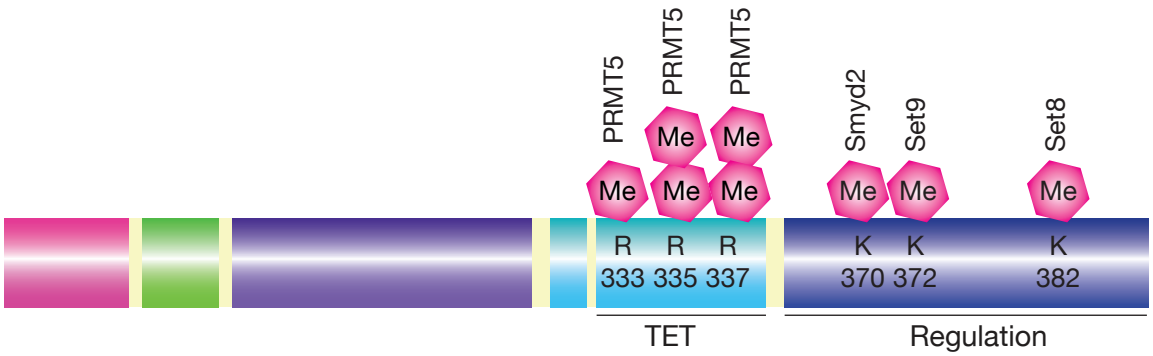

Figure 1-17: p53 is methylated at the C-terminus.
p53 is mono-and di-methylated in the tetramerisation domain (TET) and the C-terminus.

Three lysine residues in p53's C-terminus are not only acetylated, but also methylated: Lysine 370 is targeted for mono-methylation by the SET and MYND domain containing 2 (Smyd2), resulting in repression of p53-mediated transactivation[276]. Methylation of lysine 370 is inhibited by acetylation of the neighbouring lysine 372.

Lysine 372 is methylated by the SET domain methyltransferase Set9, leading to increased transactivation of p21[277]. This methylation is induced by DNA damage and occurs together with acetylation of lysine 382.

Lysine 382 itself is mono-methylated by SET domain methyltransferase Set8, leading to repression of p53's strongly induced target genes (p21 and PUMA), while the transactivation of weaker target genes (Bax and Noxa) are not affected[278].

Three arginine residues in p53's tetramerisation domain are methylated by the Protein arginine methyltransferase 5 (PRMT5) in response to DNA damage. This modification contributes to transactivation of a number of target genes, since p53 bound less to the p21, Gadd45 and PUMA promoters after ablation of PRMT5[279].

1.3.5.4 Ubiquitin-like modifications

Ubiquitin-like modifications are posttranslational modifications of proteins by the conjugation of small proteins of the Ubiquitin family to form isopeptide bonds with ε-amino groups of lysine residues in the target protein.

Ubiquitin was the first identified and founding member of the family of Ubiquitin-like proteins. A chain of four or more Ubiquitin moieties is recognised and degraded by a large protein complex called the 26S proteasome[154]. The proteasome is a ubiquitous 670 kD complex that serves to degrade proteins in the cytoplasm, nucleus and endoplasmatic reticulum. The large structure can be subdivided into three parts: Two regulatory 19S particles serve as gatekeepers at either end, and the 20S proteasome, a central core domain of four stacked rings, forms a barrel-like structure[280]. The regulatory 19S particles recognise and bind Ubiquitin chains. The Ubiquitin molecules are removed from the target protein by 19S-associated deubiquitinating enzymes and the substrate is unfolded with the help of ATPases. The linearised protein is then passed into the core, where it is cleaved by 20S-associated proteases[281].

Ubiquitin can form a number of different chains. The 76 amino acid residue Ubiquitin protein contains seven lysine residues: K6, K11, K27, K29, K33, K48 and K63 and every single one of these lysines can be used for conjugation of a further Ubiquitin molecule[282]. Indeed, analysis of auto-ubiquitinated MDM2 in combination with the E2 conjugation enzyme Ubch5 identified all possible Ubiquitin chain-linkages and even forked chains as result of the conjugation of 2 Ubiquitin molecules to two different lysines in one Ubiquitin[282]. However, certain chain linkages prevailed and K11, K48 and K63 linked chains were predominantly found[282]. The specific chain-linkage is influenced by the E3 ligases: In a different study, MDM2 was found to predominantly produce K48 linked chains while MDM2 in complex with MDMX conjugated K6, K11 and K48 linked chains[283].

The K48-linked chain is classically associated with proteasomal degradation. However, K63-linked chains are recognised by the 26S proteasome equally well as K48-linked chains, but deubiquitinating enzymes dissemble K63 chains six times faster than K48 linkages, possible explaining why proteins with K63 chains are less efficiently degraded[284]. While pure K11-linked chains can serve as degradation signal for mitochondrial proteins[285], K11 and K63 mixed chains can serve as a signal for internalisation of cell membrane proteins[286]. Also linear Ubiquitin chains, where the N-terminal amino group is used for conjugation instead of the ε-amino group of a lysine

residue, have been reported[287]. The NF-κB essential modulator (NEMO) was found to possess a binding motif that specifically recognises such linear Ubiquitin chains[288].

MDM2 ubiquitinates p53 on lysine residues in the DNA-binding domain, nuclear localisation domain, tetramerisation domain[141] and C-terminus[139]. In addition to promoting p53 ubiquitination (described in 1.3.2.1), MDM2 was shown to bind to the 19S proteasome, delivering p53 for degradation[289].

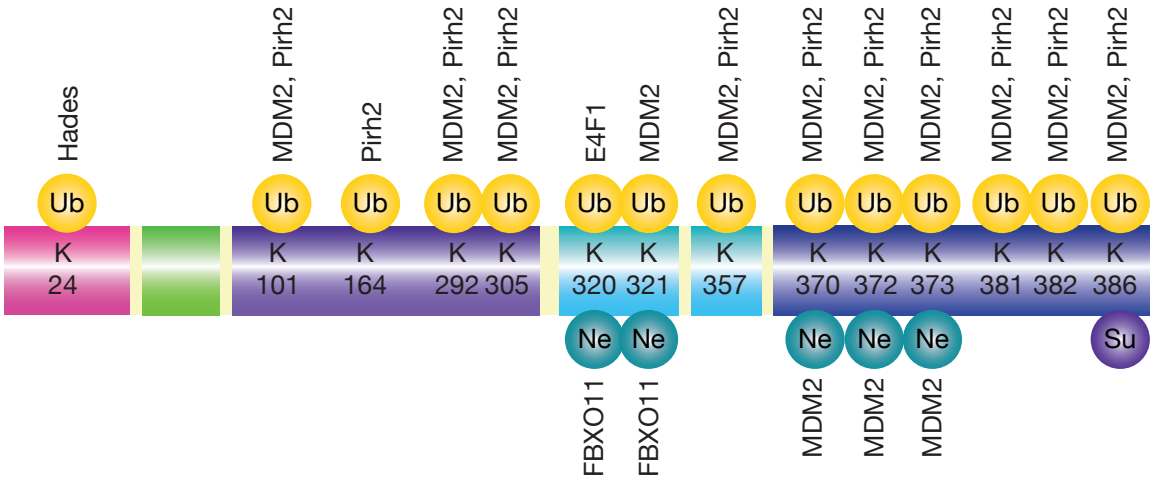

Figure 1-18: The central and C-terminal regions of p53 are ubiquitinated, neddylated and SUMOylated.
Many different E3-ligases target p53 for ubiquitination, only some of which are indicated here. Distinct lysine residues in the C-terminus and nuclear localisation domain are neddylated by MDM2 and FBXO11 respectively. Only lysine K386 has been reported to be SUMOylated. A number of different proteins have been shown to promote this modification of p53 (see chapter 1.4.3).

Additionally, roles for other Ubiquitin E3 ligases for p53 are emerging. The oldest known Ubiquitin ligase for p53 is the human papillomavirus protein E6-AP. While all types of HPVs encode an E6 protein that can bind to p53, only the E6 proteins of HPV 16 and 18 can also target p53 for degradation. Strikingly, these HPV types induce the formation of malignant lesions, while the other HPV types only trigger the formation of benign lesions[290,291]. The E6 protein itself does not harbour any catalytic activity, but associates with the host protein E6-AP (E6-associated protein), which is the founding member of the HECT-domain (homologous to E6-AP C-terminus) Ubiquitin ligases[292]. A p53-protein without the C-terminal lysine residues is resistant to E6-AP-mediated degradation, suggesting that the C-terminal lysine residues are the target of E6-AP mediated ubiquitination[139]. The adenoviral E1B 55K protein together with E4orf6 also promotes the degradation of p53[293,294]. By contrast, the

simian virus 40 (SV40) large T antigen does bind p53, but rather stabilises the protein and inhibits its transactivation[295-297]. Indeed, p53 was first discovered in a screen for SV40 binding proteins[298,299].

Non-viral proteins other than MDM2 target p53 for ubiquitination as well. The RING domain E3s Pirh2[300] and COP1[301] target p53's C-terminal lysine residues for poly-ubiquitination and subsequent proteasomal degradation, while themselves being upregulated by p53, forming a negative feedback loop with p53, just like in the case of MDM2. Pirh2 furthermore targets some lysine residues in the DNA binding domain for ubiquitination, most of them overlapping with MDM2, apart from Lys 164, which is exclusively modified by Pirh2[141]. Another RING finger ligase called Topors was also reported to induce ubiquitination and degradation of p53[302]. The HECT Ubiquitin ligase ARF-BP1 shows Ubiquitin E3-ligase activity towards p53[303]. Moreover, the Caspase associated RING proteins (CARPs) target unmodified p53, as well as p53 phosphorylated at serine 15 and serine 20, which cannot be ubiquitinated by MDM2, for degradation[304]. UHRF2 is the most recent addition to the p53-E3-ligases. It contains Ubiquitin-like PHD and RING domains and has been shown to poly-ubiquitinate p53, possibly contributing to its degradation[305].

Strikingly, a Ubiquitin-independent degradation mechanism for p53 has also been described, as unmodified p53 is constantly degraded by the 20S proteasome. However, upon treatment with ionising radiation, p53 interacts with the 20S proteasome-associated NQO1[306], thereby preventing p53 degradation and contributing to the stabilisation of p53 upon stress[307].

Four other Ubiquitin ligases modulate p53, but do not contribute to p53 degradation: Cullin 7 in complex with the small RING finger protein ROC1 mono- and di-ubiquitinates p53, thereby repressing the transactivation of its target genes[308]. The HECT domain E3 ligase WW-domain containing protein WWP1 does not degrade, but rather stabilises p53 by sequestering it in the cytoplasm[309]. The atypical Ubiquitin ligase E4F1 does not target the C-terminal lysine residues, but ubiquitinates lysine 320, not affecting protein stability, but inducing cell cycle arrest by promoting activation of the p53 target genes p21 and Gadd45[310]. Finally, the cytoplasmic E3-

ligase Hades interacts with p53 at the mitochondria, poly-ubiquitinating lysine 24 and inhibiting p53 interaction with anti-apoptotic Bcl-2[311].

Protein ubiquitination is a reversible reaction and deubiquitinating enzymes (DUBs) play a role in controlling p53 stability by counter-acting the work of Ubiquitin ligases. The deubiquitinating enzyme HAUSP was first reported as p53-stabilising enzyme, since it could remove Ubiquitin chains from p53[312]. However, it was subsequently found that HAUSP also deubiquitinated and stabilised MDM2, and that disruption of HAUSP ultimately lead to stabilised p53[313,314]. By contrast, the deubiquitinating enzyme USP42 targets p53 but not MDM2, contributing to p53-stabilisation during the early phase of a stress-response[315].

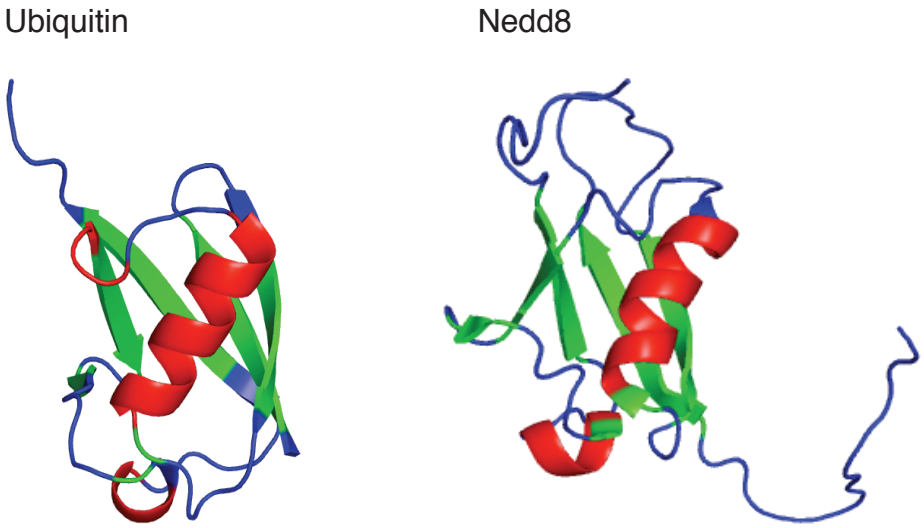

Figure 1-19: The Ubiquitin-like modifiers share a β-barrel structure.
Nedd8 is structurally homologous to Ubiquitin. Protein database accession numbers: Ubiquitin 1UBQ[316], Nedd8 1NDD[317].

Two Ubiquitin-like modifications are known to be conjugated to p53's C-terminal lysines as well: Nedd8 and the small Ubiquitin-related modifier SUMO (also called Sentrin). While SUMO only displays 18% sequence homology to Ubiquitin, Nedd8, with 58% sequence homology, is much more closely related to Ubiquitin. Although also conjugated via an E1, E2 and E3 cascade, conjugation of Nedd8 requires a different set of enzymes than ubiquitination with APPBP1-Uba3 serving as E1 enzyme and Ubc12 as E2 enzyme. The Ubiquitin-ligase MDM2 also shows Nedd8-E3 activity towards p53 and promotes neddylation of lysine residues 370, 372 and 373, leading to inhibition of p53-mediated transcriptional activation[318]. Another

protein, the F-Box protein family member FBXO11, was later shown to induce neddylation of the C-terminal lysines and two other residues (lysine 320 and 321), also inhibiting the transactivation of p53's target genes[319]. The Nedd8-protease NEDP1 was furthermore shown to specifically remove Nedd8, but not Ubiquitin from p53[320].

1.4 Small Ubiquitin-related modifiers

Although not apparent at amino acid level (18% homology), SUMO is indeed very similar to Ubiquitin in its three-dimensional conformation[323]. Major differences are the presence of an N-terminal tail in SUMO and a different surface charge distribution, suggesting distinct binding partners for SUMO and Ubiquitin[324].

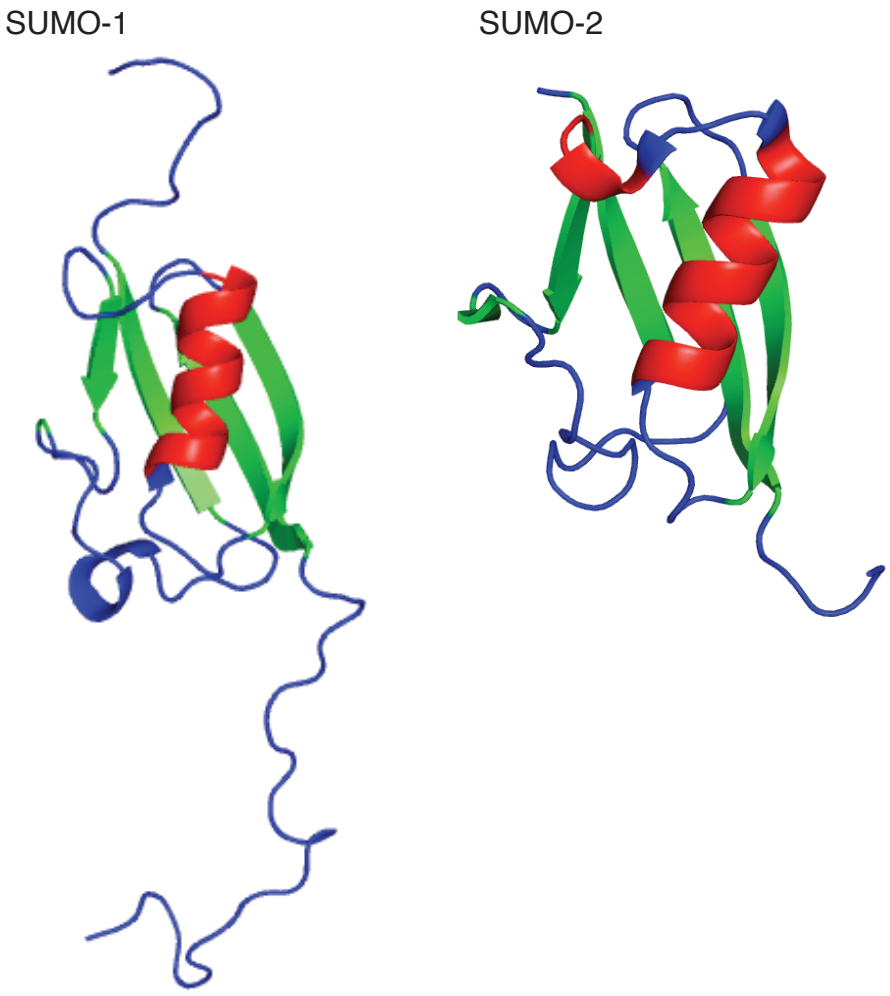

Figure 1-20: SUMO-1 and SUMO-2 ribbon models.
SUMO-1 and SUMO-2 have 50% identical amino acids and share the characteristic Ubiquitin-like fold of a barrel of β-sheets with one helix.
Protein database accession numbers: SUMO-1 1A5R[321], SUMO-2 1WM2[322]

In 1995, the first SUMO protein (SMT3) was discovered in baker's yeast as a suppressor of the centromeric protein Mif2 and was functionally linked to mitosis[325]. In the following year, a human homologue was found[326]. SUMO is a ubiquitously expressed protein of about 100 amino acid length and 10 kD weight in mammals.

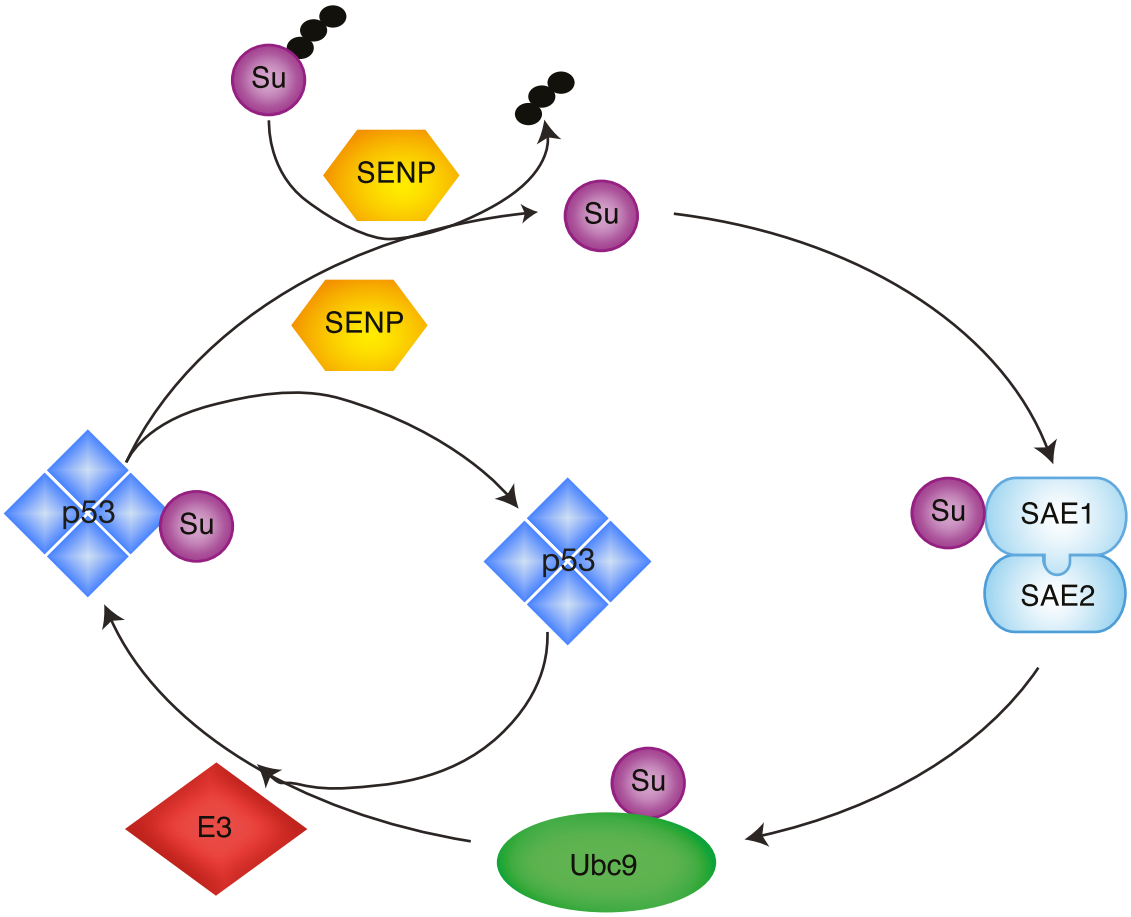

Figure 1-21: SUMO is conjugated to p53 with the help of the SUMO-E1 SAE1/SAE2 and SUMO E2 Ubc9.
The Sentrin-specific proteases (SENP) cleave the SUMO precursor, exposing the C-terminal diglycine motif, which is used to conjugate the SUMO protein to amino-groups of lysines in the target proteins.

SUMO, like all Ubiquitin-like modifiers, is synthesised as inactive precursor and needs to be cleaved by proteases in order to be activated. The cleavage by Sentrin specific proteases (SENPs) results in the exposure of a diglycine motif, which is then used for the conjugation reaction to the ε-amino-group of a lysine residue in the target protein. All SUMO family members are conjugated to their target proteins via the same E1 - a dimer of SUMO-activating enzymes SAE1 and SAE2, which activates the cleaved SUMO by forming a thioester-bond between the C-terminal glycine of SUMO and its own active cysteine residue in an ATP-consuming step[327]. From here, SUMO is transferred to the conjugating E2-enzyme Ubc9, which forms the

isopeptide bond between the C-terminal glycine residue of SUMO and ε-amino-group of a lysine residue in the target protein with the help of an E3-ligase[328].

Mammals have four SUMO-family members; the best studied being SUMO-1, which is about 50% homologous to the almost identical SUMO-2 and 3. SUMO-4 is structurally more closely related to SUMO-2/3 than SUMO-1. SUMO 1, 2 and 3 have a broad tissue distribution and the expression of SUMO-4 is limited to certain organ types, being predominantly expressed in the kidney[329]. It is yet unclear, whether SUMO-4 can be conjugated to any target proteins because the SUMO-4 precursor contains a proline residue in close proximity to the diglycine motif, which may prevent activation via protease cleavage[330].

SUMOylation predominantly takes place on lysine residues in a specific consensus motif, consisting of the four amino acid sequence ΨKxE, Ψ being a hydrophobic residue, x being any amino acid[331]. However, not all of these amino acid sequences are modified and most SUMOylated lysines reside in unfolded areas or extended loops, allowing access for the conjugating enzymes.

In contrast to Ubiquitin E3 ligases, SUMO E3 ligases are less essential for SUMO conjugation, as E1 and E2 are enough to promote SUMOylation in vitro[332]. Other than the Ubiquitin E2s, the SUMO-E2 Ubc9 contains a substrate recognition site that directly interacts with the SUMO consensus motif with the target lysine residue fitting into a hydrophobic groove in Ubc9. Nevertheless, several SUMO E3 ligases have been identified, including RanBP2, the PIAS family proteins and Pc2[333]. While the PIAS proteins contain a RING finger similar to the Ubiquitin E3-ligases, RanBP2 contains neither a RING nor a HECT domain[334]. It is possible that E3 ligases have a role in determining the substrate specificity, as not all lysine residues in SUMO consensus motifs are actually SUMOylated. Furthermore, the E3-ligases might influence which SUMO isoform is conjugated to which substrate, as SUMO-1 and SUMO-2/3 proteins have distinct and overlapping target proteins. Exclusive modification with either SUMO-1 or SUMO-2/3 cannot be explained without the activity of an E3 ligase, as the SUMO-E2 ligase Ubc9, which can directly bind to the SUMO consensus[335], does not discriminate between the SUMO isoforms. Ubc9's

target discrimination might be influenced by SUMOylation of its own lysine 14 residue[336].

SENPs, which activate the inactive precursor proteins, also cleave the conjugated SUMO proteins off the target protein, resulting in a highly dynamic turnover of SUMO on and off its target proteins[337]. Humans have six different SENPs with different subcellular distribution and a varying degree of isoform specificity[337]. SENP1 is located throughout the nucleus and SENP2 localises to the nuclear pore[338]. SENP1 and SENP2 are the only SENPs that can process and deconjugate all SUMO isoforms from their targets. Both proteins can process the precursor forms of all isoforms, but SENP1 more efficiently catalyses the processing of SUMO-1[339], while SENP2 preferentially cleaves SUMO-2[340]. All other SENPs exclusively use SUMO-2/3 as their substrate. SENP3 and SENP5 locate to both nucleus and nucleolus and hydrolyse both the SUMO-2/3 C-terminal peptide and the SUMO-diglycine - target-lysine isopeptide bond[341,342]. SENP6 and SENP7 are located in the nucleoplasm and do not process unconjugated SUMO, but edit poly-SUMO-2/3 chains[343-345]. SENP6 was found to also cleave a SUMO-1 cap off a SUMO-2/3 chain, although less efficiently than editing pure SUMO-2/3 chains[346]. Recently, a new kind of SUMO protease called DeSI-1 was described, which seems to target a distinct set of SUMOylated proteins in the cytoplasm[347].

Although SUMO-1 and SUMO-2/3 are conjugated via the same set of enzymes, they can preferentially target specific proteins, for example RanGAP1 is only SUMO-1 conjugated[348]. These isoforms are all nuclear, but show different distribution: SUMO-1 accumulates at the nuclear envelope and in nucleoli, while SUMO-2/3 does not[349]. Furthermore, SUMO-2/3 is more abundant in a large free pool than SUMO-1 and is also much more rapidly conjugated and removed[348], consistent with a potential role for SUMO-2/3 in stress responses[350]. It is becoming apparent that the modification of proteins with SUMO-2/3 may have different consequences to the modification with SUMO-1[351]. Strikingly, only SUMO-2/3 contains an internal SUMO consensus motif around lysine 11 and can therefore be conjugated in chains, whereas SUMO-1 cannot form chains and might act as chain-terminator on SUMO-2/3 chains[352]. SUMO chain formation was observed in vitro and in vivo[353] and distinct roles for SUMO-chains are beginning to emerge[353].

Much of SUMO's function is most likely mediated through creation of a new interaction surface on the SUMOylated protein and recruitment of binding partners, which would not bind the unmodified protein. This model became an exciting new area of research with the discovery of SUMO interaction motifs.

1.4.1 SUMO interaction motifs

Analysis of SUMO-interacting proteins revealed that a stretch of amino acids with non-linear aliphatic side chains could interact non-covalently with all SUMO isoforms[354]. The SUMO interaction motif (SIM) was then defined as V/I-X-V/I-V/I or V/I-V/I-X-V/I, which forms a β-sheet that interacts with the β_2-sheet of SUMO, in parallel or antiparallel direction[355]. A hydrophobic pocket on the SUMO surface formed by amino acids valine 30, phenylalanine 32 and isoleucine 34 in SUMO-3 interacts with the hydrophobic side chains of the SUMO interacting motif[356,357]. Many SIMs are located in close vicinity to a stretch of acidic amino acids, which were thought to assist the SUMO recognition. However, it later emerged that this stretch incurred some isoform specificity, since SIMs with an acidic stretch preferentially bind to SUMO-1, while SUMO-2/3 is predominantly recognised by SIMs without acidic stretch. Possibly, the amino group of SUMO-1's lysine 78, which is not present in SUMO-2/3, forms a salt bridge with the carboxyl group of the acidic amino acids next to the SIM[358]. Interestingly, some SUMO interacting motifs contain a stretch of serine residues instead of acidic amino acids, which can also introduce the negative charge when they are phosphorylated[359].

Many SUMO-conjugating enzymes contain SIMs, among them the SUMO-activating E1 enzyme that contains two SIMs, although the functional consequences on its E1-activity remain unclear[360]. In contrast, the SUMO-1 specific E3 ligase RanBP2, which harbours neither a RING nor a HECT domain, contains a SUMO interaction motif that is strictly required for its SUMO-E3 activity[361]. The PIAS E3 ligases, also require their SIM domain for the SUMO-promoting activity, while their SP-RING domain can be dispensable[362]. Moreover, SUMO interacting motifs are found in SUMO-targets and provide a plausible explanation for SUMOylation of non-consensus sites[363]. Analogous to the SUMO system, presence of Ubiquitin-binding domains can also target proteins for ubiquitination without the need for a Ubiquitin-E3[364].

The presence of SUMO interacting motifs allows a vast array of proteins, which are not directly involved in the SUMO conjugation process to be recruited to SUMOylated proteins. An example for a SUMO-recruited protein is the Ubiquitin-ligase RNF4, which harbours a row of SUMO interaction motifs and is therefore recruited by poly-SUMO chains. The recruited Ubiquitin ligase then mediates the ubiquitination and degradation of the poly-SUMOylated target proteins, and is for example involved in the degradation of SUMOylated PML[365,366]. Furthermore, SUMO-dependent recruitment of RNF4 to the mediator of DNA damage checkpoint MDC1, was recently shown to play a central role in homologous recombination after DNA damage[367,368]. The hypoxia inducible factor HIF-2α was also reported to be poly-SUMOylated and subsequently degraded by RNF4 and von Hippel-Lindau VHL[369]. However, no SUMO-interacting motif could be found in VHL, making it an unlikely SUMO-targeted Ubiquitin ligase[370].

1.4.2 SUMO function

SUMO clearly plays a significant role in development, since knockout mice of the SUMO-E2 Ubc9 are embryonic lethal[371] and some SUMO-1 heterozygous mice are born with a cleft lip[372]. But the SUMO system is also emerging as a critical modulator in tumourigenesis and cancer treatment. Tumours engineered to overexpress a dominant-negative SUMO-E2 Ubc9 (with a point mutations at the critical cysteine residue 93) show increased drug sensitivity[373], reduced tumour growth and increased apoptosis[374]. Importantly, expression of Ubc9 and polymorphisms of the SUMO systems have also been associated with risk[375] and treatment prognosis[376] of certain types of cancers.

In line with its role in cancer, SUMOylation influences many crucial cellular functions such as the mitosis[377], DNA repair[378] and the induction of senescence[379]. Many of the SUMO target proteins are transcription factors, whose activity is generally thought to be repressed by SUMOylation[380]. However, SUMOylation can also induce transcriptional activity of some transcription factors[381]. Strikingly, SUMO-1 modification of chromatin was recently observed upstream of the transcriptional start of actively transcribed housekeeping genes[382], suggesting that SUMO might also play a role in the epigenetic regulation of gene transcription.

Particularly the highly dynamic conjugation of SUMO-2/3 is thought to play an important role in response to stress. Global SUMOylation is sensitive to even physiological levels of reactive oxygen species, which trigger a reversible disulfide bond between the E1 and E2 enzyme to be formed, completely blocking any SUMO-conjugation[383]. Furthermore, presence of SUMO-2 and SUMO-3 was found to be essential for survival of heat shock, which resulted in a global increase in SUMOylation[384]. A possible mechanism for this increased global SUMOylation is the finding that deSUMOylating enzymes are inactivated under heat shock[385]. It was furthermore described that misfolded proteins were generally marked by SUMO-2 modification[386]. The transfer of SUMO from the E1 to the E2 enzyme was recently found to be regulated by SUMOylation of lysines in close proximity to the catalytic cysteine residue 173 in the SUMO-activating enzyme SAE2[387]. Upon heat shock, the SUMO-E1 was deSUMOylated, leading to more efficient SUMOylation of its substrates[387].

While poly-ubiquitination is frequently associated with proteasomal degradation, such a defined role for SUMOylation does not exist. However, as mentioned earlier SUMOylation can also influence other posttranslational modifications, for example when recruiting SUMO-targeted Ubiquitin ligases. But SUMOylation is part of a complex crosstalk not just with ubiquitination, but also phosphorylation and acetylation.

The discovery of SUMO-targeted Ubiquitin ligases like RNF4[365], which were first identified in yeast[388], brought the Ubiquitin- and SUMO pathways closer together. In addition, many players of the Ubiquitin-system are regulated by SUMOylation: The Ubiquitin E2 E2-25K is SUMO-1 modified on lysine 14, resulting in inhibition of its Ubiquitin chain formation activity[389]. By contrast, the Ubiquitin-E3-ligase activity of BRCA1 is enhanced after SUMOylation by PIAS1 and PIAS4 in response to DNA damage[390,391]. Some deubiquitinating enzymes are also regulated by SUMOylation: The USP25 is modified by SUMO-2/3 within its ubiquitination interaction motif, which impairs its catalytic activity of hydrolysing Ubiquitin chains[392]. Another way, by which SUMOylation influences ubiquitination is by occupying the same lysine residue, which would usually be targeted for ubiquitination. Through this mechanism SUMOylation stabilises IκBα, an inhibitor of NF-κB[393].

Phosphorylation of serine and threonine residues within a phospho-dependent SUMO interaction motif can regulate the discrimination of the SUMO isoforms[358]. Moreover, phosphorylation of serine residues close to the SUMO consensus motif was observed to regulate the conjugation of SUMO to its targets. The phosphorylation-dependent SUMO motif consists of an extended classical consensus ΨKxExxSP, in which the serine residue is a target of proline-directed kinases[394]. It was proposed that phosphorylation introduced a negative charge interacting with a basic patch on the surface involving lysines 65, 74 and 76 of the SUMO-E2 Ubc9[395]. Via this mechanism, phosphorylation increases the SUMO-conjugation to lysines within phosphorylation-dependent SUMO motifs, as observed for example for the SUMOylation of heat shock factor HSF1[396] and PIAS1[359]. Phosphorylation outwith the extended consensus motif can also influence the rate of SUMOylation. In the case of p53 it was reported that phosphorylation at serine 20 impairs its association with Ubc9[397]. The notion of phosphorylation-dependent SUMOylation opens up new possibilities SUMO conjugation in stress response and signal transduction.

Like ubiquitination, acetylation also takes place on many of the SUMO-modified lysine residues. Interestingly, the SUMO conjugation system targets important players of the acetyltransferase system. For example SENP1 deSUMOylates the deacetylase HDAC1, which results in repression of its deacetylase activity towards the androgen receptor[398], while SENP3 deSUMOylates the acetyltransferase p300 and contributes to its activation[399]. Regulation of p300-mediated repression was furthermore found to be dependent on SUMO-1 modification, which initiates recruitment of deacetylase HDAC6[400]. In the case of p53, which is SUMOylated at lysine 386 in close proximity to lysine residues acetylated by p300, SUMO-1 modification was shown to block p300-mediated acetylation of its lysine residues 373 and 382, while acetylated p53 could still be SUMOylated[401]. Furthermore, it was reported that the N-terminus of SUMO-1 contains a domain similar to the acetylated region in p53's C-terminus and SUMO-1 itself was found to be acetylated at lysine residues 37, 38, 39, 45, 46 and 48. The conjugation of acetylated SUMO-1 to p53 had different consequences on p53's transcriptional activity than conjugation of non-acetylated SUMO-1[402]. Indeed, further studies revealed that acetylation of lysine residues K37, K39 and K46 in SUMO-1 and K33, K35, K42 in SUMO-2 alters the

basic surface of the SUMO protein, preventing its recognition by the negatively charged stretches in proximity of SUMO interacting motifs[403].

1.4.3 SUMO and p53

Most studies on p53-SUMOylation have so far focused on SUMO-1, which was shown to modify a single lysine (K386) residue residing within a SUMO consensus motif in the p53's C-terminus. However, despite 13 years of research consequences of SUMO-1 modification of p53-activity are still a matter for debate[404]. The first publications reported that SUMO-1 modification of p53 led to augmented transcriptional activity, showing increased activation of PG13 Luciferase when p53 was over-expressed together with SUMO-1[405,406]. Although the overall conclusion from both initial reports was that SUMOylation activated p53 transcriptional activity, it was noted in one report that the SUMO-site mutant p53 K386R activated the PG13 Luciferase reporter more strongly than wild-type p53[405], suggesting that disruption of the SUMO site could also increase transcriptional activity of p53. However, it needs to be taken into account that other modifications such as ubiquitination, neddylation and acetylation also take place on this particular lysine residue, with the K386R mutation affecting all of them. Surprisingly, the second study showed equal activation of a p21 Luciferase construct with wild-type and p53 K386R[406]. These discoveries sparked several contradicting reports on the functional implications of p53 SUMOylation such as showing no SUMO-dependent change of p53's activity in a CAT assay[407] and reduced induction of apoptosis by p53 K386R[408].

PIAS1 was the first protein identified as promoting the SUMO-1 conjugation of p53. It was furthermore observed that PIAS1 required its RING domain for this activity, however the functional implications of p53-SUMOylation were not investigated[409]. Another report, which identified PIAS1 and PIAS2 (also known as PIASx) as SUMO-E3 ligases for p53, found that overexpression of SUMO-1 and PIAS1/2 repressed p53's ability to activate a pRGC Luciferase reporter. Strikingly, the same down-regulation was observed when the SUMO-site mutant p53 K386R was used instead of wild-type p53, indicating that this repression of transcriptional activity was not due to SUMO-1 modification of lysine 386[410]. Interestingly, PIAS4 (also known as PIASy) had just been reported to prevent p53 from binding to DNA[411] and it is possible that

a similar mechanism led to the observed repression in p53-mediated transactivation in the presence of PIAS1 and PIAS2. Later, the expression of PIAS4 was shown to induce p53 modification with both SUMO-1 and SUMO-2. Co-expression of PIAS4 enhanced p53's activation of a p21 Luciferase reporter and SUMOylation of both p53 and Rb was linked to the induction of senescence[412]. Mono-ubiquitination of p53 by MDM2 was demonstrated to enhance SUMO-1 conjugation by PIAS4, leading to nuclear export of p53[413]. These observations were consistent with subsequent studies of a p53-SUMO-1 fusion protein, which showed partial cytoplasmic localisation[414].

Proteins outside the PIAS family were also reported to induce SUMO-1 modification of p53: Overexpression of the Ubiquitin-E3 ligase MDM2 together with its negative inhibitor $p14^{ARF}$ was shown to increase SUMO-1 conjugation of p53 in vivo, but not in vitro[415]. It was concluded, that the $p14^{ARF}$-MDM2 complex was relocating p53 to the nucleolus, where it would be SUMO-1 modified, however the cryptic nucleolar localisation signal in $MDM2^{214}$ was never mutated in order to fully test this hypothesis. A different RING-domain ligase called Topors, which also possesses Ubiquitin-E3 ligase activity towards $p53^{302}$, was shown to promote SUMO-1 conjugation of p53 in vivo and in vitro[416]. While ubiquitination was dependent on its RING finger, mutants without RING domain could still promote SUMOylation of p53[416]. Although co-expression of high levels of Topors induced p53's activation of the PG13 Luciferase reporter system, it was concluded that this was not due to p53 SUMOylation, since the p53-SUMO-1 level had already reached its maximum at lower Topors concentration which did not induce p53's transcriptional activity[416]. Furthermore, an adenovirus E1B 55-Kilodalton protein was also reported to confer SUMO-1 conjugation to p53, leading to its nuclear export and repression of its transcriptional activities[417,418]. New insights into other proteins not previously involved in the field of Ubiquitin like modifications came from a study on the protein kinase, interferon-inducible double stranded RNA dependent activator PRKRA (also known as RAX/PACT), which was found to efficiently induce SUMO-1 modification of p53. PRKRA co-expression enhanced p53's activation of a BP100 Luciferase reporter construct, while K386R mutation of p53 prevented double stranded RNA activated protein kinase PKR mediated phosphorylation at serine 392. The group therefore proposed a model, in which PRKRA-induced SUMO-1 conjugation of p53 stimulated

PKR-mediated phosphorylation of serine 392, leading to stabilisation and activation of p53 and ultimately cell cycle arrest in G_1[419].

Further advances on delineating the functional impact of p53-SUMOylation were made recently: An intricate study on SUMO-1 modification of p53 and its crosstalk with acetylation revealed that, while acetylated p53 can be SUMOylated, SUMO-1 conjugation blocks p300-mediated acetylation of the C-terminus[401]. Furthermore, SUMO-1 modified p53 could not bind DNA, while p53 that was both acetylated and SUMOylated did bind DNA. In line with these findings, the investigators found that p53 K386R induced a p21- Luciferase construct more strongly than wild-type p53 and was more present at the endogenous p21 promoter in a CHIP experiment[401], linking SUMOylation to repression of p53 activity. Complicating the SUMO-acetylation crosstalk even further, another group reported that SUMO-1 itself could be acetylated at its N-terminus and could thereby mimic C-terminal acetylation of p53. These investigators found that conjugation of non-acetylated SUMO-1 to p53 led to the activation of p53's cell cycle arrest target genes, while conjugation of acetylated SUMO-1 increased the apoptotic target genes[402]. Recently, Tip60-mediated acetylation of lysine 120 in p53 in combination with SUMO-1-modification of K386 was shown to act as a signal for cytoplasmic accumulation of p53 and lead to induction of autophagy[420].

In contrast to the plentiful studies on SUMO-1, relatively few studies have addressed SUMO-2/3 modification of p53. Overexpression of SUMO-2/3 was shown to induce senescence in a p53 and Rb-dependent manner[421]. Both proteins were found to be SUMO-2/3 conjugated and SUMO-2/3 modification of p53 on lysine 386 was reported to be induced by H_2O_2 treatment of cells[421]. In line with these findings, overexpression of PIAS4 also induced senescence via p53 and Rb[412]. But while PIAS4 was shown to modify p53 with both SUMO-1 and SUMO-2[412], the viral protein K-bZIP from Kaposi's sarcoma associated herpes virus showed specificity towards SUMO-2/3 conjugation of p53[422]. Co-expression of K-bZIP enhanced p53's induction of the 4xBS2WT Luciferase reporter, dependent on the presence of its SUMO-2/3 specific SIM[422]. A recent report demonstrated a transcription-independent role for SUMO-3 conjugation of p53 in disturbed-flow induced apoptosis of endothelia cells: the protein kinase PKCζ was induced by peroxynitrite

and subsequently promoted SUMO-3 conjugation of p53. The SUMO-3 conjugated p53 was then exported into the cytoplasm where it bound pro-apoptotic Bcl-2, leading to the induction of apoptosis[423].

Clearly, SUMO-2/3 conjugation of p53 is a poorly understood area with opportunities for exciting discoveries. Furthermore, the p53 regulators MDM2 and $p14^{ARF}$ also play a significant role in the SUMO system.

1.4.4 MDM2, $p14^{ARF}$ and SUMO

The investigation of MDM2-SUMOylation was unfortunately misled by a report published by Buschmann et al in 2000 claiming that MDM2 was SUMO-1 modified at lysine 446, leading to MDM2's stabilisation and increased ubiquitination of p53. The results subsequently turned out to be non-reproducible and the paper was retracted[424]. Although some attempts were made since, the site of SUMO-conjugation has still not been definitively identified.

One study, reporting that $p14^{ARF}$ induces SUMO-1 conjugation of MDM2 limited the SUMO-site to an area in the N-terminus between amino acids 134 and 212[425]. Other investigators used PIAS1, PIAS2 and RanBP2 to enhance SUMO-1 modification of MDM2 and found that mutation of lysine 182 to arginine stopped SUMOylation in vivo, but not in vitro. However, this effect was probably due to the K182R mutant protein's cytoplasmic localisation, since this lysine residue is located in the nuclear localisation motif[426].

It was later observed that MDMX could inhibit $p14^{ARF}$-mediated MDM2-SUMOylation[427] and around the same time a group discovered that $p14^{ARF}$ was involved not only in the SUMOylation of MDM2, but stimulated the SUMOylation of many of its binding proteins including E2F1 and HIF-1α by directly interacting with the SUMO-E2 Ubc9[428]. The role of $p14^{ARF}$ in the SUMO system was further strengthened by the notion that it could induce the degradation of the deSUMOylating enzyme SENP3, resulting in global changes of SUMOylation[429].

A different deSUMOylating enzyme, SUSP4, a mouse homologue of SENP2, was found to remove SUMO-1 from MDM2 upon UV treatment. The deSUMOylation

induced MDM2 auto-ubiquitination and stabilisation of p53[430]. Other proteins found to enhance MDM2-SUMOylation were the TRIM family members, in particular TRIM27[431], and the proto-oncogene SKI[432]. Consistent with the destabilisation of MDM2 upon deSUMOylation, both reports observed an enhanced MDM2 stability when SUMOylated and a subsequent decrease in p53.

In contrast to p53, where the SUMO site was identified straight away, but the functional consequences are still a matter of debate, multiple studies agree that SUMOylation of MDM2 affects its stability, yet the site of SUMO modification is still unclear. Surprisingly, no reports of SUMO-2/3 modification of MDM2 exist to this date.

1.5 The p53 family

Two p53-related genes called p63[433] and p73[434] were identified 20 years after the discovery of p53 and classed as a family of transcription factors based on their homology with p53. p63 and p73 can induce some p53 target genes, but also have other roles for example in the embryonic development.

All p53 family members, are active as tetramers[435,436]. Strikingly, the structure of the p63 and p73 tetramerisation domains contains an additional α-helix, which is not present in the p53 protein. This allows p63 and p73 to form heterotetramers (heterodimers of two homodimers), while p53 cannot oligomerise with either of them[437]. However, cancer-associated mutants of p53 do bind and inhibit p63 and p73 and this is a potential mechanism, by which tumours can silence all three transcription factors at once by only mutating one gene.

1.5.1 p63

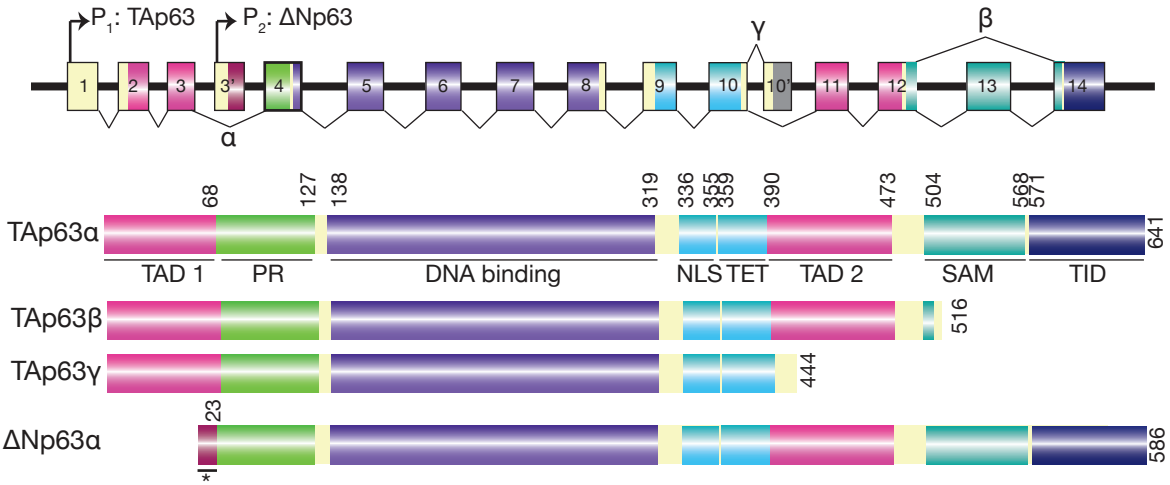

Figure 1-22: p63 isoforms.
Isoform β results from skipping of exon 13, resulting in alternative reading of exon 14, which presents a stop codon after 5 codons. Isoform γ lacks exons 11, 12, 13 and 14, ending on a 38 amino acid sequence from intron 10. ΔNp63 is transcribed from an alternative promoter in intron 3 and the resulting protein lacks the first 69 amino acids of TAp63, but starts with 26 different amino acids, harbouring an alternative transactivation domain (*).
TAD: transactivation domain, PR: proline rich domain, NLS: nuclear localisation signal, TET: tetramerisation domain, SAM: sterile α motif, TID: transcription inhibitory domain.

The p63 gene is located at chromosome 3q27-26 and contains 265822 nucleotides spanning 14 exons[433,438-440]. The gene gives rise to a transcription factor with a domain structure similar to p53: an N-terminal transactivation domain, a central DNA binding domain and a tetramerisation domain. Particularly the DNA binding domain is highly homologous to p53 with 58% identical amino acids. The active transcription factor forms a dimer of dimers, just like p53[435]. The full-length p63 protein is considerably longer than p53 (641 amino acids) and contains a sterile α motif (SAM) at its C-terminus, which is not present in p53. The SAM domain forms a bundle of five helices that serve as platform for protein interactions[441]. Interestingly, SAM-like domains are often found in proteins involved in development[442].

Many different isoforms can be derived from the p63 gene due to an internal promoter and alternative splicing of the C-terminus. An internal promoter is located in intron 3 and gives rise to ΔNp63 isoforms, which lack the transactivation domain present in the full-length TAp63 isoforms, but contain an alternative stretch of 26 amino acids not present in the TAp63 isoforms[433]. Two different coding sequences for the TAp63 isoform are available in the nucleotide sequence collection database, differing in the ATGs used for translation. The shorter protein, starting at the second

ATG, resulting in a 39 amino acid shorter N-terminus (accession number AF075430 for TAp63α) is considered to be the expressed full-length TAp63 in this study, since it results in a protein with a transactivation domain homologous to p53 and p73. The TAp63 isoforms can bind the p53 response element and induce p53 target genes such as p21[440]. However, the preferred TAp63 response element differs slightly from the p53 response element: While p53 preferentially binds to RRRCATGYYY, TAp63 preferentially binds to RRRCGTGYYY[443] and consequently p63 target genes were identified, which could be induced by TAp63, but not p53, for example Bullous pemphigoid antigen (BPAG1)[444]. At first, the ΔNp63 isoforms were handled as purely negative regulators of the TAp63 isoforms, since they lacked a transactivation domain[433] and could not bind the p53 response element. However, specific ΔNp63-inducible target genes were discovered[445,446] and consistently, two alternative transactivation domains were identified, one in the first 26 amino acids of the ΔNp63 isoforms[445] and another one between amino acids 410 and 512 in the C-terminus[447].

Alternative splicing in the C-terminus gives rise to 3 different isoforms called α, β and γ with α being the longest isoform[448]. p63β lacks exon 13, which encodes for the SAM domain and ends after the second transactivation domain, since the alternative reading of exon 14 presents a stop, resulting in a protein roughly 100 amino acids shorter than p63α. p63γ lacks exons 11, 12, 13 and 14, ending after the tetramerisation domain. TAp63γ is the isoform most similar to p53. Two more isoforms have been predicted, but not yet been confirmed: p63δ as a product from skipping exons 12 and 13 and p63ε resulting from termination of transcription after exon 10, where intron 10 presents a stop codon[448]. All C-terminal splice variants can occur in the TA or ΔN version, resulting in 10 different p63 isoforms. Only the p63α isoforms contain the SAM domain and a following inhibitory sequence, which was shown to fold back to interact with the N-terminal transactivation domain, rendering TAp63α a much less potent transcription factor than TAp63β and TAp63γ[449]. The inhibitory core element in TAp63α has been narrowed down to an amino acid stretch RFTLRQTISF between amino acids 604 and 613[450]. Strikingly, this inhibitory domain can be cleaved off by Caspases in response to stress[451].

While p63 can induce cell cycle arrest and apoptosis by up-regulating p53-target genes, it clearly has a critical role distinct from p53 in regulating epithelial

development. p53 null mice are predominantly born without major developmental malformations other than a neural tube closure defect in about 20% of female embryos[452], and survive until they develop tumours, whereas p63 null mice are born with limb truncation, craniofacial malformation and no epidermis and only survive a few days after birth[453,454]. The importance of p63's role in limb development is mirrored by a number of human diseases caused by mutations in the p63 gene. Heterozygous germ-line missense mutations in the p63 DNA binding domain can lead to the EEC syndrome with ectrodactyly (hand/feet malformation), ectodermal dysplasia and facial clefts[455], while amino acid substitutions in the SAM domain lead to the AEC syndrome with ankyloblepharon-ectodermal dysplasia and clefting[456]. The scale of developmental abnormalities caused by this mutation is remarkable, since mutations in the SAM domain only affect the p63α isoforms.

Consistent with the role of p63 in skin development, p63 expression was initially found to be restricted to the nuclei of the basal cells in epithelia[433]. Furthermore, ΔNp63 was shown to be required to maintain the proliferative potential of keratinocytes[457] and stem cells of stratified epithelia[458]. Most tissues express higher levels of the ΔNp63 isoforms, however more recently TAp63 has been found to be highly expressed in oocytes and shown to have a role in protecting the female germ-line by inducing apoptosis in oocytes upon DNA damage[459,460].

Studies using isoform-specific mouse models have allowed to delineate the roles of TAp63 and ΔNp63 in further detail. A conditional TAp63 knockout mouse (Exon 2) in the epidermis showed no abnormality, however a germ-line knockout of TAp63 led to premature aging of the skin with formation of blisters and abnormal hair morphogenesis. The TA-specific knockout did not result in any limb developmental defect and survived into adulthood, although premature ageing resulted in a decreased life span compared to wild-type littermates[461]. Inducibly deleted ΔNp63 mice (by induction of ΔNp63-specific siRNA) were also viable, but showed skin fragility due to an abnormal basement membrane[462]. An isoform-specific reconstitution study revealed that expression of ΔNp63 could restore epidermis formation of p63 null mice, while TAp63 expression could not[463].

In contrast to p53, p63 is rarely mutated in cancer[464], although it is frequently found to be deregulated. A large fraction of squamous cell carcinomas of the head and neck express high levels of ΔNp63[465]. Despite the high expression levels in tumours, ΔNp63 amplification was correlated with good treatment response[466]. TAp63 levels are not detectable in many neoplasias, but lymphomas form an exception expressing only TAp63 and no ΔNp63[467]. While p63 null mice do not live long enough to develop cancers, p63 heterozygous mice were shown to develop some spontaneous tumours, mostly squamous cell carcinomas and histiocytic sarcomas[468]. Importantly, most of the tumours had lost the wild-type p63 allele, suggesting that p63 poses a barrier to tumour development. This is consistent with recent work that loss of p63 is associated with an invasive phenotype[469]. Indeed, 50% of tumours arising in $p53^{+/-}$;$p63^{+/-}$ mice were metastatic while only 5% of tumours arising in $p53^{+/-}$ mice had metastasised[468].

1.5.2 p73

The p73 gene is located on chromosome 1p36.3 and includes 80728 nucleotides spanning 14 exons. Like p63, the p73 gene also gives rise to a transcription factor highly homologous to p53 with 62% identical amino acids in the DNA binding domain, 18% in the N-terminal transactivation domain and 22% in the tetramerisation domain[470].

A number of different isoforms are transcribed from the p73 gene. The full-length protein TAp73 can induce p53-responsive genes such as p21, MDM2 and PIG3, just like the other p53-family members[471-473]. Overexpression of p73 was shown to induce apoptosis with some isoforms being even more efficient than p53[471,474]. Surprisingly, the ΔNp73 isoforms, which are product of an internal promoter at intron 3 and lack the N-terminal transactivation domain, were only described five years after the identification of the p73 gene[475]. Just as in the case of ΔNp63, the ΔNp73 isoforms were initially shown to inhibit the transcriptional activity of TAp73, and specific ΔNp73-responsive genes were identified later[476]. In line with this finding, two domains required for ΔNp73's transactivation capability were identified: one comprising the unique first 13 amino acids, which are not present in TAp73[476] and another one in the C-terminus between amino acids 381 and 399[477]. Interestingly, while the N-terminal

transactivation domain of TAp73 was found to be crucial for the induction of apoptotic target genes, the C-terminal transactivation domain was sufficient to induce cell cycle arrest[477]. Two further N-terminally truncated splice variants of p73 were described, which are the result of alternative splicing skipping exon 2 or exons 2 and 3 resulting in proteins lacking the first 48 and 71 amino acids, respectively[478].

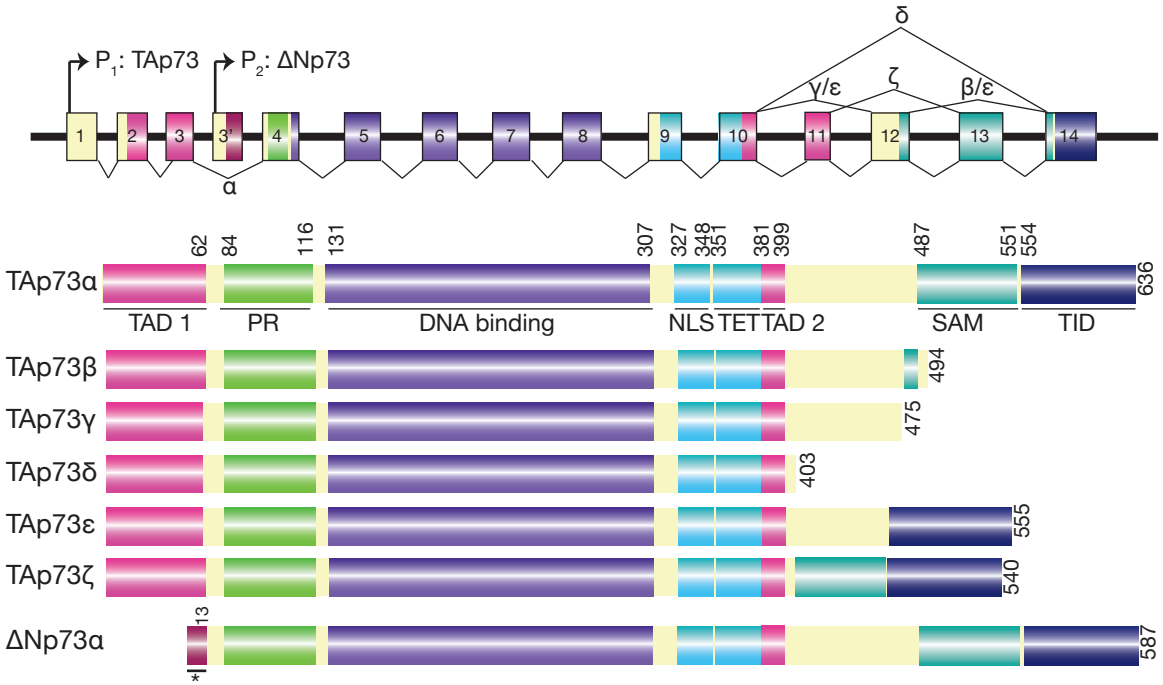

Figure 1-23: p73 isoforms
Isoform β results from skipping of exon 13, resulting in alternative reading of exon 14, which presents a stop codon. Isoform γ lacks exons 11, reading exons 12, 13, and 14 in an alternative reading frame. Isoform δ lacks exons 12, 13 and 14. Isoform ε lacks isoform 11, resulting in alternative translation of exons 12, additional splicing of exon 13 reverts the frame-shift. Isoform ζ lacks exon 12. ΔNp73 is transcribed from an alternative promoter in intron 3 and the resulting protein lacks the first 62 amino acids of TAp73, but starts with 13 different amino acids, harbouring an alternative transactivation domain (*).
TAD: transactivation domain, PR: proline rich domain, NLS: nuclear localisation signal, TET: tetramerisation domain, SAM: sterile α motif, TID: transcription inhibitory domain.

Like the entire p53-family, p73's C-terminus is subject to alternative splice events giving rise to the full-length α-variant and five other shorter isoforms named β (lacking exon 13)[434], γ (lacking exon 11), δ (lacking exons 11, 12 and 13)[479], ε (lacking exons 11 and 13) and ζ (lacking exons 11 and 12). Combined with the four N-terminal variations at least 24 different proteins can be translated from the p73 gene. Deletion of exon 11 causes a frame-shift, due to which p73γ expresses a different 75 amino acid C-terminal tail. A frame-shift, which results in an alternative exon 12 reading is reverted by deletion of exon 13 in p73ε. p73ζ has an internal deletion of amino acids 400 to 496, but retains the α-tail, while isoforms β, γ and δ terminate on a short

alternative translation of exon 14[480]. Like p63, full-length p73 also contains a sterile α motif. Exon 13 encodes the SAM domain and consequently only the α and ζ isoforms contain this motif. Similar to the TAp63α isoform, TAp73α is less transcriptionally active than TAp73β. In contrast to TAp63α, whose C-terminal inhibitory domain was shown to interact directly with the N-terminal transactivation domain, both the SAM domain and a following inhibitory domain are required for repressing the activity of TAp73α. Surprisingly, the inhibitory core sequence identified in p63α's C-terminus[450] is not present in the p73α tail and the C-terminus of p73α does not seem to contact the N-terminus[481]. Indeed, TAp73α is still much more potent in activating its target genes than TAp63α, possibly representing this difference in their C-terminal inhibitory domains.

Similarly to p53, the p73 protein is stabilised by proteasome inhibitors, suggesting that it is constantly degraded. While MDM2 can interact with p73 (see 1.5.4), it is not ubiquitinated by this E3 ligase[482,483]. Instead, the p73 protein is targeted for degradation via ubiquitination by the E3-ligases Itch[484], which also targets p63[485], and Pirh2[486,487], which also ubiquitinates p53[300]. p53 induces expression of Pirh2, thereby negatively controlling expression levels of p73, while p73 induces MDM2, leading to degradation of p53[488]. However, ΔNp73 protein levels decrease in response to DNA damage, suggesting a separate regulation from TAp73[489]. Indeed, Pirh2 only affects TAp73, but not ΔNp73 protein levels[486,487]. In contrast, the Ubiquitin-ligase p73-induced RING2 protein PIR2, only targets ΔNp73 for degradation[490]. TAp73 not only induces degradation of ΔNp73, but also transcriptionally activates its own internal promoter, inducing expression of ΔNp73[491].

p73's activity is induced by oncogenes like Myc, E1A and E2F1[492] and in response to DNA damage, thereby supplementing p53 function. E2F1 directly initiates transcription of p73[493], while in response to γ-irradiation, p73's tyrosine 99 is phosphorylated by ATM-induced kinase c-Abl, promoting p73-dependent apoptosis[494,495]. Additionally, p73 activity is regulated by the cell cycle due to p73's Cyclin-binding motif. In the G_2/M phase threonine 86 of p53 is phosphorylated by Cdk1 and Cdk2[496], inhibiting p73's transcriptional activity and preventing a p73-induced cell cycle arrest while the DNA is being replicated and therefore vulnerable.

However, p73 also fulfils functions distinct from p53, particularly in the neuronal development. This role is demonstrated by p73-null mice that showed hippocampal dysgenesis with a selective loss of bipolar Cajal-Retzius neurons[497]. The animals furthermore suffered form cerebrospinal fluid hyper-secretion and abnormal reproductive and social behaviour, reflecting dysfunction in the pheromone sensory pathway due to a defect in the vomeronasal organ, which expresses high levels of ΔNp73[497]. High levels of ΔNp73 were also found in the mouse brain and it was suggested that this inhibitory p73-isoform protected neurons from undergoing p53-induced apoptosis[498]. Consistently, ΔNp73 null neurons were found to be hypersensitive to DNA damage, while ΔNp73 was proposed to directly localise to sites of DNA damage, where it prevented ATM-induced p53-activation[499]. Interestingly, an isoform-specific TAp73-knockout mouse still developed some hippocampal defects, albeit not as severe as the p73 null mouse, suggesting that TAp73 contributes to the neuronal development[500]. Strikingly these mice also showed infertility, genomic instability and development of spontaneous tumours[500]. Although the initial p73 null mouse was reported not to be tumour-prone[497], some p73 heterozygous mice were found to develop benign and malignant lesions, most commonly lung adenocarcinomas, haemangiosarcomas and thymic lymphomas[468]. Strikingly, 45% of tumours arising in $p53^{+/-};p73^{+/-}$ were metastatic as compared to 5% metastatic tumours in $p53^{+/-}$ mice, suggesting that p73 just like p63 has a role in protecting from metastasis[468].

Despite the p73 gene being identified in a chromosomal region frequently lost in neuroblastoma[434], the second p73 allele is rarely in lost cancers. No tumour-associated p73-mutations could be identified and on the contrary many ovarian cancers were found to overexpress p73[501,502]. In contrast to normal tissue, which predominantly expresses p73α and p73β, neoplastic lesions frequently express shorter C-terminal splice variants[503] or ΔNp73[504]. Strikingly, ΔNp63 amplification in head and neck squamous carcinoma was shown to silence TAp73 activity[505] and it was found that N-terminally truncated isoforms of p63 and p73 can each inhibit both TAp63 and TAp73. In some cases, particularly in lymphomas and leukaemias, the p73 promoter is hyper-methylated and p73 transcription ablated without loss or mutation of the gene[506,507].

p73 activity can furthermore be counteracted by a panel of interacting proteins, such as iASPP, which inhibits activation of p53- and p73 induced apoptosis[508], mTOR[509], certain p53 mutants (see 1.6.2) and MDM2, which inhibits p73's transcriptional activity[482,483,510].

1.5.3 MDM2 and p63/p73

MDM2's N-terminus interacts with the p53 N-terminus, thereby shielding p53's transcriptional domain and promoting ubiquitination of the p53 protein[107,108,114,135]. Despite relatively low homology (around 30%) of the transactivation domain across the p53 family proteins, the three amino acid residues phenylalanine 19, tryptophan 23 and leucine 26, which were identified to be crucial for p53's interaction with MDM2[163,511], are also present in the N-termini of the TAp63 and TAp73 proteins. Consequently, several studies were conducted, analysing the ability of p63 and p73 to interact with MDM2.

The majority of reports suggest that MDM2 can interact with both TAp73α and TAp73β via their N-termini homologous to p53 and that this interaction silences TAp73's transcriptional activity, but does not result in TAp73 degradation[482,483,510]. While it is conceivable that TAp73's transactivation domain is simply shielded just as in the case of p53[114], another model suggests that MDM2 prevents the co-activator p300 from binding to TAp73's C-terminus and thereby impairs transactivation[483,512]. A recent study revealed that MDM2 could promote neddylation of TAp73β, which resulted in its nuclear export, possibly contributing to the silencing of p73's transcriptional activity[513]. Curiously, MDM2 overexpression was not previously shown to induce p73 export, although it is well known to induce export of p53[153,167]. In contrast, MDM2 co-expression with TAp73 triggered accumulation of both proteins in nuclear speckles distinct from PML bodies[514,515]. While some publications suggest that p73 protein levels are not affected by MDM2[482,510], other studies found MDM2 co-expression to increase TAp73 levels[483,516] with one study consequently reporting MDM2-mediated induction of TAp73α and TAp73β activity[516]. By contrast, a different recent study found MDM2 to promote association of p73 with the E3-Ubiquitin ligase Itch to promote TAp73α degradation[517]. However, the MDM2-Itch complex and

subsequent p73 degradation was only observed in HeLa cells, but not in H1299 cells, suggesting, that this might be a cell-type specific effect[517].

In contrast to TAp73, TAp63-MDM2 interaction was only observed in a few studies[518,519], while most reports agree, that MDM2 does not form a complex with TAp63 in cells[520-523]. The most compelling evidence comes from an intricate biochemical analysis of the p53, p63 and p73 transactivation domain peptides binding to MDM2. A number of different in vitro assays revealed that the p63 peptide bound MDM2 with at least one order of magnitude weaker affinity than p73 and p53, making it unlikely for the interaction to occur under endogenous circumstances[523]. This finding is striking, since the p63 transactivation domain contains the same conserved amino acids as p73 and p53, which were found to be critical for interaction with MDM2.

1.6 Mutant p53: an oncogene?

p53 poses a major barrier against cellular transformation and all cancers have escaped p53's control by some mechanism. In the majority of tumours, p53 is inactivated by mutations in the coding sequence. This accounts for 50% of all cases, although the prevalence of p53 mutations are dependent on the tumour type, from only 10% of leukaemia to 60% of ovarian cancers and colorectal cancers harbouring p53 mutations[524]. Other mechanisms of p53 inactivation were also observed: Around 7% of tumours contain MDM2 gene amplifications[525] and the prevalence depends on the tumour type: up to 30% of soft tissue sarcomas were shown to harbour MDM2 amplifications[526]. Another tumour strategy to avoid induction of p53, is losing the MDM2-inhibitor $p14^{ARF}$, which is involved in sensing oncogene activation. $p14^{ARF}$ is expressed as alternative reading frame the INK4A locus, which is mutated in roughly 50% of all tumours[527], leading to loss of expression of both $p16^{INK4A}$ in the Rb pathway and the MDM2-inhibitor $p14^{ARF}$. The $p14^{ARF}$ promoter is furthermore frequently found to be epigenetically silenced by hyper-methylation[528].

While most cancer patients harbour somatic p53 mutations in their lesions, very few individuals express a mutant p53 allele in all their tissues since they carry a heterozygotic germline p53 mutation. Germline p53 mutations are associated with the Li-Fraumeni-syndrome[529], which is defined by development of sarcomas under

the age of 45, with first and second degree relatives also suffering from early-onset cancers[530]. The most common tumours observed in Li-Fraumeni families are sarcomas, brain and breast cancers and leukaemia[531]. Strikingly, somatic p53 mutations occur with a different prevalence spectrum than germline p53 mutations, codon R337 in p53's tetramerisation domain is frequently mutated[532], while the most frequently somatic mutations all occur in the DNA binding domain.

The vast majority of p53 mutations (74%) are missense mutations leading to the expression of a full-length p53 protein with a single amino acid exchange[524]. Indeed, expression of these mutant p53 proteins is so common that the initially identified p53 was thought to be an oncogene[298,299], since it harboured a mutation in the DNA binding domain[533]. In the case of somatic mutations, most amino acid exchanges occur within exons 5 to 8 encoding for the DNA binding domain. Although every single amino acid of the DNA binding domain was observed to be mutated, 30% of these mutations are very rare[534]. However, the mutation frequency in the p53 database is biased towards the DNA binding domain, since frequently only the area of the DNA binding domain was sequenced when mutational status was assessed, missing out on the roughly 14% of mutations which occur outside exons 5 to 8[535].

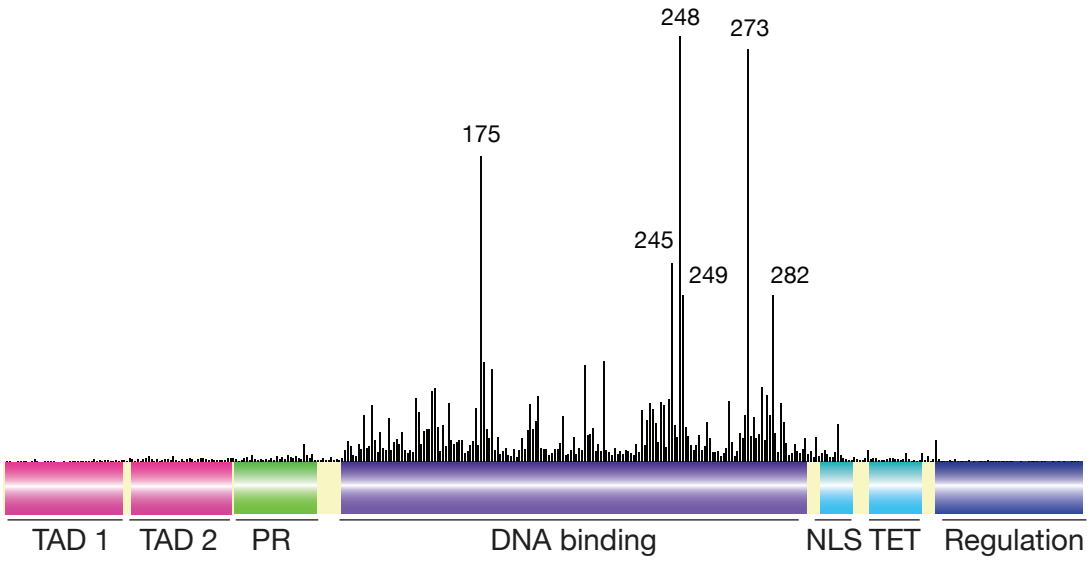

Figure 1-24: Prevalence of somatic p53 mutations.
Most p53 mutations cluster to codons of the DNA binding domain. Particularly frequently mutated codons, the so-called "hotspots" are annotated with their respective codon numbers.

Only 6 amino acids in the DNA binding loops L2 and L3 account for a third of all somatic p53 mutations observed. These “hotspot” codons are arginine 175, glycine 245, arginine 248, arginine 249, arginine 273 and arginine 282 (Figure 1-24).

Mutants can be separated into 2 categories: amino acids, which are directly contacting the DNA, such as arginine 273 and arginine 248, and amino acids which are structurally critical with their mutation resulting in changes of local or overall conformation such as glycine 175, arginine 245 and arginine 282[116]. These mutations have in common that they disrupt p53’s sequence-specific DNA binding and therefore lose wild-type p53’s ability to transcriptionally regulate its target genes.

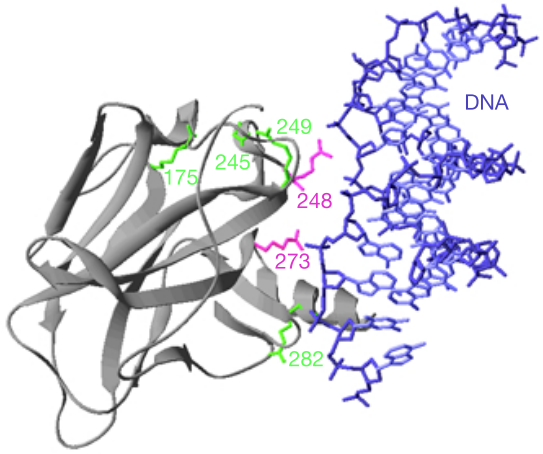

Figure 1-25: Ribbon model of p53 DNA binding domain in complex with DNA.
DNA contacting amino acids R248 and R273 in pink and structurally important amino acids 175, 245 and 282 in green. Protein database accession number 1TUP[116].

The p53 mutant proteins are often expressed to very high levels in tumours[536], leading to the assumption that the mutant p53, in contrast to wild-type p53 is a stable protein. Since wild-type p53 induces MDM2, but mutant p53 has lost this ability, it seems plausible that mutant p53 could accumulate to higher levels, since it has lost this feedback regulatory mechanism. Indeed, high p53 staining as assessed by immunohistochemistry (IHC) is often judged as an indication for p53 mutations[537]. However, a study comparing staining by IHC to sequencing of the gene found that p53 protein levels only correctly indicated p53 mutations in 70% of all cases[538]. In line with this analysis, mutant p53 was found not to be intrinsically stable in normal tissue of Li-Fraumeni patients[539] and knock-in mouse models[540,541]. Actually, mutant p53 could still be degraded by MDM2[107,542-544] and knockout of MDM2 stabilised mutant

p53 in the R172H (mouse equivalent of R175H) p53 knock-in mouse[545]. The mechanism, by which transformed cells stabilise the mutant p53 protein is not clear yet, it was hypothesised that similarly to wild-type p53, mutant p53 can be stabilised by stress, which prevails in tumour cells[540].

1.6.1 Loss of function

Consistent with the observation that cancer-associated p53 mutations abrogate the transcription factor's sequence-specific binding, hotspot mutant p53 proteins could not induce p53 target genes, while other mutants of the DNA binding domain retained the activity to induce some promoters[546]. It was observed that mutant p53 could also inhibit the induction of p53-target genes in the presence of wild-type p53[547-549]. This suggested that mutant p53 acted in a dominant negative fashion, not only losing its own function, but also suppressing the wild-type p53 activity. Mechanistically this could be due to mixed wild-type - mutant p53 tetramers. Indeed, studying complexes of wild-type and the temperature sensitive A135V mutant p53, it was revealed that wild-type p53 adopted an unfolded conformation in a complex with mutant p53[550]. Conformation of the p53 DNA binding domain can be assessed by two conformation-specific antibodies (Figure 1-26): pAb 1620 recognises an epitope on the protein surface of the natively folded DNA binding domain created by arginine 156, leucine 206, arginine 209 and asparagine 210[551] and pAb 240 recognises an epitope on the S7 β-strand, which is buried in the wild-type conformation, but exposed upon unfolding of the DNA binding domain[552].

By contrast, a study expressing equal levels of wild-type and mutant p53 from a bicistronic plasmid revealed that mutant p53 (mutations at codons 143, 245 or 258) could not suppress wild-type p53 activity[553]. Consistently, it was later reported that three mutant proteins harbouring the R273H or R249S mutations were needed to inactivate a tetramer, while one p53 lacking the N-terminal domain was enough to inactivate a tetramer[554]. This suggests that mutant p53 is only dominant negative when expressed at high levels that are frequently found in neoplastic lesions. The suppressive activity of p53 mutants might not just be dependent on the type of mutation, but also on the target gene promoter, since a study using equal wild-type and mutant p53 expression found, that some p53 mutants (mutations at codons

245, 248 or 273) could only act dominantly negatively on the Bax promoter, but not on the p21 promoter[555].

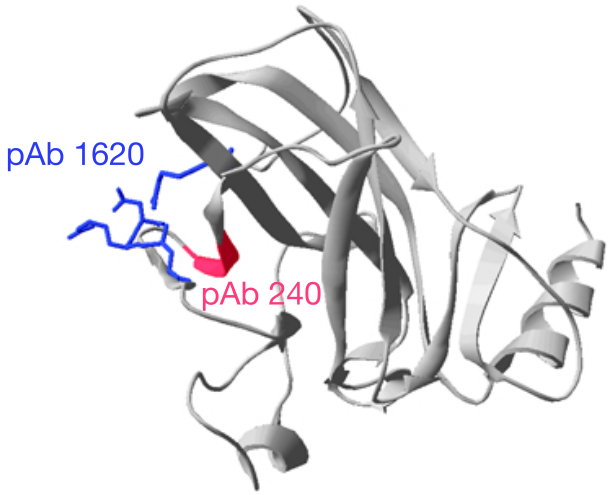

Figure 1-26: Conformational epitopes on p53's DNA binding domain.
pAb1620 detects an epitope present only on natively folded p53 (blue), while pAb 240 detects an epitope only exposed in the unfolded conformation (pink). Protein database accession number 1TUP[116]

The notion that mutant p53 is not as dominant as initially assumed is supported by the observation that the wild-type p53 allele is often lost in tumours expressing mutant p53[556]. Indeed, survival of heterozygous knock-in mice expressing one wild-type p53 allele and one R172H mutant p53 allele[540,541] resembled the heterozygous knockout mice with 15 months median survival rather than p53 null mice with 4 months median survival[25,557], suggesting that one mutant p53 allele was not enough to silence the wild-type p53 activity. Furthermore, MDM2-null mice can be rescued by knockout of both copies for p53, but not by knock-in of one R172H mutant p53 allele[540].

Despite the same survival of the heterozygous knock-in and the $p53^{+/-}$ mice, a different tumour spectrum was observed. Animals with a p53 R172H allele (equivalent to the human p53 R175H) developed osteosarcomas and those expressing p53 R270H (equivalent of human p53 R273H) developed adenocarcinomas[541]. These tumours were rarely seen in p53 knockout mice, which develop predominantly lymphomas and sarcomas[25,557]. Furthermore, 25% of tumours were found to metastasise, particularly in animals harbouring the p53 R172H allele[540,541], while tumours of $p53^{+/-}$ mice did not metastasise[25,557]. This observation

raises the possibility of the mutant p53 protein acquiring new oncogenic potential rather than simply losing and suppressing wild-type p53 function.

1.6.2 Gain of function

It had already been noted a decade earlier that introducing mutant p53 into p53 null cells increased their transformation potential, which was described as gain of function[558]. The notion that a single amino acid exchange can turn a tumour suppressor into an oncogene is striking and raises questions as to how mutant p53 molecules acquire new functions such as increased tumourigenicity[558], proliferation[559], interference with cell differentiation[560], genomic instability[561], resistance to drug treatment[562] and increased aggressiveness and metastasis[540,541]. Several mechanisms seem to contribute to mutant p53's oncogenic activity and a number of mutant-p53 specific binding proteins and target genes have been identified.

Supporting the model of mutant-p53 induced transcription of genes was the observation that the mutant p53 protein required its N-terminal transactivation domain to exert its gain of function effect. Deleting two glycine residues (glycines 22 and 23), which were shown to interact with the transcription machinery[563], suppressed the increased tumourigenicity conferred by the mutant p53 protein[562]. It is a striking thought that a protein with an unfolded DNA binding domain (or which lost DNA-contacting amino acids) could be able to act as a transcription factor in the traditional sense. A mutant p53 response element could not be identified due to lack of similar regions in the promoters of activated genes and this struggle is consistent with the view that mutations in the DNA binding domain eradicate the sequence-specific binding of p53. However, mutant p53 activated genes commonly contain AT-rich unfolded matrix attachment regions[564] or G/C rich G-quadruplex structures[565] and it is now thought, that mutant p53 recognises secondary DNA structures rather than binding to specific sequence-determined response elements[566].

Mutant p53 was found to upregulated the Multi-drug transporter MDR1[567], which can confer chemoresistance by pumping drug molecules out of cells. Activation of MDR1 by mutant p53 depended on an intact transactivation domain, as a p53 D281G mutant with additional glycine 22/23 mutation could not induce MDR1[568] anymore.

The activation of MDR1 also required mutant p53 to interact with the ETS-1 transcription factor[569]. Thus, binding other transcription factors could be another mechanism for mutant p53 to circumvent its deficiency in sequence-specific DNA binding. A striking example of the complete inversion of regulation comparing wild-type and mutant p53 is the regulation of G_2/M cell cycle genes via the NF-Y transcription factor. While wild-type p53 binds NF-Y and recruits the Histone deacetylase HDAC1 to repress genes such as Cdk1, Cyclin A_2 and Cyclin B_1, contributing to a G_2-arrest[240], the mutant p53 protein also binds NF-Y, but recruits p300 instead of HDAC1, thereby initiating the target genes and driving proliferation[570].

Interestingly, many oncogenic functions acquired by mutant p53 require the presence of its C-terminus[571]. A number of protein interactions were mapped to this region, leading to gain of function via the transcription factor ETS-2[572] and Plk2 kinase[573]. Induction of c-Myc by mutant p53 also required p53's C-terminal domain[574].

Two other transcription factors interacting with mutant p53 are its family members p63 and p73. While wild-type p53 cannot bind either of these proteins, multiple studies have shown that mutant p53 proteins can interact with both TAp63[575-577] and TAp73[134,575,577-581]. Importantly, binding of mutant p53 suppresses activation of p53 target genes induced by both TAp63 and TAp73[575,576,578,579]. Since both p63 and p73 also have roles in inducing apoptosis[582], mutant p53 not only abrogates wild-type p53 function, but also inhibits its "back-up" partners p63 and p73[583], thereby leading to a worse outcome than loss of p53 alone. One study suggested that the polymorphism at codon 72 could affect the mutant-p53 interaction[134] and it was consequently found that mutant p53 R72, which bound more strongly to p73, resulted in a worse response to chemotherapy[584]. However, other studies could not find a link between the polymorphism and p73 binding[580] or therapy response[585].

Several reports agree, that the core domain of p53[575,576,579,581] interacts with the DNA binding domains of p63[576] and p73[579], however the extent to which p53's DNA binding conformation influences this interaction has been controversial. While some groups reported that both conformational and DNA contact mutants (R273 and

R248) interacted with p63 and p73[576,578], other groups found a correlation of the DNA binding domain unfolding (measured by reactivity with the pAb240 antibody) with the extent of interaction with p63 and p73 and reported DNA contact mutants to bind less efficiently[134,575]. Even wild-type p53, which was experimentally unfolded was shown to bind p73 and the conformation of the DNA binding domain was postulated as only determinant of the interaction[581]. Although the interaction is taking place at the core domain, the C-terminus of mutant p53 was required for promoting invasion towards EGF via TAp63[586]. The repression of TAp63α-activated Luciferase was also affected by deletion of mutant p53's C-terminus[586], suggesting that other proteins binding here or posttranslational modifications taking place in this region could play a role in the repression of TAp63α.

More recently, it was reported that the structural mutations in the DNA binding domain (such as R175H and R282) exposed an aggregation motif located at amino acids 251 to 257 in the DNA binding domain. This motif, which is located on the S7 β-strand, is buried in the wild-type conformation and its exposure led to aggregation of p53 and also p63 and p73, which contain the same motif within their DNA binding domains. Deletion of the aggregation motif and a single point mutation at isoleucine 254 within the motif prevented this aggregation and alleviated mutant p53 induced repression of p63 and p73[577]. Aggregation of the thermodynamically relatively unstable wild-type p53 DNA binding domain had previously been observed in vitro[587]. The kinetics of p53 aggregation in vitro was determined to follow two-step sequential first order lag kinetics. The first rate-limiting step was found to be the formation of an aggregation competent state, followed by a rapid second polymerisation step[588]. Mutant p53 could also co-aggregate wild-type p53, however the kinetic analysis suggested that aggregates of mutant p53 could not serve to nucleate wild-type p53 aggregation[589]. While it is possible that aggregation is involved in the dominant negative and gain of function mechanism of mutant p53[590], it is important to consider that most aggregation studies were carried out in vitro and p53 aggregates are only rarely observed in cells.

Patients who carry tumours harbouring p53 mutations, commonly face worse clinical prognosis due to therapy resistance and more invasive tumour behaviour[591-593]. Since p63 and p73 heterozygous deletions in the $p53^{+/-}$ mouse led to a dramatic increase

in metastasis[468], it is possible that suppression of p63 and p73 by mutant p53 is responsible for the increase of metastasis observed in the mutant p53 knock-in mice[540,541]. Indeed, p63 and p73 were found to be bound to R172H p53 and silenced in tumour cells derived from the R172H knock-in mice[540]. It is however not clear, whether the extent of mutant p53 binding fully correlates with the extent by which p63/p73 function is repressed as observed in one study[575]. After all, conformational and DNA contact mutants both show a gain of function for example in mouse models, although the $p53^{R172H/+}$ knock-in mouse showed more metastasis than the $p53^{R270H/+}$ knock-in[541].

More evidence on how mutant p53 could induce invasion and metastasis through inhibition of p63 was collected recently: Mutant p53 relieved the TAp63-mediated suppression of α5β1 integrin and EGFR recycling, stimulating invasion towards EGF[586]. A similar effect of mutant p53 on Met recycling and invasion towards HGF was observed later, although this was not strictly TAp63 dependent[594]. Moreover, induced by TGFβ, SMAD bridges a complex of mutant p53 and p63, which results in repression of SHARP1 and Cyclin G2, associated with higher metastasis[595]. Furthermore, mutant p53 represses p63-induced expression of miR-205, which controls the ZEB-1 transcription factor, regulating epithelial to mesenchymal transition[596].

However, expression of mutant p53 does not simply reflect loss of p63 and p73, exemplified by the tumour spectra of the double heterozygous $p53^{+/-};p63^{+/-}$ and $p53^{+/-};p73^{+/-}$mice, which are very different from the $p53^{+/R172H}$ mouse which did not develop any squamous cell carcinoma or hepatocellular carcinoma[468]. But mutant p53 also binds to other proteins than p63 and p73. Mutant p53 was recently reported to disrupt the structure of mammary epithelial cells by upregulating the mevalonate pathway via the SREBP transcription factors[597]. Some functions of mutant p53 were also transcription factor independent: It was for example shown recently that mutant p53 interacts with Nardilysin NRD1 to promote invasion towards HB-EGF[598]. Analysing the mechanisms, by which mutant p53 induces the aggressiveness of cancers is crucial in light of cancer therapy. Despite the mouse survival curves of p53 null and mutant p53 mice being similar, the induced metastasis and possibly also chemoresistance would certainly lead to a poorer

treatment prognosis in patients with mutant p53-containing tumours and it would therefore be beneficial to be able to silence mutant p53 activity.

1.7 Targeting p53 for cancer treatment.

Considering p53's multiple role in eliminating faulty cells via apoptosis or senescence, it is clear that it would be beneficial for cancer patients to activate p53 in their tumour cells in order to trigger their destruction. Classic chemo- or radiotherapy relies on p53-induced apoptosis[599]: While radiation induces DNA strand breaks, drugs such as the pyrimidine analogues 5-Fluorouracil (thymidine) and Gemcitabine (cytidine) or the DNA intercalating Doxorubicin and DNA-crosslinking agent Cisplatin interfere with DNA synthesis and mitosis, all of which leads to p53 activation. Since tumour cells are derived from normal cells, it has been challenging to develop therapy strategies, which discriminate between tumour and healthy cell and only target the tumour cells. During systemic delivery of cytotoxic drugs, p53 also induces apoptosis in healthy dividing tissues such as the intestinal epithelium, the pluripotent cells in the bone marrow and hair follicles, leading to nausea, neutropenia and hair loss. It is therefore a priority to develop more targeted therapies with less severe side effects. The fact that every tumour cell has altered its p53 pathway in some way can be used to discriminate tumour cells from normal cells and makes p53 an ideal drug target.

Initially, it was not clear whether restoration of p53 without any further activating DNA damage would lead to regression of tumours. Mouse models demonstrated that p53 restoration even in advanced in tumours could lead to an impressive tumour regression. A switchable p53-knockin triggered apoptosis of tumour cells in the Eμ-Myc lymphoma model[600] and even brief reactivation of p53 expression (via conditional RNA interference) in a liver carcinoma model led to senescence and clearance of tumour cells by macrophages[601]. Restoring p53 in p53 null animals, which had developed spontaneous sarcomas and lymphomas also led to tumour regression and triggered apoptosis in lymphomas and senescence in sarcomas[602]. Thus, although the specific response to p53 restoration seems to depend on the tissue of origin, in all cases, tumours responded and regressed, suggesting that restoration of p53 is a strategy worth pursuing.

1.7.1 Tumours expressing wild-type p53

Tumours that retain wild-type p53 suppress the transcription factor's activity by other means, for example preventing p53's accumulation by MDM2 overexpression. The p53-MDM2 interaction is well defined[163,511] and inhibiting MDM2, which is amplified in 7% of all cancers[525] would alleviate the constant p53 degradation. Indeed, blocking the p53 binding site in MDM2 was shown to be sufficient for activation of p53[603]. A small molecule MDM2 inhibitor called Nutlin-3a, displacing p53 at MDM2's hydrophobic pocket was developed by Roche and shown to reduce tumourigenicity in xenografts[604] and is currently in Phase I clinical trials. Efforts have been made by a number of groups to develop other compounds, also targeting the p53-MDM2 interaction. While computational design led to the development of compound YH265[605], another group successfully developed spirooxindole derivatives, which showed promising activity in mouse models and are scheduled to enter clinical trials later this year[606].

A reciprocal inhibitor, binding to the MDM2-binding domain in p53, called RITA has also shown anti-tumour activity in xenografts[607]. However, the specificity of this compound is still under debate, since it was also found to activate a DNA-damage pathway exclusively in wild-type p53 cells[608].

Systemic treatment with an MDM2-inhibitor would inhibit MDM2 and thus possibly activate p53 in all tissues. Thus, concerns over toxicity of this strategy were voiced, particularly after modelling this scenario in the mouse: An MDM2 null mouse with switchable p53 showed extensive damage to bone marrow, thymus, spleen and intestinal epithelium after switching p53 on[609], suggesting that MDM2-inhibitors could induce severe side effects similar to chemotherapy. However, a further mouse model with a hypomorphic MDM2 allele demonstrated that reduction of MDM2 up to 50% was tolerated[610]. Importantly, the extent of MDM2 reduction was sufficient to convey resistance to intestinal adenoma formation in the $APC^{min/+}$ mouse[610]. Hence, side effects seem to depend on the extent of MDM2 inactivation and a level of inhibition, which is tolerated, could still exhibit anti-tumour activity. In line with these preclinical models, updates from the phase I clinical trials with the Nutlin-3a-derived compound RG7112 in haematological malignancies and solid tumours suggest that toxicity is

limited. It remains to be determined, whether long-term treatment with MDM2-inhibitors could incur other unpleasant side-effects, since a mice with slightly hyperactive p53 showed premature aging[611].

Instead of targeting the MDM2-p53 interaction, a screen for inhibitors of MDM2's Ubiquitin E3 ligase activity identified a number of compounds called HLI89[612], which inhibit MDM2 by binding to its RING domain and proved to be a successful alternative approach for stabilising p53[613].

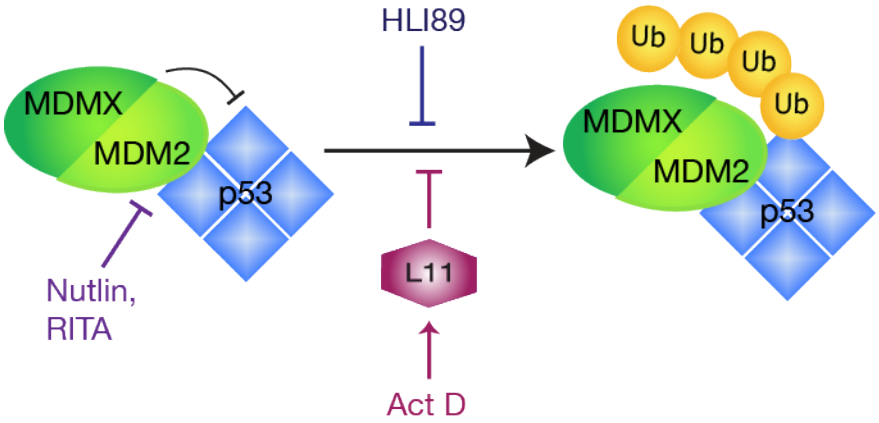

Figure 1-27: Treatment strategies for stabilising wild-type p53.
Different strategies can be implemented to stabilise wild-type p53, either disrupting p53-MDM2 interaction or inhibiting MDM2's ubiquitination activity.

Another way to release MDM2's negative regulation of p53 is via ribosomal stress, since ribosomal proteins are known to interact with MDM2's acidic domain and inhibit its ubiquitination activity. Inhibition of RNA Polymerase I, which leads to nucleolar disruption, was recently shown to activate p53 and selectively kill B-cell lymphoma cells[614]. Furthermore, the RNA polymerase II inhibitor Actinomycin D was reported to induce a specific p53 response at low doses without triggering DNA damage[615]. Promisingly, treatment of tumour cells resulted in p53-dependent apoptosis, while normal fibroblasts only underwent a temporary G_1 arrest[615]. Many systemic therapies seem to induce a different response in normal tissue than in tumour tissue. Nutlin-3a treatment was shown to induce a reversible a cell cycle arrest in normal tissue[616], while induction of apoptosis could be observed in tumours[617]. Tumour cells can be considered on the brink of death due to accumulation of genomic alterations, and the high level of stress signalling in this environment might lead to a different activation of p53 than in unstressed normal

tissues. Thus, treatment might activate p53 to induce apoptosis only in cancerous cells but not normal tissue[618].

In line with the drug mechanism, the major determinant for response to MDM2-inhbitors is the p53 status[619]. In fact, since mutant p53 can also be degraded by MDM2, treatment of mutant p53 lesions could potentially lead to an increase in mutant p53 levels[544]. Moreover, even patients with wild-type p53 tumours could harbour p53 mutations in sun-exposed skin[620] or premalignant skin lesions such as moles[621], and the dormant mutant p53 could potentially be activated by MDM2 inhibitors.

Alongside mutation of p53[622], overexpression of MDM2's binding partner MDMX also conveys resistance to treatment with MDM2-inhibitors[623]. Surprisingly, although the N-termini of MDM2 and MDMX are very similar, Nutlin-3a does not interact with MDMX and it would therefore be useful to develop an inhibitor targeting both MDM2 and MDMX[624]. MDMX is frequently amplified in retinoblastoma and two different MDMX inhibitors were developed, which were able to activate p53 and induce apoptosis[625,626]. Furthermore, 65% of melanomas overexpress MDMX and a stabilised α-helical peptide, derived from the p53 N-terminus[627], was recently shown to efficiently inhibit MDMX, reactivate p53 and sensitise melanomas to cytotoxic therapy[628].

1.7.2 Tumours expressing no p53

Not all cells harbouring a mutation in their p53 gene actually express a full-length p53 protein. While 74% of mutations recorded in the p53 database are missense mutations, 8% are nonsense mutations and 9% of mutations lead to a frameshift[524]. Even if truncated proteins might be expressed, these fragments are generally thought not to carry any p53 activity and it can therefore be assumed that around 8.5% of all cancers (if 50% of cancers harbour a p53 mutation, of which 17% contain a frameshift or nonsense mutant) are functionally deficient for p53.

An obvious strategy to treat p53-null tumours is to reintroduce wild-type p53 by gene transfer. The first attempt was made in 1996, when a p53 gene in a retroviral vector was locally injected into non small lung cancer[629]. While no adverse effects were

observed, gene integration efficacy was poor. The idea was further developed and a Phase I clinical trial launched to treat gliomas by injecting p53 in an adenoviral vector[630]. Delivery to every tumour cell transpired to be difficult to achieve and no effect on survival could be noted. However, treatment in head and neck squamous carcinoma was more successful, particularly in combination with radiotherapy[631] and the p53 adenovirus was approved for drug administration in China and launched under the name of Gendicine[632].

A different approach was developed at the same time, also involving viruses, but this time making use of the fact that only the tumour cells lack p53 expression, while the normal tissue contains wild-type p53. The investigators engineered an adenovirus to lack the E1B-55K gene, which is required to target p53 for degradation. Usually viral gene transfer will activate p53 and the cell cycle will stop, preventing virus replication. Therefore the adenovirus needs to inactivate the p53 gene in the infected cell, which it does by inducing degradation of the p53 protein via the Ubiquitin-ligase complex of E1B-55K and E4orf6[294]. Thus, a virus lacking the p53-silencing mechanism will not be able to replicate in wild-type p53-containing cells. However, it will be able to replicate in cells lacking p53, i.e. tumour cells, and lead to their lysis[633]. Indeed, the engineered virus named Onyx 015 showed promising tumour-restricted destruction in Phase II clinical trials in head and neck cancers[634] and was found to be synergistic with Cisplatin and 5-FU[635]. However, it was noted that Onyx 015 activity did not entirely correlate with p53 status[636], yet the virus still specifically targeted tumour cells, possibly due to differences in their ability to export viral RNA compared to normal cells[637]. The main problem with Onyx 015, which did not live up to its expectations in Phase III trials, was the limited life cycle due to deletion of the E1B 55K protein[638]. Nevertheless, the concept was further developed and a similar oncolytic virus called H101 was approved in China[639].

Strikingly, the MDM2 inhibitor Nutlin-3a also showed some efficacy in p53 null cells. Since the p53-family member p73 can bind to MDM2 as well and it was proposed that Nutlin-3a treatment releases and induces p73[640-643]. MDM2 inhibition might therefore not be restricted to wild-type p53 tumours. Nutlin-3a could also improve response to chemotherapy via p73 in cells expressing mutant p53[644,645] despite the concerns of stabilising mutant p53 by the MDM2-inhibitor. Indeed, Nutlin-3a was

shown to promote apoptosis independent of p53 in combination with Gemcitabine in cholangiocarcinoma cells[644] and Doxorubicin in hepatocellular carcinoma cells[645].

1.7.3 Tumours expressing mutant p53

The majority of p53 mutations in cancers (74%) are missense mutations and result in expression of a full-length protein with a single amino acid exchange[524]. In most cases, this mutant p53 protein will be present in the tumour, mostly to very high levels, but not in any other tissues, rendering it an ideal target for therapy discriminating between normal and neoplastic tissue. Furthermore, since the mutant p53 protein confers more advantage to the tumour than just a pure p53 loss, abrogation of mutant p53 already leads to reduced tumour malignancy[646].

With mutant p53 accumulating to high levels in tumour cells, p53-vaccination was attempted as cancer therapy. Since p53-specific T-lymphocytes were observed in the blood of cancer patients, p53 expressed to high levels seemed to be immunogenic, despite its presence at low levels in normal tissue. Mouse models showed remarkable tumour regression induced by p53-specific cytotoxic T-lymphocytes and T-helper cells[647,648]. p53 peptides were successfully used to stimulate a specific response against p53 in colorectal cancer patients[649], however the tumour response was disappointing. Instead, immunising with multiple epitopes, for example p53 in combination with Survivin and Telomerase at once seems to be more promising[650]. A challenge for this therapy approach is that combination with chemo- or radiotherapy is limited by the factor that cytotoxic therapy tends to be immunosuppressant.

A fascinating observation about mutant p53 folding was made by structural chemists in that a second mutation could refold the DNA binding domain of certain structural mutants to adopt a wild-type like conformation[651]. Based on these observations, a small molecule called PhiKan083 was developed, which stabilises the conformation of the p53 Y220C mutant[652]. Although this codon is not a “hot-spot” mutant, the Y220C mutation accounts for 1.8% of all somatic mutations reported in the p53 database[524]. Residue 220 lies outside the DNA-binding surface, peripheral to the β-sandwich with the mutation resulting in the formation of a large destabilising cleft, which is an ideal target for the development of small binding molecules[653].

However, ideally, mutant p53 restoring drugs could be applied to more than one specific mutation. The low molecular weight molecule PRIMA-1 was identified in functional screen as suppressing growth of mutant p53 cells[654]. Strikingly, this compound reactivates a range of mutants, of both contact and conformational kind. The initial screen was performed using a p53-null cell line stably expressing the p53 R273H mutant. While restoration of DNA binding domain folding by a binding molecule is understandable, it is surprising, that this molecule also activates the transcriptional activity of DNA contact mutants such as p53 R273H. One explanation might be, that PRIMA induces cell death independent of transcriptional activation by promoting Bax translocation to the mitochondria[655]. Furthermore, PRIMA-1 was shown to induce transcription of apoptotic genes such as caspase-2, Bax and Noxa in mutant p53 cells[656-658]. Decomposition products of the PRIMA-1 drug were found to alkylate cysteine residues in the p53 DNA-binding domain, providing a mechanism for stabilisation of conformational mutants[659]. A PRIMA-1 analogue, PRIMA-1^{MET}, in combination with Cisplatin showed convincing tumour regression of xenografts harbouring both p53 R175H and p53 R273H[660] and the drug is currently in Phase I/II trials in haematological malignancies.

Thiosemicarbazone compounds were recently shown to inhibit tumour growth in xenografts in a mutant p53 dependent mechanism. p53 R175H was reactivated, possibly by chelating the zinc ion, which is required to maintain the structure of p53's DNA binding domain and is lost as a result of the R175H mutation[661].

A different screen in A431 cells, which express p53 R273H, identified the small molecule RETRA, which was found to stimulate induction p21 and PUMA. This drug seems to delay tumour formation by releasing p73 from its repression by mutant p53[662].

A different approach to treating mutant p53 (or p53 null) tumours is by taking advantage of their inability to induce p53 in a setting termed cyclotherapy. Treatment with MDM2-inhibitors such as Nutlin-3a or low dose Actinomycin D results in a reversible G_1 arrest in normal tissue, however the tumour cells lacking wild-type p53 continue to proliferate. Therapy targeting dividing cells, such as the spindle poison paclitaxel now only affects tumour cells, while eliminating the adverse effects on the

intestinal epithelium and bone marrow, since these cells are temporarily arrested. Consistent with this model, Nutlin-3a pre-treatment protected mice from PLK1-inhibitor induced neutropenia[663] and importantly did not compromise the response of tumour cells to Paclitaxel[664]. Pre-clinical results are looking promising and further studies are under way to determine the best combination of p53-activator as tissue protector and mitotic poison as tumour killer[665,666].

1.8 Objectives

p53 plays a central role in both tumour formation and cancer therapy. Furthermore, p53 activity in the wrong moment (such as induction of apoptosis of healthy cells) as well as inactivity, when its actions are needed (such as letting cells with genomic alterations multiply) can have serious consequences. p53 can take up some transcription-independent functions, however most of its actions are controlled via activating and repressing a plethora of different target genes. Much of p53's activity is regulated by complex sets of posttranslational modifications. While phosphorylation, acetylation and ubiquitination of p53 have been extensively studied, the consequences of p53 SUMOylation are still a matter of debate. Particularly, the modification with SUMO-2/3, which is more dynamically conjugated and deconjugated and available in a larger free pool than its close relative SUMO-1, is not well understood. Therefore, this study is aimed at determining how SUMO-2/3 conjugation of p53 could be promoted, particularly what role the Ubiquitin- and Nedd8 E3 ligase MDM2 plays in this pathway, and what the consequences of SUMO-2/3 modification are on p53's activity.

Half of all tumour cells do not retain wild-type p53 and expression of mutant p53 is often associated with a poor prognosis. Several studies have suggested that phosphorylation of mutant p53 can modify its oncogenic activity[573,667,668], but the impact of other modifications such as SUMOylation on mutant p53 activity have not been explored. In order to block the detrimental gain of function conferred by mutant p53, it is criticial to understand the mechanisms of function of mutant p53 in more depth, including its interaction with the family members p63 and p73. While multiple studies have shown mutant p53 in complex with p63 and p73, it is not clear where this interaction takes place, how binding of the mutant p53 proteins translate into

repression of p63 and p73 or whether this interaction can be regulated. Furthermore, most studies have focused on the interaction with the TA isoforms, however most tumours express the ΔN isoforms of p63 and p73 at much higher levels. Although initially treated as pure inhibitors of the TA isoforms, identification of additional transactivation domains have raised the complexity of roles that the ΔN isoforms can play.

MDM2 inhibitors such as Nutlin-3a not only lead to accumulation of p53, but concomitantly to an increase in MDM2 levels. In the light of MDM2-inhibitors moving into the clinic, the role of MDM2 binding to p63 and p73 and its impact on their activity is important to bear in mind. Again, while TA isoforms were investigated previously, no study to date has addressed the interaction of MDM2 with the ΔN isoforms of p63 and p73. Furthermore, the impact of MDM2 on the mutant p53/p63 or mutant p53/p73 complexes is entirely unknown.

In brief, the main goals of this work were to

- Analyse the mechanism of SUMO-2/3 modification of p53 (Chapter 3).
- Assess the consequences of SUMO-2/3 conjugation on p53 activity (Chapter 4).
- Determine the interplay between mutant p53, p63/p73 and MDM2 (Chapter 5).

2 Materials and Methods

2.1 Materials

2.1.1 General Reagents

Reagent	Source
Acetonitrile	Sigma-Aldrich
Acrylamide 29:1 (40%w/v)	National diagnostics
Agar	Sigma-Aldrich
Agarose	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
APS (Ammonium persulfate)	Sigma-Aldrich
Bacto Tryptone	BD Biosciences
Benzonase	Sigma-Aldrich
Blasticidin	Sigma-Aldrich
Blasticidin S Hydrochloride	Sigma-Aldrich
BSA (bovine serum albumin)	Sigma-Aldrich
Calcein	Life Technologies
Complete Protease inhibitor cocktail	Roche
Cycloheximide	Sigma-Aldrich
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich
DMEM (Dulbecco's modified eagle medium)	Life Technologies
DMSO (dimethyl sulfoxide)	Sigma-Aldrich
DTT (dithiothreitol)	Sigma-Aldrich
EDTA	Sigma-Aldrich
Effectene	Qiagen
Ethanol	Thermo Fisher Scientific
Fetal Calf Serum	GE Healthcare
Fibronectin	Sigma-Aldrich
Formaldehyde 16% w/v	TAAB labs
Formic Acid	Sigma-Aldrich
GeneJuice	Novagen, Merck
Glutamine	Life Technologies
HLI 373	A Weissman
Iodacetamide	Sigma-Aldrich
IPEGAL CA-630 (NP-40 equivalent)	Sigma-Aldrich
Kanamycin	Sigma-Aldrich
KCl	Thermo Fisher Scientific
KH_2PO_4	Thermo Fisher Scientific
Leptomycin B	Sigma-Aldrich
Methanol	Thermo Fisher Scientific

MG132	Sigma-Aldrich
$MgCl_2$	Sigma-Aldrich
Na_2HPO_4	Thermo Fisher Scientific
NaCl	Thermo Fisher Scientific
Nutlin-3a	Sigma-Aldrich
Opti-MEM	Life Technologies
Orange G	Sigma-Aldrich
Penicillin-Streptomyicin	Life technologies
PI (Propidium Iodide)	Sigma-Aldrich
Pierce ECL (enhanced chemiluminescence) reagent	Thermo Fisher Scientific
Polybrene	Sigma-Aldrich
SDS (sodium dodecyl sulphate)	Thermo Fisher Scientific
TEMED (Tetramethylethylenediamine) Sigma	Sigma-Aldrich
Trichloroacetic acid	Sigma-Aldrich
Triethyl Ammonium Bicarbonate buffer (TEAB)	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100 Sigma	Sigma-Aldrich
Trypsin 2.5%	Life Technologies
Tween-20 Sigma	Sigma-Aldrich
Urea	Sigma-Aldrich
Vectashield Hard Set	Vector Laboratories
Whatman Nitrocellulose membrane Protran 0.2µm	VWR
Yeast Extract	Sigma-Aldrich
β-Mercaptoethanol	Sigma-Aldrich

Table 2-1: General Reagents

2.1.2 Solutions and Buffers

Lysogeny Broth (LB)
- 1% Bacto-Tryptone
- 86 mM NaCl
- 0.5% Yeast Extract

LB Agar
- 1% Bacto-Tryptone
- 86 mM NaCl
- 0.5% Yeast Extract
- 1.5% Agar

Phosphate Buffered Saline (PBS)

- 170 mM NaCl
- 3.3 mM KCl
- 1.8 mM Na_2HPO_4
- 10.6 mM KH_2PO_4
- pH 7.4

Tris Buffered Saline (TBS)

- 25 mM Tris-HCl pH 7.4
- 147 mM NaCl
- 5 mM KCl

TBS-Tween

- 25 mM Tris-HCl pH 7.4
- 147 mM NaCl
- 5 mM KCl
- 0.1% Tween 20

Tris-EDTA (TE)

- 10mM Tris-HCl pH 8.0
- 1mM EDTA

3x SDS Sample Buffer

- 15% β-Mercaptoethanol
- 30% Glycerol
- 9% SDS
- 188 mM Tris pH 6.8
- 0.1% Orange G

SDS-PAGE Running Buffer

- 0.1% SDS
- 192 mM Glycine
- 25 mM Tris-HCl pH 8.3

Electroblotting Buffer

- 192 mM Glycine
- 25 mM Tris-HCl pH 8.3
- 20% Methanol

NP-40 Buffer

- 150 mM NaCl
- 50 mM Tris pH 8.0
- 1% IPEGAL (NP-40 equivalent)

HUNT Buffer

- 20 mM Tris pH 8
- 120 mM NaCl
- 1 mM EDTA
- 0.5% IPEGAL

SUMO assay lysis buffer 1
- 1% SDS
- 25 mM Tris-HCl pH 7.4
- 147 mM NaCl
- 5 mM KCl

SUMO assay lysis buffer 2
- 1.5% Triton X-100
- 25 mM Tris-HCl pH 7.4
- 147 mM NaCl
- 5 mM KCl

Resolving Gel
- 8-12% acrylamide
- 375 mM Tris-HCl pH 8.8
- 0.1% SDS
- 0.1% APS
- 50 mM TEMED

Stacking Gel
- 5% acrylamide
- 0.4% SDS
- 500 mM Tris pH 6.8

2.2 Methods

2.2.1 Cells

H1299 cells (p53-null human non-small-cell lung adenocarcinoma cells), HCT116 cells with and without wild-type p53 (human colorectal carcinoma cells), A2780 cells (wild type p53 expressing ovarian cancer cells), U2OS cells (wild type p53 expressing human osteosarcoma cells), Hek293T cells (human embryonic kidney cells, transformed with SV40 large T antigen) and Phoenix cells expressing the ecotropic receptor were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine and antibiotics.

2.2.2 DNA preparation

Competent E. coli DH5α bacteria (Molecular Services, Beatson Institute) were transformed with plasmid DNA by mixing 0.5 µg DNA with 50 µl competent bacteria. After incubation on ice for 30 min, the bacteria-DNA mix was heat-shocked at 42ºC

for 45 seconds. 200 µl LB was added and incubated shaking at 37ºC for an hour. The bacteria suspension was spread on LB agar plates containing 100 µg/ml Ampicillin or 50 µg/ml Kanamycin upside down over night at 37ºC to grow colonies.

Single colonies were picked and left shaking over night at 37ºC in LB with 100 µg/ml Ampicillin or 50 µg/ml Kanamycin using 5 ml for small scale DNA preparation (mini prep) and 200 ml for large scale DNA preparation (maxi prep).

Small-scale preparation of DNA was performed by the Beatson Molecular Technology Services with the QIAgen BioRobot 9600 according to the QIAprep 96 Plus Miniprep protocol. Large-scale DNA preparations were carried out by the Beatson Molecular Technology services using the Invitrogen Purelink plasmid filter purification kit according to the manual.

2.2.3 Plasmids

The following plasmids were used in this study:

Plasmid	Source
pcDNA3 empty	Life Technologies
pcB6+ empty	from L Lamis
pcDNA3 p53 (72R)	published[413]
pcDNA3 p53 (72P)	site-directed mutagenesis
pcDNA3.1 mouse p53	from K Ryan
pcDNA3 p53 K386R	published[413]
pcDNA3 p53 E388A	published[413]
pWZLneo p53	from K Ryan
pWZL neo p53 K386R	site-directed mutagenesis
pWZL neo p53 E388A	site-directed mutagenesis
pWZL blast p53	from A Vigneron
pWZL blast p53 R175H	site-directed mutagenesis
pWZL blast p53 R273H	site-directed mutagenesis
pWZL p53 R248W	site-directed mutagenesis
pWZL p53 I254R	site-directed mutagenesis
pcB6+ flag p53 (72R)	published[544]
pcB6+ flag p53 (72P)	published[669]
pcB6+ p53 ΔI	published[669]
pcB6+ p53 ΔII	published[669]

pcB6+ p53 ΔNLS (L305A, R306A, L319-321A)	published[413]
pcB6+ p53 ΔNES (L348A, L350A)	published[413]
pcB6+ flag p53 R175H	published[544]
pcB6+ p53 R273H	published[544]
pcB6+ flag p53 I254R	site-directed mutagenesis
pcB6+ flag p53 C277Y	site-directed mutagenesis
pcB6+ flag p53 R248W	site-directed mutagenesis
pcB6+ flag p53 R175H I254R	site-directed mutagenesis
pcB6+ p53 R273H I254R	site-directed mutagenesis
pcB6+ flag p53 Δ96-312	site-directed mutagenesis
pcB6+ flag p53 R175H Δ95-150	site-directed mutagenesis
pcB6+ flag p53 I254R Δ150-200	site-directed mutagenesis
pcB6+ flag p53 R175H Δ201-250	site-directed mutagenesis
pcB6+ flag p53 R175H Δ251-312	site-directed mutagenesis
pcB6+ flag p53 R175H Δ251-257	site-directed mutagenesis
pcB6+ p53 ΔTET	= pcB6+ p53 ΔNES
pcB6+ p53 R175H ΔTET	site-directed mutagenesis
pcB6+ p53 R273H ΔTET	site-directed mutagenesis
pcB6+ flag p53 R175H Δ347	published[586]
pcB6+ flag p53 R175H Δ363	mutated by L. Jobeili
pcB6+ flag p53 R175H Δ370	published[586]
pcB6+ flag p53 R175H Δ380	mutated by L. Jobeili
pcB6+ p53 R273H Δ347	published[586]
pcB6+ p53 R273H Δ363	mutated by L. Jobeili
pcB6+ p53 R273H Δ370	published[586]
pcB6+ p53 R273H Δ380	mutated by L.Jobeili
pCHDM1A MDM2	published[113]
pCHDM1A MDM2 Δ58-89	published[113]
pCHDM1A MDM2 ΔAD (Δ222-437)	published[670]
pCHDM1A MDM2 ΔRING (1-440)	published[670]
pCHDM1A MDM2 C464A	published[136]
pCHDM1A MDM2 NoLS (466-473 Ala)	published[213]
pCHDM1A MDM2 ΔAD NoLS	site-directed mutagenesis
pCHDM1A MDM2 C305F	site-directed mutagenesis
pCHDM1A MDM2 C319R	site-directed mutagenesis
pCHDM1A MDM2 ΔAD K185R	site-directed mutagenesis
pCHDM1A MDM2 ΔAD ΔNLS (181T 183L)	site-directed mutagenesis
pCHDM1A MDM2 Δ9 (483 stop)	published[174]
pCHDM1A MDM2 ΔAD SIM1 (L107S V110D)	site-directed mutagenesis
pCHDM1A MDM2 ΔAD SIM2 (L199R I202R)	site-directed mutagenesis

pCHDM1A MDM2 ΔAD SIM1+2 (L107S V110D L199R I202R)	site-directed mutagenesis
pCHDM1A MDM2 ΔAD Δ9 (483 stop)	site-directed mutagenesis
pCHDM1A MDM2 Δ58-89 C464A	site-directed mutagenesis
pcDNA3 flag MDM2 ΔAD	site-directed mutagenesis
peGFP MDM2	cloned by R. Ludwig
peGFP MDM2 ΔAD (Δ222-437)	site-directed mutagenesis
peGFP MDM2 C464A	site-directed mutagenesis
peGFP MDM2 Δ9	site-directed mutagenesis
peGFP MDM2 ΔAD Δ9	site-directed mutagenesis
pWZL Blast MDM2	from K Ryan
pWZL Blast MDM2 ΔAD	site-directed mutagenesis
pcDNA3 myc MDMX	published[671]
pcDNA3 $p14^{ARF}$	published[672]
flag-L11	published[188]
pMT123 HA-Ubiquitin	from R Hay[544]
pcDNA3 HA-SUMO-3	from R Hay[673]
pcDNA3 HA-SUMO-1	from R Hay[393]
pcDNA3 HA-SUMO-3 QFI (Q30A F31A I33A)	site-directed mutagenesis
pcDNA3 HA-SUMO-3 K11R	site-directed mutagenesis
p53-SUMO-3	from S Carter
p53-SUMO-3 QFI (Q30A F31A I33A)	site-directed mutagenesis
pcDNA3 SV5-Ubc9	from R Hay
pCMX PML1	published[674]
pBOS YFP-RNF4	from R Hay[675]
pCMV CD20	published[676]
pcDNA3 TAp63α	from C de Fromentel[677]
pcDNA3 HA-TAp63α	from G Melino[678]
pcDNA3 HA-ΔNp63α	from G Melino[679]
pcDNA3 HA-TAp63α Δ70-161	site-directed mutagenesis
pcDNA3 HA-TAp63α Δ161-261	site-directed mutagenesis
pcDNA3 HA-TAp63α Δ263-361	site-directed mutagenesis
pcDNA3 HA-TAp63α Δ362-461	site-directed mutagenesis
pcDNA3 HA-TAp63α Δ462-561	site-directed mutagenesis
pcDNA3 HA-TAp63α ΔQQ (Δ398-411)	site-directed mutagenesis
pcDNA3 HA-TAp63α ΔDBD (Δ138-319)	site-directed mutagenesis
pcDNA3 HA-ΔNp63α ΔDBD (Δ84-265)	site-directed mutagenesis

pcDNA3 HA-TAp73α	from G Melino[479]
pcDNA3 HA-TAp73α Simian	published[482]
pcDNA3 HA-ΔNp73α	from G Melino
pcDNA3 HA-TAp73α Δ47-130	site-directed mutagenesis
pcDNA3 HA-TAp73α ΔDBD (Δ131-307)	site-directed mutagenesis
pcDNA3 HA-TAp73α Δ308-386	site-directed mutagenesis
pcDNA3 HA-TAp73α Δ387-500	site-directed mutagenesis
pcDNA3 HA-TAp73α Δ131-223	site-directed mutagenesis
pcDNA3 HA-TAp73α Δ224-307	site-directed mutagenesis
pcDNA3 HA-ΔNp73α ΔDBD (Δ82-258)	site-directed mutagenesis
Bax Luciferase	published[482]
PG13 Luciferase	published[30]
BPAG1 Luciferase	from G Melino[680]
K14 Luciferase	from G Melino[679]
TK Renilla	published[586]

Table 2-2: Plasmids

2.2.4 Site-directed Mutagenesis

Site-directed mutagenesis was performed using the KOD Hot Start Master Mix (MERCK Biosciences). 60 ng of plasmid DNA was mixed with 10 µM forward and reverse mutagenesis primer and 25 µl KOD Master Mix (containing reaction buffer with $MgSO_4$, deoxynucleotides and KOD Hot Start Polymerase) in a 50 µl reaction. PCR-cycles were run according to the following programme: 2 min 95° hot start and 20 cycles of 20 sec denaturation at 95°, annealing at 55° and 4 min elongation at 70°. Mutagenesis primers were synthesised and purified by Eurofins MWG Operon.

p53 K386R fw	aaa ctc atg ttc agg aca gaa ggg cct gac
p53 K386R re	gtc agg ccc ttc tgt cct gaa cat gag ttt
p53 E388A fw	ctc atg ttc aag aca gca ggg cct gac tca gac
p53 E388A re	gtc tga gtc agg ccc tgc tgt ctt gaa cat gag
p53 72P fw	gag gct gct ccc ccc gtg gcc cct gc
p53 72P re	gca ggg gcc acg ggg gga gca gcc tc
p53 R175H fw	acg gag gtt gtg agg cac tgc ccc cac cat gag cgc tgc t
p53 R175H re	agc agc gct cat ggt ggg ggc agt gcc tca caa cct ccg t
p53 R273H fw	act ggg acg gaa cag ctt tga ggt gca tgt ttg tgc ctg tcc tgg g
p53 R273H re	ccc agg aca ggc aca aac atg cac ctc aaa gct gtt ccg tcc cagt
p53 I254R fw	gaa ccg gag gcc cat cct cac ccg cat cac act gga aga ctc c

p53 I254R re	gga gtc ttc cag tgt gat gcg ggt gag gat ggg cct ccg gtt c
p53 C277Y fw	gag gtg cgt gtt tgt gcc tat cct ggg aga gac cgg cgc ac
p53 C277Y re	gtg cgc cgg tct ctc cca gga tag gca caa aca cgc acc tc
p53 R248W fw	ctg cat ggg cgg cat gaa ctg gag gcc cat cct cac cat cat c
p53 R248W re	gat gat ggt gag gat ggg cct cca gtt cat gcc gcc cat gca g
p53 Δ96-312 fw	ccc tcc tgg ccc ctg tca tct agc tcc tct ccc cag cca aag
p53 Δ96-312 re	ctt tgg ctg ggg aga gga gct aga tga cag ggg cca gga ggg
p53 Δ95-150 fw	ccc cct cct ggc ccc tgt cac ccc gcc cgg cac ccg cgt ccg
p53 Δ95-150 re	cgg acg cgg gtg ccg ggc ggg gtg aca ggg gcc agg agg ggg
p53 Δ151-200 fw	cag ctg tgg gtt gat tcc aca ttg cgt gtg gag tat ttg g
p53 Δ151-200 re	cca aat act cca cac gca atg tgg aat caa ccc aca gct g
p53 Δ201-250 fw	ctt atc cga gtg gaa gga aat atc ctc acc atc atc aca ctg
p53 Δ201-250 re	cag tgt gat gat ggt gag gat att tcc ttc cac tcg gat aag
p53 Δ251-312 fw	cgg cat gaa ccg gag gcc cag ctc ctc tcc cca gcc aaa ga
p53 Δ251-312 re	tct ttg gct ggg gag agg agc tgg gcc tcc ggt tca tgc cg
p53 Δ251-257 fw	ggc ggc atg aac cgg agg ccc gaa gac tcc agt ggt aat cta c
p53 Δ251-257 re	gta gat tac cac tgg agt ctt cgg gcc tcc ggt tca tgc cgc c

Table 2-3: p53 primers

MDM2 Δ222-437 fw	cta cag gga cgc cat cga att gtg tga ttt gtc aag gtc g
MDM2 Δ222-437 re	cga cct tga caa atc aca caa ttc gat ggc gtc cct gta g
MDM2 C464A fw	cag gac atc tta tgg cct gct tta cag ctg caa aga agc taa
MDM2 C464A re	tta gct tct ttg cag ctg taa agc agg cca taa gat gtc ctg
MDM2 Δ9 fw	gta gac aac caa ttt aaa tga ttg tgc taa c
MDM2 Δ9 re	gtt agc aca atc att taa att ggt tgt cta c
MDM2 Δ58-89 fw	gaa aga ggt tct ttt tta tct tag ctt ctc tgt gaa aga gca c
MDM2 Δ58-89 re	gtg ctc ttt cac aga gaa gct aag ata aaa aag aac ctc ttt c
MDM2 ΔNLS fw	ctg gtg aac gac aaa caa aac tcc aca aat ctg ata g
MDM2 ΔNLS re	cta tca gat ttg tgg agt ttt gtt tgt cgt tca cca g
MDM2 K185R fw	gaa aac gcc aca gat ctg ata gta
MDM2 K185R re	tac tat cag atc tgt ggc gtt ttc
MDM2 C305F fw	cta ttg gaa att cac ttc atg caa tg
MDM2 C305F re	cat tgc atg aag tga att tcc aat ag
MDM2 C319 fw	ctt cca tca cat cgc aac aga tgt tg
MDM2 C319 re	caa cat ctg ttg cga tgt gat gga ag
MDM2 SIM1 fw	cat gat cta cag gaa ctc ggt agt aga caa tca gca gga atc
MDM2 SIM1 re	gat tcc tgc tga ttg tct act acc gag ttc ctg tag atc atg
MDM2 SIM2 fw	gat gaa agc ctg gct cgg tgt gta aga agg gag ata tgt tg
MDM2 SIM2 re	caa cat atc tcc ctt ctt aca cac cga gcc agg ctt tca tc

Table 2-4: MDM2 primers

SUMO-3 K11R fw	caa gga ggg tgt gag gac aga gaa tga c
SUMO-3 K11R re	gtc att ctc tgt cct cac acc ctc ctt g
SUMO-3 QFI fw	ggc tcc gtg gtg gcg gcc aag gcc aag agg cac a
SUMO-3 QFI re	tgt gcc tct tgg cct tgg ccg cca cca cgg agc c

Table 2-5: SUMO primers

TAp63α Δ70-161 fw	gac tgt atc cgc atg cag gac ctc tac tgc caa att gca aag
TAp63α Δ70-161 re	ctt tgc aat ttg gca gta gag gtc ctg cat gcg gat aca gtc
TAp63α Δ162-261 fw	gta ttc cac tga act gaa gaa aac agt ctt gta caa ttt cat g
TAp63α Δ162-261 re	cat gaa att gta caa gac tgt ttt ctt cag ttc agt gga ata c
TAp63α Δ263-361 fw	tgg cac tga att cac gac att ata ctt acc agt gag ggg c
TAp63α Δ263-361 re	gcc cct cac tgg taa gta taa tgt cgt gaa ttc agt gcc a
TAp63α Δ362-461 fw	gat ccc cag atg atg aac tgg cca aca ttc cca tga tgg g
TAp63α Δ362-461 re	ccc atc atg gga atg ttg gcc agt tca tca tct ggg gat c
TAp63α Δ462-561 fw	cca ttc ctg gca tgg gaa tcc tgg acc acc ggc agc tc
TAp63α Δ462-561 re	gag ctg ccg gtg gtc cag gat tcc cat gcc atc agg aat gg
TAp63α ΔQQ (Δ398-411) fw	cac aca att gaa acg tac agg acc tca ata cag tct cca tc
TAp63α ΔQQ (Δ398-411) re	gat gga gac tgt att gag gtc ctg tac gtt tca att gtg tg
TAp63α Δ138-319 fw	ccc agg ccc gca cag ttt cat cag aaa gca gca agt ttc g
TAp63α Δ138-319 re	cga aac ttg ctg ctt tct gat gaa act gtg cgg gcc tgg g

Table 2-6: p63 primers

TAp73α Δ47-130 fw	ggt ggt ggg cgg aac gga ttt cca gca gtc cag cac ggc c
TAp73α Δ47-130 re	ggc cgt gct gga ctg ctg gaa atc cagt tcc gcc cac cac c
TAp73α Δ131-307 fw	ccc acc act ttg agg tca ctc act acc ggg agc agc agg c
TAp73α Δ131-307 re	gcc tgc tgc tcc cgg tag tga gtg acc tca aag tgg tgg g
TAp73α Δ308-386 fw	ccg aaa agc tga tga gga cga ctc cta tcg gca gca gca g
TAp73α Δ308-386 re	ctg ctg ctg ccg ata gga gtc gtc ctc atc agc ttt tcg g
TAp73α Δ387-500 fw	ggt gcc gca gcc act ggt ggg gtg tcc aaa ctg cat cga g
TAp73α Δ387-500 re	ctc gat gca gtt tgg aca ccc cac cag tgg ctg cgg cac c
TAp73α Δ131-223 fw	cca cca ctt tga ggt cac tca gta tgt gga tga ccc tgt c
TAp73α Δ131-223 re	gac agg gtc atc cac ata ctg agt gac ctc aaa gtg gtg g
TAp73α Δ224-307 fw	gga agg caa taa tct ctc gca cta ccg gga gca gca ggc c
TAp73α Δ224-307 re	ggc ctg ctg ctc ccg gta gtg cga gag att att gcc ttc c

Table 2-7: p73 primers

2.2.5 DNA Sequencing

Correct site-directed mutagenesis products were confirmed by DNA sequencing. DNA was sequenced by the Beatson Molecular Technology Services on an Applied Biosystems 3130xl genetic analyser. The results were analysed using the ApE plasmid editor v1.17.

The following sequencing primers were used (synthesised and purified by Eurofins MWG Operon):

Primer name	Sequence
CMV fw	cgc aaa tgg gcg gta ggc gtg
p53 fw	ggt tca ctg aag acc cag gtc c
p53 121	tct gtg act tgc acg tac
p53 221	gag ccg cct gag gtt ggc
MDM2 3 fw	gtg caa tac caa cat g
MDM2 317	ctt ggt agt agt caa tca gca
MDM2 430 re	cct gaa gct ctt gta caa ggt
MDM2 628	gca gta gca gtg aat cta cag g
MDM2 663 re	atc cgg att cga tgg cgt c
MDM2 946	cat cac att gca aca gat gt
MDM2 1248	gaa gaa acc caa gac aaa g
MDM2 1476 re	cta ggg gaa ata agt tag cac aat c
p63A fw	ccc tcg ccc tac gca cag ccc agc tcc
p63A re	gga gct ggg ctg tgc gta ggg cga ggg
p63 B fw	cag tct tgt aca att tca tgt gta aca gc
p63 B re	gct gtt aca cat gaa att gta caa gac tg
p63 C fw	cct caa tac agt ctc cat ctt cat atg g
p63 C re	cca tat gaa gat gga gac tgt att gag g
p63 D fw	cct gag caa ttt cga cat gcg gtc tgg aag
p63 D re	ctt cca gac cgc atg tcg aaa ttg ctc agg
TAp73 103 fw	agc cgg ggg aat aat gag gt
TAp73 403 fw	agc acg gcc aag tca gcc ac
TAp73 717 fw	cta tga gcc acc aca ggt gg
TAp73 1079 fw	gag gcc ggg aga act ttg ag
TAp73 1418 fw	agt cca tgg tct cgg ggt cc

Table 2-8: Sequencing primers

2.2.6 Transfections

Cells were transfected with Effectene (QIAgen) or GeneJuice (MERCK Biosciences). For SUMOylation studies p53 and MDM2 plasmids were transfected in a ratio of 3:1. For co-immunoprecipitation studies plasmids encoding the interaction partners were transfected in a 1:1 ratio.

For Effectene transfections 7.5 x 10^5 cells per 10 cm plate were seeded in 10 ml medium and grown for 24 hours. 2 µg plasmid DNA was diluted in DNA-condensation buffer EC to a total volume of 300 µl. 16 µl Enhancer was added, mixed by vortexing and incubated 5 minutes at room temperature. 60 µl Effectene reagent was added, mixed by vortexing and incubated for 10 minutes at room temperature to allow formation of the transfection-complexes. Medium on the cell dishes was replaced by 4 ml fresh medium. The Effectene transfection mix was diluted in 1 ml medium and added drop-wise to the cell dishes. Cells were harvested 24 hours later.

For GeneJuice transfection 7.5 x 10^5 cells were seeded per 10 cm plate in 6 ml medium. 24 hours later, cells were transfected with 5-6 µg DNA. 15 to 18 µl GeneJuice was incubated in 300 µl OptiMem (Life Technology) and incubated for 5 minutes. DNA was added, mixed by pipetting up and down and incubated for 10 minutes. The transfection mix was added to the plates drop-wise and cells were harvest 20-24 hours later. Smaller dishes were transfected with the same medium-DNA-GeneJuice ratio, amounts were scaled down according to plate surface area.

For retroviral infections 10 cm plates of Phoenix cells were transfected with GeneJuice. The virus-containing supernatant was collected 36, 48 and 60 hours later, 4 µg/ml Polybrene was added and transferred to 10 cm plates growing H1299 cells at 50% confluency for retroviral infection.

2.2.7 SDS-PAGE and Western Blotting

SDS-Polyacrylamide gel electrophoresis was performed as previously described[681]. Samples were boiled in sample buffer for 5 min at 99ºC and loaded on SDS-polyacrylamide gels with 8-12% acrylamide content depending on the protein size.

Electrophoresis was performed in SDS-PAGE buffer at 90-120 V on Hoefer Mighty Small vertical units SE250 (Amersham). Protein was transferred to nitrocellulose membrane in Blotting buffer by Western blotting as previously described[682,683] using the Hoefer TE22 Mini transfer tank (Amersham) at 200 mA for 2 hours. Membranes were blocked in 5% milk powder in TBS-T for one hour and incubated with primary antibodies over night at 4ºC. The following antibodies were used for blotting (1:1000 dilution) and immunoprecipitation (5 µl):

target	antibody name and supplier
human p53	DO-1 (aa 20-25) (Beatson Molecular Services)[684]
human p53	1801 (aa 46-55) (Beatson Molecular Services)[685]
wild-type p53	1620 (Calbiochem)[686]
unfolded p53	240 (Calbiochem)[687]
murine p53	1C12 (Cell Signaling Technology)
MDM2	Ab-1 (Calbiochem, Merck)
p21	C19 (Santa Cruz, Insight Biotechnology)
$p14^{ARF}$	4037, raised in rabbit[672]
flag-tag	Flag-M2-HRP (Sigma-Aldrich)
HA-tag	16B12 (Covance, Cambridge Bioscience)
SV5-tag	SV5-PK1 (AbD Serotec)
SUMO-1	PW8330 (Enzo Life Sciences)
SUMO-2/3	PW9465 (Enzo Life Sciences)
MDMX	A300-287A (Bethyl Laboratories, Cambridge Bioscience)
GCN5	H-75 (Santa Cruz, Insight Biotechnology)
LDHB	2H6 (Sigma-Aldrich)
PML	H-238 (Santa Cruz, Insight Biotechnology
p63	BC4A4 (Santa Cruz, Insight Biotechnology)
Actin	C4 (Chemicon, Merck)
GFP	7.1/13.1 (Roche)

Table 2-9: Primary antibodies

Rat anti mouse IgK Light Chain HRP	BD Biosciences
Rabbit anti goat IgG HRP-linked	Amersham, GE Healthcare
Donkey anti rabbit HRP-linked	Amersham, GE Healthcare
Sheep anti mouse IgG HRP-linked	Amersham, GE Healthcare

Table 2-10: Secondary antibodies

After three washes in TBS-T, membranes were incubated with horseradish-peroxidase (HRP) conjugated secondary antibodies at 1:10 000 dilution for 2 hours. Proteins were visualised by Pierce ECL reagent, using Fuji Medical X-Ray Film Super RX 18x24 on an AGFA classic E.O.S film processor.

2.2.8 SUMOylation and Ubiquitination of p53 in cells

Cells were seeded in 10 cm plates to reach 70% confluency 24 hours prior to transfection with Effectene (QIAgen) or GeneJuice (MERCK Biosciences) reagent. 24 hours later cells were scraped off the plate, washed with PBS and lysed in 200 µl SUMO assay lysis buffer 1. The lysate was boiled for 10 minutes with intermediate vortexing and diluted with 400 µl SUMO assay lysis buffer 2. Lysates were pre-cleared with 25 µl Protein G Sepharose FastFlow (Sigma) for 1 hour and p53 was immunoprecipitated with 5 µl DO-1 antibody and 30 µl Protein G beads over night. Beads were washed 3 times with HUNT buffer and taken up in 3x sample buffer.

2.2.9 Immunoprecipitation under native conditions

Cells were transfected at 70% confluency in 10 cm plates with 2-3 µg DNA for each protein (5-6 µg total DNA per plate) using GeneJuice. 24 hours later, cells were scraped off the plates, washed in PBS and lysed in NP-40 buffer with proteasome inhibitor cocktail. The suspension was frozen and thawed three times and debris spun down. Immunoprecipitation was carried out with 5 µl antibody and 30 µl Protein G Dynabeads (Invitrogen) over night, rotating at 4°C. Beads were washed three times with NP-40 buffer and resuspended in 3x sample buffer.

2.2.10 Immunofluorescence staining

Cells were grown on coverslips in 12-well plates and transfected when cells were 30-40% confluent. 24 hours after transfection coverslips were rinsed with PBS. Cells were fixed in 4% w/v formaldehyde in PBS for 15 min at 4ºC. Coverslips were washed 3 times with PBS and cells permeabilised with 0.2% Triton X-100 in PBS for 5 min. Coverslips were washed 3 times with PBS and incubate in 5% BSA in PBS for 30 min to block unspecific binding. Proteins were stained with 150 µl primary antibody solution (p53: 1:200 DO-1, MDM2: 1:100 Ab1 and Ab2, PML: 1:100 PML

H-238) in 5% BSA/PBS over night. Coverslips were washed three times with PBS and incubated with 150 µl secondary antibody in 5% BSA (1:150 Alexa Fluor 594 (red), Alexa Fluor 488 (green) and DAPI 1:1000 for 60 min. After further 3 PBS washes, coverslips were mounted on slides with Vectashield hard set. Confocal images were taken at an Olympus FV100 microscope.

2.2.11 Cellular fractionation

Fractionation into cytoplasmic and nuclear fraction was carried out with Epigentek's Nuclear Extraction Kit. Cells were seeded to 70% confluency in 10 cm dishes the day before transfection. Transfection was performed with GeneJuice. 24 hours later, cells were trypsinated off the plate and spun down (5 min, 1000rpm). The outer membrane was lysed in 200 µl NE1 buffer supplemented with protease inhibitor cocktail and 10 µM iodacetamid to prevent deSUMOylation and incubated on ice for 20 min. Organelles and debris were spun down at 800g for 5 minutes and supernatant collected as cytoplasmic fraction. The pellet was washed twice in 200 µl NE1 buffer and resuspended in 200 µl NE2 buffer containing proteinase inhibitor, benzonase and 10 µM iodacetamid. During 15 minutes of incubation on ice the mix was vortexed every 3 minutes, then cell debris was spun down at full speed for 10 minutes and supernatant collected as nuclear fraction. Successful fractionation was confirmed using LDHB as cytoplasmic and GCN5 as nuclear marker. For subsequent SUMO-analysis both cytoplasmic and nuclear fractions were then boiled in 1% SDS and subject to an in vivo SUMO assay.

2.2.12 Analysis of half-life by Cycloheximide treatment

Cells were transfected with p53, SUMO and MDM2 using GeneJuice. 24 hours later, medium was removed and replaced with medium containing 200 µg/ml Cycloheximide to block protein translation. In order to assess protein stability, dishes were harvested at 0, 1.5, 3 and 4.5 hours time points after translation block. Samples were subjected to SDS-PAGE and Western Blotting and primary antibody incubation as usual. Membranes were then incubated with the infrared fluorescently labelled IRDye 800CW Goat anti-mouse secondary antibody (Licor) and infrared fluorescence intensity was quantitated with the Licor Odyssey imaging system.

2.2.13 Oligomerisation Assay

HCT116 $p53^{-/-}$ cells were transfected with MDM2 ΔAD and either wild-type or SUMO consensus mutant p53 (E388A, K386R). Cells were lysed in 1 ml NP40 buffer supplemented with 10 µM Iodacetamide to block deSUMOylation. Half of the lysate was cross-linked with 0.01% Glutaraldehyde for 20 minutes on ice. Cross-linking was stopped by addition of 3x SDS sample buffer. Samples of lysates with and without cross-linking reagent were run on a 6% polyacrylamide gel.

2.2.14 Luciferase assays

Cells were seeded to 70% confluency into 24-wells for transfection using GeneJuice (Fig 4-9: 10 or 100 ng p53, 30 ng MDM2 ΔAD, 20 ng TK Renilla Luciferase and 100 ng PG13 or Bax Luciferase; Fig: 5-6 and 5-7: 25 or 100 ng MDM2 C464A, 75 ng p53, p63 or p73, 25 ng TK Renilla and 75 ng BPAG1 Luciferase; Fig 5-25: 25 or 100 ng p53 constructs, 50 ng TAp63α, 25 ng Renilla, 100 ng K14 Luciferase). 24 hours after transfection, cells were lysed in 100 µl lysis buffer provided by the Promega Renilla Luciferase Kit for 30 minutes at 4°C. 20 µl lysate were transferred to luminometer plates and readings with both Renilla and Luciferase substrate (Promega Dual Luciferase Kit) were carried out at the Veritas Microplate luminometer (Turner Biosystems) using the Glomax Software. Relative Luciferase units were determined by dividing the Luciferase readings by the values obtained for Renilla Luciferase to correct for cell number and transfection efficiency. Data was plotted as fold change to p53-null control readings. Error bars represent the standard error of the mean for 3 independent experiments.

2.2.15 RNA extraction and Realtime-PCR

H1299 cells were retrovirally infected with pWZL blast MDM2 ΔAD and selected for 5 days in 5 µg/ml Blasticidin. RNA was extracted using the RNeasy kit from QIAgen, following the manufacturer's instructions. cDNA was synthesised from 1 µg RNA using Oligo d(T) primers and the DyNAmo SYBR Green two-step kit (Finnzymes) according to the manufacturer's instructions. The Realtime PCR reaction was performed on 5 µl cDNA, diluted 1:20 using the DyNAmo SYBR Green two-step kit (Finnzymes).

The amount of fluorescent PCR product accumulating during the PCR programme (15 min 95°C hot start, 40 cycles of 20 sec denaturing at 94°C, 30 sec annealing at 60°C and 30 sec elongation 72°C; final elongation 10 min 72°C) was detected by the Chromo4 Reader (Bio-Rad) and analysed using the Opticon Monitor 3 software. Gene expression was quantified relative to the housekeeping genes β_2-microglobulin and ribosomal protein, large, P0 (RPLP0) according to the comparative $\Delta\Delta C_t$-method. Results are presented relative to target gene induction by wild-type p53. Error bars represent the standard error of the mean of three independent experiments.

The following primers were used (all primers only amplified one product):

RPLP0 fw	gca atg ttg cca gtg tct g
RPLP0 re	gcc ttg acc ttt tca gca a
B2M fw	gtg ctc gcg cta ctc tct c
B2M re	gtc aac ttc aat gtc gga t
p21 fw	ctg gag act ctc agg gtc gaa a
p21 re	gat tag ggc ttc ctc ttg gag aa
MIC-1 fw	gtt gca ctc cga aga ctc ca
MIC-1 re	gag aga tac gca ggt gca gg
Bax fw	ggg ttg tcg ccc ttt tct act t
Bax re	cagc cca tga tgg ttc tga tca g
Cdk1 fw	ctt gcc aga gct ttt gga ata c
Cdk1 re	ttc tga atc ccc atg gaa aa
Cyclin A_2 fw	cct gca aac tgc aaa gtt ga
Cyclin A_2 re	tgc tgt ggt gct ttg agg ta
Cyclin B_2 fw	ttg cag tcc ata aac cca ca
Cyclin B_2 re	gaa gcc aag agc aga gca gt

Table 2-11: Quantitative Realtime PCR primers

2.2.16 MIC-1 ELISA

The MIC-1 ELISA was performed using the human GDF-15 Quantikine ELISA Kit (R&D Systems). Medium of H1299 cells retrovirally infected with empty vector, wild-type p53, p53 K386R or p53 E388A and MDM2 ΔAD was collected. 50 µl medium from each condition (in triplicate) or standards were mixed with 100 µl assay diluent RD1-9 and incubated on the microplate for 2 hours at room temperature. Wells were

washed four times with 400 µl wash buffer and plate dried upside down on paper towels. 200 µl MIC-1 conjugate was added to each well and incubated for one hour. Wells were washed four times with wash buffer. 100 µl Substrate solution A and 100 µl Substrate solution B were added to each well and incubated in the dark for 30 minutes. 50 µl of stop solution was added to each well and optical density at 450 nm determined on a microplate reader. Measurements were corrected for plate imperfections by subtracting readings at 540 nm. A standard curve was generated using MIC-1 concentrations in a range of 23.4 pg/ml to 1500 pg/ml.

2.2.17 Flow Cytometry

Cells were transfected with empty vector or p53 constructs and MDM2 ΔAD, SUMO-3 and CD20 using GeneJuice 24 hours prior to harvesting. Media was collected from each plate and plates were washed with 2 ml PBS containing 2.6 mM EDTA (PBS/EDTA). PBS was collected and combined with previously collected media. Cells were incubated with 2 ml PBS/EDTA at 37ºC until cells were lifting from the plate. Cells were collected and added to previously collected media.

Cells were spun down for 5 min at 2000 rpm, the supernatant aspirated and the pellet washed with 2 ml cold PBS. Cells were spun down again and the supernatant was aspirated. The pellet was resuspended in 20 µl anti FITC-conjugated CD20 antibody (BD Biosciences) and incubated on ice for 30 min. Antibody incubation was stopped by addition of 2 ml cold PBS containing 1% serum. Cells were spun down and resuspended in 500 µl PBS. 5 ml cold methanol was added while vortexing and the mix was incubated over night at 4ºC to fix the cells.

Cells were rehydrated in 500 µl PBS/1%FCS for an hour at room temperature. After another spin and removal of supernatant pellets were resuspended in 400 µl PBS containing 125 µg/ml RNase and 50 µg/ml Propidium Iodine. Cells were analysed by flow cytometry after 30 minutes incubation in the dark. CD20 status was assessed in the FL1 channel and CD20-positive cells (representing the transfected fraction) were gated for PI analysis. DNA content was analysed in the FL3 channel determined by PI intensity. The FL3 chromatogram of the CD20 positive cell population was analysed with FlowJo software in order to determine the percentage of cells in each cell cycle phase according to DNA content.

2.2.18 Flag-Elution and Mass-Spectrometry of MDM2 interacting proteins

Hek293T cells were transfected with 5 µg flag MDM2 or flag MDM2 ΔAD using GeneJuice and lysed in 600 µl NP40 buffer 24 hours after transfection. MDM2 was immunoprecipitated using 30 µl flag-beads (Sigma-Aldrich) over night, rotating at 4ºC. Beads were washed three times using 150 mM Tris pH 7.5. Flag-tagged protein was eluted using 100 µl 150 mM Tris pH7.5 containing 100 µg/ml flag peptide. Supernatant was collected after 30 min incubation rotating at 4ºC. All remaining protein was eluted with 1% SDS and collected separately as elution efficacy control. 20 µl of input, flag-elution and SDS-elution were run on a polyacrylamide gel to confirm protein expression and immunoprecipitation.

The flag-peptide eluate was analysed by mass spectrometry by the Beatson Advanced Technologies Unit. 30 µl of the sample were dried down and re-dissolved in 30 µl 1% SDS, 50 mM Triethyl Ammonium Bicarbonate buffer (TEAB) and 10 mM DTT. The solution was boiled and incubated at room temperature with 15 µl 0.2M ioadacetamide for 30 min to alkylate free cysteine residues in order to prevent formation of disulfide bonds. Protein was precipitated with 0.5 ml 20% trichloric acid on ice for 15 min and pelleted by spinning at 13000 rpm for 10 min. The pellet was washed with 0.5 ml 10% trichloric acid and pelleted at 13 000 rpm, followed by further three washes with 1 ml H_2O.

The pellet was dissolved in 10 µl 8 M urea and diluted with 70 µl 50 mM TEAB. Trypsin was added to 5 µg/ml and protein digested over night at 30ºC. The digested protein mix was diluted with 20 µl 2.5% formic acid/water. 10 µl out of the 100 µl sample were analysed on an LTQ Orbitrap Velos ion trap mass spectrometer (Thermo Scientific) using the 60 min Top10 method:

The tryptic digests were analysed by LC-MS-MS using a Proxeon Easy-LC connected to an LTQ Orbitrap Velos system via a Proxeon nanospray source fitted with a New Objective FS360-20-20 uncoated emitter. 10 µl of the tryptic digest was injected onto 20 x 0.1mm C_{18} guard column equilibrated in buffer A (2% acetonitrile/ 0.1% formic acid in water) at 7 µl/min. After washing the injector loop with 30µl buffer A, the guard column was switched in line with a 150 x 0.075 mm PepMap C18

column (Dionex) equilibrated in buffer A at 300 nl/min. The column was developed with a 60 min discontinuous gradient of buffer B (80% acetonitrile/0.1% formic acid in water).

Nanoelectrospray was performed by applying a voltage of 1.6 kV to the emitter and the Orbitrap was set to perform a survey scan of m/z 350-1800 at a resolution of 60000 and the top 10 multiply charged ions (minimum intensity 10000 cps) were selected for collision induced dissociation in the LTQ and then excluded for 30 sec after 2 occurrences. The raw data was converted to a Mascot Generic File (mgf) using Raw2msm program (a gift from M Mann) and searched using Mascot 2.3 run on a local server. The data was searched using the following criteria:

Database = SwissProt
Species= Human
Enzyme= Trypsin (1 missed cleavage permitted)
Fixed modification = Carboxyamidomethylation of cysteine
Variable Modification = Oxidation of Methionine
Precursor mass tolerance = 10ppm, MSMS mass tolerance = 0.8 Da.

Significant peptide assignments were based on a minimum peptide score of above 20 and proteins were identified on the basis of at least 2 peptides meeting these minimum criteria.

2.2.19 Inverted Transwell Invasion Assays

H1299 cells were retrovirally infected with empty vector, or p53 mutants and selected with 7.5 µg/ml Blasticidin for 3-4 days.

Transwells (Corning Life Sciences) were filled with 60 µl Matrigel batch A6520 (BD Bioscience) diluted 1:1 in PBS, containing 25 ng/ml Fibronectin and incubated 45 min at 37ºC to allow polymerisation. Transwells were inverted and 100 µl of a 2.5 x 10^5 cells/ml cell suspension were pipetted onto the filter. Cells were allowed to settle onto the filter for 5 hours at 37ºC. Transwells were washed in serum-free medium and transferred (upright) to a 24-well plate containing 1ml serum-free medium. 100 µl of medium containing serum and 10 ng/ml HGF were pipetted into the centre of the transwell on top of the set matrigel to attract cells from underneath the matrigel plug.

Cells were allowed to migrate for 3 days and stained in medium containing 4 nM Calcein for one hour.

Migration was visualised on a Leica TCS SP2 laser scanning confocal microcope, taking images in serial sections every 15 µm starting at the membrane and moving up into the matrigel plug until no more cells could be detected. The number of pixels with intensity of 100 or above was quantified in each binary confocal image using ImageJ. The percentage of invading cells was calculated by dividing the sum of pixels at or beyond 45 µm by the sum of pixels in all images.

3 MDM2 promotes SUMO-2/3 modification of p53.

3.1 SUMO-2/3 conjugation by MDM2.

MDM2 is a well-known E3-Ubiquitin ligase for p53, promoting its mono[153]- and poly-ubiquitination[107,108] . More recently, MDM2 was also reported to promote conjugation of the ubiquitin-family members Nedd8[318] and SUMO-1 (in complex with $p14^{ARF}$)[415] to p53.

Most studies on p53-SUMOylation have so far focused on SUMO-1, which has been shown to modify a single lysine (K386) residue residing within a SUMO consensus motif in the p53's C-terminus. However, in contrast to SUMO-1 the SUMO-2/3 isoforms are more abundant in a large dynamic pool in the cell[348], suggesting an interesting and distinct role for modification with SUMO-2/3.

In this part of the study, how the Ubiquitin- and Nedd8 ligase MDM2 promotes SUMO-2/3 modification of p53 was investigated. The requirements of functional domains in p53, MDM2 and SUMO for successful SUMOylation were analysed in more detail.

3.1.1 MDM2, but not MDMX promotes SUMO-3 conjugation of p53.

Since MDM2 can induce ubiquitination, neddylation and conjugation of SUMO-1 (in complex with $p14^{ARF}$), its ability to promote conjugation of SUMO-2/3 to p53 was tested. Overexpression of p53, MDM2 and SUMO-3 induced modification of p53 with SUMO-3 in HCT116 cells (Figure 3-1 A) and H1299 cells (Figure 3-1 B). MDM2 can form a heterodimer with MDMX, a protein of the MDM2-family with a very similar p53-binding domain to MDM2[688]. The MDM2/MDMX heterodimer was found to be more stable[146] and a more efficient ubiquitin-E3-ligase than the MDM2-homodimer[147]. However, MDMX overexpression had no effect on p53-SUMOylation, either alone (Figure 3-1 A and B) or together with MDM2 (Figure 3-1 B).

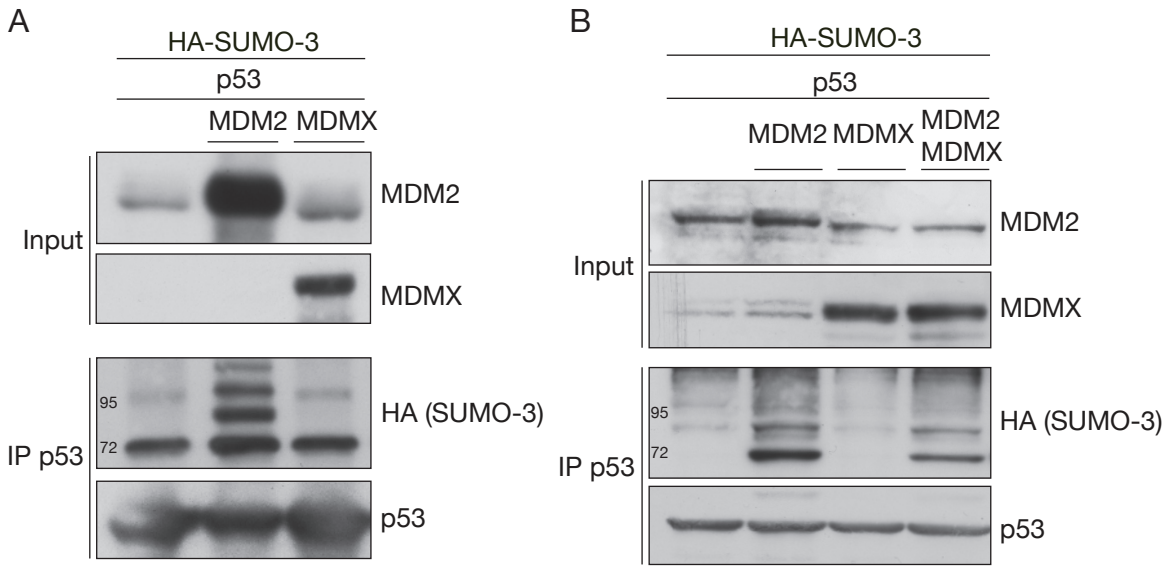

Figure 3-1: MDM2 promotes SUMOylation of p53, while MDMX does not.
HCT116 cells (A) and H1299 cells (B) were transfected with indicated plasmids. Cells were lysed under denaturing conditions and p53 was immunoprecipitated (IP) with the DO-1 antibody.

These data suggest that MDM2 on its own efficiently promotes SUMO-3 modification of p53, but presence of MDMX does not further stimulate MDM2-mediated SUMO-3 conjugation. Since MDMX had been reported to promote MDM2's ability to conjugate Ubiquitin to p53[147], but this was not the case for SUMO-3 modification, the importance of functional domains of MDM2, which are known to be required for ubiquitination, in promoting SUMOylation of p53 were also analysed.

3.1.2 MDM2-p53 interaction is required for efficient SUMOylation.

Both p53 and MDM2 consist of clearly structured functional domains (Figure 3-2).

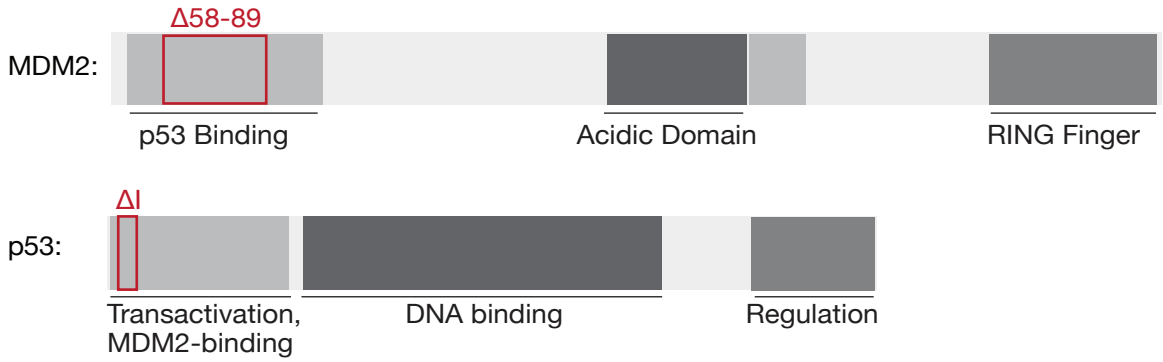

Figure 3-2: Representation of p53 and MDM2 interacting domains.
Regions deleted in the mutants MDM2 Δ58-89 and p53 ΔI (conserved box I: amino acids 13-18) are indicated in red.

The interaction of MDM2 and p53 was mapped to their N-termini, where a hydrophobic pocket of MDM2 contacts an α-helix in p53's N-terminus[163,511]. Interaction of the N-terminal domains was shown to be critical for efficient ubiquitination of p53[136]. Therefore, the role of p53-MDM2 binding on SUMO-3 modification of p53 was examined. An MDM2 protein that does not contain the N-terminal p53-binding domain (MDM2 Δ58-89) could not induce SUMOylation of p53 (Figure 3-3, lane 3). Similarly full-length MDM2 (wt) could not enhance SUMOylation of a p53 protein (p53 ΔI) lacking the MDM2-binding domain (Figure 3-3, lane 4).

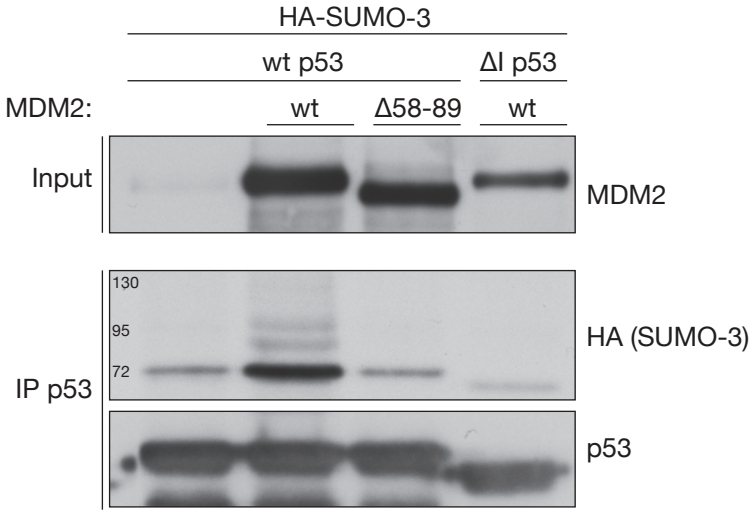

Figure 3-3: MDM2 binding to p53 is necessary for its SUMO-3 modification.
U2OS cells were transfected with the constructs indicated, lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53).

These observations indicate that direct interaction of MDM2 and p53 via their N-terminal binding regions has to take place in order for MDM2 to efficiently promote SUMO-3 conjugation onto p53. This is a prerequisite similar to p53 modification with other Ubiquitin-like proteins by MDM2[318].

As well as the N-terminus, the central and C-terminal domains of MDM2 also play a critical role in promoting p53 ubiquitination. Hence, the role of these functional domains in SUMOylating p53 was analysed next.

3.1.3 Promoting SUMOylation and ubiquitination are two distinct activities of MDM2.

Although the RING domain of MDM2 on its own can show Ubiquitin ligase activity and can function in auto-ubiquitination assays[175], both the N-terminal p53-binding domain[157] and the central acidic domain with adjacent zinc finger[168,169] are also necessary for MDM2's Ubiquitin ligase activity towards p53 (Figure 3-4).

Figure 3-4: Representation of domains contributing to MDM2's ubiquitination activity. Regions lacking in the deletion mutants MDM2 ΔAD (amino acids 222-437) and MDM2 ΔRING (amino acids 441-491) are illustrated in red. Point mutation of cysteine 464 is also indicated.

In order to compare the role of the different functional domains in SUMOylation and ubiquitination of p53, ubiquitination and SUMOylation assays were performed in parallel with the same MDM2 constructs, deleted for particular functional domains. In line with previous observations, MDM2 mutants deleted of the RING domain (MDM2 ΔRING) or harbouring mutation of a structurally critical cysteine residue within the RING (MDM2 464A) both lost the ability to ubiquitinate p53 (Figure 3-5 A). Similarly, deletion of the central part of MDM2 (MDM2 ΔAD) strongly reduced, although did not completely abrogate, the ability of MDM2 to drive ubiquitination of p53 (Figure 3-5 A). By contrast, when repeating the assay with SUMO-3 instead of Ubiquitin, the RING deletion and point mutants (MDM2 ΔRING and C464A) did not impede the SUMO-3 modification of p53 by MDM2, suggesting that the ability to promote SUMOylation is distinct from the Ubiquitin ligase activity (Figure 3-5 B). Deletion of the acidic domain of MDM2 (MDM2 ΔAD) also did not prevent SUMOylation of p53, consistent with previous work showing that this region is not necessary for SUMO-1 modification of p53 by MDM2[415].

The finding that the integrity of the RING domain of MDM2 was not required for promoting SUMO-3 conjugation was very surprising, since this domain is strictly required for promoting both Ubiquitin[157,181] and Nedd8[318] conjugation to p53.

Interestingly, the RING domain was also found to be dispensible for SUMO-1 conjugation to p53 mediated by an MDM2-p14^{ARF} complex[415].

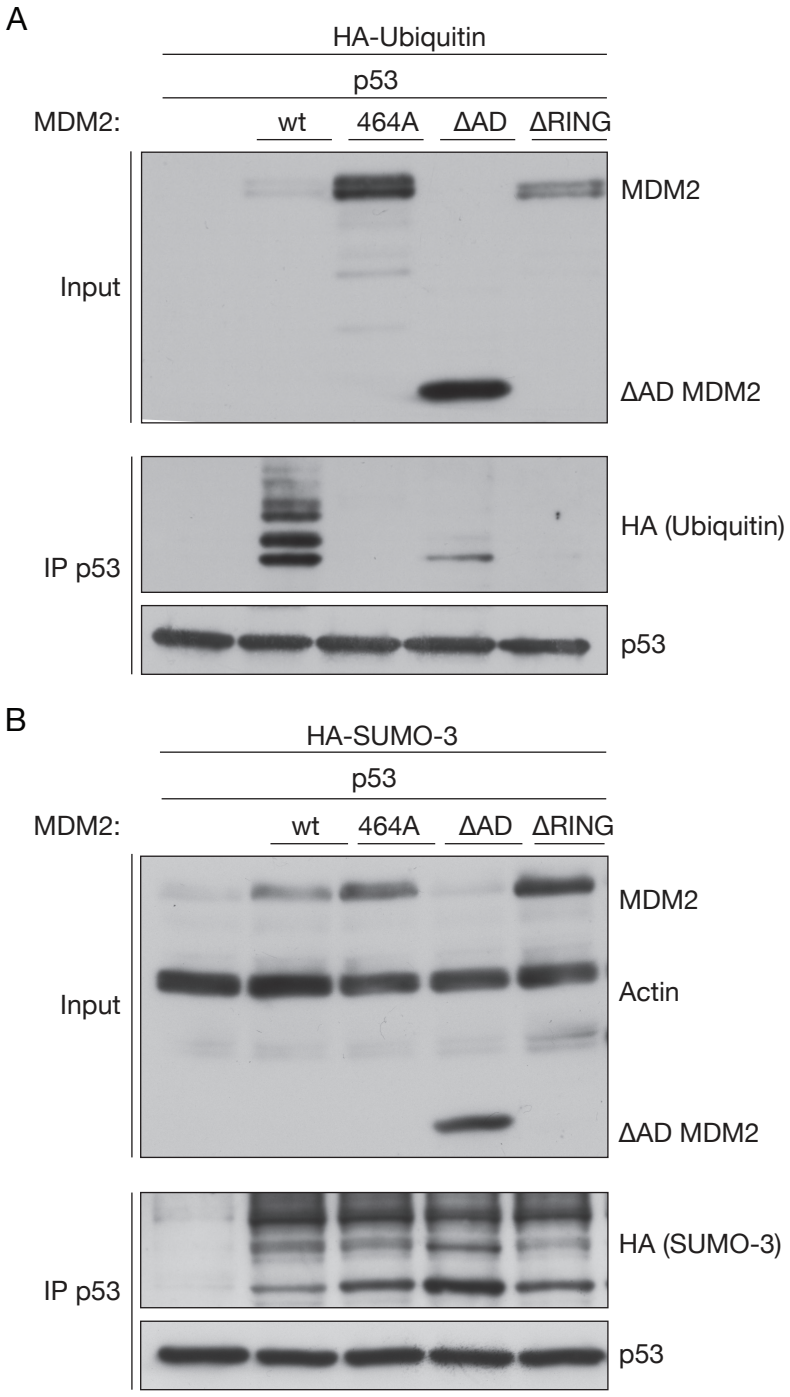

Figure 3-5: MDM2-domains required for ubiquitination (A) and SUMOylation (B) of p53.
HCT116 p53$^{-/-}$ cells were transfected with p53, MDM2 mutants and either HA-Ubiquitin (A) or HA-SUMO-3 (B) as indicated. Cells were lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

3.1.4 SUMOylation can be observed with endogenous protein.

Most previous studies of SUMO-1 modification of p53 by MDM2 have utilised ectopic expression of tagged SUMO, as in these experiments so far. However, overexpression of MDM2 could also promote conjugation of endogenous SUMO-2/3 to p53 (Figure 3-6 A). Using the MDM2 mutants deleted for functional domains, even stronger endogenous SUMO-2/3 conjugation on p53 with MDM2 ΔAD and the RING domain point mutant of MDM2 mutants (MDM2 464A) was observed. The MDM2 ΔRING protein showed a modest activity, while an MDM2 protein lacking the N-terminal p53-binding pocket (MDM2 Δ58-89) was once again unable to drive p53 SUMOylation (Figure 3-6 B).

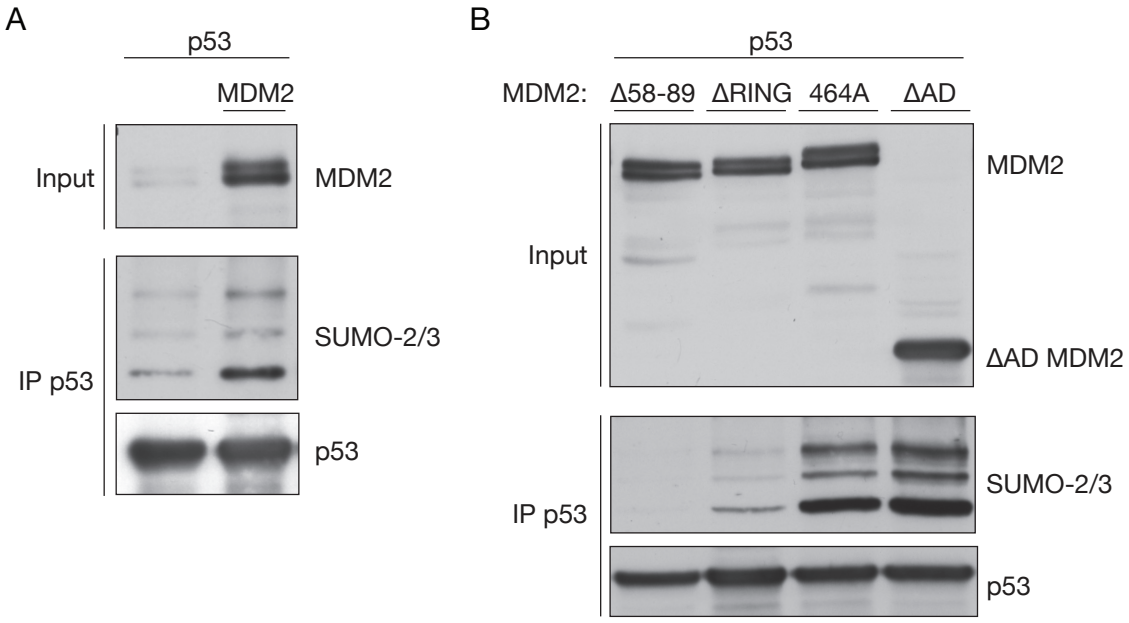

Figure 3-6: Endogenous SUMO-2/3 is conjugated to p53 by MDM2.
U2OS cells were transfected with p53 and either wild-type MDM2 (A) or MDM2 mutants (B). Cells were lysed under denaturing conditions and p53 was immunoprecipitated using the DO-1 antibody (IP p53).

The effect of MDM2 on the SUMO-2/3 modification of endogenous p53 was also tested. Efficient conjugation of endogenous SUMO-2/3 by MDM2 ΔAD and MDM2 464A, the latter of which was expressed at lower levels in this experiment, were observed (Figure 3-7). No clear SUMOylation activity of wild-type MDM2 was detected here, although this is likely to reflect the confounding effect of the ubiquitination and degradation of p53 by this protein, resulting in low p53 levels

(Figure 3-7). MDM2 ΔAD was therefore used to enhance SUMO-2/3 modification of p53 for further experiments, since this MDM2 mutant promotes the highest level of p53 SUMOylation.

Having analysed the SUMO-promoting ability of overexpressed MDM2, the activity of endogenous wild type MDM2 was also investigated. In order to study the function of endogenous MDM2, small molecule inhibitors of MDM2-mediated ubiquitination of p53 were used. Treatment of cells with either Nutlin-3a (which inhibits the N-terminal MDM2/p53 interaction)[604] or HLI373 (which directly inhibits MDM2's Ubiquitin ligase activity)[641] stabilised MDM2, as previously shown (Figure 3-8). p53 levels remained equal despite MDM2-inhibitor treatment, since p53 was overexpressed in this experiment.

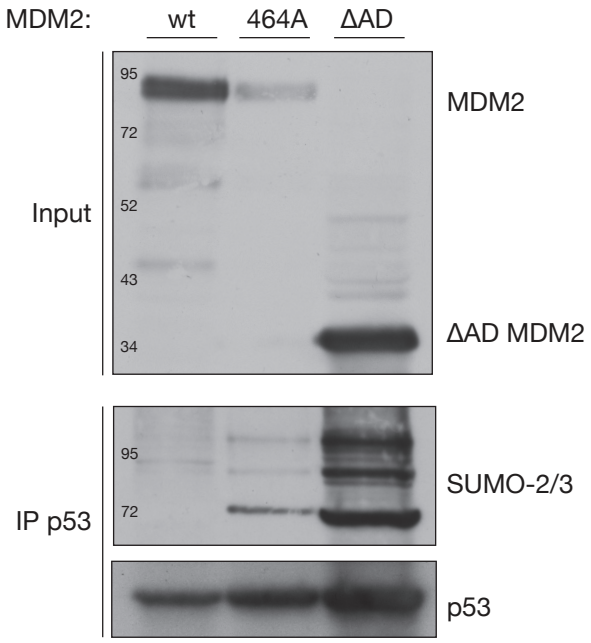

Figure 3-7: Endogenous SUMO-2/3 is conjugated to p53 by MDM2.
U2OS cells were transfected with p53 and either wild-type MDM2 or MDM2 mutants. Cells were lysed under denaturing conditions and p53 was immunoprecipitated using the DO-1 antibody (IP p53).

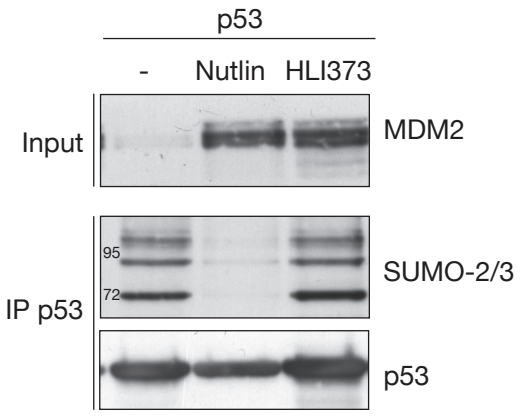

Figure 3-8: Nutlin-3a disrupts SUMOylation, but HLI373 does not.
A2780 cells were transfected with p53 and treated with 20 μM Nutlin-3a or 7.5 μM HLI373 over night. Cells were lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53).

Nutlin-3a treatment completely abolished p53-SUMOylation, confirming the requirement of endogenous MDM2 to interact with p53 for efficient p53 SUMOylation. By contrast, HLI373 treatment did not affect the SUMOylating activity of endogenous MDM2 (Figure 3-8). These data confirm that the p53-MDM2

interaction is absolutely required for SUMOylation. Furthermore, endogenous MDM2 could still drive the SUMO-2/3 modification of p53 when the Ubiquitin ligase activity was inhibited, which is consistent with the finding that the RING domain of MDM2 is dispensable for SUMOylation.

3.2 A single SUMO-2/3 is conjugated to lysine 386 in human p53.

In contrast to ubiquitination, SUMOylation is often restricted to certain lysine residues, which are surrounded by a SUMO consensus motif, mediating the direct binding to SUMO-E2 ligase Ubc9[335]. p53 contains one classical SUMO consensus motif in its C-terminus, around lysine 386. This SUMO consensus was analysed and the effect of p53 localisation on MDM2-mediated SUMOylation was also studied. Furthermore, it was investigated, whether p53 is poly-SUMOylated.

3.2.1 The SUMO consensus around lysine 386 is essential.

In most cases SUMOylation is restricted to lysine residues in the environment of a SUMO motif ΨKxE[405,406].

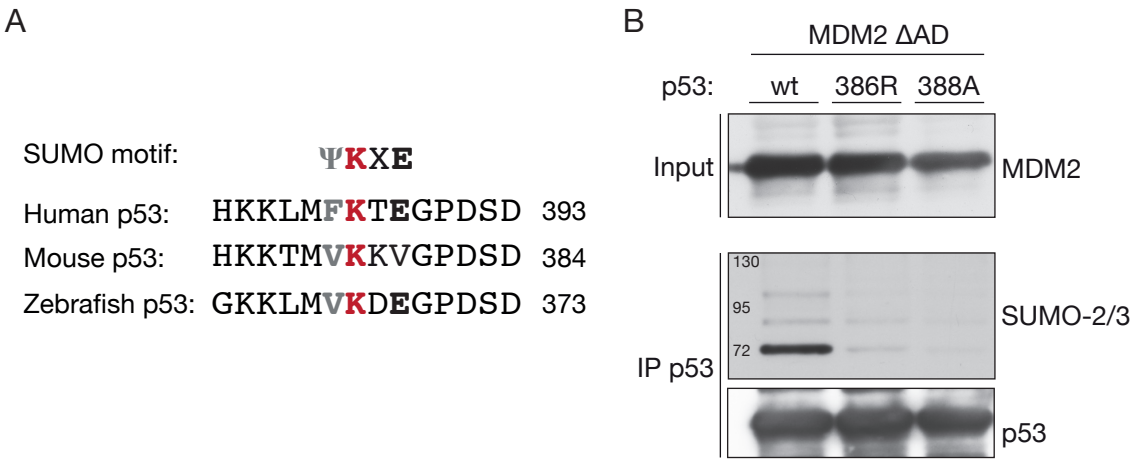

Figure 3-9: p53 requires an intact SUMO motif to be SUMOylated.
(A) Alignment of p53 C-termini across species. The SUMO-accepting lysine (red) is conserved in all species, however the glutamic acid residue 2 codons down-stream (bold) is not present in the mouse sequence. Ψ: hydrophobic amino acid, X: any amino acid. SwissProt accession numbers: human p53: P04637; mouse p53: P02340; zebrafish p53: P79734.
(B) H1299 cells were transfected with the wild-type or SUMO site mutant p53 and MDM2 ΔAD and lysed under denaturing conditions. p53 was immunoprecipitated with the DO-1 antibody (IP p53).

Previous studies showed that both SUMO-1[405] and SUMO-2/3[421] were exclusively conjugated to p53 on lysine 386 of the human p53 protein within the canonical

SUMO motif (Figure 3-9 A). In line with these previous studies, p53 K386R, which lacks the SUMO-accepting lysine residue, could not be SUMOylated (Figure 3-9 B). Furthermore, mutation of the glutamic acid residue 388 in p53's SUMO consensus motif also abrogated SUMOylation of p53 by MDM2 ΔAD (Figure 3-9 B). Since lysine 386 is not only SUMOylated, but also ubiquitinated and acetylated, the E388A mutation served as a useful tool, since it specifically abrogates SUMOylation of K386 while sparing lysine 386 for other modifications.

Surprisingly, alignment of the p53 C-termini across different vertebrate species revealed that despite the conservation of the SUMO-accepting lysine residue in mice, the required glutamic acid two residues downstream is not present in the murine p53 protein (Figure 3-9 A). Hence, it was investigated, whether mouse p53 could still be SUMOylated.

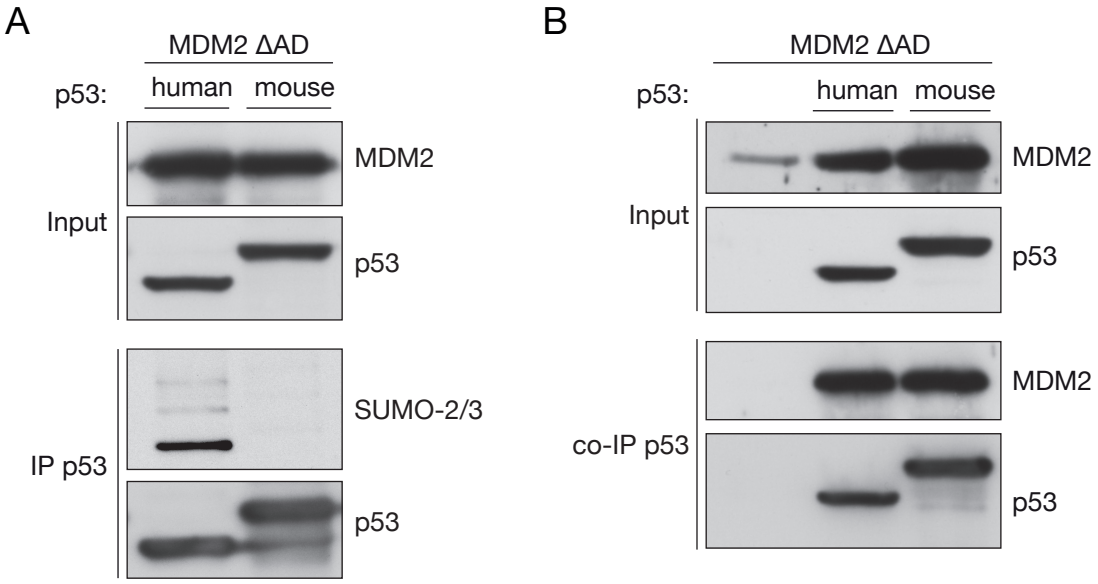

Figure 3-10: Mouse p53 is not SUMOylated.
HCT116 $p53^{-/-}$cells were transfected were transfected with MDM2 ΔAD and either human or mouse p53. Cells were lysed (A) under denaturing conditions to assess SUMOylation or (B) under mild conditions to assess p53-MDM2 interaction. p53 was immunoprecipitated with the DO-1 antibody (IP p53 (A) and co-IP p53 (B)).

Consistent with the observation that mutation of glutamic acid residue 388 in the human protein disrupts SUMOylation, human MDM2 did not modify mouse p53 with SUMO-2/3 (Figure 3-10 A). In order to confirm that this lack of SUMOylation was not due to a deficiency in binding of the human MDM2 protein to murine p53, a co-immunoprecipitation assay of mouse p53 and human MDM2 ΔAD was performed

(Figure 3-10 B). Human MDM2 could bind mouse p53 equally well as human p53, therefore the absence of SUMOylation of mouse p53 is unlikely to be due to a lack of interaction and can be ascribed to the missing canonical SUMO consensus site in the mouse p53 protein.

3.2.2 Both genetic variants of p53 are SUMOylated.

The p53 gene contains a common polymorphism at codon 72 in the proline-rich domain, which can encode for either an arginine or proline residue[689]. The polymorphism has biochemical implications for the p53 activity[690] and differences in regulation of apoptosis[691] and cell cycle progression[692] were reported. To avoid potential polymorphism effects affecting the results, so far only the 72R variant of p53 has been used in this study.

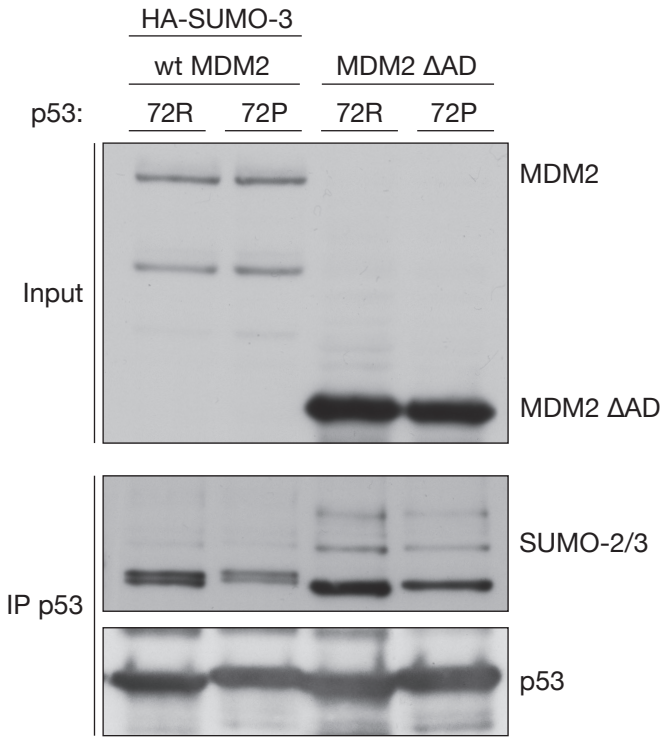

Figure 3-11: p53 72P is SUMOylated slightly less than p53 72R.
HCT116 $p53^{-/-}$cells were transfected with both variants of p53 and either wild-type MDM2 and HA-SUMO-3 or MDM2 ΔAD. Cells were lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

Since the polymorphism can influence the interaction with p53-binding proteins, it was of interest to study, whether the polymorphism can have an impact on p53

SUMOylation. Both variants were therefore tested in an in vivo SUMO assay (Figure 3-11).

The p53 72P variant not only migrated more slowly in the gel, which had also been observed by others[693], but was also SUMOylated slightly less well by both full-length MDM2 (wt) and MDM2 ΔAD compared to the p53 72R variant (Figure 3-11). Although the proline-rich domain of p53, where this polymorphism is located, is not directly involved in interacting with MDM2, p53 72R was shown to bind more strongly to MDM2 in previous studies, resulting in more efficient ubiquitination[691]. Hence, it is likely that the moderate difference observed between the two isoforms is a reflection of their extent of MDM2 binding. Since the p53 72R variant was SUMOylated more efficiently, it was used for all further experiments.

3.2.3 p53's nuclear localisation signal is dispensable, but the nuclear export signal is required for SUMOylation.

p53 shuttles in and out of the nucleus, using its nuclear localisation (NLS) and nuclear export signals (NES), which are located between the DNA-binding and the C-terminal regulatory domain[120]. Most SUMOylated proteins are localised in the nucleus and a nuclear localisation signal was described as prerequisite for efficient SUMO-conjugation[331]. In order to disrupt the nuclear localisation signal in p53, lysine residues 305, 319, 320 and 321 and arginine 306 were mutated to alanine, creating p53 ΔNLS, which is defective in entering the nucleus. Similarly, leucines 348 and 350 in the nuclear export signal were replaced with alanine residues resulting in p53 ΔNES, which is defective for export (Figure 3-12 A). The p53 ΔNLS and p53 ΔNES mutants had been shown by others to be defective for import and export, respectively, resulting in exclusive cytoplasmic or nuclear immunofluorescence staining[413].

The impact of p53 localisation on its SUMOylation was analysed using p53 mutants, which could not enter (p53 ΔNLS) or could not leave the nucleus (p53 ΔNES), with the p53 K386R mutant serving as negative control. Strikingly, mutation of the nuclear export signal in p53 ΔNES abrogated SUMOylation completely (Figure 3-12 B). While the observed effect could be a result of the lack of nuclear export, it could also reflect the requirement of p53 to oligomerise in order to be SUMOylated, because the

nuclear export signal is located within the oligomerisation domain and this p53 ΔNES mutant was previously reported to disrupt tetramerisation of p53[694]. Since the nuclear export signal is an integral part of the oligomerisation domain, it is difficult to discriminate between the two activities. However, it would be possible to block nuclear export with compounds such as the CRM1-inhibitor Leptomycin B[695]. If pharmacological inhibition of nuclear export did not affect p53 SUMOylation, the observed effect of the ΔNES mutation would most likely be due to lack of oligomerisation. Interestingly, loss of p53 oligomerisation was found to abrogate C-terminal acetylation[694]. Furthermore, MDM2 could not ubiquitinate p53, if it was not tetrameric[696], however poorly ubiquitinated monomeric p53 could still be degraded by MDM2[697].

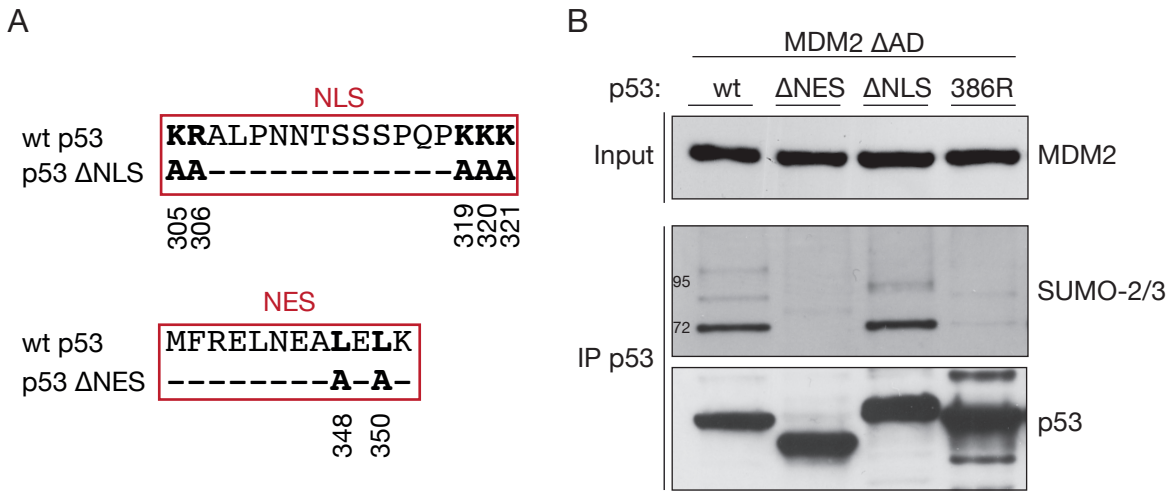

Figure 3-12: Mutation of the NES abrogates SUMOylation.
(A) Representation of the p53 amino acid sequence of the nuclear localisation (NLS) and nuclear export signals (NES) with mutations indicated in bold. SwissProt accession number: p53 P04637.
(B) H1299 cells were transfected with MDM2 ΔAD and the p53 plasmids indicated. Cells were lysed under denaturing conditions and p53 was immunoprecipitated using the DO-1 antibody (IP p53).

Surprisingly, mutation of the nuclear localisation signal of p53 did not stop SUMOylation of the protein, but altered the band pattern from three to two bands (Figure 3-12 B, lane 3). While the ladder of bands could represent different states of mono- and poly-SUMOylation, it could also be an indication for SUMOylation and ubiquitination on the same p53 molecule, since both modifications result in a shift of the modified product of 11-20 kD. Four of the mutated residues in p53 ΔNLS are lysine residues, which could be subject to modification with UBLs themselves. The lysine cluster K319, K320 and K321, which is mutated in p53 ΔNLS, was shown to

be targeted for ubiquitination by E4F1[310] and more recently also by MDM2[141]. Since MDM2 ΔAD can still mono-ubiquitinate p53, albeit less strongly than wild-type p53, a missing ubiquitination on the K319-321 lysine cluster could be the cause for the altered band pattern (Figure 1-12 B). However, it is still unclear how p53 can be SUMOylated without being localised to the nucleus, unless p53 ΔNLS still enters the nucleus by another mechanism. It has previously been observed that MDM2 can import p53 ΔNLS into the nucleus[698]. In order to prevent p53 from entering the nucleus in a complex with MDM2, the NLS in MDM2 ΔAD was also disrupted by mutating arginine residues 181 and 183 to threonine and leucine, respectively[699], resulting in the MDM2 ΔAD ΔNLS protein (Figure 3-13 A).

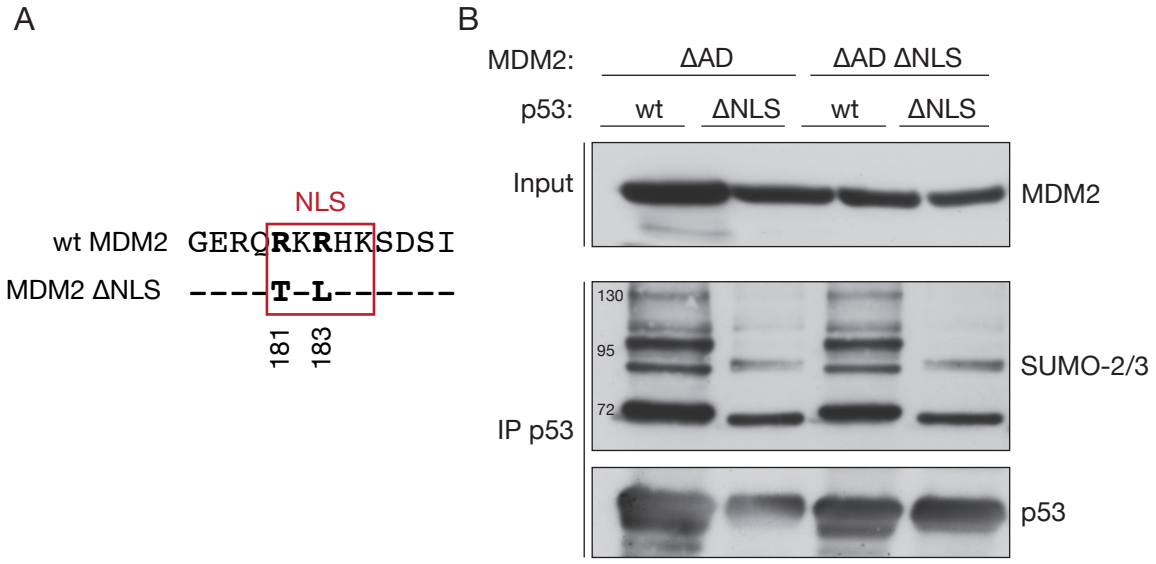

Figure 3-13: p53 ΔNLS is still SUMOylated by MDM2 ΔAD ΔNLS.
(A) Representation of the nuclear localisation signal (NLS) in MDM2 (red). Amino acids mutated in the MDM2 ΔNLS protein are indicated in bold.
(B) HCT116 $p53^{-/-}$ cells were transfected with the plasmids indicated. Cells were lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53).

Strikingly, p53 ΔNLS could still be SUMOylated by an MDM2 protein, lacking the NLS (MDM2 ΔAD ΔNLS) (Figure 3-13 B). It is possible that the nuclear localisation is not necessary for p53 SUMOylation, analogous to ubiquitination, which can still be promoted by MDM2 on p53 ΔNLS in the cytoplasm[696]. Nevertheless, to determine whether these NLS mutations really prevented nuclear import, their subcellular distribution was assessed by cellular fractionation (Figure 3-14).

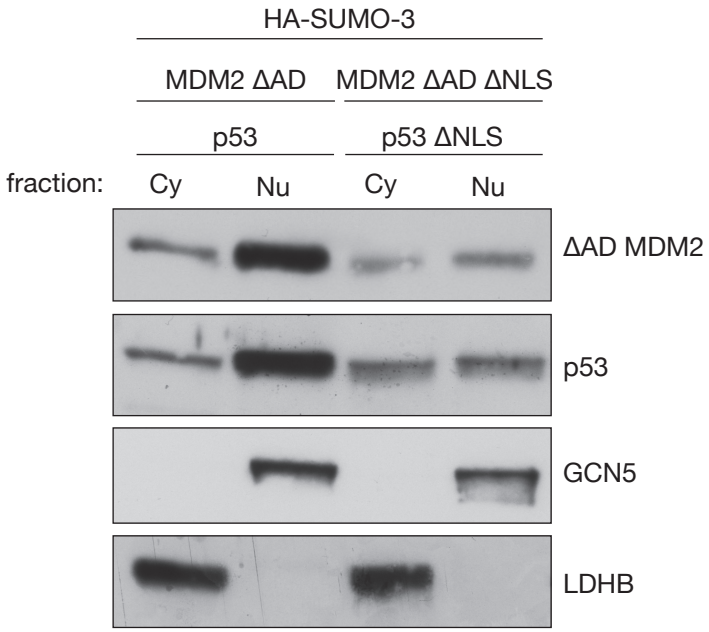

Figure 3-14: p53 ΔNLS and MDM2 ΔAD ΔNLS can enter the nucleus.
HCT116 $p53^{-/-}$ were transfected with the plasmids indicated. Cells were fractionated into cytoplasmic (Cy) and nuclear (Nu) fractions. Efficient fractionation was confirmed using GCN5 as nuclear and LDHB as cytoplasmic marker.

Fractionation revealed that both p53 ΔNLS and MDM2 ΔAD ΔNLS could still enter the nucleus and were evenly distributed between cytoplasm and nucleus in contrast to the mainly nuclear and partially cytoplasmic wild-type proteins (Figure 3-14). Although the nuclear fraction of p53 was largely reduced compared to the wild-type protein, no reduction in SUMOylation of p53 ΔNLS had been observed (Figure 3-13 B). However, since no complete nuclear exclusion was achieved, no firm conclusion can be drawn as to whether nuclear localisation is required for SUMOylation. It is possible that the NLS mutations are not sufficient to disrupt the import signal completely. Two further nuclear localisation signals have been described around amino acids 370 and 380 of p53[118]. Since these regions were left intact in the p53 ΔNLS protein, partial nuclear import could be allowed via these alternative nuclear localisation signals. In order to determine whether exclusively cytoplasmic p53 could be SUMOylated, a p53 protein mutated for all nuclear localisation-associated sequences should be tested.

Since a change in the band pattern of SUMOylated p53 ΔNLS was observed, it was investigated whether the ladder of bands represented poly-SUMOylation of p53.

3.2.4 SUMO-3 does not form a chain on p53.

In contrast to SUMO-2/3, which contains a SUMO consensus motif around lysine 11 and can form chains[353], SUMO-1 does not contain the necessary hydrophobic amino acid (Ψ) next to the lysine residue and cannot form chains (Figure 3-15 A). Poly-SUMOylation has functionally distinct consequences to mono-SUMOylation[700], for example in the recruitment of SUMO-interacting proteins. When blotting for SUMOylated p53, a ladder of three bands in about 20 kD intervals, which would be consistent with a chain of up to three SUMO molecules, was frequently observed. The transfected HA-SUMO-3 in the SUMO assay was therefore replaced by a mutant HA-SUMO-3 K11R, in which the chain-accepting lysine 11 is substituted for an arginine residue (Figure 3-15 B).

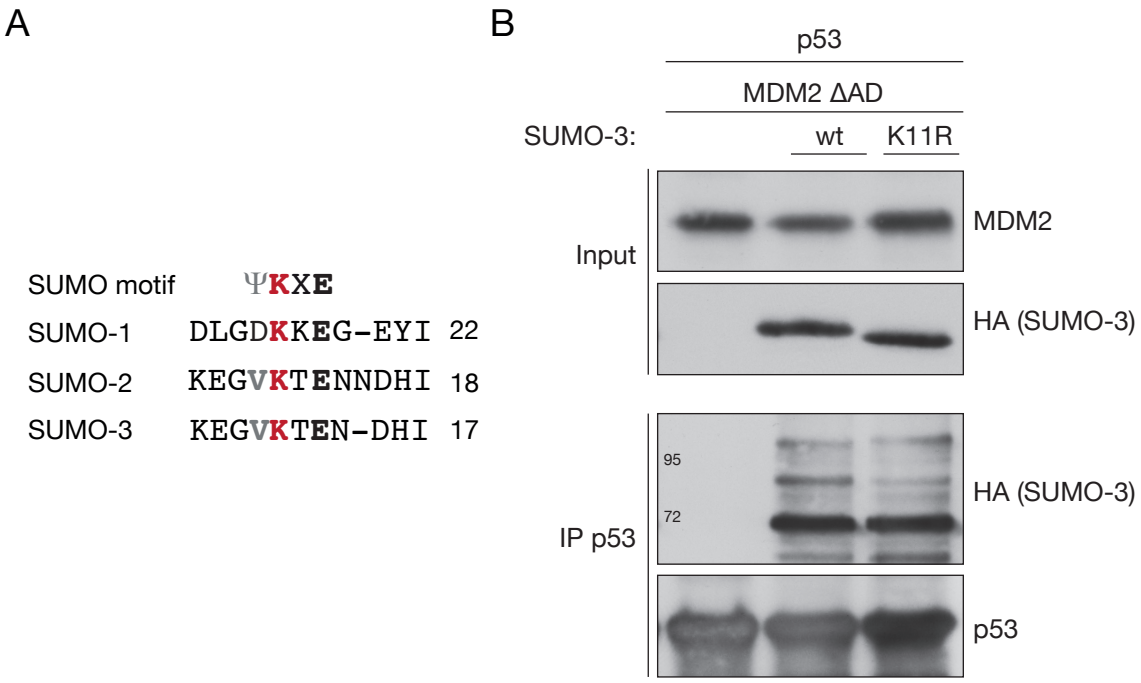

Figure 3-15: p53 is not poly-SUMOylated.
(A) Alignment of human SUMO-1, 2 and 3 around lysine 11 in SUMO-2/3. SwissProt accession numbers: SUMO-1: P63165; SUMO-2: P61956; SUMO-3: P55854
(B) U2OS cells were transfected with p53, MDM2 ΔAD and either SUMO-3 or SUMO-3 K11R. Cells were lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53).

The band-pattern of immunoprecipitated p53 blotted with the HA-specific antibody consistently did not change in ten repeat experiments, whether or not SUMO-3 had an intact SUMO motif (Figure 3-15 B). This suggests that the upper bands represent a combination of different modifications, possibly SUMO and ubiquitin on multiple

lysine residues in one p53 molecule, rather than a SUMO chain. This finding is consistent with the observation that mutation of multiple lysine residues (305, 319, 320 and 321) as in p53 ΔNLS resulted in an altered band pattern of SUMOylated p53 (see Figure 3-12).

3.2.5 SUMO-2 and 3 are preferentially conjugated to p53.

p53 was first identified as a SUMO-1 target[405,406] and MDM2 in complex with p14[ARF] was reported to promote SUMO-1 conjugation to p53[415]. Clearly, MDM2 can also promote SUMO-2/3 conjugation of p53. However, it is unclear, whether MDM2 preferentially conjugates one of the SUMO isoforms. Despite the same set of E1-and E2 enzymes conjugating the three different SUMO-isoforms, certain targets have been shown to be preferentially modified by particular isoforms[348].

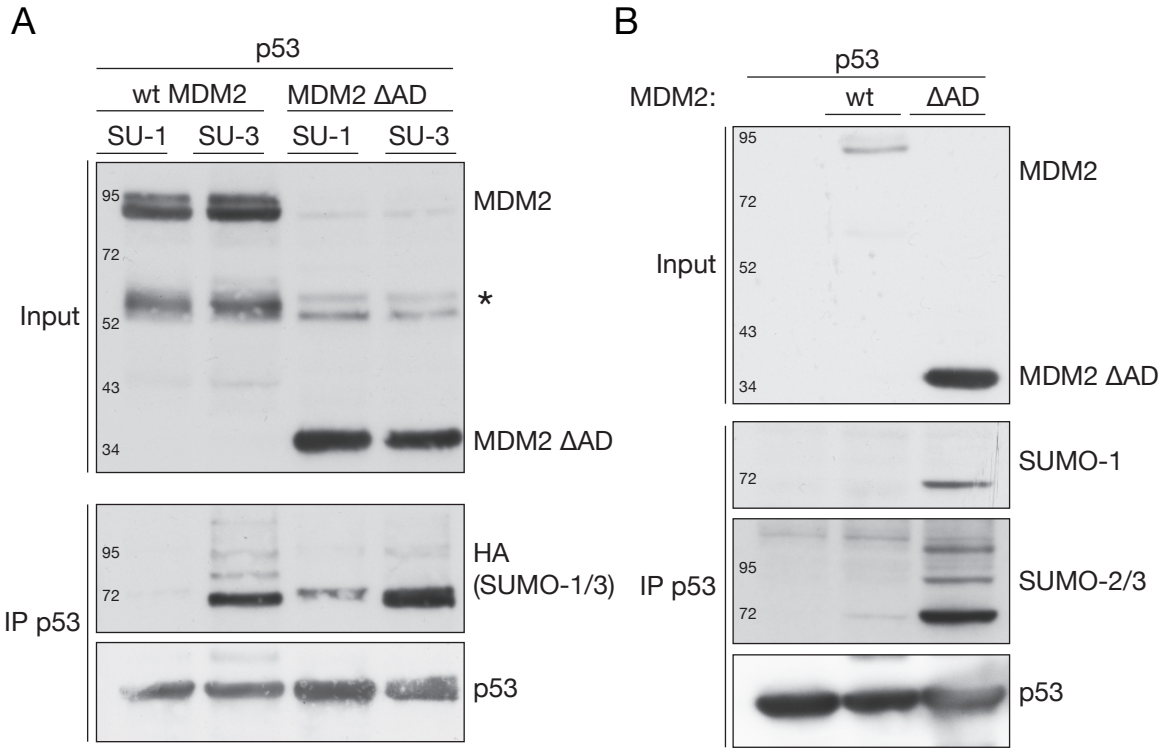

Figure 3-16: MDM2 preferentially SUMOylates p53 using SUMO-2/3.
HCT116 cells were transfected with either (A) p53, MDM2 and SUMO-1 (SU-1) or SUMO-3 (SU-3) or (B) p53 and MDM2 constructs. Cells were lysed under denaturing conditions and p53 was immunoprecipitated using the DO-1 antibody (IP p53). SUMOylation was assessed with either (A) HA-antibody in case of overexpressed SUMO or (B) SUMO-1 and SUMO-3 specific antibodies in the case of endogenous SUMO. *indicates smaller MDM2 isoform.

HA-tagged SUMO-1 and SUMO-3 were overexpressed in the presence of p53 and MDM2. Wild-type MDM2 only conjugated HA-SUMO-3, but not HA-SUMO-1 to p53

(Figure 3-16 A, lanes 1 and 2), while MDM2 ΔAD modified a considerable amount of p53 with SUMO-1. However, MDM2 ΔAD still conjugated substantially more SUMO-2/3 to p53 (Figure 3-16 A, lanes 3 and 4). Although it remains to be verified that both SUMO-1 and SUMO-3 isoforms were expressed to the same level, this data suggests that MDM2 mainly drives p53 modification with SUMO-2/3 and only to a limited extent modification with SUMO-1.

Since SUMO-2/3 is naturally much more abundant as free pool in a cell than SUMO-1, overexpression of either isoform, but particularly the more restricted SUMO-1, can shift the natural balance and skew results due to a non-physiological isoform representation[701]. Demonstrating the importance of using the endogenous pool of UBLs, a recent study showed that Nedd8 was being used by the conjugation system instead of Ubiquitin if overexpressed to unphysiological levels[702]. Thus, MDM2-induced SUMOylation of p53 with endogenous SUMO isoforms was assessed with isoform-specific antibodies. Conjugation of SUMO-2/3, but not SUMO-1 to p53 was observed with wild-type MDM2. However, overexpression of the stronger SUMOylator MDM2 ΔAD led to more SUMO-2/3-conjugated p53 and also some SUMO-1-conjugated p53 (Figure 3-16 B). This result suggests that while MDM2 does not completely discriminate against SUMO-1, it still preferentially drives SUMO-2/3 conjugation to p53.

Having studied the functional domains in p53 required for efficient SUMOylation, the role of MDM2 in the SUMOylation process was also addressed in more detail.

3.3 Regulation of MDM2's SUMOylation activity.

The data presented here suggest that the ubiquitination and SUMOylation activity are distinct features in the MDM2 protein. Thus, the effect of known ubiquitination regulators such as the MDM2-inhibitors $p14^{ARF\ 672}$ and $L11^{188}$ on the SUMOylation activity of MDM2 was analysed. Furthermore, it was investigated why certain MDM2 mutants, particularly the protein deleted for the central domain including acidic domain and zinc finger (MDM2 ΔAD), were able to promote SUMOylation of p53 more efficiently than the wild-type MDM2 protein.

3.3.1 The MDM2-inhibiting proteins p14^{ARF} and L11 stimulate SUMOylation.

The interaction of MDM2 with p14^{ARF} leads to the inhibition of ubiquitination of p53[672], but was shown to promote p53's SUMO-1 modification[415]. A similar result was observed for endogenous SUMO-2/3 conjugation to p53, which was enhanced by addition of p14^{ARF}, both under endogenous and overexpressed MDM2 levels (Figure 3-17 A, lane 5 and 6). However, p14^{ARF} was also shown to associate with the SUMO-E2 Ubc9, promoting SUMOylation of p14^{ARF}-interaction partners[428], and to inhibit deSUMOylation of proteins by increasing the turnover of the sentrin-specific protease SENP3[429]. It is therefore difficult to discriminate between specific p53-related and global SUMOylation effects of p14^{ARF}.

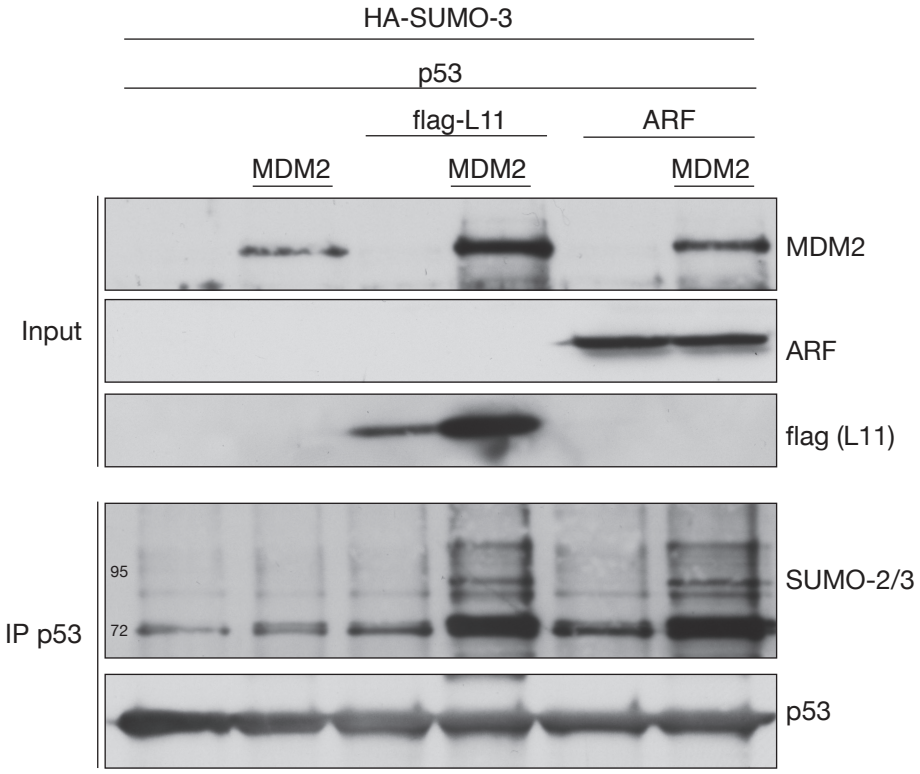

Figure 3-17: MDM2 strongly SUMOylates p53 in complex with p14^{ARF} and L11.
U2OS cells were transfected with the plasmids indicated. Cells were then lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

The ribosomal protein L11 functions similarly to p14^{ARF} by binding to the acidic domain of MDM2 and inhibiting its ubiquitination activity[188], but has not yet been linked to any SUMOylating or deSUMOylating activity. Hence, the effect of L11 co-

expression with MDM2 was investigated. Similar to p14^{ARF} co-expression of L11 also strongly induced the SUMOylation of p53 (Figure 3-17 A, lane 3 and 4).

Previous studies suggested that the ability of MDM2 and p14^{ARF} to drive SUMO-1 modification of p53 was depending on nucleolar relocalisation of MDM2[415]. A cryptic nucleolar localisation (NoLS) signal between amino acids 466 and 473 in MDM2, which is exposed upon p14^{ARF} binding, was identified and characterised earlier[214]. The ribosomal protein L11 was also reported to recruit MDM2 to nucleoli[188]. Deletion of the central domain of MDM2 also exposed this nucleolar localisation signal, resulting in nucleolar accumulation of MDM2 ΔAD[214]. In order to assess whether the strong SUMOylation promoted by MDM2 ΔAD was due to its relocalisation to the nucleoli, a mutation disrupting the nucleolar localisation signal (Figure 3-8 A), was introduced into MDM2 ΔAD (MDM2 ΔAD ΔNoLS). Mutation of lysine residues 466, 467, 469, 470, 471 and 473 was previously shown to prevent p14^{ARF}-induced nucleolar relocalisation of MDM2[214].

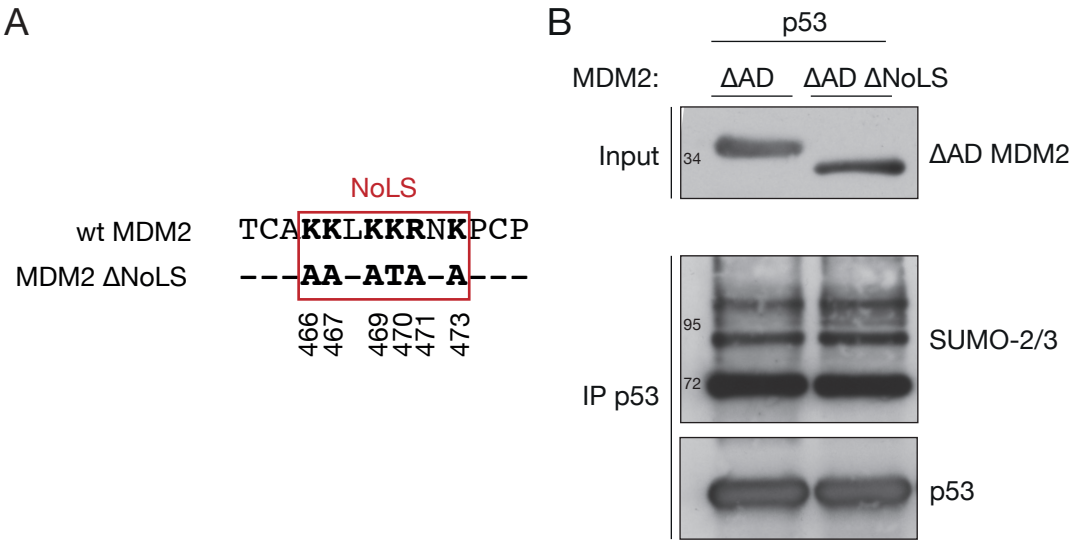

Figure 3-18: MDM2 SUMOylates p53 independently of nucleolar localisation.
(A) Representation of the nucleolar localisation in MDM2 (in red). Amino acids replaced with uncharged residues in the MDM2 ΔNoLS protein are indicated in bold.
(B) H1299 cells were transfected with p53 and wither MDM2 ΔAD or MDM2 ΔAD ΔNoLS. Cells were then lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

However, mutation of the NoLS did not affect the ability of MDM2 ΔAD to promote SUMO-2/3 conjugation of p53 (Figure 3-18 B). This finding is consistent with the observation that the ΔRING MDM2 mutant, which only consists of amino acids 1-

440 and therefore lacks the nucleolar localisation signal[214], can still efficiently SUMOylate MDM2 (Figure 3-5 B).

Analysing cancer-associated missense MDM2 mutation in liposarcomas[703], lymphomas and hepatocellular carcinomas[161] revealed that many point mutations affect the structurally critical cysteine residues of the zinc finger in MDM2's central domain (C305, C308 and C319). One of these mutations (C305F) was subsequently reported to abrogate L11 binding to MDM2, while not affecting binding of $p14^{ARF}$ [162]. Interestingly, the MDM2 C305F mutant was deficient in degrading p53, however retained its ability to promote p53 ubiquitination[162].

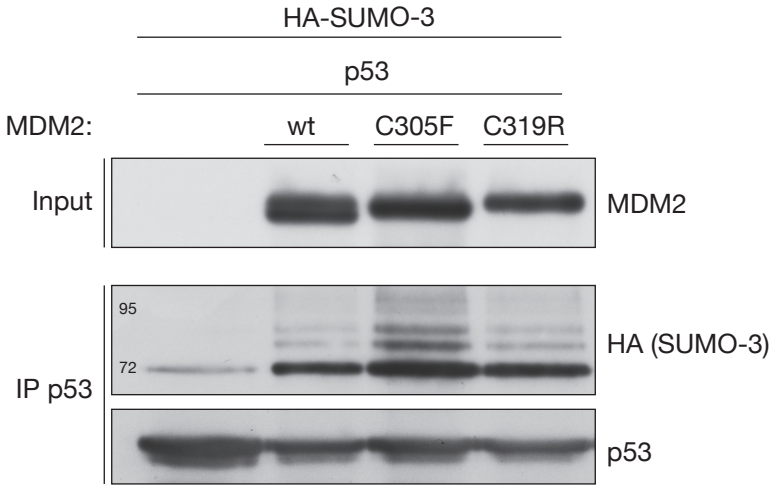

Figure 3-19: Cancer-associated zinc-finger MDM2 mutants SUMOylate p53.
U2OS cells were transfected with the plasmids indicated. Cells were then lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53)

Since L11 had a profound impact on MDM2's SUMOylation activity, the ability of two cancer-associated zinc-finger mutants C305F and C319R MDM2 to SUMOylate p53 was studied. Both cancer-associated MDM2 mutants could SUMOylate p53 well, even slightly better than wild-type MDM2 (Figure 3-19). The data suggests that, although presence of L11 enhanced MDM2-mediated SUMO-2/3 modification of p53, binding of endogenous L11 to MDM2 is not a prerequisite for SUMOylation of p53. Interestingly, mutation of zinc-coordinating cysteine residues leads to slightly stronger SUMOylation of p53.

3.3.2 MDM2 does not require SUMO interaction motifs to SUMOylate p53.

One important mechanism, by which conjugation of SUMO can modify the interactome of the target protein, is by providing a new binding surface for SUMO-interacting motifs (SIMs). The SIM motif has been described to consist of a cluster of hydrophobic amino acids V/I-X-V/I-V/I[354] or V/I-V/I-X-V/I/L[355] with a following stretch of negatively charged amino acids, which later turned out to be more crucial for SUMO-1 and are thought to be dispensable for SUMO-2/3 recognition[358].

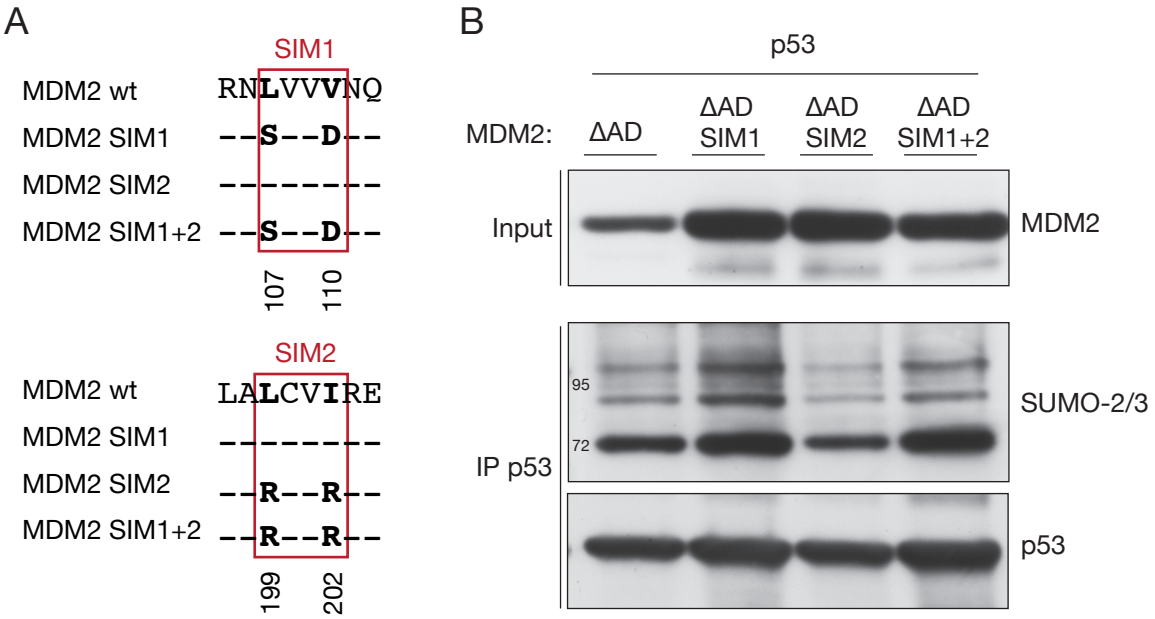

Figure 3-20: MDM2 proteins without putative SIM domains SUMOylate p53.
(A) Representation of the putative SIMs identified in MDM2, indicated in red. Numbers represent the amino acids replaced by hydrophilic amino acids (in bold) in the SIM mutant proteins. SwissProt accession number: MDM2 Q00987.
(B) HCT116 $p53^{-/-}$ cells were transfected with p53 and MDM2 ΔAD with two, one or no SIM motifs. Cells were lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53)

Many proteins involved in the SUMO pathway, for example the SUMO E3 ligase RanBP2[361], rely on SUMO interacting motifs as means to associate with SUMO. Indeed MDM2 interaction with SUMO-2/3 could be observed (data not shown) and it was therefore investigated whether the SUMO binding motif also played a role in the process of SUMO-2/3 modification of p53 via MDM2. Analysis of the MDM2 protein sequence revealed four hydrophobic amino acid clusters, two of which lie outside the acidic domain and could potentially act as SUMO interaction motif in the MDM2 ΔAD protein. Although none of the sequences were perfect matches to the published SIM

consensus, since the first amino acid was a leucine rather than the proposed valine or isoleucine residue, the potential SUMO interaction motifs were mutated. In order to disrupt the motifs, two of the amino acids hydrophobic amino acids within each proposed SIM was replaced with non-hydrophobic residues resulting in the mutant proteins MDM2 ΔAD SIM1, MDM2 ΔAD SIM2 and MDM2 ΔAD SIM1+2 (Figure 3-20 A).

All SIM mutant MDM2 proteins still SUMOylated p53 to a comparable extent (p53 is expressed slightly less in the third lane), suggesting that the mutated domains are either not serving as SUMO-interaction motifs or that the non-covalent SUMO-SIM interaction is not required for p53 SUMOylation by MDM2 (Figure 3-20 B).

3.3.3 MDM2 does not need to dimerise in order to SUMOylate.

Previously, it was observed that MDM2 mutants, which were impaired for ubiquitination, were able to SUMOylate p53 more efficiently. The ability of MDM2 to dimerise was shown to be important for conjugation of Ubiquitin mediated by MDM2[176]. The RING domain of the MDM2 protein can form dimers with another MDM2 RING[156] or the RING domain of the related MDMX protein[146]. Interestingly, mutations affecting the MDM2-dimerisation not only affect its ability to promote ubiquitination, but also neddylation of p53[704].

Figure 3-21: Representation of MDM2 mutants tested in the dimerisation assay.
The region deleted or mutated in the MDM2 mutants are indicated in red.

In contrast to ubiquitination of p53, co-expression of MDMX did not induce MDM2's ability to promote SUMO-3 modification of p53 (Figure 3-1). Furthermore, the data presented here suggest that MDM2 mutants that cannot ubiquitinate tend to SUMOylate p53 more strongly (e.g. C464A MDM2 and MDM2 ΔAD). Not only the RING domain, but also the acidic domain was reported to influence MDM2's dimerisation[705]. Thus, it is possible, that all MDM2 mutants shown to strongly

SUMOylate p53 fail to dimerise, leading to a model of dimeric MDM2 driving ubiquitination of p53 and monomeric MDM2 promoting SUMOylation of p53.

In order to investigate whether the dimerisation of MDM2 mutants correlates with their ability to SUMOylate p53, the dimerisation capability of a number of MDM2 mutants was assessed. An MDM2 protein deleted for the acidic domain and lacking the last 9 amino acids (MDM2 ΔAD Δ9) in the C-terminal tail behind the RING domain (Figure 3-21) was included as a control. This MDM2 mutant was previously reported to neither dimerise nor ubiquitinate p53[174].

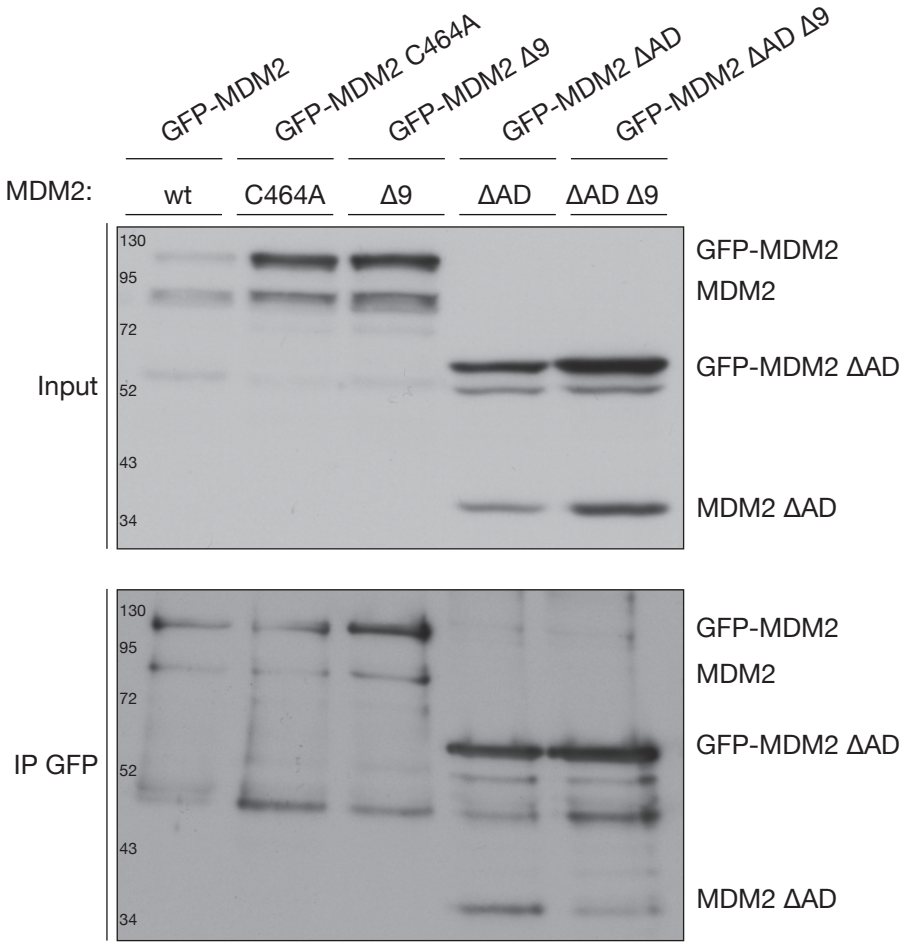

Figure 3-22: MDM2 C464A and MDM2 ΔAD dimerise.
HCT116 cells were transfected with untagged wild-type or mutant MDM2 constructs and their matching GFP-tagged counterparts. Cells were lysed under mild conditions and the tagged MDM2 was immunoprecipitated using a GFP antibody (IP GFP). MDM2 dimerisation was assessed via co-immunoprecipitation of the untagged MDM2.

In this dimerisation assay, untagged and GFP-tagged versions of the MDM2 mutants, which run 30 kD higher on an SDS poly-acrylamid gel, were co-expressed.

Having immunoprecipitated tagged MDM2 with a GFP antibody under native lysis conditions, the amount of un-tagged MDM2 co-immunoprecipitated with the tagged MDM2 was analysed as indication of their ability to dimerise.

Consistent with previous reports that this MDM2 mutant fails to dimerise, the MDM2 protein deleted for the acidic domain and the C-terminal tail (GFP MDM2 ΔAD Δ9) co-immunoprecipitated significantly less untagged protein (Figure 3-22, lane 5) compared with the GFP MDM2 ΔAD protein (Figure 3-22, lane 4). However, all other MDM2 mutants tested, including the RING domain mutant MDM2 C464A still co-immunoprecipitated untagged MDM2 proteins to an extent similar to wild-type MDM2. This observation was surprising, since the RING structure is clearly distorted by the C464A mutation. It is possible that a second interaction via the acidic domain is responsible for the dimerisation observed in this experiment. The MDM2 ΔAD mutant was the strongest promoter of p53 SUMOylation, but dimerised well in this assay. This data suggests that the extent of dimerisation and strength of SUMOylating activity do not correlate.

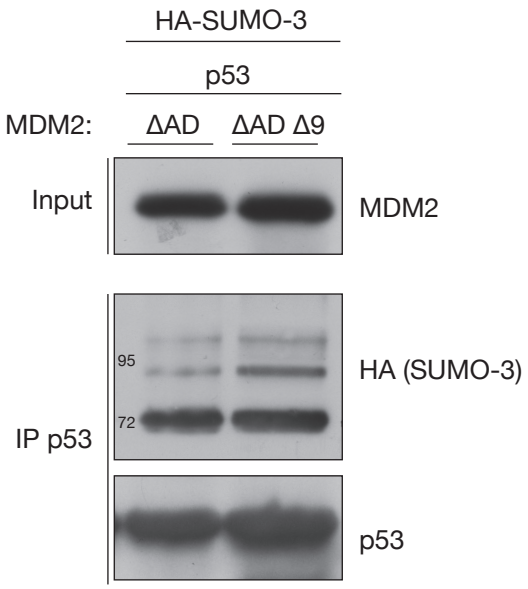

Figure 3-23: MDM2 dimerisation is not required for SUMOylation of p53.
HCT116 cells were transfected with the plasmids indicated and lysed under denaturing conditions. p53 was immunoprecipitated using the DO-1 antibody (IP p53).

However, it was not clear, whether MDM2 dimerisation was a pre-requisite for SUMOylation as in the case of ubiquitination. In order to assess, whether monomeric

MDM2 could promote SUMO-2/3 modification of p53, the monomeric MDM2 ΔAD Δ9 was tested in a SUMO assay (Figure 3-23). This mutant has previously been reported to fail to induce ubiquitination of p53[174], however it can still efficiently promote SUMO-2/3 conjugation (Figure 3-23). Dimerisation of MDM2 is thus not necessary for its SUMOylation activity.

In order to investigate in more detail, why some MDM2 are such efficient promoters of p53 SUMOylation, the interaction of MDM2 with the SUMO-E2 Ubc9 was studied next.

3.3.4 MDM2 interacts with SUMO-E2 Ubc9.

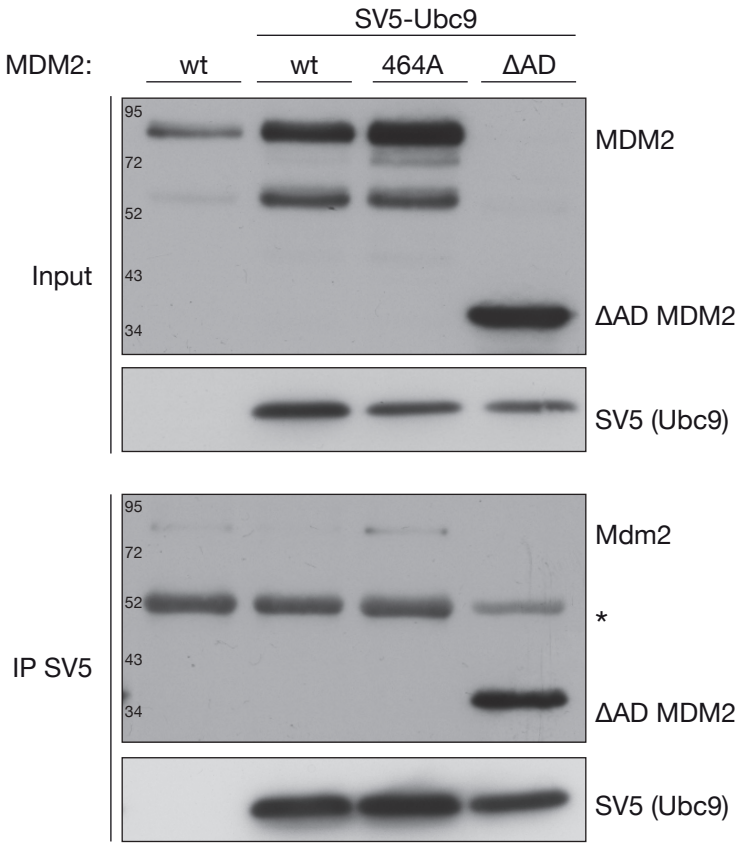

Figure 3-24: MDM2 ΔAD interacts strongly with the SUMO-E2 Ubc9.
HCT116 cells were transfected with the MDM2 constructs indicated and either SV5-tagged Ubc9 or an empty vector. Cells were lysed under mild conditions and Ubc9 immunoprecipitated with an SV5 antibody (IP SV5). The ability of MDM2 to interact with Ubc9 was assessed via the amount of co-precipitated MDM2 protein. * indicates the IgG heavy chain.

SUMO is conjugated to target proteins via a 3-step enzyme cascade similar to conjugation of Ubiquitin. In contrast to the multiple ubiquitin-E2 enzymes described, the only SUMO-conjugating enzyme reported so far is Ubc9[328]. Since MDM2 can promote p53-SUMOylation, the interaction of MDM2 with the SUMO-E2 Ubc9 was analysed.

While hardly any wild-type MDM2 was immunoprecipitated with Ubc9, a weak interaction with MDM2 C464A and a much more pronounced interaction with MDM2 ΔAD were observed (Figure 3-24). It is a striking finding that deletion of the central part of the MDM2 protein leads to such dramatic changes in interaction with the SUMO-E2 Ubc9. This interaction with Ubc9 could explain why the MDM2 ΔAD mutant promotes SUMOylation of p53 particularly efficiently.

3.3.5 MDM2 and MDM2 ΔAD interacting proteins.

Encouraged by the observation that MDM2 ΔAD bound more strongly to Ubc9 than wild-type MDM2, it was investigated whether any other proteins, which could potentially be linked to SUMOylation, also bound differentially to the two proteins. On the one hand it is possible that MDM2 ΔAD not only interacts better with the SUMO-E2 Ubc9, but also cooperates with other SUMO-E3 ligases or factors that promote SUMOylation. On the other hand, the acidic domain of MDM2 could serve as binding platform for deSUMOylating enzymes, which could counteract the SUMO-conjugating activity of the full-length MDM2 protein, but would not be recruited to the MDM2 ΔAD protein. The deSUMOylating enzyme SENP3 was recently reported to bind MDM2 between amino acids 222-274[706], which are missing in the MDM2 ΔAD protein. MDM2 was furthermore shown to interact with SUSP4, the mouse homologue of SENP2[707], although it is not known where this interaction takes place.

Proteins interacting with MDM2 and MDM2 ΔAD were identified using mass spectrometry on eluates from an MDM2 immunoprecipitation experiment. In brief, MDM2 and its interacting proteins were immunoprecipitated with flag-antibody-coupled beads and eluted with triple-flag peptide using lysates from cells overexpressing either flag-tagged full-length MDM2 or flag-tagged MDM2 ΔAD. Flag-peptide elution was used to circumvent elution of proteins binding to the beads or

the tubes, which would be present in the sample if MDM2 had been eluted in SDS instead.

First, the eluted proteins were denatured and trypsin-digested, then the resulting peptides were separated by liquid chromatography. This involved capturing peptides from the digest on a C_{18} column and eluting them according to their polarity with a water/acetonitrile gradient. The peptides eluted from the reversed phase liquid chromatography column were automatically sprayed into an LTQ Orbitrap Velos MS-MS system. In this system, ionised peptides first pass through an orbitrap mass spectrometer in a survey scan, certain peptides are then selected for collision in a linear trap quadropole (LTQ) and their fragment spectra recorded in another Orbitrap.

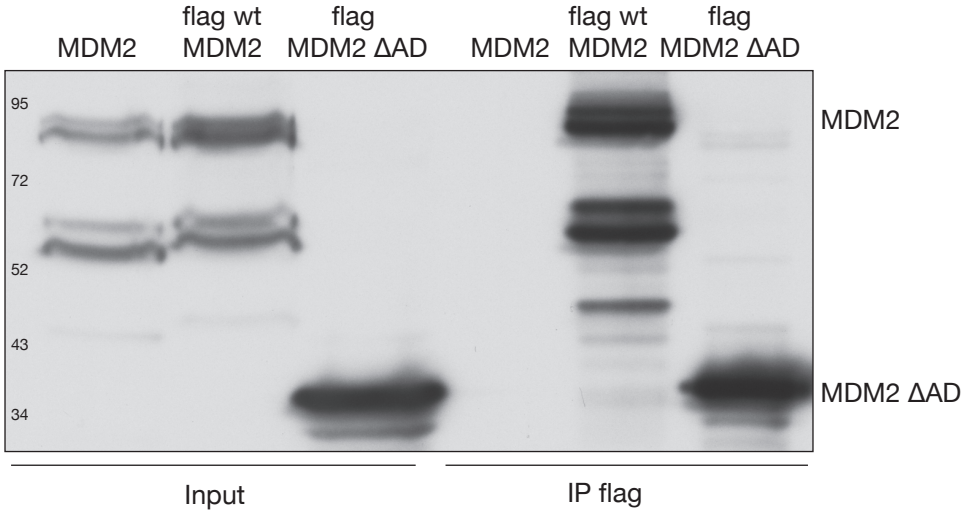

Figure 3-25: MDM2 is efficiently eluted from the flag beads.
Hek293T cells were transfected with flag MDM2 or flag MDM2 ΔAD (untagged MDM2 was included as control, but not used for analysis by mass-spectrometry). Cells were lysed under native conditions and MDM2 immunoprecipitated using flag beads. Elution from the beads with the flag-peptide was achieved efficiently (IP flag).

In an Orbitrap mass spectrometer, ions are injected perpendicular to an electric field between a central inner electrode and a barrel-shaped outer electrode. The ions begin to rotate around the central electrode (orbital trapping) and the path of oscillation is dependent on the mass to charge ratio (m/z) of the peptide. Thus, the m/z peaks, which are used to identify the peptides in the sample, can be calculated from the oscillation path of the ions in the Orbitrap[708]. Recording a spectrum of the peptide fragments after collision in a second round of mass spectrometry helps to identify the peptides corresponding to the m/z peak corresponding to the unfragmented peptide recorded in the first round of mass spectrometry. In this

system, the linear trap quadrupole (LTQ) was used to select the ions giving rise to the ten most intense peaks for collision.

The mass spectra were used to find matches for the trypsin-cleaved peptides in the human SwissProt database. Although this experiment was not a quantitative assay, if a number of peptides of the same protein are exclusively identified in one sample, this could be an indication that the protein predominantly interacts with either wild-type MDM2 or MDM2 ΔAD. Hits, which had 5 or more unique peptides identified in one sample and none or only one in the other sample were therefore treated as proteins preferentially binding to either full-length MDM2 or MDM2 ΔAD.

Identified proteins	Accession Number	Unique peptides	
		MDM2 ΔAD	wt MDM2
E3 Ubiquitin-protein ligase MDM2	Q00987	9	19
Cellular tumour antigen p53	P04637	13	16
60S ribosomal protein L5	P46777	10	15
40S ribosomal protein S3	P23396	8	12
60S ribosomal protein L11	P62913	3	8
60S ribosomal protein L23	P62829	3	7
Nucleophosmin	P06748	6	7
Nucleolin	P10338	10	5

Table 3-1: Well-established MDM2-binding proteins identified in both samples.
Proteins well established to interact with MDM2 identified in both samples by detection of at least 5 unique peptides in one and at least 3 in the other sample. Numbers represent unique peptides identified.

Serving as positive control for a successful assay setup, MDM2 itself and its binding partner p53 were identified in both samples. Since MDM2 ΔAD is considerably shorter than full-length MDM2, it is not surprising that less unique peptides were identified in the cells expressing MDM2 ΔAD. A number of ribosomal proteins are well established binding partners for p53, such as L5[223], L11[188], L23[222] and more recently also S3[709]. Interaction of MDM2 with the ribosomal protein S3[709] and the proteins Nucleolin[710] and Nucleophosmin[711] were found to take place outside the acidic domain, consistent with the identification of peptides in both samples (Table 3-1). However, although L5, L11 and L23 were described to bind to the MDM2 acidic domain[220-222], interaction with MDM2 ΔAD was also observed in this proteomics analysis (Table 3-1). It is possible that MDM2 ΔAD could have dimerised with

endogenous full-length MDM2 and co-immunoprecipitated these ribosomal proteins via the acidic domain of the endogenous protein. However, since overexpression levels were very high, the likelihood for exogenous/endogenous MDM2 dimers to occur is very low. Furthermore, the acidic domain binding protein $p14^{ARF}$ was exclusively identified in cells expressing full-length MDM2, but not MDM2 ΔAD (not included in list since only 4 peptides were identified), suggesting that differences in binding can be picked up with this experimental setup.

Identified proteins	Accession Number	Unique peptides	
		MDM2 ΔAD	wt MDM2
PHD finger protein 6	Q8IWS0	0	**16**
40S ribosomal protein S4	P62701	1	**10**
DNA ligase 3	P49916	0	**10**
Splicing factor 3B subunit 2	Q13435	0	**8**
Proliferation-associated protein 2G4	Q9UQ80	1	**7**
Ubiquitin carboxyl-terminal hydrolase 7	Q93009	1	**6**
60S ribosomal protein L10	P27635	0	**6**
40S ribosomal protein S3a	P61247	0	**6**
40S ribosomal protein S2	P15880	1	**5**
60S ribosomal protein L26-like 1	Q9UNX3	0	**5**
40S ribosomal protein S11	P62280	0	**5**
Developmentally-regulated GTP-binding protein 1	Q9Y295	0	**5**
40S ribosomal protein S25	P62851	1	**4**
E3 Ubiquitin-protein ligase HUWE1	Q7Z6Z7	**14**	0
Protein phosphatase 1G	O15355	**9**	1
Sodium/potassium-transporting ATPase subunit alpha	P05023	**9**	1
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	**5**	1

Table 3-2: Proteins binding selectively to either full-length MDM2 or MDM2 ΔAD.
Proteins identified in one sample by detection of at least 5 peptides in the mass-spectrometer, with no more than one peptide identified in the other sample were selected for this list. Numbers represent unique peptides identified.

A number of other ribosomal proteins S2, S3a, S4, S11, S25, L10 and L26L1 exclusively interacted with full-length, but not MDM2 ΔAD (Table 3-2). While many of these proteins have never previously been described to interact with MDM2, S25[712] and L26[713] were recently reported to bind to MDM2. Since many other ribosomal

proteins, such as S7[226], S14[714], S20[715] and S27a[716] have only recently been discovered to bind to MDM2, it is possible that the interaction with other ribosomal proteins, such as the ones identified here, had not yet been described. Since ribosomal proteins are part of large protein complexes, it is furthermore conceivable that some of these proteins do not directly interact with MDM2, but are pulled down in a complex with other ribosomal proteins.

Several non-ribosomal proteins were identified that bind exclusively to full-length MDM2, such as PHD finger protein 6, DNA ligase 3, Splicing factor 3B subunit 2, Proliferation-associated protein 2G4, Ubiquitin carboxyl-terminal hydrolase 7 and Developmentally regulated GTP-binding protein 1. While none of these proteins are involved in the SUMO-pathway, the Ubiquitin carboxyl-terminal hydrolase 7, also known as USP7 or HAUSP, is known to deubiquitinate both p53 and MDM2[313,314]. In contrast to one study mapping the HAUSP interaction to MDM2's amino acids 147 and 159[717], a preferred interaction with the full-length MDM2 protein was observed here, suggesting that the acidic domain of MDM2 might be supporting the interaction with HAUSP. Consistent with these results, a different structural study mapped the HAUSP interaction region to amino acids 222-232, which are deleted in our the MDM2 ΔAD protein[718]. However, HAUSP seems to be specific for the removal of Ubiquitin and no SUMO-related activities have been reported to date. It is possible though, that posttranslational modifications such as ubiquitination of MDM2 regulate its activity to SUMOylate p53 or Ubiquitin-modification of p53 could regulate its availability for subsequent SUMOylation and it would therefore be interesting to investigate whether HAUSP could modulated MDM2's ability to promote SUMO-2/3 modification of p53.

Another notable hit was the PHD finger protein 6 (PHF6) that exclusively bound to full-length MDM2, but not MDM2 ΔAD. PHD domains contain zinc-coordinating structures similar to RING fingers[719]. The PHD domain of the Kip1 protein has recently been demonstrated to confer intramolecular SUMO-E3 ligase activity[720] and it is possible that the PHD finger 6 protein also plays a role in SUMOylation.

The Proliferation-associated protein 2G4, also known as ErbB3 binding protein EBP1, is a part of a large ribonucleotide protein complex and was previously

observed to bind MDM2, promoting its interaction with p53[721]. Since MDM2-mediated p53 ubiquitination was also induced by EBP1, it was described as ubiquitination cofactor[721]. It could be informative to investigate whether this binding factor exclusively promotes ubiquitination, or also drives SUMOylation, since the p53-MDM2 interaction was promoted by EBP1. No association with MDM2 or the UBL pathway are known for DNA ligase 3, Splicing factor 3B subunit 2 or developmentally-regulated GTP-binding protein.

By contrast, peptides of a number of proteins (E3 Ubiquitin ligase HUWE1, Protein phosphatase 1G, Sodium/potassium-transporting ATPase subunit alpha (ATP1B1) and heterogeneous nuclear ribonucleoproteins A2/B1) were exclusively identified in the sample overexpressing MDM2 ΔAD. No associations of ATP1B1 with MDM2 or SUMOylation could be found. While no direct interaction of Protein phosphatase 1G (PPM1G) and MDM2 has been described, the phosphatase was recently found to dephosphorylate HAUSP, leading to downregulation of its deubiquitinating activity towards MDM2[722]. MDM2's SUMOylation activity could potentially be regulated by modification with ubiquitination, and it is furthermore possible that MDM2 is a PPM1G target and that MDM2's E3 functions are regulated by phosphorylation. Strikingly, the HECT-domain containing E3 Ubiquitin ligase HUWE1 bound exclusively to MDM2 ΔAD, but not to wild-type MDM2. To date, no HECT-domain ligases have been described to promote SUMOylation, however it would still be informative to test whether HUWE1 could aid p53-SUMOylation.

While the proteomics analysis did not reveal any obvious explanation as to why MDM2 ΔAD promotes SUMOylation so efficiently, a number of hits, most of which are associated with ubiquitination, were identified and could form the basis for future studies.

Having studied the SUMO-2/3 conjugation to p53, the potential of MDM2 to be modified by SUMO-2/3 itself was investigated.

3.4 SUMO-2/3 modification of MDM2.

SUMO-1 conjugation to MDM2 was previously reported[425,426,431,432] , yet no definite SUMO-site has been identified to date. Since MDM2 is a Ubiquitin-ligase not only for p53, but also for itself[157,181] it was investigated, whether MDM2 could also be SUMO-2/3 modified itself.

Unlike ubiquitination, SUMOylation is in most cases restricted to lysine residues in the environment of a SUMO motif ΨKxE[331]. However, not every SUMO motif is SUMOylated and sometimes also lysine residues outwith the canonical consensus sequence are modified by SUMO. Analysis of the MDM2 amino acid sequence with the SUMO site prediction tool SUMOsp 2.0[723] revealed one classical SUMO consensus motif (ΨKxE) around lysine 346 and two non-canonical SUMO sites at lysine 185 and 422 (Figure 3-26). A study from David Lane's lab had previously narrowed the SUMO-1 conjugation site down to an area between amino acids 134 and 212[425].

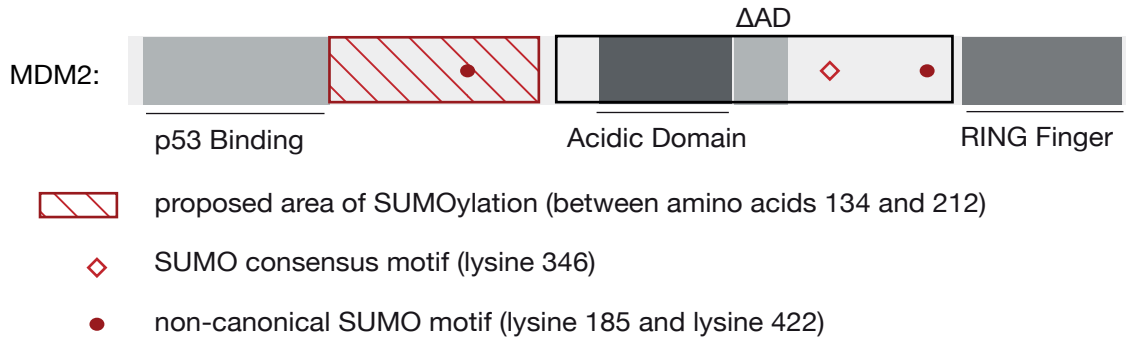

Figure 3-26: Potential SUMO sites on MDM2.
Representation of the MDM2 protein with SUMO sites predicted by SUMOsp2.0 prediction tool and previous studies (in red). The area deleted in the MDM2 ΔAD protein is indicated in black.

All studies on MDM2-SUMOylation have so far been conducted with SUMO-1, and it was therefore assessed whether any SUMO-2/3 conjugated MDM2 could be detected after overexpressing SUMO-3 with an MDM2 mutant, which had been found to drive strong SUMOylation of p53 in previous experiments. Indeed, MDM2 ΔAD showed a higher band doublet in the SUMO assay, which was shifted up by roughly 20 kD and could represent conjugation of a single SUMO-3 moiety (Figure 3-27 A).

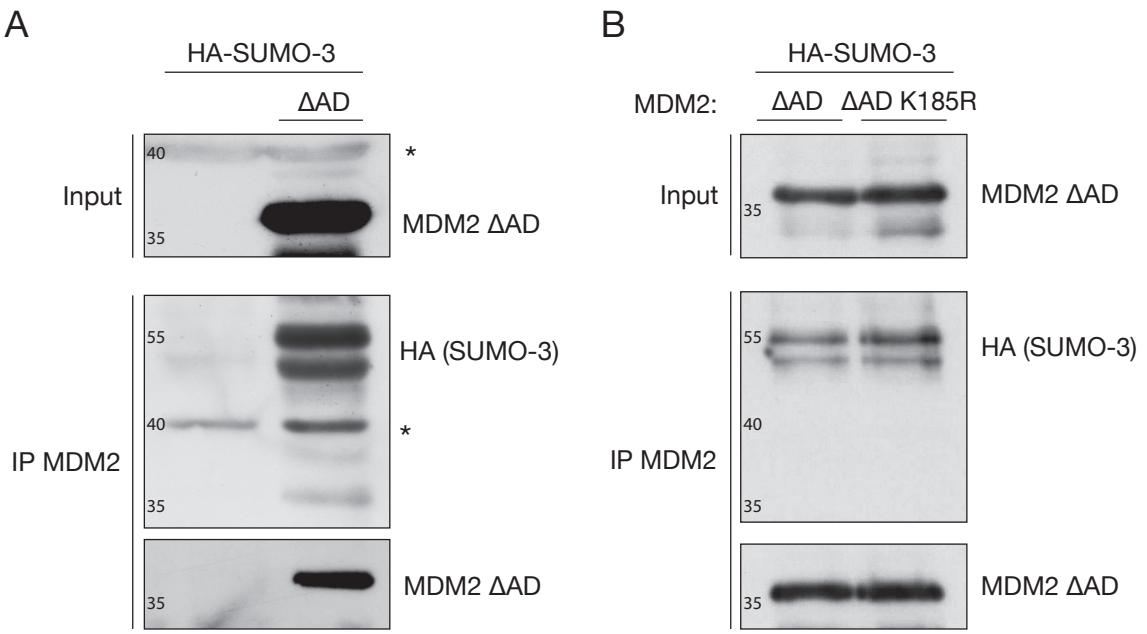

Figure 3-27: MDM2 ΔAD MDM2 is SUMO-2/3 modified.
HCT116 cells were transfected with the indicated MDM2 mutants and SUMO-3. Cells were lysed under denaturing conditions and MDM2 was immunoprecipitated using the Ab-1 antibody (IP MDM2). * indicates an Actin band. Size marker is included as guidance, numbers indicate protein size in kD.

Since MDM2 ΔAD is efficiently SUMOylated, both the canonical SUMO consensus site at lysine 346 and the non-canonical site at lysine 422 were excluded as potentially SUMOylated residues, because all amino acids between 222 and 437 are deleted in this protein. The only remaining proposed SUMO site lies at lysine 185, which also fulfils the previously set requirement of SUMOylation taking place between amino acids 134 and 212[425]. MDM2's lysine 185 was thus mutated to arginine and its ability to be SUMOylated analysed. The resulting MDM2 ΔAD K185R protein was still SUMOylated equally well as MDM2 ΔAD (Figure 3-27 B), suggesting that SUMO-2/3 is conjugated to a different lysine residue in MDM2 ΔAD.

Taken together, these data show that MDM2 is not only SUMO-1 modified, but also SUMO-2/3 conjugated. At least one SUMO site is located outside the region deleted in the MDM2 ΔAD mutant, however the predicted SUMO site around lysine 185 is not the SUMOylated residue. Three other lysine residues (K136, K146 and K182) are located in the region proposed to be SUMOylated by Xirodimas et al[425] and it could be of interest to determine whether mutation of any of these lysines could abrogate SUMO-2/3 modification.

3.5 Summary and Discussion

Taken together, these data show that, in addition to promoting Ubiquitin conjugation to p53, MDM2 can also enhance modification by SUMO-2/3 and that the requirements are different to those previously reported for SUMO-1.

MDM2-mediated conjugation of SUMO-2/3 to p53 depends on the p53-MDM2 interaction (Figure 3-3), but is independent of the integrity of the RING domain (Figure 3-5), which is essential for p53 ubiquitination[181]. Although the Ubiquitin-E3 ligase activity is not required for SUMOylation of p53, MDMX, which binds p53 in a manner similar to MDM2, cannot promote p53 SUMOylation (Figure 3-1). Furthermore, a monomeric MDM2 is sufficient to drive SUMOylation (Figure 3-23), while a homodimer of MDM2 or heterodimer of MDM2 and MDMX is required for efficient ubiquitination[174]. Interestingly, the SUMO-E2 can directly bind to the SUMO consensus in the target protein[335] in contrast to Ubiquitin E2 enzymes that do not tightly associate with the target protein. In light of this different role of the conjugating enzyme in the SUMOylation cascade, it is conceivable that a potential SUMO-E3 ligase would also require different activities than a Ubiquitin-E3 ligase. The PIAS family SUMO-E3 ligases contain SP-RING domains related to Ubiquitin-E3 RINGs, and it was suggested that they act similarly to the Ubiquitin-E3 RINGs by binding to the E2-UBL thioester and activating the thioester-bond for transfer to the target[724]. However, even though RING fingers (or similar structures like PHD fingers) or HECT domains seem to be strictly required for the activity of Ubiquitin E3 ligases, SUMO-E3s do not always rely on this functional domain to promote SUMOylation of their targets. Indeed, the first identified SUMO-E3 ligase RanBP2 contains neither RING- nor HECT-domain like structures[333], but harbours its catalytic activity in two internal repeat domains[361]. While PIAS4 requires an intact RING domain to promote SUMOylation of p53[412], it can promote SUMOylation of Yin Yang1 independently of the SP RING[725]. PIAS4-mediated SUMO-modification of GATA-2 also does not rely on the RING domain, but requires to bind to GATA-2[726], similar to the requirement of MDM2 to interact with p53 in order to promote SUMO-2/3 modification of p53 (Figure 3-3).

Generally, MDM2 mutants that were unable to ubiquitinate were better in promoting SUMOylation of p53 (Figure 3-6). This finding points towards distinct SUMO- and ubiquitination activities in the same enzyme and possibly a competition of both activities. The degree of SUMO conjugation activity however does not correlate with the loss of ubiquitination activity. For example, the MDM2 ΔAD mutant retains some residual ubiquitination activity, but promotes SUMOylation of p53 most efficiently (Figure 3-6). This MDM2 mutant had previously been shown to promote SUMO-1 conjugation to p53, which was linked to its relocalisation to the nucleolus[415]. However, relocalisation of MDM2 to the nucleolus is not a prerequisite for SUMO-2/3 modification of p53, since MDM2 mutants that have lost the nucleolar relocalisation signal, for example ΔRING (Figure 3-5) and MDM2 ΔAD ΔNoLS (Figure 3-18), or are not activated for nucleolar localisation, for example 464A MDM2 (Figure 3-6), show efficient SUMO-2/3 conjugating activity. Interestingly, only SUMO-1, but not SUMO-2/3 localises to the nucleolus[349], which might explain the differences observed between the requirements for MDM2-mediated SUMO-1 and SUMO-2/3 modification. Nevertheless, similar to previous observations for SUMO-1 conjugation of p53, co-expression of $p14^{ARF}$, which induces nucleolar relocalisation of MDM2 and inhibits its ubiquitination activity, markedly induced MDM2's ability to promote SUMO-2/3 conjugation of p53 (Figure 3-17). Importantly, $p14^{ARF}$ has been linked to other MDM2-independent mechanisms of promoting SUMOylation, since it binds and recruits the SUMO-E2 Ubc9[428] and induces turnover of the deSUMOylating enzyme SENP3[429]. Co-expression of the ribosomal protein L11, which also inhibits MDM2-mediated ubiquitination, but has not been linked to any further involvement in the SUMOylation pathway, also promoted SUMO-2/3 modification of p53 by MDM2 (Figure 3-17).

Rather than relying on MDM2 relocalisation to the nucleolus, I propose that the strong SUMO-2/3 conjugating activity of MDM2 ΔAD depends on MDM2-interacting proteins, as this mutant interacted particularly well with the SUMO-E2 Ubc9 (Fig 3-24). Other proteins binding to this region could have additional effects, for example it has recently been published that the Sentrin-specific protease Senp3 interacts with MDM2 between amino acids 222-274[706], which are missing in the MDM2 ΔAD protein. Although no SUMO-specific proteases known to interact with MDM2[706,727] were identified as MDM2-interacting proteins in the mass-spectrometry experiment

(Table 3-2), a number of proteins were identified to bind exclusively to either protein. In particular, the HECT-E3-ligase HUWE1, which only interacted with MDM2 ΔAD and the PHD finger protein 6, which only interacted with full-length MDM2 could form the basis for further investigations. Although these proteins have not yet been linked to SUMOylation, it would still be conceivable that they might have an effect on p53-SUMOylation since both Ubiquitin ligases, albeit to date no HECT-domain ligases, and PHD-domain containing proteins[720] have previously been found to confer SUMOylation activity.

The observation that mouse p53 cannot be SUMOylated (Figure 3-10) is crucial for the choice of an animal model. The knock-in mouse created by Krummel et al. where all C-terminal lysine residues (7 in the mouse, 6 in the human protein) were mutated to arginine[151], which strikingly did not result in any major change in p53 regulation, was thought to affect all C-terminal Ubiquitin-like modifications. But since wild-type murine p53 cannot be SUMOylated, no conclusions about the effects of SUMOylation on p53-activity can be drawn from this study. Histological co-staining of p53 and SUMO-1 in mouse tumours[402] will therefore most likely not represent SUMOylated p53 either. Strikingly, SUMOylation of p53 was recently also studied in rat brain neurons[728], however similar to mouse p53, the SUMO motif is not conserved in the rat p53 sequence and it remains to be determined, where this SUMOylation of the rat p53 took place. SUMOylation of p53 has also been studied in drosophila, but limited conclusions can be drawn form these experiments, since drosophila p53 has two SUMO motives, one in the N-terminus and the central part of the protein, but the C-terminal SUMO consensus found in the human protein is not conserved[729]. Interestingly, a lethal SUMO knockout model in zebrafish could be rescued by deletion of p53, raising the possibility that p53-SUMOylation could play a crucial role here[730]. Since zebrafish p53 does contain a C-terminal SUMO-site homologous to the human site, it would be informative to create a lysine 366 or glutamate 368 mutant knock-in zebrafish (analogous to the human K386R and E388R p53) to study functional consequences of p53 SUMOylation in vivo.

Although the same SUMO-E2 enzyme (Ubc9) is responsible for conjugating all SUMO-isoforms, some target genes are preferentially conjugated with SUMO-1 and others show much higher SUMOylation with SUMO-2/3[348,731]. While MDM2

predominantly modified p53 with SUMO-3 (Figure 3-16), it remains challenging to compare SUMO-1 and SUMO-2/3 conjugated levels. Although overexpression of tagged SUMO-isoforms might be able to eliminate possibly different isoform-specific antibody quality issues, immunoprecipitation of SUMOylated targets can only capture a snapshot picture, which might be highly flawed due to the different dynamics of SUMO-1 and SUMO-2/3[348]. Overexpression, especially of SUMO-1, which is normally mostly conjugated to proteins rather than available free in the cell, can furthermore shift the natural balance of a high free SUMO-2/3 pool and identify SUMO-1 targets which would naturally preferentially be conjugated to SUMO-2/3[349]. Despite these technical difficulties, functionally distinct consequences of SUMO-1 versus SUMO-2/3 conjugation are emerging. Isoform-specific SUMO-interacting motifs for the recruitment of interacting proteins[358] and SUMO-chain-specific functions[731] - chain formation is limited to SUMO-2/3[673] - have been described.

Despite the observation of a ladder of SUMOylated p53 bands, p53 did not seem to be poly-SUMOylated since mutation of lysine residue 11 in SUMO-3, which is required for chain formation, did not alter the band pattern (Figure 3-15). Thus, the ladder of bands probably reflects a combination of SUMO- and Ubiquitin- or Nedd8-conjugation to different lysine residues in p53. This hypothesis is consistent with the observation that mutation of multiple lysine residues in p53 ΔNLS, some of which are known to be targeted for ubiquitination[141,310] and neddylation[319], altered the band pattern of SUMOylated p53 from three main bands to two main bands (Figure 3-12). However, in light of a recent report that SUMO-3 can also form chains via lysine 41[732], the question of a SUMO chain on p53 could be re-adressed by also mutating lysine 41. Furthermore, the cells overexpressing the arginine mutant SUMO isoforms, still contain endogenous SUMO, which could still be used for conjugation of another SUMO molecule. In order to eliminate these technical limitations, it would be useful to analyse SUMOylated p53 with mass-spectrometry, where identification of branched SUMO-peptides would give a clear indication of the presence of SUMO-chains[733].

Specific SUMO-paralogues can be selected for conjugation to certain targets, mediated by SUMO-interacting motifs showing higher affinity towards particular isoforms. A stretch of hydrophobic residues is important for recognition of all SUMO-isoforms, whereas a cluster of negatively charged amino acids in close proximity was

identified to preferentially interact with SUMO-1, potentially due to interaction with a lysine residue at position 78 in SUMO-1, which is not present in SUMO-2/3[358]. Analysis of the MDM2 amino acid sequence revealed two potential SUMO interaction motifs outside the central acidic domain region. One of the hydrophobic clusters identified in MDM2 (SIM1) is followed by a number of negatively charged amino acids and serine residues (QQESSDS), which have also frequently been observed in vicinity to SIMs, and could be negatively charged after phosphorylation of the hydroxyl group. This suggests that SIM1 would possibly prefer binding to SUMO-1, which might explain why no difference in SUMO-2/3 conjugation of p53 was observed here (Figure 3-20).

Since the second hydrophobic stretch (SIM2) lacks negatively charged amino acids it would probably preferentially interact with SUMO-2/3. Indeed, SIM2 is an inverted version of the SUMO-2/3 specific SIM identified in the deubiquitinating enzyme USP25[392]. However, no reduction of SUMOylation activity was observed with the MDM2 SIM2 mutant. Many SUMO-E3 ligases, including RanBP2[734], PIAS proteins[354] and Pc2[735] contain SIMs and these motifs can contribute to their SUMO-ligase activity. Possibly, since MDM2 can directly interact with Ubc9, which would bring along the activated SUMO and p53, no non-covalent interaction with SUMO is required for MDM2-mediated SUMOylation of p53.

SUMO targets can also contain SIMs, through which they can interact with Ubc9 charged with SUMO. This provides a mechanism for SUMOylating lysine residues outwith a classical SUMO consensus, which Ubc9 would bind directly[335]. Such a SIM, which is crucial for its SUMOylation, has been identified in the BLM DNA helicase[357]. p53 also contains a hydrophobic stretch of amino acids, starting from amino acid 252 (ILTII) in proximity of negatively charged amino acids (EDSS). It would be interesting to assess, whether mutation of this potential SIM could affect SUMOylation of p53.

Furthermore, the potential SIMs identified in MDM2 could also play a role in SUMOylation of itself[425,427]. MDM2 SUMOylation has been subject to many controversies, especially after the retraction of a paper, which proposed the SUMO site to be lysine 446 (reviewed in[323]). To date the definitive SUMO site on MDM2 has

not been identified. Mutation of K185, which was a software-predicted non-canonical SUMO site[723] matching previous observations that the site of SUMOylation lies within amino acids 134 and 212[425] did not prevent SUMOylation (Figure 3-27). This finding recapitulates earlier experiments, where K185R MDM2 was also SUMO-1 modified to a similar extent as wild-type MDM2[426]. The search for the SUMO site is further complicated by the presence of the nuclear localisation signal in this region (between amino acids 181 and 185)[699]. Lysine 182 had also been suggested as SUMO site, since K182R could not be SUMOylated by PIAS1 in cells[426]. However, it needs to be considered that lysine 182 is part of the MDM2's nuclear localisation signal and the MDM2 K182R mutant protein was mostly found in the cytoplasm. Indeed, MDM2 K182R was still SUMOylated in vitro, suggesting that the change in localisation could indeed have been the factor abolishing SUMOylation of the MDM2 K182R protein in cells[426]. It is therefore difficult to determine whether lysine 182 is a SUMO accepting lysine residue. There are two more lysine residues (K136 and K146) in the region predicted to be SUMOylated between amino acids 134 and 212[425]. It would be informative to mutate these to lysine residues as well and to investigate, whether a reduction in SUMOylation could be observed. MDM2's SUMO site has still not been determined and it is possible that the identification has been hindered by the presence of multiple sites, which could explain why a single lysine replacement has not yielded any success so far.

Taken together, the data presented in this chapter suggest that rather than catalytically enhancing the SUMOylation reaction by activating the Ubc9-SUMO thioester through contact of the RING, MDM2 promotes the SUMOylation of p53 by bringing the E2, SUMO and the target into close proximity. It is therefore crucial for MDM2 to interact with both p53 and Ubc9, interactions with both of which were shown here. I therefore suggest that MDM2 does not act as a classic E3 ligase, but rather as a co-activator and scaffold to the SUMOylation reaction. Promotion of SUMOylation independent of the RING domain was often shown to require SUMO interaction motifs, as in the case of the PIAS proteins[736]. However, the potential SIMs in MDM2 were not required to promote SUMO-2/3 modification of p53. Since an interaction between MDM2 and Ubc9 was observed, it is possible that MDM2 does not directly interact with SUMO, but rather brings the SUMO-charged Ubc9 into proximity of p53.

This model is in accordance with the observation that the MDM2 RING domain and dimerisation were not required for the SUMOylation of p53. However, it is still unclear how the multiple activities of MDM2 are regulated. It was observed here, that conditions which inhibited the Ubiquitin-ligase function generally stimulated SUMOylation. It is possible that MDM2 is by default in ubiquitination mode and that a switch to SUMOylation mode is triggered by MDM2-binding proteins. For example co-expression of $p14^{ARF}$ and L11, which inhibit MDM2's Ubiquitin E3-ligase activity, enhanced its ability to SUMOylate. It is furthermore possible, that posttranslational modifications play a role in regulating the multiple functions of MDM2. It was shown that the Ubiquitin- and SUMO-E3 ligase Topors switches from ubiquitination to SUMOylation of p53 after Topors has become phosphorylated by PLK1[737]. A similar scenario could be proposed for MDM2 and it would be useful to test the impact of serine 386 and serine 429 phosphorylation, which were reported to disrupt MDM2 dimerisation[205], on MDM2's ability to promote SUMOylation of p53. Also, acetylation of lysine residues K466 and K467 in MDM2's RING domain was reported to inhibit p53 ubiquitination by MDM2[738] and it would be informative to test the impact of these modifications on MDM2-mediated SUMOylation of p53.

It is still unclear, when p53 would become SUMOylated in a cell. Both p53 and SUMO-2/3 are involved in stress response pathways and it is possible that SUMOylation has a role in modulating p53's transcriptional output in response to certain stresses. DNA damage is unlikely to induce p53 SUMOylation, since many phosphorylation events modify the N-terminus of p53, thereby disrupting its interaction with MDM2, which is clearly required for efficient SUMO-2/3 modification. Indeed, phosphorylation of p53 has previously been reported to impair its SUMOylation[408]. However, since L11 and $p14^{ARF}$ were found to stimulate p53 SUMOylation, SUMO-2/3 modification of p53 could be induced as part of the responses to oncogenic and ribosomal stress.

Clearly, many posttranslational modifications have important roles in modulating p53's activity. Therefore the functional implication of p53 SUMOylation was studied in the following chapter.

4 Modulation of p53 activity by SUMO-2/3 modification.

Posttranslational modifications are often involved in regulating the activity of transcription factors. It was therefore investigated whether SUMO-2/3 modification on p53 affected its activity as a transcription factor. Although more studied than SUMO-2/3, consequences of SUMO-1 modification on p53 activity are still a matter for debate[404] (the early p53-SUMO results are reviewed in[739]). There have been reports that SUMO-1 modification of p53 results in increased[405,406], decreased[410] and unchanged transcriptional activity[407]. A more recent study has shown that SUMO-1 modification of p53 plays a role in inhibiting C-terminal acetylation, thereby repressing DNA binding and transcriptional activity[401]. Other investigators reported that conjugation of acetylated SUMO-1 to p53 can modulate the transcriptional activity of p53 differently than conjugation of unmodified SUMO-1[402]. The few studies, which have addressed SUMO-2/3 conjugation of p53, linked H_2O_2[421] or K-bZIP induced[422] SUMO-2/3 modification to increased p53 transcriptional activity. However, SUMOylation has generally more often been associated with transcriptional repression[380]. In order to study the consequences of SUMOylation, the SUMO isoforms were often overexpressed together with p53. This approach harbours caveats, because SUMO-overexpression would affect SUMOylation of many other proteins as well, making it difficult to link observed differences back to p53. Others used a p53 protein mutated for the SUMO-accepting lysine residue, p53 K386R. While this approach will certainly yield more p53-specific results, it is difficult to determine the impact of SUMO-modification of this lysine residue, since lysine 386 is also acetylated and ubiquitinated. SUMOylation is in most cases dependent on the presence of a SUMO motif, particularly a glutamic residue in proximity to the SUMOylated lysine, mutation of which abrogates SUMOylation at the lysine (Figure 3-9), but most probably does not impair other modifications of the lysine residue. Thus, if p53 K386R and p53 E388A show the same phenotype, the effect can most likely be attributed to disruption of SUMOylation of p53. Surprisingly, none of the above mentioned studies used the p53 E388A mutation to specifically inhibit SUMOylation of p53, while sparing lysine 386 for other modifications.

In order to determine the effect of MDM2-induced SUMO-2/3 conjugation on p53 localisation, protein stability, tetramerisation and transcriptional activity, the assays performed here used both p53 K386R and p53 E388A as non-SUMOylatable p53. While mutating the SUMO-site cannot distinguish between the effects of the different

SUMO-isoforms, most of these experiments were carried out in the presence of MDM2 ΔAD, which predominantly promotes SUMO-2/3 modification of p53.

4.1 SUMOylation does not affect p53 localisation.

p53 activity can be regulated by its localisation, since it cannot act as a transcription factor on the promoters of its target genes, if it is excluded from the nucleus.

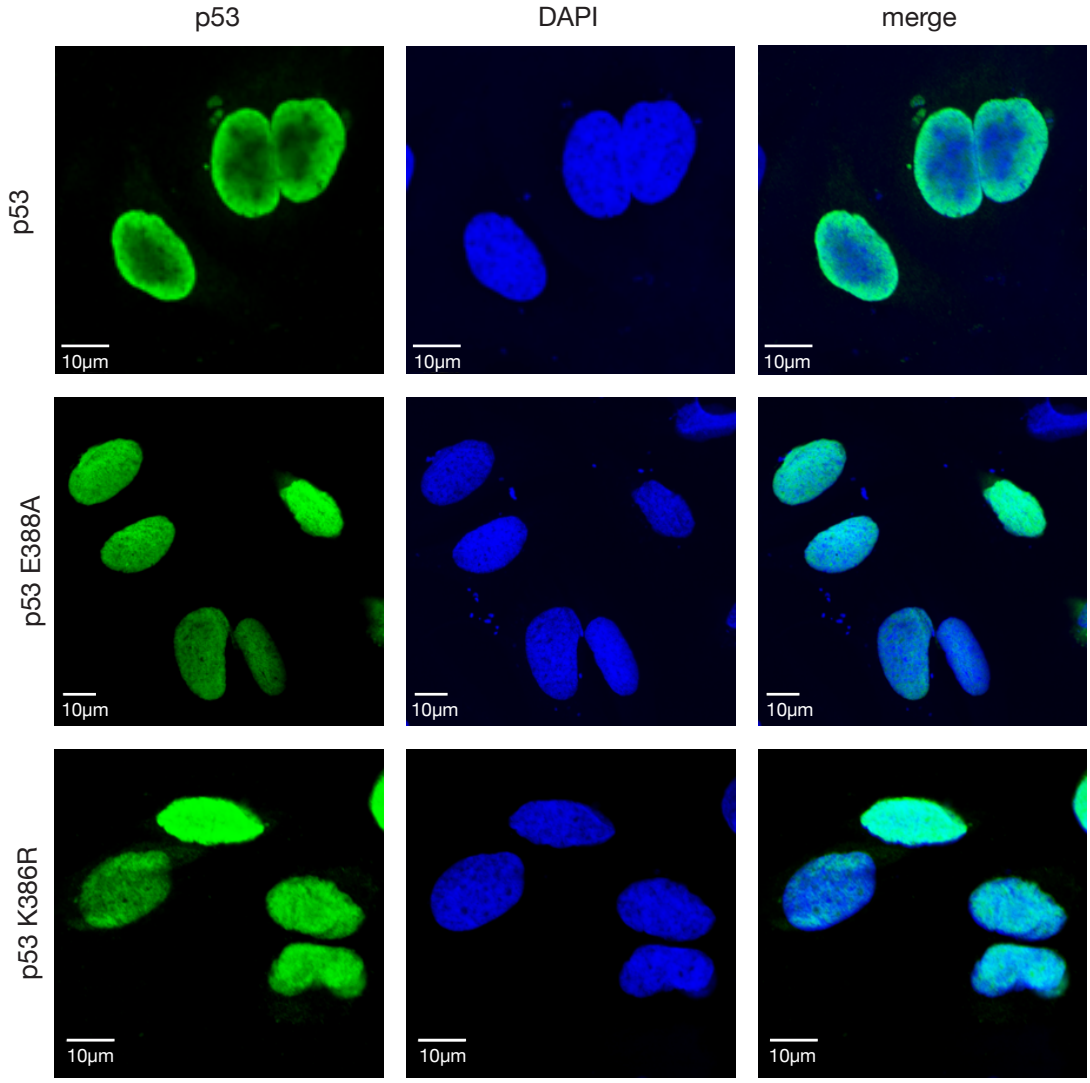

Figure 4-1: p53 proteins with and without SUMO site localise to the nuclei.
H1299 cells were transfected with wild-type or SUMO-consensus mutant (K386R and E388A) p53. p53 was stained with DO-1 primary and Alexa Fluor 488 secondary antibody. Nuclei were stained with DAPI.

Mono-ubiquitination of p53 is thought to promote the protein's nuclear export[740] and previous studies of p53-SUMO-1 fusion proteins also suggested a role for SUMO-1 in promoting p53 nuclear export[413]. It was therefore investigated whether the two

SUMO-consensus mutants of p53 (K386R and E388A) would show a different cellular distribution than wild-type p53 when overexpressed in H1299 cells.

All p53 proteins, with and without SUMO consensus, localised exclusively to the nuclei (Figure 4-1). It is possible that the fraction of SUMOylated p53 was too small to observe any differences of total p53 protein localisation in this setup. Localisation of the p53 proteins was therefore assessed under co-expression of MDM2 ΔAD in order to enhance SUMO-2/3 modification of p53. Localisation of the p53 proteins was assessed by subcellular fractionation.

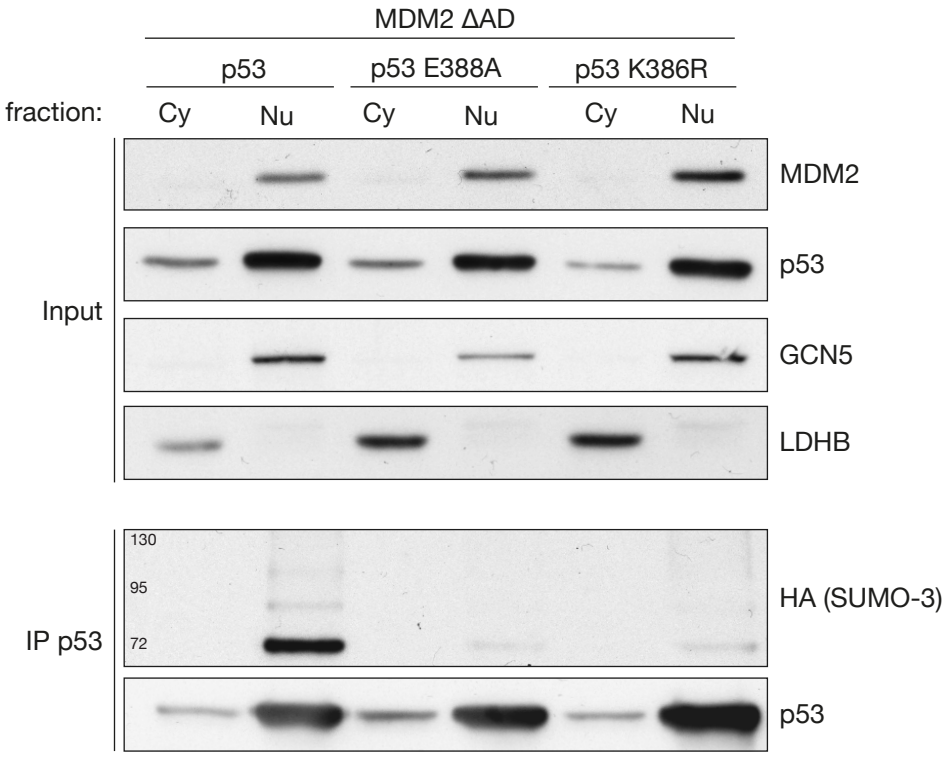

Figure 4-2: SUMO-2/3 modified p53 is exclusively nuclear.
HCT116 $p53^{-/-}$ cells were transfected with MDM2 ΔAD and wild-type or SUMO-consensus mutant p53 (E388A and K386R). Cells were fractionated into cytoplasmic (Cy) and nuclear (Nu) fractions in the presence of 10 µM Iodacetamid to prevent deSUMOylation. p53 was immunoprecipitated using the DO-1 antibody under denaturing conditions (IP p53). GCN5 served as nuclear and LDHB as cytoplasmic marker.

In the presence of MDM2 ΔAD, localisation of p53 was mainly nuclear and the distribution pattern was identical for wild-type p53, p53 K386R and p53 E388A (Figure 4-2, input).

Since SUMOylation is a highly dynamic process, analysing total p53 levels might conceal a change of localisation of only the modified fraction of p53. In order to study

the localisation of the SUMOylated fraction, p53 was immunoprecipitated from the fractionated lysates under denaturing conditions. Notably, SUMO-2/3 modified p53 was exclusively found in the nuclear fraction (Figure 4-2, IP). Thus, SUMO-2/3 conjugated p53 is not exported out of the nucleus. It is however possible that SUMOylation affects the sub-nuclear distribution of p53.

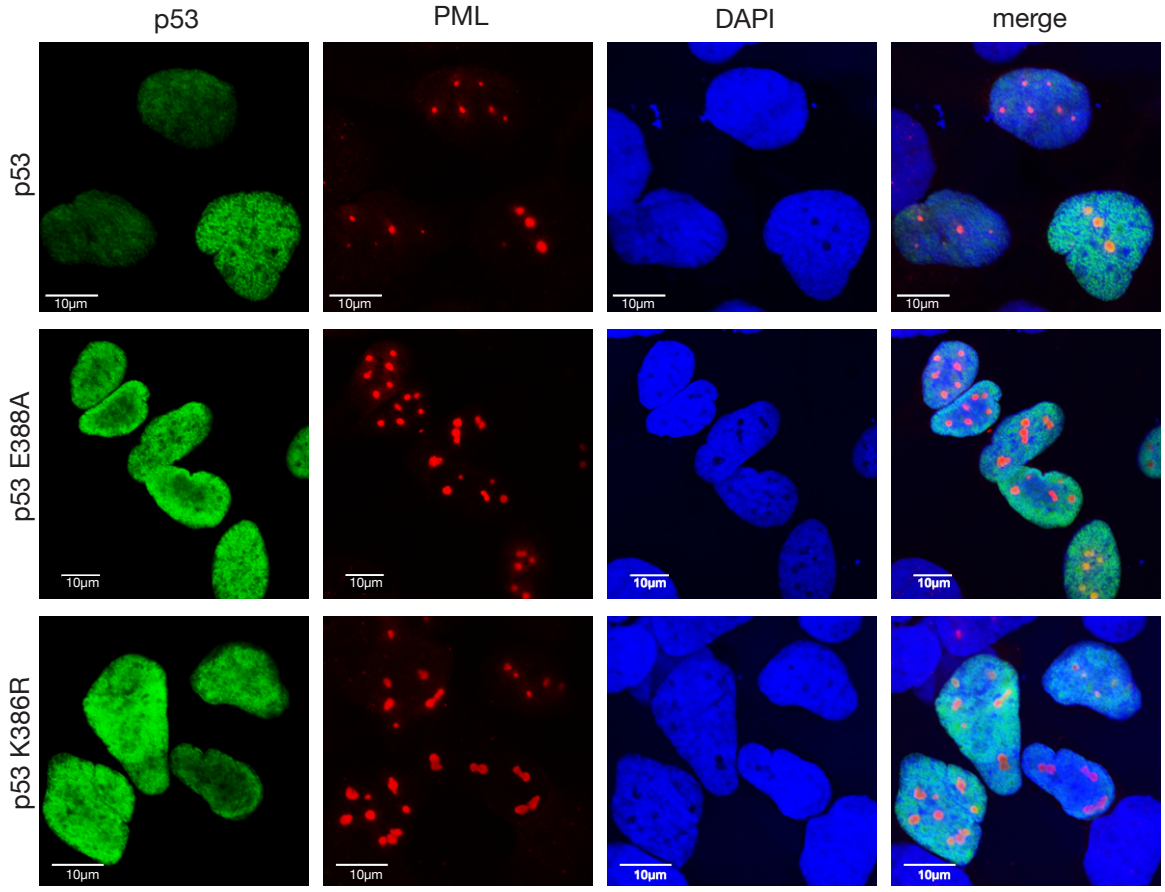

Figure 4-3: SUMOylation does not alter intranuclear localisation of p53.
U2OS cells were transfected with PML and wild-type p53, p53 E388A or p53 K386R. p53 was stained with DO-1 and Alexa Fluor 488 secondary antibodies (p53), PML was stained with PML rabbit (Santa Cruz) and Alexa Fluor 594 secondary antibodies (PML). Nuclei were stained with DAPI.

The p53 protein can localise to PML bodies[741] and PML bodies have been suggested to serve as a hub for SUMOylated proteins[742]. In order to investigate whether SUMO-2/3-modification influences p53 localisation at the PML bodies, PML was over-expressed together with p53 proteins with and without SUMO motif and localisation was assessed by confocal microscopy of immunofluorescently stained cells overexpressing p53 and PML.

PML formed characteristic punctae representing PML bodies in the nucleus and these PML bodies were excluded from DAPI staining, since they are free of DNA

(Figure 4-3). Bright p53 punctae co-localising with the PML bodies would indicate accumulation of p53 at the PML bodies, while appearance of dark dots in the p53 staining overlapping with the PML bodies (similar to the DAPI staining) would indicate that p53 was excluded from the PML bodies. However, wild-type p53, p53 K386R and p53 E388A all showed equal nuclear distribution with some darker spots, which did not overlap with the PML bodies (Figure 4-3). It can therefore be concluded that all p53 proteins tested (with and without SUMO motif) are neither enriched at nor excluded from the PML bodies. These data suggest that modification with SUMO-2/3 does not affect sub-nuclear localisation of p53. This is in line with previous reports suggesting that p53 is recruited to PML via its core domain without involvement of the C-terminus[743] and that lysine 386 is not required for p53 localising to PML bodies in the context of SUMO-1[407]. It would be informative to repeat this experiment in the presence of MDM2 ΔAD in order to stimulate SUMO-2/3 modification of wild-type p53.

Taken together, SUMO-2/3 modification of p53 does not appear to alter localisation of the p53 protein. However, it is still possible that the fraction of SUMOylated p53 was too small to observe an effect on total p53. Indeed, the p53-SUMO-1 fusion proteins were used as models for SUMOylated p53 in the past. The p53-SUMO-1 protein was observed to promote cytoplasmic p53 localisation[414] and therefore the localisation of a p53-SUMO-3 fusion protein was also analysed. The fusion protein is expressed from a vector containing the SUMO-3 sequence cloned in frame after the p53 sequence, resulting in a C-terminal fusion to the p53 protein. SUMO is usually conjugated to its substrates via a C-terminal diglycine motif, which is free in the fusion protein. Previous studies have shown that the presence of the diglycine motif can affect the localisation of a p53-SUMO-1 fusion protein: p53-SUMO-1 with the diglycine motif was localised to distinct sub-nuclear structures partially overlapping with PML bodies, while p53-SUMO-1 without the diglycine motif was present at the nuclear envelope and in some cells also to the cytoplasm[414]. It was concluded that the p53-SUMO-1 fusion protein was conjugated to other proteins instead of free SUMO-1 and the fusion protein without diglycine was proposed to be a more accurate model of SUMO-1-modified of p53[414]. Hence, SUMO-3's diglycine motif in the p53-SUMO-3 fusion protein was removed so that it could not be conjugated to any target proteins instead of free SUMO-3.

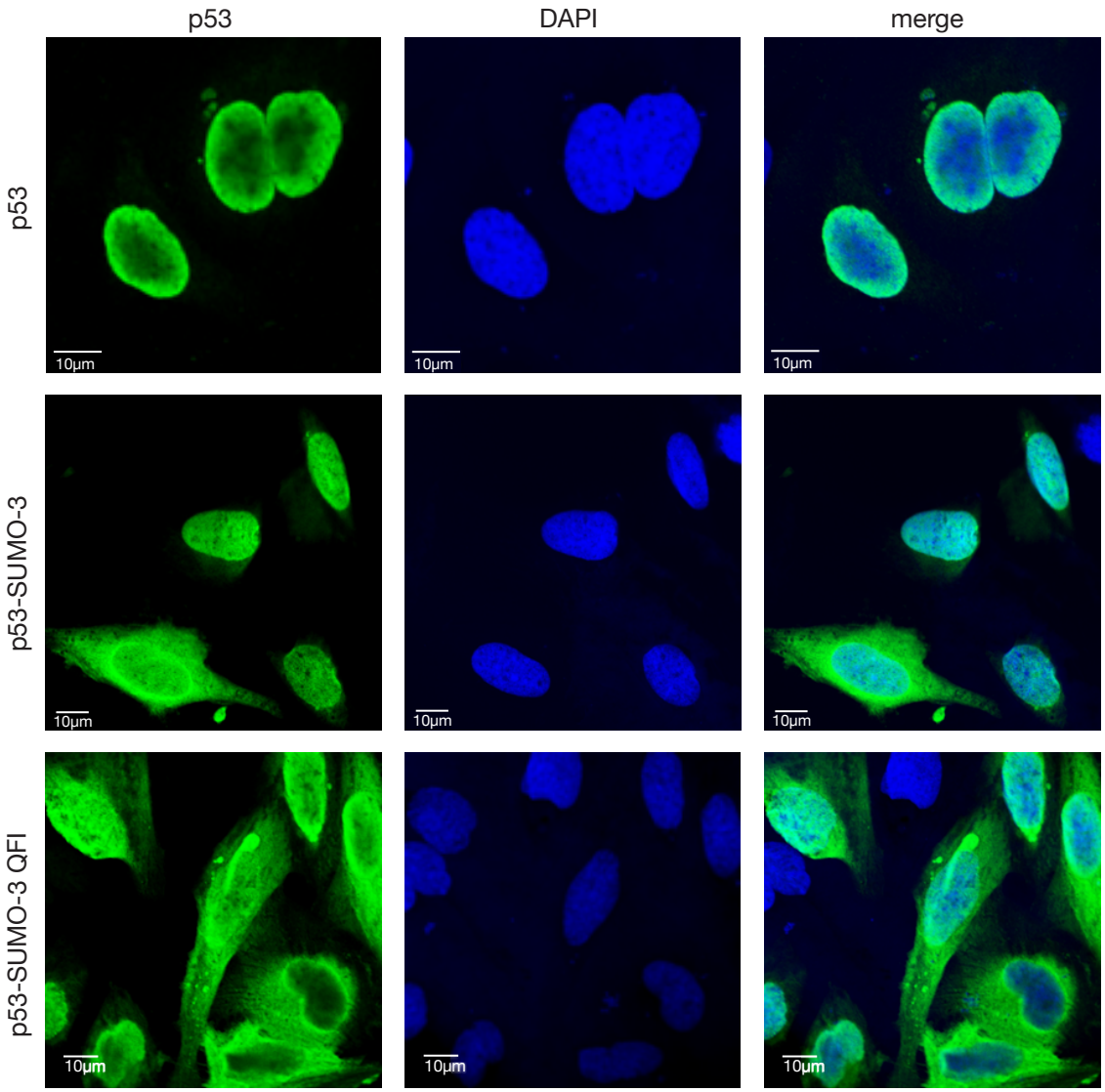

Figure 4-4: p53-SUMO-3 fusion proteins locate to the cytoplasm in some cells.
H1299 cells were transfected with wild-type p53, p53-SUMO-3 or p53-SUMO-3 QFI fusion. p53 was stained with the DO-1 primary and Alexa Fluor 488 secondary antibodies. Nuclei were stained with DAPI.

While wild-type p53 was exclusively nuclear, the p53-SUMO-3 fusion protein localised to both the nucleus and the cytoplasm in some cells, but was predominantly nuclear in most cells (Figure 4-4). This is similar to the localisation of p53-SUMO-1, which is cytoplasmic in 40% of cells[414]. Although the number of cells with cytoplasmic staining was not quantified here, the impression from confocal microscopy analysis was that significantly less than half of all cells showed cytoplasmic p53-SUMO-3, probably more in the range of 20%.

Many functions of SUMOylation are mediated by recruitment of proteins that contain SUMO-interaction motifs. It is possible that interaction of SUMO-2/3 conjugated p53 with a SUMO-binding protein is necessary for the export of the fusion protein. For a

SIM to recognise and non-covalently bind SUMO, the surface provided by the β_2-strand (amino acids 31-39 of SUMO-3) is required[356]. The surface recognition can be abolished by mutating Q30, F31 an I33 in SUMO-3[357], resulting in the SUMO-3 QFI protein (Figure 4-5 A).

Surprisingly, the p53-SUMO-3 QFI protein showed cytoplasmic staining in almost all transfected cells (Figure 4-4). In order to confirm these results with another assay, cells overexpressing either wild-type p53, the p53-SUMO-3 or the p53-SUMO-3 QFI fusion protein were fractionated and nuclear and cytoplasmic fractions assessed by western blotting.

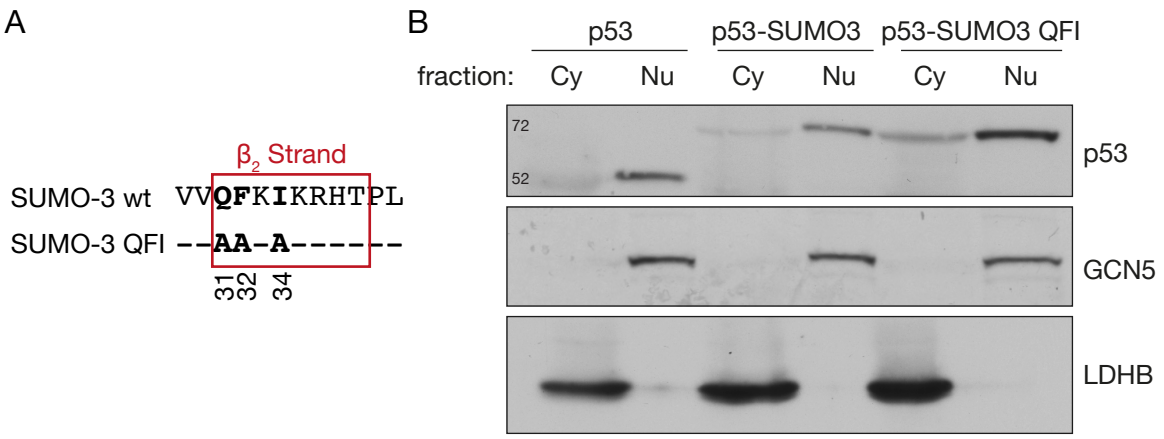

Figure 4-5: Fusion of SUMO-3 QFI increases the cytoplasmic fraction of p53.
(A) Representation of the amino acids in the β_2 strand of SUMO-3 (in red). Amino acids replaced to make the QFI mutations are indicated in bold. SwissProt accession number: P55854.
(B) HCT116 $p53^{-/-}$ cells were transfected with either p53 or p53-SUMO fusion proteins. Cells were fractionated into cytoplasmic (Cy) and nuclear (Nu) fraction. GCN5 served as nuclear and LDHB as cytoplasmic marker.

Although all p53 constructs led to expression of a mainly nuclear protein, a fraction of the p53-SUMO-3 QFI fusion protein was cytoplasmic (Figure 4-5), which is consistent with the immunofluorescence staining of these two proteins (Figure 4-4). However, the p53-SUMO-3 fusion with intact β_2-strand was hardly present in the cytoplasmic fraction (Figure 4-5), although some cells with cytoplasmic p53-SUMO-3 had been observed in the immunofluorescence staining (Figure 4-4).

Taken together, these data suggest, that the export of the p53-SUMO-3 fusion protein does not require SUMO's β_2-strand surface. Since a SUMO protein without the SUMO-interaction surface (p53-SUMO-3 QFI) was more prominently located in the cytoplasm than the p53-SUMO-3 fusion, it is possible that non-covalent

interaction of a SUMO-binding protein counteracts the export of the p53-SUMO-3 protein. Since SIMs can be SUMO-isoform specific, this could also explain, why a larger fraction of the p53-SUMO-1 fusion than of the p53-SUMO-3 fusion was exported, suggesting that a protein with a SUMO-2/3 specific SIM retains SUMO-2/3 modified p53 in the nucleus.

The finding that SUMO-2/3 modified p53 was only present in the nuclear fraction (Figure 4-2), but the SUMO-3 fusion-protein was also observed in the cytoplasm (Figure 4-4), albeit less evident in the fractionation experiment (Figure 4-5), seem contradictory and raise questions as to whether the fusion protein is an appropriate model for SUMOylated p53. Clearly, the structure of the fusion protein is different than the SUMOylated protein, since it is conjugated in a linear fashion to the C-terminus of p53, rather than conjugated to the ε-amino group of lysine 386. However, the observed effect is not likely to be purely an artefact of adding a small protein to the C-terminus of p53, since previous studies of p53-UBL fusion proteins deonstrated that only p53-Ubiquitin and p53-SUMO-1 fusions were exported, while p53-Nedd8 fusions showed no change in localisation[414]. It is possible that SUMO-3 promotes export of p53 and that the SUMO modification is removed on its journey into the cytoplasm (SUMO-2/3 modified p53 was only observed in the nucleus) and that no change in overall p53 localisation was evident, because the SUMOylated fraction was too small. However, since the majority of p53-SUMO-3 expressing cells also showed nuclear staining and only p53-SUMO-3 QFI was cytoplasmic in the majority of cells, it can be concluded that SUMO-3 modification of p53 does not evidently affect protein localisation as long as the β_2-interaction surface of SUMO-3 is present.

4.2 SUMOylation does not alter p53 protein stability.

p53 is a very short-lived protein and much of its activity is regulated by protein degradation. The most important regulator of p53 stability is MDM2, which poly-ubiquitinates p53[135,136] thereby labelling it for subsequent proteasomal degradation[107,108].

While no direct induction of proteasomal degradation by SUMOylation has been reported, it has become evident that the SUMO-pathway crosstalks with the Ubiquitin-system in a number of ways. For example SUMOylation of IκBα at lysine residue K21, which is also targeted for ubiquitination, prevents the protein's proteasomal degradation[393]. It has also recently become apparent that SUMOylation can recruit Ubiquitin-ligases such as RNF4 via their SUMO-interaction motif in order to promote the degradation of the SUMO-target[365,388,744-746].

Hence, the stability of wild-type p53 was compared to the stability of p53 proteins harbouring mutations in the SUMO consensus (K386R and E388A p53). p53 was co-expressed with wild-type MDM2 and SUMO-3, in order to enhance SUMOylation, yet still allow degradation of p53. Protein synthesis was blocked with Cycloheximide and cells were harvested 0, 90, 180 and 270 minutes later to follow the decreasing levels of p53 protein. Band intensity on the western blot was quantified using the Odyssey Licor infrared fluorescent system.

The stability of all p53 proteins tested was very similar, no matter whether they could be SUMOylated (wild-type p53) or not (K386R and E388A p53) (Figure 4-6). Although these data suggest that SUMO-2/3 modification does not affect p53 protein stability, it is possible that the small fraction of SUMOylated protein was not sufficient to allow observation of an effect on total protein level. In order to avoid the problem of only a small SUMOylated protein fraction, the stability of the p53-SUMO-3 protein could be assessed.

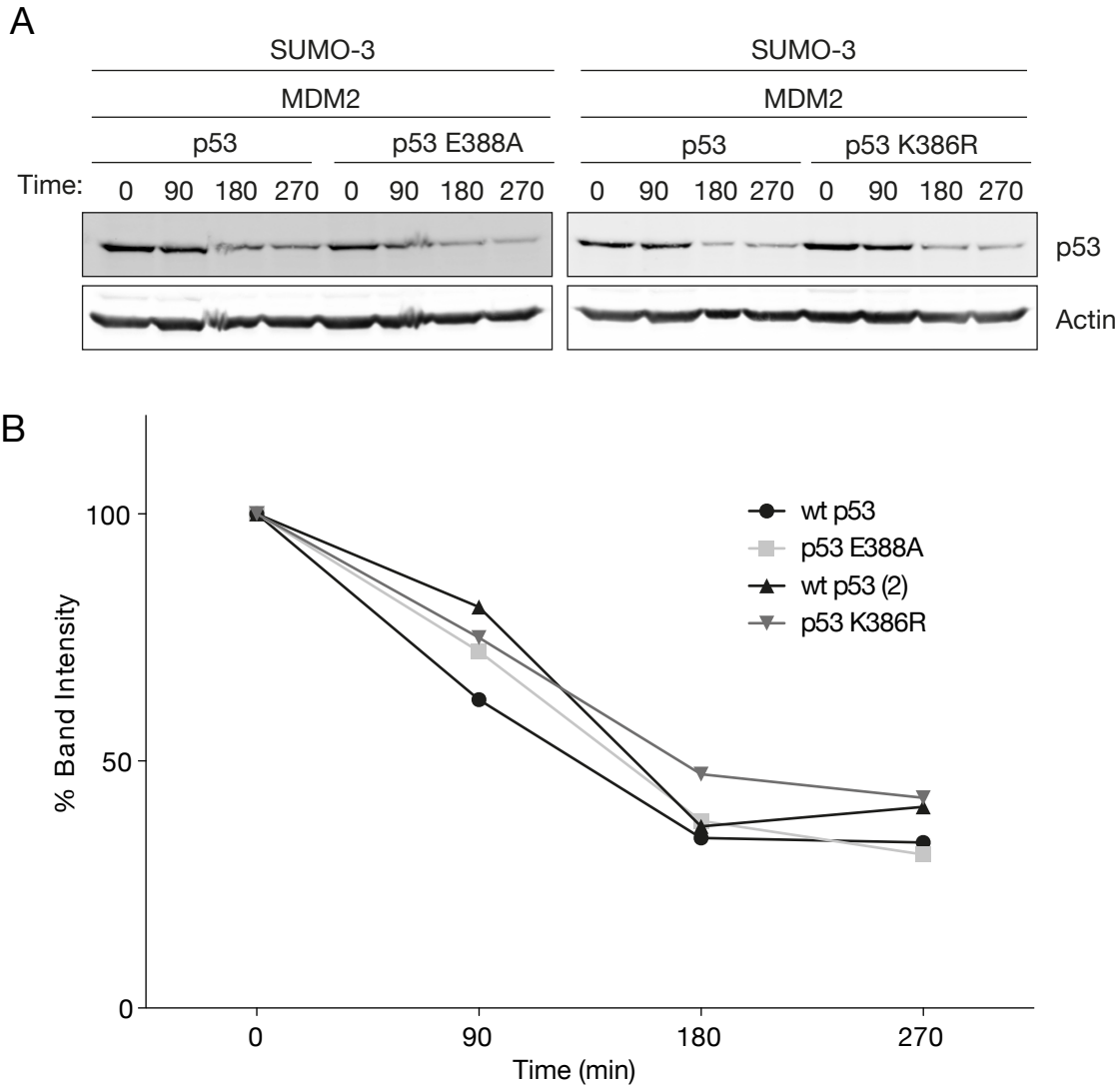

Figure 4-6: SUMOylation does not affect p53 protein stability.
HCT116 $p53^{-/-}$ cells were transfected with MDM2, SUMO-3 and either wild-type p53, p53 E388A or p53 K386R. Cells were treated with 200 µg/ml Cycloheximide for indicated times (in minutes). Wild-type p53 lysates were loaded onto 2 gels in order to run a control on each gel. Band intensities were quantified with the Licor Odyssey Imager and are plotted relative to the band intensity at 0 min.

Particularly, since MDM2 promotes SUMOylation more efficiently when it cannot ubiquitinate p53, it is difficult to assess the MDM2-mediated degradation of SUMO-modified p53. In order to address the degradation of SUMOylated p53 by another E3-ligase, levels of p53 SUMO-2/3-modified by wild-type MDM2 or MDM2 ΔAD, were analysed in the presence of the SUMO-2/3-targeted Ubiquitin ligase RNF4[365,388].

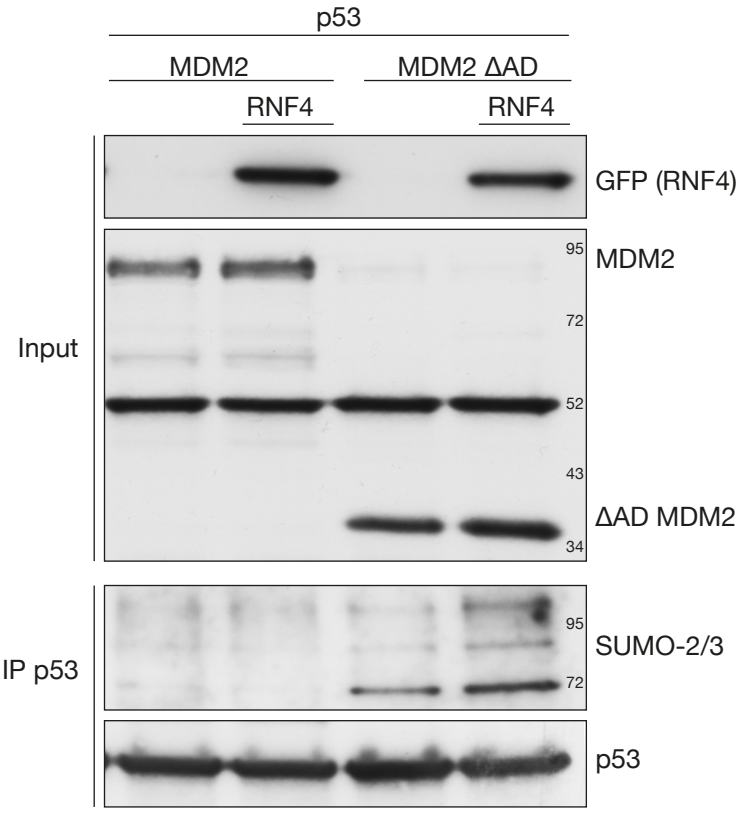

Figure 4-7: RNF4 does not degrade SUMOylated p53.
HCT116 p53$^{-/-}$ cells were transfected with p53 and MDM2 or MDM2 ΔAD and either empty vector or YFP-tagged RNF4. Cells were lysed under denaturing conditions and p53 was immunoprecipitated with the DO-1 antibody. Expression of RNF4 was detected with a GFP antibody.

Overexpression of RNF4 did not affect levels of SUMOylated p53, induced by full-length MDM2 or MDM2 ΔAD (Figure 4-7), suggesting that RNF4 does not induce degradation of SUMO-2/3 modified p53. RNF4 contains multiple SIMs in a row and was reported to be specifically recruited by SUMO-2/3 chains[365]. No poly-SUMO-chains on p53 were observed (Figure 3-25), hence RNF4 is probably not recruited to SUMOylated p53.

4.3 SUMOylation does not prevent p53 tetramerisation.

The transcription factor p53 is predominantly present as a tetramer[747]. Importantly, tetramerisation not only forms a prerequisite for sequence-specific DNA binding[748], but also for interaction with MDM2[749]. p53 contains a nuclear export signal within the oligomerisation domain. The export signal is masked in tetramers[120], whereby tetramerisation can influence p53 localisation.

Tetramerisation can be influenced by posttranslational modifications of the C-terminus, for example phosphorylation of serine 392 was reported to stabilise p53 tetramers[257], while acetylation of lysines 370 to 382 was shown to prevent tetramerisation[750]. Since SUMOylation occurs in the same region of the p53 protein, the effect of SUMO-2/3 modification on p53 oligomerisation was studied.

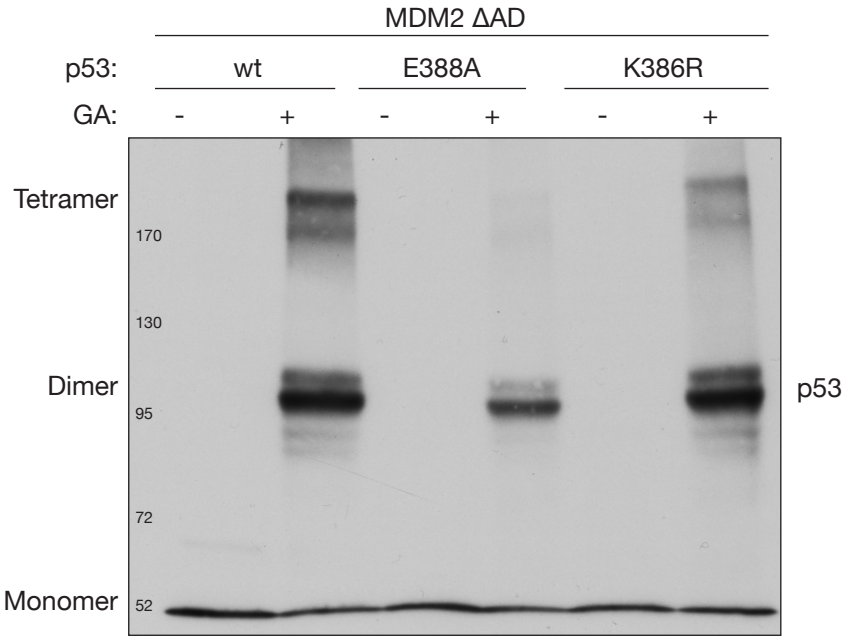

Figure 4-8: SUMOylation does not hinder p53 tetramerisation.
HCT116 $p53^{-/-}$ cells were transfected with MDM2 ΔAD and either wild-type or SUMO consensus mutant p53 (E388A, K386R). Cells were lysed under mild conditions with 10 μM Iodacetamide to block deSUMOylation. Half of the lysate was crosslinked with 0.01% Glutaraldehyde (GA) for 20 minutes. Samples of lysates with (+) and without (-) crosslinking reagent were run on a 6% polyacrylamide gel.

p53 proteins with or without the SUMO site were expressed together with MDM2 ΔAD in order to promote SUMOylation of wild-type p53. After cross-linking with Glutaraldehyde, monomers, dimers and tetramers of p53 were observed in all three samples (Figure 4-8). Although slightly under-loaded, oligomers of p53 E388A could be detected and these were much more clearly seen for the second SUMO-site mutant p53 K386R. These studies show clearly that SUMOylation does not hinder p53 tetramerisation, similar to reports that SUMO-1 modified p53 exists in tetramers[401]. It could also be investigated whether SUMO-2/3-modified tetramers have the same affinity for DNA as unmodified tetramers, since SUMO-1-modified p53 was found to bind less well to DNA in in vitro experiments[401].

4.4 SUMOylation modulates p53's transcriptional activity.

p53 plays a major role as stress-induced transcription factor and its activity has been shown to be influenced by many posttranslational modifications in the C-terminal domain, such as phosphorylation[256], acetylation[260], methylation[277,278] and neddylation[318]. The effect of SUMO-1 modification on p53 transcriptional has been studied before, yet the outcome remains controversial[405-407,410]. Here, the effect of SUMO-2/3 conjugation on p53's transcriptional activity was studied.

In order to assess the influence of SUMOylation on the activation of classic p53 target genes, Luciferase assays with two p53-responsive reporter gene constructs were performed. The PG13 Luciferase construct is regulated by a series of 13 p53 binding sites[30] and the Bax-Luciferase construct is under the control of a naturally occurring p53 binding site in the Bax-promoter[482]. Induction of the PG13 Luciferase can be a surrogate for induction of the p21 promoter. The PG13 Luciferase was used instead of a p21 Luciferase, since PG13 Luciferase has lower background Luciferase activity. The PG13 and Bax Luciferase constructs were chosen since they represent subsets of differentially activated target genes. p53 rarely induces apoptosis and cell cycle arrest at the same time and many binding proteins or posttranslational modifications of p53 have been shown to induce one of the two, but not the other[751]. Interestingly, several p53 mutants activate the p21, but not the Bax promoter[752] and wild-type p53 was also shown to have a higher affinity for the p21 than for the Bax promoter[753].

Wild type p53 and both mutants of the SUMO consensus, p53 K386R and p53 E388A, were titrated in the presence of MDM2 ΔAD for maximal SUMOylation. The Luciferase assays (plotted as RLU: (firefly Luciferase units relative to constitutively active TK Renilla as correction for cell number) fold change relative to cells not expressing any p53) revealed that both SUMO-site mutants of p53 induced the PG13 promoter significantly more efficiently than wild-type p53 (Figure 4-9 A).

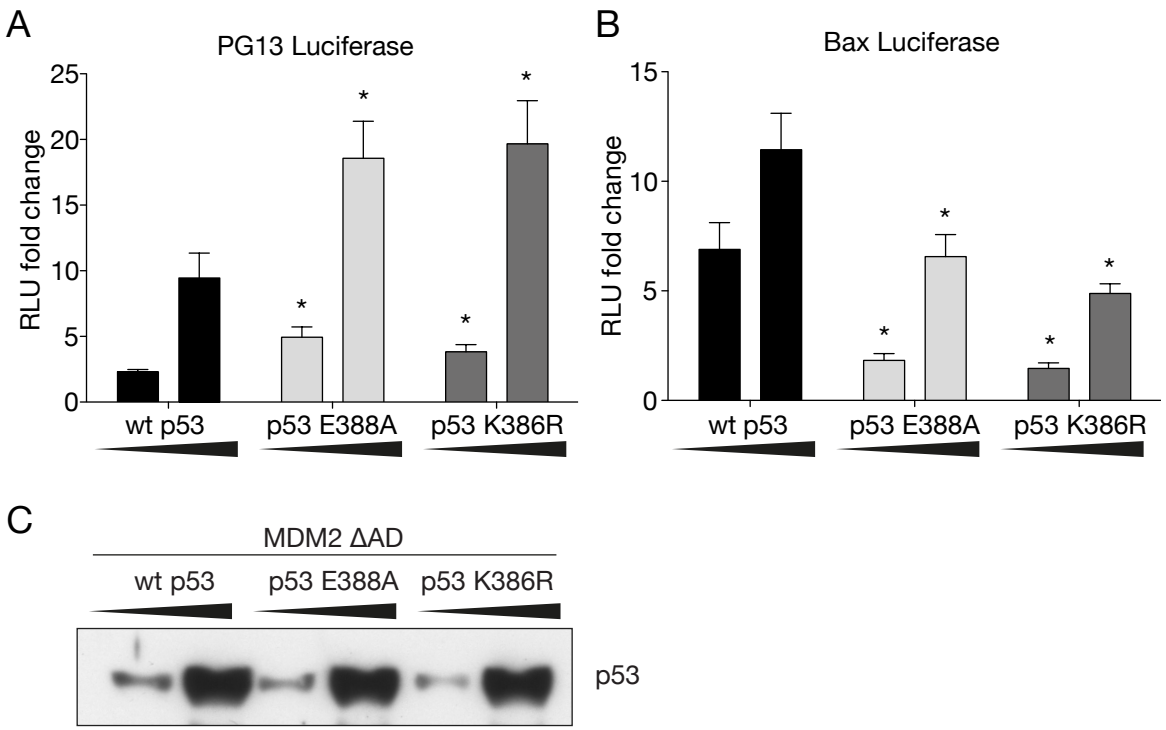

Figure 4-9: SUMOylation modulates p53's activation of Luciferase reporters.
HCT116 $p53^{-/-}$ cells were transfected with either PG13 Luciferase (A) or Bax Luciferase (B), MDM2 ΔAD and 10 or 100 ng of the p53 constructs indicated. Activation of the promoters was assayed using the Promega Luciferase system. Data was plotted as Relative Luciferase Units (RLU) (Firefly Luciferase readings divided by Renilla Luciferase readings) fold change relative to p53-null control. The diagrams represent the mean of three independent sets of triplicates with error bars as standard error of the mean. * indicates a p-value < 0.02 as results of an unpaired two-tailed Student t-test compared to readings from cells expressing wild-type p53. Equal transfection was confirmed by western blot (C).

The opposite effect was observed on Bax-Luciferase, which was more strongly induced by wild type p53 than the SUMO-site mutants p53 E388A and p53 K386R (Figure 4-9 B), suggesting that SUMOylation can both positively and negatively modulate p53 transcriptional activity depending on the target promoter. Equal p53 protein level in the Luciferase assays were confirmed by western blotting (Figure 4-9 C).

It was investigated next, whether linear conjugation of SUMO-3 as in the p53-SUMO-3 fusion protein, could also modulate p53's transcriptional activity.

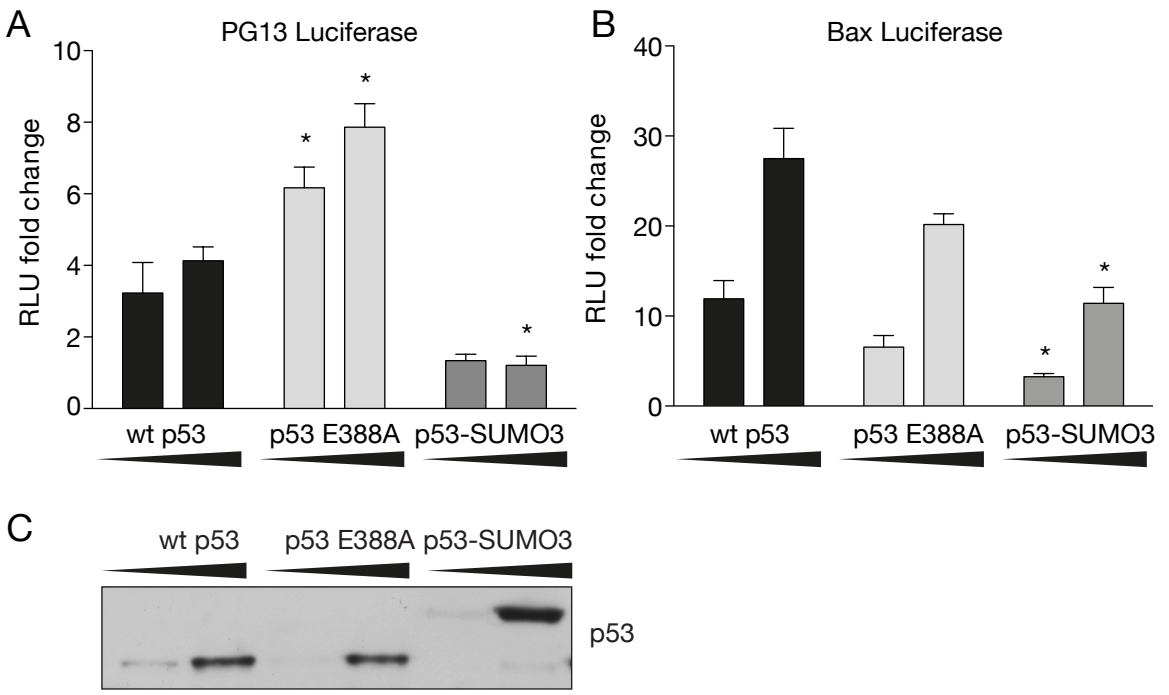

Figure 4-10: p53-SUMO-3 fusion proteins are impaired in inducing Luciferase reporters.

HCT116 $p53^{-/-}$ cells were transfected with either PG13 Luciferase (A) or Bax Luciferase (B) and 10 or 100 ng of the p53 constructs indicated. Activation of the promoters was assayed using the Promega Luciferase system. Data is plotted as Relative Luciferase Units (RLU) (Firefly Luciferase readings divided by Renilla Luciferase readings) fold change relative to p53-null control. The diagrams represent the mean of two independent sets of triplicates with error bars as standard error of the mean. * indicates a p-value < 0.02 as results of an unpaired two-tailed Student t-test, compared to readings from cells expressing wild-type p53. Equal transfection was confirmed by western blot (C).

As expected, a p53 protein without the SUMO consensus site (p53 E388A) induced PG13-Luciferase significantly more strongly than wild-type p53. Consistent with the conclusion that SUMOylation dampens p53-induced PG13 Luciferase activation, the p53-SUMO-3 fusion protein could not induce PG13 Luciferase at all (Figure 4-10 A). Surprisingly, induction of Bax-Luciferase, which would have been expected to be strongly induced by SUMOylated p53, was also significantly impaired by the SUMO-3 fusion (Figure 4-10 B). However, in contrast to the PG13 Luciferase reporter, which could not be induced, even by high levels of p53-SUMO-3 (Figure 4-10 A), a small induction of Bax Luciferase activity was observed with higher levels of the p53-SUMO-3 fusion protein (Figure 4-10 B). This finding is consistent with the hypothesis that SUMOylated p53 is more likely to activate Bax than p21 and might indicate a role for SUMOylation of p53 in guiding the selective activation of a subset of p53 target genes. Since the p53-SUMO-3 fusion localised to the cytoplasm in some cells in previous experiments, while wild-type p53 was predominantly nuclear (Figure 4-4), it is possible that the lower induction of Bax-Luciferase compared to wild-type p53 is

a consequence of reduced amount of protein available for transcriptional activation of the reporter in the nucleus.

In order to investigate the impact of SUMOylation on p53-induced transcription of endogenous promoters, mRNA of p53 target genes was extracted from p53-null H1299 cells retrovirally infected with empty vector, wild-type, p53 E388A or p53 K386R in the presence of MDM2 ΔAD, transcribed into cDNA and quantified by Real-time PCR. Retroviral infection was chosen in order to achieve relatively equal expression of the p53 constructs in the maximum number of cells to allow for detection of subtle changes in the regulation of p53 targets.

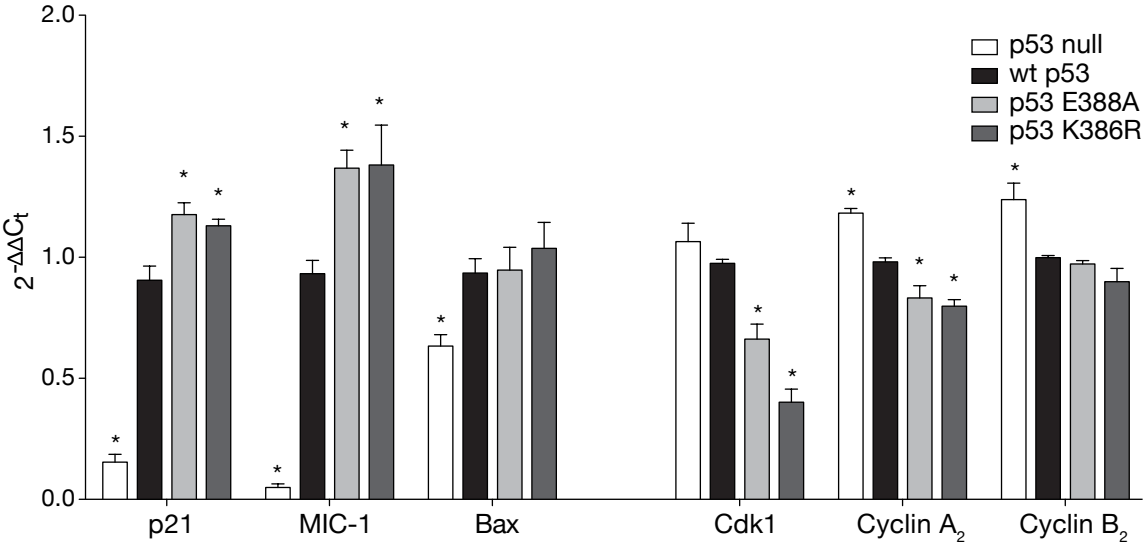

Figure 4-11: SUMOylation modulates mRNA levels of p53-activated and -repressed target genes.

H1299 cells were retrovirally infected with MDM2 ΔAD and either empty vector, wild-type (wt) or SUMO-site mutant p53 (E388A and K386R). Half the cells were used for RNA extraction and reverse transcription PCR into cDNA. Quantitative Real-time PCR was performed with a SYBR Green Mastermix for p53-activated (left panel) and -repressed (right panel) genes. Gene expression was quantified relative to the housekeeping genes B2M and RPLP0 according to the comparative $\Delta\Delta C_t$ method. Results are displayed as mean of $2^{-\Delta\Delta Ct}$ values obtained from three independent experiments. Error bars represent the standard error of the mean. * indicates a p-value ≤0.02 as calculated by an unpaired, two-tailed Student t-test compared to cells expressing wild-type p53.

Quantitative Real-time PCR showed small, yet significant differences between p21 mRNA-levels depending on p53 SUMO status with both p53 E388A and p53 K386R inducing the p21 mRNA significantly more strongly than wild-type p53 (Figure 4-11, left), following the same trend as observed with PG13 Luciferase (Figure 4-9 A). Expression of the TGFβ-family member Macrophage inhibitory cytokine-1 MIC-1 (also known as growth/differentiation factor-15 GDF-15), which is strongly induced

by p53 expression[754], was also dampened by p53 SUMOylation indicated by the SUMO-negative mutants p53 E388A and p53 K386R inducing MIC-1 to significantly higher levels than wild-type p53 (Figure 4-10, left). However, the decreased activation of Bax expression by the SUMO site mutants p53 E388A and p53 K386R observed using the Bax Luciferase reporter could not be reproduced with endogenous mRNA levels. Bax was slightly induced by p53-expression and SUMOylation status of p53 had no significant effect on its induction (Figure 4-11, left). Since the induction of the Bax mRNA by p53 was much less pronounced than induction of p21 and MIC-1, it is possible that subtle modulation of this activation by SUMOylation could not be observed in this setting.

p53 is not only a transcriptional activator, but also has an important role in repressing a range of target genes[27]. Thus, the effect of p53 SUMOylation on some established p53-repressed cell cycle genes was also studied. p53 is known to repress both Cyclin-dependent Kinase 1 (Cdk1)[42] and Cyclin B_2[40] that form a complex, required for progression through the G_2/M checkpoint. Cyclin A_2, which is necessary for the S/G_2-transition together with Cdk1, was also found to be repressed by p53[41].

Both non-SUMOylatable mutants p53 E388A and p53 K386R repressed the S- and G_2-phase genes Cdk1 and Cyclin A_2 significantly more strongly than wild type p53, suggesting that SUMO-2/3 modification of p53 can alleviate p53-mediated repression (Figure 4-11, right). Strikingly, a similar p53-repressed gene Cyclin B_2 did not show significant differences in repression levels depending on p53-SUMOylation status (Figure 4-11, right), suggesting that the SUMO-induced alleviation of p53-mediated repression is confined to a subset of p53-respressed target genes. Equal p53 protein expression was confirmed by western blotting (Figure 4-12, A).

Taken together, these results suggest that SUMO-2/3 conjugation can dampen both activation and repression of p53 targets and that modulation of p53 activity by SUMO-2/3 modification is limited to a subset of p53 target genes.

Since SUMO-dependent differences in induction of the secreted p53 target MIC-1 were observed at mRNA level, media, in which the cells used for mRNA-extraction had grown, were collected and MIC-1 protein concentration was determined using an ELISA-Kit.

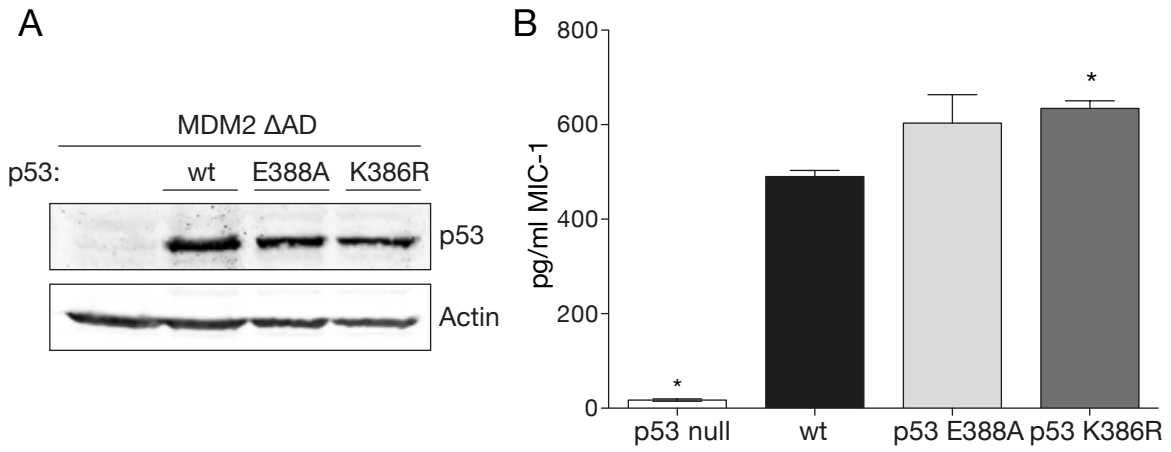

Figure 4-12: SUMO-status of p53 determines levels of secreted MIC-1 protein.
H1299 cells were retrovirally infected with MDM2 ΔAD and p53 constructs indicated.
(A) Cells were lysed in sample buffer and p53-expression levels were analysed via SDS-PAGE.
(B) Medium from the infected cells was collected and levels of secreted MIC-1 assessed using an ELISA-Kit. Protein concentration was determined with a standard curve. Diagram represents the mean of triplicate measurements of MIC-1 protein concentration in the medium. Error bars represent standard error of the mean. * indicates a p-value <0.02 as calculated by an unpaired, two-tailed Student t-test compared to cells expressing wild-type p53.

In accordance with the levels of MIC-1 mRNA as assessed by Real-time PCR, concentration of secreted MIC-1 protein was significantly higher in medium collected from cells expressing p53 K386R than in medium collected from cells with wild-type p53 (Figure 4-12 B). Cells expressing the SUMO consensus mutant p53 E388A showed the same trend as those expressing p53 K386R, although the MIC-1 concentration was not found to be significantly different to wild-type p53 (Figure 4-12 B).

Following on from the finding that modulation of p53's transcriptional activity by SUMOylation can also be detected on protein level in the case of MIC-1, the effect of p53-SUMOylation on the expression of p21 at the protein level was also investigated. Using MDM2 ΔAD to promote SUMOylation of p53, increasing amounts of both wild-type and SUMO-consensus mutant p53 E388A were titrated and endogenous p21 protein levels were analysed by western blot.

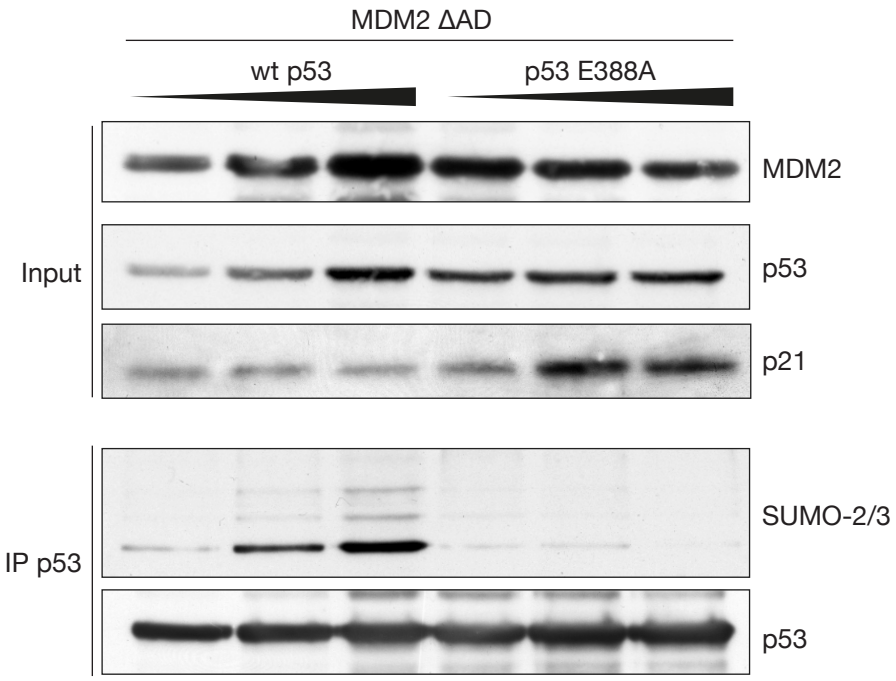

Figure 4-13: SUMOylation of p53 represses activation of p21 at protein level.
H1299 cells were transfected with MDM2 ΔAD and either 0.2, 0.5 or 1.0 µg of wild-type p53 (wt) or p53 E388A. Cells were lysed under denaturing conditions and p53 immunoprecipitated using the DO-1 antibody (IP p53).

Analysis of the protein levels revealed that SUMOylated wild-type p53 was impaired in driving the expression of p21 protein compared to p53 E388A, which is not SUMOylated (Figure 4-13), just like wild-type also induced PG-13 Luciferase and p21 mRNA-levels less well than the SUMO-consensus mutant p53 E388A. It can be concluded that the modulation of p53's transcriptional activation by SUMO-2/3 modification results in altered expression of p53's targets at protein level.

4.5 Consequences of p53 SUMOylation on the cell cycle.

The transcription factor p53 regulates the cell cycle via multiple mechanisms. Induction of its target gene p21, which efficiently inhibits the G_1/S-specific Cdk/Cyclin complexes[29,755], prevents Rb hyper-phosphorylation by the Cdk2/Cyclin E complex leading to a cell cycle arrest in G_1. Furthermore, p53 represses the S/G_2-specific Cdk1 and Cyclins A and B, contributing to a G_2-arrest[756]. Since SUMOylation of p53 impairs its ability to activate p21 fully (see Figure 4-13) and alleviates repression on the cell-cycle genes Cdk1 and Cyclin A_2 (see Figure 4-11), the implication of SUMO-2/3 modification of p53 on the cell cycle was studied.

Cells were transfected with either empty vector, wild-type p53 or SUMO-consensus mutants p53 E388A and p53 K386R in the presence of MDM2 ΔAD and SUMO-3 to maximise SUMOylation. CD20 was included as a marker to select for transfected cells. Flow cytometry of PI-stained cells with subsequent FlowJo cell cycle analysis revealed induction of apoptosis (sub-G_1), a moderate, albeit not significant G_1-arrest and reduction of cells in S-phase after p53 overexpression compared to p53-null cells. No accumulation in G_2 could be observed (Figure 4-14).

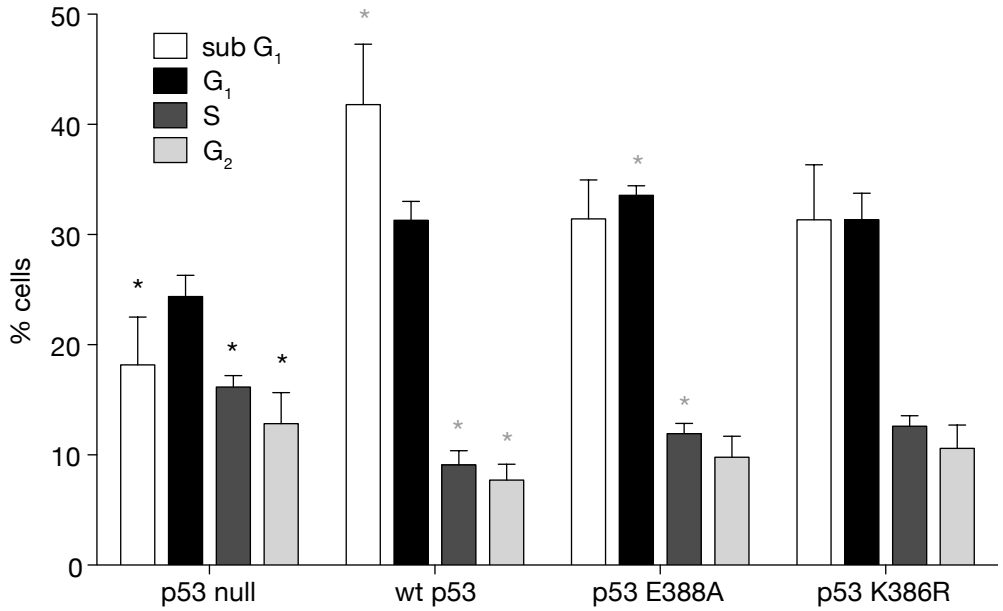

Figure 4-14: SUMO-status of p53 has a small impact on the cell cycle.
HCT116 $p53^{-/-}$cells were transfected with either empty vector, wild-type (wt) or SUMO-consensus mutant p53 (E388A and K386R) together with MDM2 ΔAD, SUMO-3 and CD20. Cells were fixed and stained with a FITC-conjugated CD20-specific antibody. PI was used to assess DNA content. Cells were analysed by flow cytometry and cell cycle distribution of CD20-positiv cells (as transfection control) according to FlowJo analysis of FACS chromatogram was plotted as mean of three independent experiments with error bars representing the standard error of the mean. * indicates a p-value <0.05 as calculated by an unpaired, two-tailed Student t-test compared to cells expressing wild-type p53.
* indicates a p-value <0.05 compared to p53 null cells.

Expression of p53 harbouring mutation of the SUMO-accepting lysine (K386R) or the SUMO consensus (E388A) resulted in a cell cycle distribution not significantly different from wild-type p53, although the sub-G_1 population appeared to be reduced. Due to the observation that p53 K386R and p53 E388A induced p21 more strongly, a stronger induction of a G_1-arrest had been expected in this scenario. However, the G_1-arrest induced by p53 expression was not very pronounced (35% cells in p53 E388A cells in G_1 compared to 25% of p53-null cells in G_1), it is possible that no subtle differences between the p53 proteins could be observed here. For

technical reasons resolution of S- and G_1 phases might not have been sufficiently reached with PI-staining alone and it would be informative to repeat the cell cycle analysis with BrdU and PI double staining. BrdU was not used in this experiment because the FL1 channel on the FACS was already occupied with CD20-staining to select for transfected cells. However, using retroviral infection with subsequent selection of infected cells could circumvent the need for CD20 staining and BrdU staining could be used together with PI staining in such an experiment.

Importantly, the SUMOylated wild-type p53 induced a significant apoptotic response and only a moderate (non-significant) induction of G_1. This observation is in line with our previous observations that SUMOylated p53 might be more inclined to activate Bax than p21 (Figure 4-10). Furthermore, p53 E388A induced a significant increase of the G_1, but not the sub-G_1 population, suggesting that unSUMOylated p53 induces G_1 arrest rather than apoptosis. Cells expressing p53 K386R showed a trend similar to those expressing p53 E388A, however, the errors were higher in this sample and the cell cycle profile was not significantly different from the p53-null cells (Figure 4-14).

Taken together the data presented in this chapter reveals a role for SUMO-2/3 modification of p53 in modulating transcriptional activation of a subset of p53-activated and -repressed genes, and SUMO-2/3 modification might have a role in channelling the p53 response towards induction of apoptosis over cell-cycle arrest.

4.6 Summary and Discussion.

Analysis of localisation, oligomerisation and transcriptional activity of p53 revealed interesting roles for SUMO-2/3 modification in modulating p53 activity. Importantly, mutations of the SUMO consensus as in p53 K386R and p53 E388A disrupt both SUMO-1 and SUMO-2/3 conjugation of p53, but most of the experiments presented here were performed under conditions where SUMO-2/3 conjugation was predominant. The similar behaviour of both p53 K386R and p53 E388A rules out effects of other modifications on lysine 386.

SUMOylation is frequently associated with changes in subcellular localisation. Indeed, the SUMO-E2 Ubc9 localises to the nuclear pore complex, suggesting the proteins being imported or exported from the nucleus are modified as they pass through[757]. For example, SUMOylation induces nuclear export of the transcription factor Smad3[758] and PIASy-mediated conjugation of SUMO-3 to p53 was also recently reported to contribute to its nuclear export in endothelial cells[423]. However, in the experiments described here, p53 cellular distribution was mainly nuclear in immunofluorescence (Figure 4-1) and subcellular fractionation experiments (Figure 4-2), whether or not p53 could be SUMOylated. It needs to be taken into consideration that the immunofluorescence experiment was performed without co-expression of MDM2 ΔAD and therefore not a large fraction of p53 might have been SUMO-2/3 modified (Figure 4-1). However, p53 distribution of cells clearly harbouring SUMO-2/3-modified p53 as assessed by subcellular fraction, also did not change according to its SUMO status (Figure 4-2). Furthermore, SUMOylated p53 was exclusively detected in the nucleus (Figure 4-2), suggesting that SUMO-2/3-conjugated p53 is not exported.

It had previously been reported that a SUMO-1-fusion to p53 leads to nuclear export of p53[414] and a similar, albeit less pronounced, induction of cytoplasmic staining was observed with a p53-SUMO-3 fusion protein. This observation was not consistent with the data obtained with posttranslationally SUMO-2/3 modified p53, but could represent a model, according to which SUMOylation induces nuclear export of p53, with the posttranslational SUMO-modification being removed during or shortly after the export. Since only a minority of p53-SUMO-3 fusion expressing cells showed

cytoplasmic staining, other factors most likely influence whether export of p53 is triggered. A p53-SUMO-3 fusion protein with disrupted SUMO-interaction surface (p53-SUMO-3 QFI) showed cytoplasmic staining in a much larger fraction of cells (Figure 4-4). Thus, nuclear export of the p53-SUMO-3 fusion protein could be restricted by proteins non-covalently interacting with SUMO-2/3. Posttranslational modifications of the p53-fusion proteins might also take place, further influencing its localisation. Mono-ubiquitination of p53 promotes nuclear export[740] and this modification could be enhanced by the SUMO-3 fusion, similar to previous reports that mono-ubiquitination of p53 contributes to the export of a p53-Ubiquitin fusion protein[413]. Lysine residues in SUMO-3 proximal to the amino acids replaced in the QFI mutation were recently shown to be acetylated[403] and it is thus also possible that posttranslational modification of the SUMO-2/3 protein influences the export of SUMO-2/3 modified p53.

The emerging field of Ubiquitin-SUMO crosstalk with reports of mixed SUMO-Ubiquitin chains and identification of SUMO-targeted Ubiquitin ligases[370] raised the possibility that SUMOylation of p53 modulates the protein stability. However, no differences between protein stability of wild-type p53 and SUMO-consensus mutant p53 could be noted (Figure 4-6), suggesting that p53 protein stability is not regulated via SUMO-2/3 modification. It is possible that the fraction of SUMOylated p53 induced by MDM2 was not large enough to reveal differences in overall protein stability. In order to assess p53 stability in the presence of a larger fraction of SUMO-2/3 conjugated p53, it might be more informative to express p53 in the presence of both MDM2 ΔAD to promote p53 SUMOylation and full-length MDM2 to promote p53 ubiquitination and degradation. Furthermore, the influence of p53-SUMO-2/3 modification on degradation mediated by other known Ubiquitin-ligases for p53 such as the viral protein E6AP[292] or non-viral E3 ligases such as Pirh2[300] and COP1[301] could be tested in combination with MDM2 ΔAD. Stability of the SUMOylated p53 fraction could be tested by conducting a Cycloheximide assay with subsequent immunoprecipitation under denaturing conditions to visualise the SUMO-2/3 modified p53.

Co-expression of the SUMO-targeted Ubiquitin-ligase RNF4 did not affect the levels of SUMO-2/3 modified p53, neither did it alter the band pattern of SUMOylated p53,

which could have indicated modification with Ubiquitin without subsequent degradation (Figure 4-7). However, RNF4 contains four SUMO interaction motifs in a row and was shown to only be recruited to poly-SUMOylated target proteins. Indeed, the SIMs of RNF4 were successfully used as a tool to enrich for poly-SUMOylated proteins[759]. Consistent with the conclusion that p53 was not poly-SUMOylated (Figure 3-15) RNF4 co-expression did not modify p53-SUMOylation (Figure 4-7 and it can be concluded that SUMO-2/3 modified p53 is not a target for RNF4-mediated ubiquitination.

SUMO-3 modification of p53 did not hinder tetramerisation of the protein (Figure 4-8). This is in contrast to p53-Nedd8 fusions, which were observed to form more high-molecular weight aggregates[414,760]. It could be informative to test the p53-SUMO-3 fusion in an oligomerisation assay, although the fusion might not be able to recapitulate the structure of posttranslational SUMO-3 conjugation. Interestingly, a recent in vitro study showed that SUMO-1 modified p53, which blocked its subsequent acetylation, had a lower affinity towards DNA than non-SUMOylated p53[401]. Therefore, despite not affecting the oligomerisation of p53, SUMO-3 modification might influence p53's ability to bind DNA.

Many posttranslational modifications such as phosphorylation, acetylation and methylation, ubiquitination and neddylation influence transactivation by p53 and specific modifications of particular amino acid residues have been shown to be involved in steering p53 towards the activation of certain panels of target genes. Many transcription factors are modified by SUMOylation and SUMO-modification has often been associated with transcriptional repression of their target genes[356,380,761]. The data presented here show that SUMO-2/3 modification of p53 reduces activation of both p21 and MIC-1 at mRNA- and protein level, since p53-proteins with a disrupted SUMO consensus were inducing these target genes more strongly than wild-type p53 (Figures 4-11, 4-12 B and 4-13). Although never shown for p53 E388A before, enhanced activation of p21 after p53 K386R expression had previously been observed on Luciferase-reporters[401,405] and is consistent with a report stating that the SUMO-site mutant p53 K386R is more present at the p21 promoter in a chromatin immunoprecipitation experiment[401]. Importantly, other studies have also shown the opposite effect of SUMOylation on p53's induction of

p21[405,406,412] and while it is possible that SUMO-2/3 modification of p53 has a different impact on its transcriptional activity than SUMO-1 modification, the p53 response is known to be divergent in different tissue types and results might therefore also be influenced by the choice of cell line studied.

The effect of SUMO-2/3 modification of p53 on the induction of Bax was less straightforward. Mutation of the p53 SUMO consensus significantly enhanced activation of a Bax-Luciferase reporter (Figure 4-9 B), however no significant difference could be observed at Bax mRNA-level (Figure 4-11). Furthermore, expression of a p53-SUMO-3 fusion protein induced activation of the Bax-reporter construct significantly less than p53 (Figure 4-10 B), which is contrary to what would have been expected, if SUMO-3 modification of p53 promoted its activation of Bax. However, induction of the Luciferase activity by high levels of p53-SUMO-3 was observed only on the Bax-reporter and not at all on the PG13 promoter (Figure 4-10). It is thus possible, that the fusion protein is generally less active than wild-type p53 (possibly since it can be exported from the nucleus or the artificial fusion makes it more prone to misfolding than wild-type p53), but selectively induces the Bax, but not the p21 reporter. This would be consistent with the observation that the SUMO-site mutants p53 K386R and p53 E388A induced PG13 Luciferase more strongly than wild-type p53, while inducing Bax-Luciferase less efficiently (Figure 4-9). I therefore suggest a model of SUMO-2/3 modification of p53 preventing p21-activation and favouring activation of Bax.

Nevertheless, it is still unclear, why wild-type p53, p53 K386R and p53 E388A induced similar levels of Bax mRNA (Figure 4-11). Since expression of all p53 proteins only resulted in a very small induction of Bax mRNA (less than two-fold), the impact of a small SUMOylated fraction of p53 on its induction might have been missed in this experiment. Other members of the lab had also observed that Bax mRNA was not strongly induced purely by p53 overexpression and found that further stresses (e.g. Adriamycin treatment), probably inducing posttranslational modifications such as phosphorylation and acetylation of p53, were required to achieve substantial Bax mRNA induction. p53 has a lower affinity towards the Bax promoter than towards the p21 promoter[753] and it is conceivable that either higher levels of p53 or DNA-binding-enhancing posttranslational modifications of p53 would

have been required to induce Bax more efficiently. The Realtime PCR experiment was performed with retroviral infection, which leads to a more uniform and generally lower p53 expression in the infected cells. By contrast, the Luciferase assay was performed with transient transfection, which can lead to very high p53 expression in some cells (only the transfected cells are measured in this assay, since only these cells contain the reporter construct and therefore equal expression in all cells is not required). Furthermore, Luciferase reporters can be much more sensitive than endogenous promoters. Therefore, the observed differences between Bax-activation in Luciferase assays and Realtime PCR experiments could be explained by technical differences. Furthermore, only a small selection of p53-induced genes was tested and while the effect on the Bax promoter might not have been very pronounced, the data indicate that SUMOylation of p53 does not dampen the activation of all p53 target genes, but only of some genes such as p21 and MIC-1 and it is possible that other target genes are more strongly induced by SUMOylation of p53, similar to the effects observed on the Bax Luciferase. This study could be expanded to test more p53 target genes, for example conducting a ChIP-sequencing experiment with wild-type p53, p53 K386R and p53 E388A could lead to the identification of target genes, which are regulated by SUMOylation of p53.

Since SUMOylation has often been reported to contribute to the assembly of repressor complexes[356,380], but its effect on p53-mediated repression had not yet been investigated, the impact of SUMO-2/3 conjugation on p53-repressed target genes was also studied. In contrast to the transcription factor Ikaros, which is dependent on SUMOylation in order to repress its target genes[762], the SUMO-site deficient p53 mutants (p53 K386R, p53 E388A) repressed some targets (Cdk1 and Cyclin A_2) even more efficiently than wild-type p53, while having no effect on another (Cyclin B_2) (Figure 4-11). Cdk1, Cyclin A_2 and Cyclin B_2 have previously been shown to be repressed by p53[40-42], yet the proposed mechanism of repression remains controversial (reviewed in[763]). It has been proposed that p53 binds to the CCAAT binding factor NF-Y to recruit chromatin remodeling Histone deacetylases[42,240], but it is also possible that the repression might be mediated more indirectly via the p53-target gene Cyclin-dependent kinase-inhibitor p21, which prevents hyper-phosphorylation of retinoblastoma-protein Rb by the Cdk2/Cyclin E complex, thereby enhancing Rb-mediated repression of E2F target genes[764]. E2F1 was found to bind

the Cyclin A and Cdk1 promoters[765,766], but not the Cyclin B_2 promoter, which is bound by E2F4 instead[767]. Since differences in p21-expression according to the SUMO-status of p53 were observed, the stronger repression of the Cyclin and Cdk genes could also be an indirect consequence of E2F1 repression by Rb via p21. The quantitative Real-time PCR experiment was therefore repeated under treatment with the ribonucleotide reductase inhibitor Hydroxyurea[768]. Under these conditions cells arrest in S-phase since DNA replication is stalled, but at this point in the cell cycle Rb has already been hyper-phosphorylated and can therefore not inhibit the E2F transcription factors. Exactly the same repression pattern (data not shown) was observed when cells were treated with Hydroxyurea and the possibility that differences in repression of p53-target genes dependent on SUMO-status are indirect effects via p21/Rb/E2F-mediated repression can thus be excluded.

Instead, I propose a model in which SUMOylation affects interaction with the transcription factor NF-Y, which has been shown to interact with C-terminal part of p53[240]. Indeed, C-terminal acetylation of p53 was shown to be required for p53-mediated repression via NF-Y[240]. Acetylation of p53 could be affected by the SUMO status of p53, since SUMO-1 modification was reported to inhibit p300-mediated acetylation of p53[401]. However, it is still unclear why repression of Cyclin B_2 that also contains CCAAT boxes, which are recognised by the NF-Y transcription factor, was not affected by p53 SUMOylation. It would be interesting to also study the effect of SUMO-3-modification on repression of p53 target genes that are repressed independent of NF-Y, such as PLK-1[769].

Contrary to the common assumption that SUMOylation of transcription factors is associated with repression of their transcriptional activity, these data show that SUMO-3 modification dampens the activation of some p53-activated genes, while possibly inducing others and furthermore alleviating the repression of a number of p53-repressed target genes. A microarray study with a p53-SUMO-1 fusion protein found that while p53 repressed 634 target genes, the p53-SUMO-1 fusion protein only repressed 168 target genes and concluded that SUMO-modification alleviated p53-mediated repression[402]. This study also found that the p53-SUMO-1 fusion protein induced Noxa more strongly than wild-type p53, which would be consistent with a model of SUMOylation targeting p53 towards its apoptotic target genes.

Cell cycle analysis of cells expressing no p53, wild-type p53, p53 K386R or p53 E388A showed no significant differences in cell cycle distribution between cells expressing wild-type p53 or the SUMO-site mutants p53 K386R and p53 E388A. However, a slightly smaller, albeit not significant, fraction of cells expressing the SUMO-consensus mutants p53 K386R and p53 E388A had sub-G_1 DNA content, which would be consistent with SUMOylation of p53 promoting a stronger apoptotic response. Importantly, others had also observed that p53 K386R had a reduced apoptotic potential compared to wild-type p53[408]. Furthermore, expression of wild-type p53 in the p53-null cells did not induce a significant G_1-arrest, but did result in a significant increase in apoptosis (Figure 4-14), while expression of p53 E388A resulted in a significant G_1-arrest, but no significant induction of apoptosis. Thus, under the conditions used to promote p53-SUMOylation (MDM2 ΔAD expression), induction of apoptosis seems to be favoured over induction of cell cycle arrest, suggesting a role for SUMO-2/3 modification modulating the p53 stress response.

50% of tumours do not express any wild-type p53, the majority of which expresses a mutant p53 protein harbouring a missense mutation. These mutant p53 proteins acquire novel oncogenic activities, such as repressing the transcription factors p63 and p73. Our lab has previously published that mutant p53 can drive invasion and that this activity is dependent on the C-terminal amino acids[586], including the SUMO-site. The activity of mutant p53 was therefore studied in the next chapter.

5 Mutant p53 interacts with p63 and p73.

Approximately half of all tumours express a mutant p53 protein, which has lost wild-type p53 activity. A significant fraction of somatic p53 mutations observed in cancer patients result in expression of point-mutated p53 proteins, which additionally gain new oncogenic functions. This gain of function is partially mediated by mutant p53 binding to and repressing the p53 family transcription factors p63 and p73[134,575-581].

Both p63 and p73 can induce some p53 target genes and have been found to be required to support p53 in initiating apoptosis[582]. The transcription factors also induce sets of target genes distinct from those of p53. As well as its important role in embryonic development, p63 also represses epithelial-to mesenchymal transition[596], migration[770] and invasion. Thus, loss of p63 is often associated with more aggressive and invasive tumours[771,772]. A number of studies have recently addressed the mechanism as to how mutant p53 induces invasion via p63 in crosstalk with EGFR, Met and TGFβ signalling[586,594,595]. While p73's role in migration is less well established than p63, loss of p73 has also been associated with more invasive tumours in some studies[773]. Loss of p63 or p73 in p53 heterozygous mice leads to a significant increase in invasive tumours from 5% to 50% in $p53^{+/-};p63^{+/-}$ and 45% in $p53^{+/-};p73^{+/-}$ mice[468], suggesting that p73 also plays a role in inhibiting metastasis. It is possible that rather than affecting epithelial to mesenchymal transition and motility directly, p73 suppresses metastasis by inducing anoikis and thereby posing a barrier to anchorage-independent growth[760].

5.1 The mutant p53 C-terminus is dispensable for interaction with p63.

Having studied the SUMO-2/3 modification of wild-type p53 and its consequences on p53 function, it was investigated whether mutant p53 could also be SUMOylated. Since other members of the lab recently found that mutant p53 required its C-terminus to promote invasion via p63[586], the consequences of SUMOylation on mutant p53 activity and in particular its binding to p63 were studied.

5.1.1 Mutant p53 is SUMOylated.

It has previously been shown that mutant p53 and wild-type p53 interact differently with MDM2, although mutant p53 was found to still be ubiquitinated by MDM2[544]. Since MDM2 needs to physically interact with p53 via the N-terminus for successful SUMOylation to occur, it was investigated whether conformational p53 mutants could also be SUMOylated.

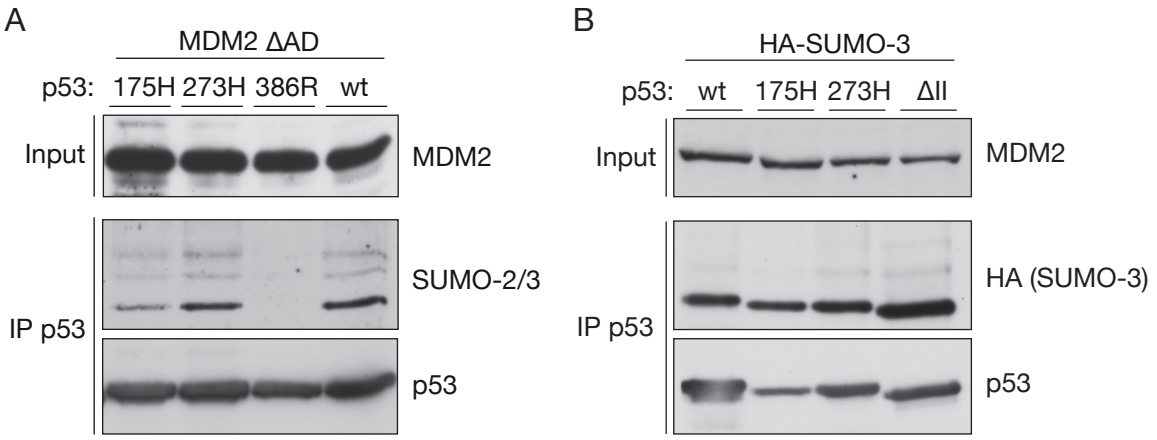

Figure 5-1: p53 hot-spot mutants are SUMOylated.
HCT116 $p53^{-/-}$cells were transfected with p53 mutants an either MDM2 ΔAD (A) or HA-SUMO-3 (B). Cells were lysed under denaturing conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

Both cancer-derived p53 mutants tested, which are impaired for DNA-binding (273H) or overall conformation of the DNA-binding domain (175H), retain the ability to be SUMOylated (Figure 5-1 A).

While earlier studies found that MDM2 can ubiquitinate mutant p53, the level of MDM2-independent ubiquitination was much higher for mutant p53 compared to wild-type p53[544]. In order to test whether mutant p53 is also SUMOylated without MDM2 overexpression, the level of SUMOylated protein after expression of HA-SUMO-3 was assessed. It is important to note that endogenous MDM2 was still present in these cells. Both hot-spot mutants p53 R175H and p53 R273H as well as deletion of the conserved box II (ΔII), which adopts a mutant conformation, showed a similar degree of residual SUMOylation compared to wild-type p53 (Figure 3-18 B). It can therefore be concluded that SUMOylation of p53 is neither affected by p53's DNA-binding capability nor the conformation of its DNA-binding domain.

5.1.2 The SUMO site is not required for interaction with p63.

An important property of mutant p53 is its ability to interact with the transcription factors TAp63[575-577] and TAp73[134,575,577-581], preventing induction of their target genes. Since SUMOylation modulated wild-type p53 activity, its effect on mutant p53 activity was also analysed. Thus, the interaction of the p53-hotspot mutants p53 R175H and p53 R273H with TAp63α was assessed. The role of the C-terminal SUMO site in this interaction was studied by expressing mutant p53 proteins deleted for the last 14 (p53 Δ380), 24 (p53 Δ370), 31 (p53 Δ363) or 47 (p53 Δ347) amino acids (Fig 5-2 B). The latter three proteins lack the SUMO site at lysine 386.

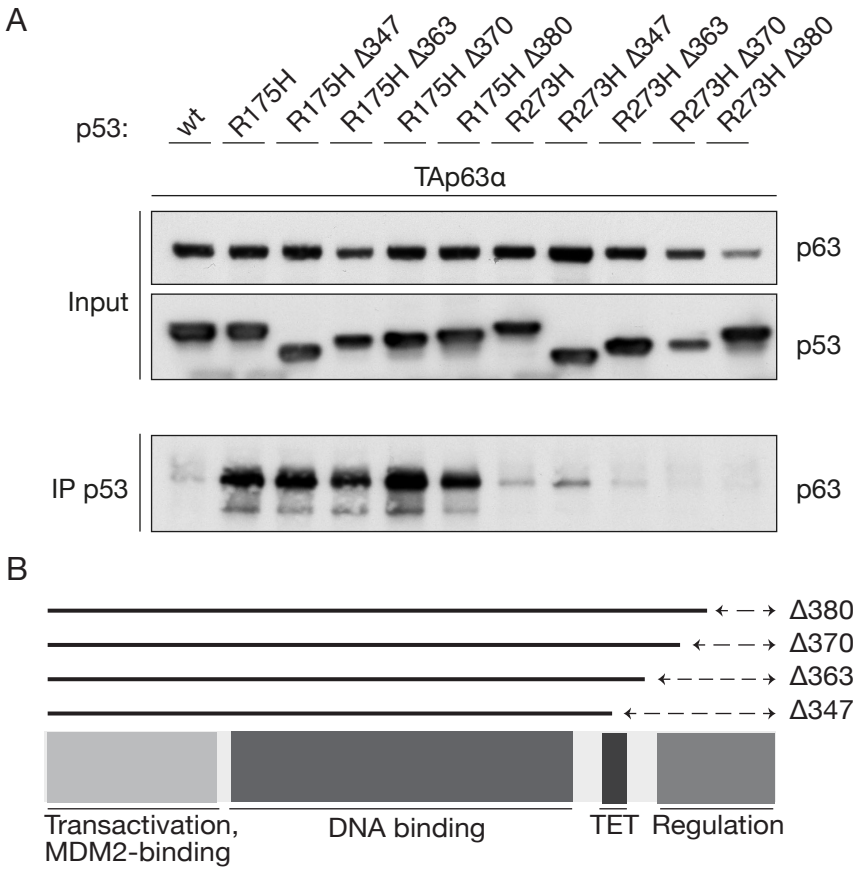

Figure 5-2: Interaction of C-terminally deleted p53 mutants with TAp63α.
(A) HCT116 p53$^{-/-}$ cells were transfected with wild-type or mutant p53 constructs and TAp63α and lysed under native conditions. p53 was immunoprecipitated with the DO-1 antibody (IP p53) and interaction was assessed as level of TAp63α protein co-immunoprecipitated with p53.
(B) Domain structure of TAp63α with indication of deletion mutants. TET: tetramerisation domain.

While hardly any binding between wild-type p53 and TAp63α was observed, the conformational p53 R175H mutant strongly interacted with TAp63α in the co-immunoprecipitation experiment (Fig 5-2 A). None of the C-terminal deletions influenced the degree of TAp63α binding. All C-terminal deletion mutants lack the SUMO-site at lysine 386. Therefore, the SUMO site of mutant p53 does not seem to be required for interaction with TAp63α. The p53 R175H Δ347 mutant lacks a part of the tetramerisation domain (Fig 5-2 B), deletion of which also did not affect TAp63α binding (Fig 5-2 A). In contrast to p53 R175H, the DNA contact mutant p53 R273H interacted only very weakly with TAp63α. Again, the C-terminal deletions did not influence the binding to a large extent, although the p53 R273H Δ347 mutant showed slightly enhanced interaction in this particular experiment, possibly since the TAp63α levels were slightly higher in this lane. While the presence of the SUMO site did not affect the ability of mutant p53 to interact with TAp63α, strong differences were observed depending on the kind of p53 mutant used.

5.2 p53 in unfolded conformation interacts with p63 and p73.

Other studies have also shown different degrees of p63 and p73 binding, depending on the particular p53 mutation. One study suggested that only p53 mutations that lead to a disruption of the wild-type p53 conformation gain the ability to efficiently interact with p63 and p73[575].

5.2.1 Different p53 mutants show different binding behaviour.

p53 hot-spot mutants are classically sub-divided into conformational and DNA contact mutants. The frequently detected R175H mutation globally alters the conformation of p53's DNA structure, disrupting binding of the critical zinc ion required for stabilising the L2 and L3 loops[774]. By contrast, mutation of the DNA-contacting residue R273 leaves the conformation of the DNA binding domain largely intact, while directly impairing binding of p53 to the DNA[651]. While the DNA binding domain structure of p53 R273H and wild-type p53 are virtually identical[651] and the mutation has little impact on the thermodynamic stability of the protein[775], mutation of other DNA-contacting side chains, for example arginine 248, was found to have

some impact on the local conformation and thermodynamic destabilisation[775], albeit much smaller than the R175H mutation. Consequently, p53 mutants were re-classified into three groups: Mutants that are highly destabilising (3 kcal/mol or above) and result in global unfolding (e.g. p53 R175H), mutants that destabilise by less than 2 kcal/mol and result in local conformational changes (e.g. p53 R248Q) and mutants that only affect DNA contacting, but do not clearly destabilise the conformation (e.g. p53 R273H).

Unfolding of the DNA binding domain can be monitored by immunoprecipitation with the conformation-specific antibodies pAb1620 and pAb240, which recognise epitopes accessible in the folded or unfolded conformation, respectively (see Figure 1-26 in the introduction).

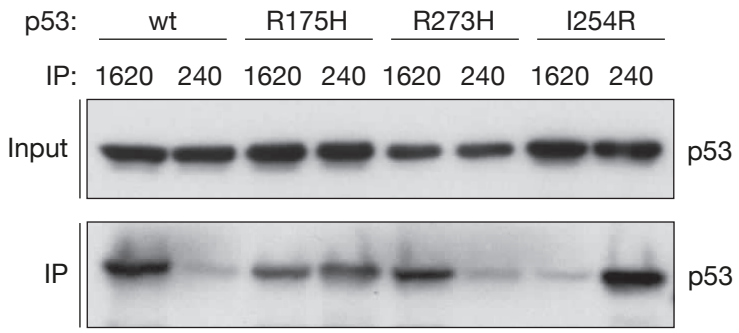

Figure 5-3: Conformation of p53 mutants.
HCT116 p53$^{-/-}$ cells were transfected with wild-type or mutant p53 constructs, lysed under native conditions and p53 was immunoprecipitated with the conformation-specific antibodies pAb 1620 and pAb 240 (IP).

Consistent with the published structural evidence[774], both wild-type p53 and the DNA contact mutant p53 R273H were predominantly immunoprecipitated with the pAb1620 antibody, recognising native folding, while a large fraction of p53 R175H was pulled down with the pAb240 antibody (Fig 5-3), which detects an epitope exposed only in the unfolded protein. Mutation of isoleucine 254, which is located in the S7 β-strand within the β-sandwich structure, occurs in 0.2% of mutant p53 tumours[524]. The I254R mutation has a very strong effect on the conformation of the p53 DNA binding domain, resulting in the p53 I254R protein being almost exclusively immunoprecipitated by the pAb240 antibody, thus destabilising the DNA binding domain even stronger than the R175H mutation (Fig 5-3).

The unfolding of the DNA binding domain and the reactivity with the pAb240 antibody were reported to correlate with p63 binding[575]. Indeed, one study on the temperature-sensitive xenopus p53 protein showed that wild-type p53 started to interact with p73, once the DNA binding domain was unfolded as a result of increased temperature[581]. Thus, the p63-binding capability of a range of p53 mutants including the highly unfolded p53 I254R and p53 R175H, and the DNA contact mutants R273H and R248W was addressed.

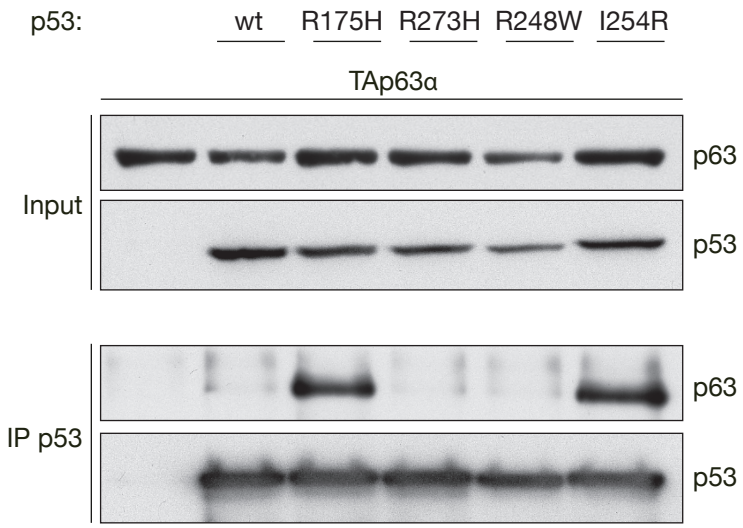

Figure 5-4: Interaction of p53 mutants with TAp63α.
HCT116 $p53^{-/-}$ cells were transfected with empty vector, wild-type or mutant p53 constructs and TAp63α and lysed under native conditions. p53 was immunoprecipitated with the DO-1 antibody (IP p53).

Strong interactions of the conformational hotspot mutant p53 R175H and the highly unfolded p53 I254R mutant with TAp63α were observed, while the DNA contact mutants p53 R273H and p53 R248W showed only marginal interaction, comparable to the level of background-interaction observed between wild-type p53 with TAp63α (Fig 5-4). This observation supports the notion that the conformation of the DNA binding domain determines the level of p63 binding. It is therefore likely that p63 binds to an epitope on p53 that is hidden in the native folding of the DNA-binding domain and becomes exposed upon unfolding.

Most previous investigations have focussed on the interaction of mutant p53 with the TA-isoforms of p63 and p73. Initially thought to act purely as a negative regulator of the full-length isoforms, the ΔN-isoforms are now also well established as activators

of distinct sets of target genes[445,446,476] through second transactivation domains in their C-termini[447,477]. Since many cancers express high levels of the shorter ΔNp63 and ΔNp73 isoforms, the interaction of mutant p53 with these shorter isoforms was also studied.

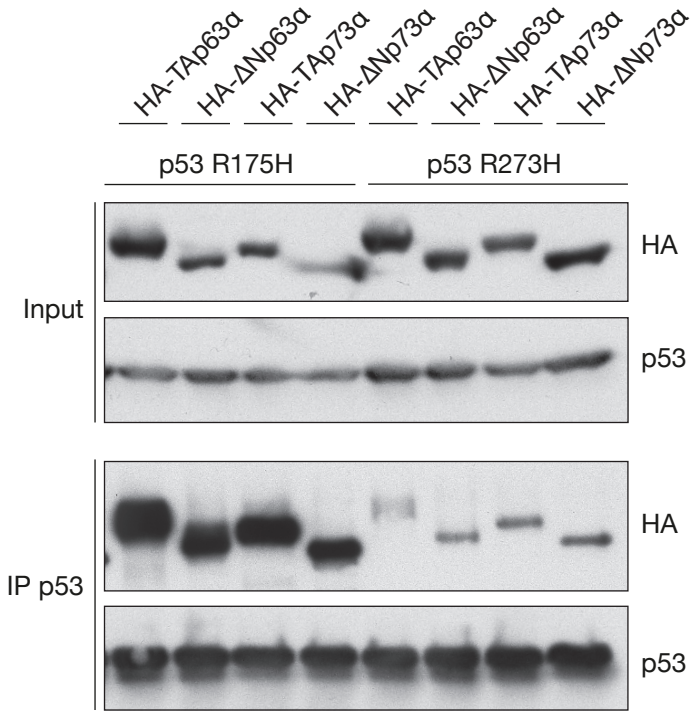

Figure 5-5: Mutant p53 interacts with the TA- and ΔN-isoforms of p63 and p73.
HCT116 $p53^{-/-}$ cells were transfected with either p53 R175H or p52 R273H and HA-tagged TAp63α, ΔNp63α, TAp73α or ΔNp73α. Cells were lysed under native conditions and p53 immunoprecipitated using the DO-1 antibody (IP p53).

The conformational mutant p53 R175H strongly interacted with the TA- and ΔN-isoforms of both p63 and p73 to a similar extent (Fig 5-5). The DNA contact mutant p53 R273H showed much weaker interaction with all isoforms and bound to the TA- and ΔN variants to a similar degree. Thus, mutant p53 does not seem to display any preference as to which isoform it interacts with. Since ΔN- and TA isoforms of p63 and p73 can play opposite roles on some target gene promoters, this broad interaction would complicate predictions of the overall outcome of mutant p53 repression in cells expressing both TA- and ΔN isoforms.

5.2.2 Mutant p53 binds p63 and p73 via the DNA-binding domain.

While p63 and p73 can form mixed tetramers via their tetramerisation domains, p53's oligomerisation domain is slightly different, missing an additional α-helix, and does not allow formation of heterotetramers with the other family members[437]. By contrast, interaction of mutant p53 with p63 and p73 is thought to take place via their core domains[576,579].

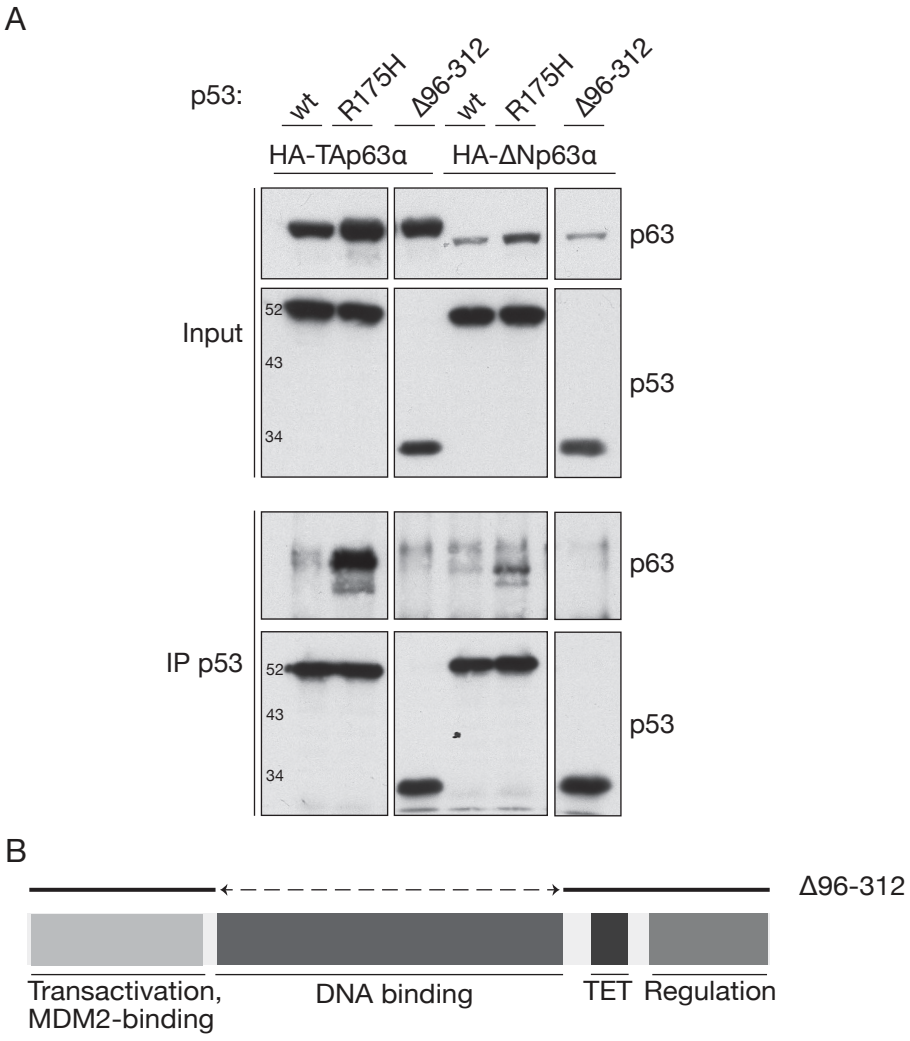

Figure 5-6: The DNA binding domain of p53 R175H is required for interaction with TAp63α and ΔNp63α.
(A) HCT116 $p53^{-/-}$ cells were transfected with p53 R175H and either full-length TAp63α or the deletion mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53). All samples were part of the same experiment and were run on the same gel, irrelevant samples were excised as indicated to produce this figure.
(B) Domain structure of TAp63α with indication of deletion mutants. TET: tetramerisation domain.

In order to investigate the contribution of the DNA binding domain to the interaction between p53 R175H and TAp63α or ΔNp63α, a deletion mutant, lacking the entire DNA binding domain (p53 Δ96-312), was co-expressed with p63 (Fig 5-6 B).

While no interaction between wild-type p53 and either TAp63α or ΔNp63α could be observed, p53 R175H bound to both p63 isoforms, although ΔNp63 was expressed at much lower levels here. Deletion of the DNA binding domain completely abrogated any interaction between p53 and TAp63α or ΔNp63α (Fig 5-6 A). These observations are in line with previous reports that the p53 R175H core domain is sufficient to interact with TAp63α[576]. In order to narrow down the region of interaction with TAp63α, a series of mutants with smaller deletions of around 50 amino acids within the DNA binding domain was tested (Fig 5-7 B).

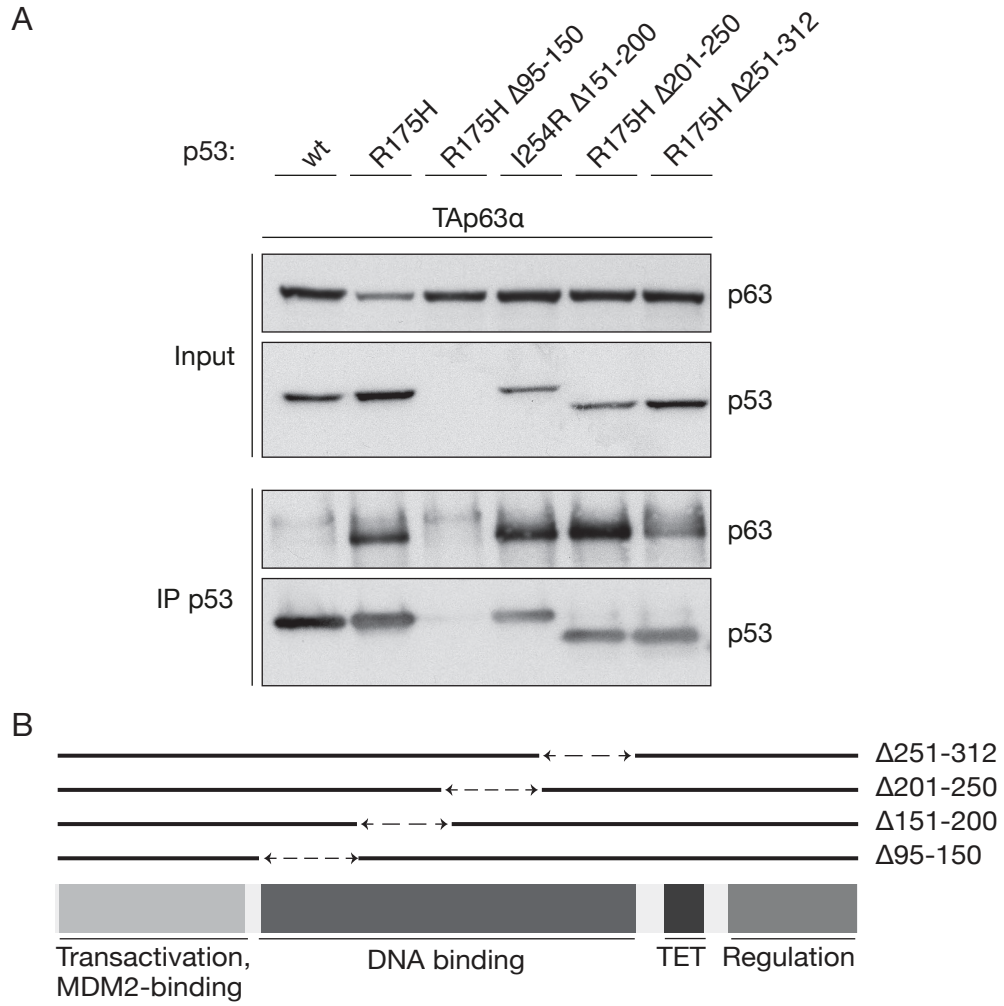

Figure 5-7: Deletion of amino acids 251-312 impairs p53 binding to TAp63α.
(A) HCT116 $p53^{-/-}$ cells were transfected with TAp63α and either wild-type or p53 R175H or the deletion mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).
(B) Domain structure of TAp63α with indication of deletion mutants. TET: tetramerisation domain

As expected, wild-type p53 did not interact with TAp63α, while p53 R175H co-immunoprecipitated TAp63α (Fig 5-7 A). The construct deleted for the first 55 amino acids of the DNA binding domain in mutant p53 (p53 R175H Δ95-150) failed to express any protein. The other three deletion mutants expressed to comparable levels. Since deletion of amino acids 150 to 200 removes the R175H point mutation, a different unfolding point mutation (I254R) was introduced into this construct (p53 I254R Δ151-200). Both p53 I254R Δ151-200 and p53 R175H Δ201-250 interacted with TAp63α to a similar degree as full-length p53 R175H, however p53 R175H Δ251-312, lacking the DNA-binding region adjacent to the C-terminal part of the protein, co-immunoprecipitated less TAp63α, suggesting that this region contributes to the interaction with TAp63α (Fig 5-7 A).

5.2.3 Mutant p53 interacts with p63 and p73 independent of aggregation.

The C-terminal part of the DNA binding domain of p53 contributes to TAp63α binding (Figure 5-7 A). This region harbours an amino acid stretch between 251 and 257 that was recently described to serve as an aggregation-nucleating segment[577]. This region is located on the S7 β-strand and is usually buried in the hydrophobic core of the protein. According to the aggregation model, this amino acid sequence becomes exposed upon unfolding of the DNA binding domain and induces aggregation of mutant p53 protein with other mutant p53 or wild-type p53 molecules. Since this amino acid stretch is located in conserved box IV, p63 and p73 also contain a similar amino acid sequence (Fig 5-8 A) and it has been proposed that mutant p53 achieves gain of function by co-aggregating with p63 and p73[577]. Deletion of amino acids 251-257 or point mutation of the central isoleucine residue I254 were reported to abrogate aggregation of unfolded p53 mutants[577]. While deletion of the last 63 amino acids of the DNA binding domain (p53 R175H Δ251-312) severely impaired binding to TAp63α consistent with earlier experiments (Fig 5-7), deletion of only the aggregation region (p53 R175H Δ251-257) surprisingly did not have any impact on the co-immunoprecipitation of TAp63α (Fig 5-8 B). Similarly, point mutation of the I254 residue (p53 R175H I254R) did not reduce interaction with TAp63α (Fig 5-8 B). It must therefore be concluded that the interaction between p53 R175H and TAp63α does not depend on p53 aggregation.

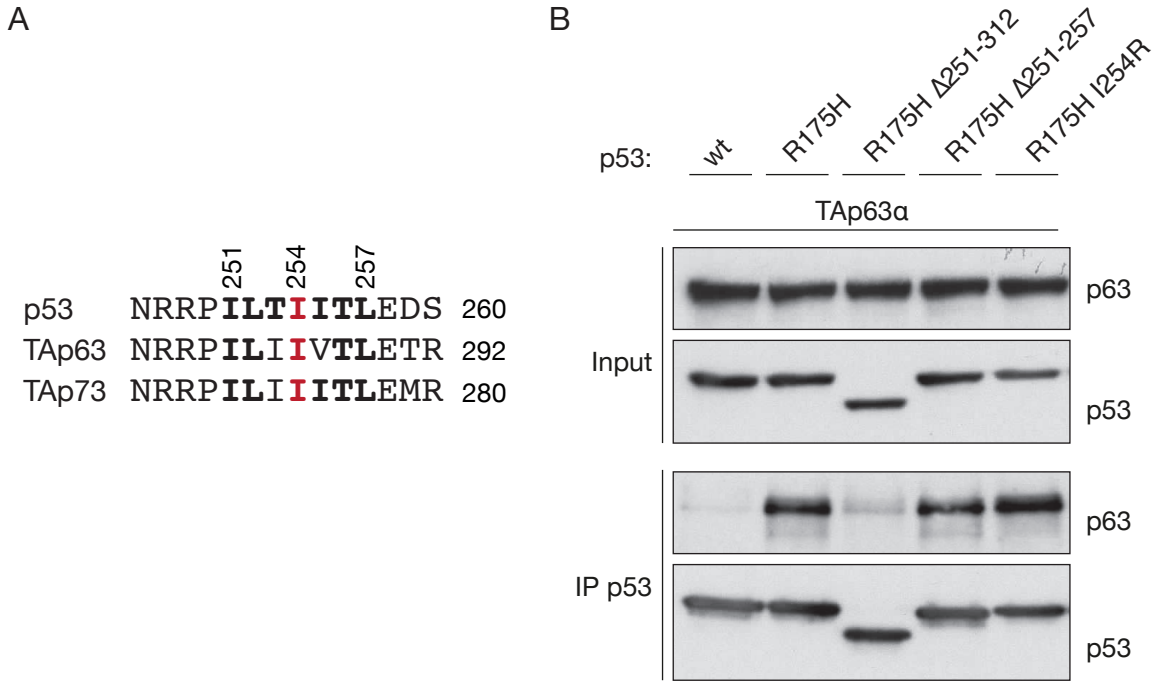

Figure 5-8: p53 interacts with TAp63α independent of an aggregation domain.
(A) Alignment of the aggregation domain in box IV across the p53-family. Amino acids identical with the aggregation domain in p53 are highlighted in bold. The central isoleucine residue is marked in red. Accession numbers: p53: P04637, TAp63α: AAF43487, TAp73α: O15350.
(B) HCT116 $p53^{-/-}$ cells were transfected with TAp63α and either wild-type or p53 R175H or the deletion mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP Do-1)

Mutation of isoleucine 254 resulted in a large pool of unfolded p53 protein in a previous experiment (see Fig 5-3) and the p53 I254R protein interacted strongly with TAp63α (Fig 5-4). Due to the recent reports that this point mutation stopped co-aggregation of unfolded p53 with p63 and p73, the effect of the I254R mutation in the background of p53 R175H and p53 R273H were also tested in the presence of ΔNp63α (Fig 5-9).

Similar to the interaction with TAp63α, the p53 I254R mutant bound strongly to ΔNp63α. Furthermore, mutation of I254R in p53 R175H did not weaken the binding of ΔNp63α (Fig 5-9), similar to the results obtained with TAp63α (Fig 5-8). In line with the finding that mutant p53 interacts with p63 via its DNA binding domain, disruption of the tetramerisation domain of p53 (p53 R175H ΔTET) did not affect the interaction with ΔNp63α either (Fig 5-9).

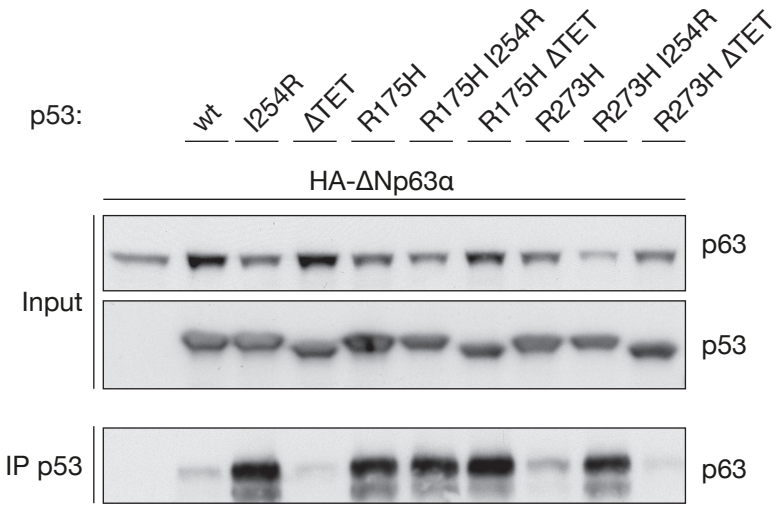

Figure 5-9: p53 R175H I254R binds strongly to ΔNp63α.
HCT116 $p53^{-/-}$ cells were transfected with ΔNp63α and either empty vector, wild-type p53 or the p53 mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53). p53 ΔTET contains the following mutations in order to disrupt tetramerisation: L348A, L350A

While p53 R273H did not co-immunoprecipitate much ΔNp63α, the p53 R273H I254R double mutant strongly bound to ΔNp63α (Fig 5-9), probably due to the unfolding effect of the I254R mutation (Fig 5-3). An intact aggregation domain is therefore not required for p53 R175H to interact with TAp63α or ΔNp63α.

The impact of the I254R mutation on p53 R175H on binding to TAp73α was assessed next. While the first lane was under-loaded in this experiment, expression levels of p53 R175H and p53 R175H I254R p53 were comparable. As seen before, p53 R175H interacted strongly with TAp73α. The p53 R175H I254R double mutant interacted with TAp73α to similar strength as p53 R175H (Fig 5-10 A). Thus, the aggregation domain is not required for mutant p53 to interact with TAp73α.

Although not dependent on the aggregation domain located between amino acids 251-257, the last 63 amino acids of the DNA binding domain were important for the interaction of p53 R175H with TAp63α (Fig 5-7 A). Next, the DNA binding domain's role in p53 R175H binding to TAp73 was examined in more detail (see Fig 5-7 B for an overview of deletion mutants used in Fig 5-10 B).

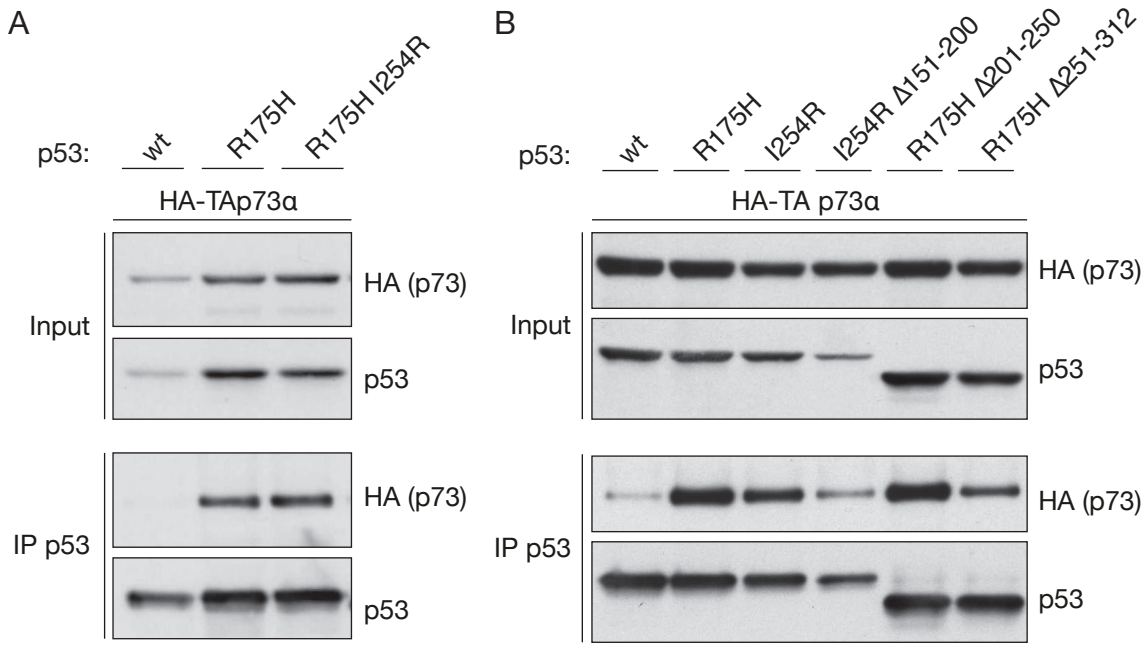

Figure 5-10: p53 R175H I254R still interacts with TAp73α.
(A) and (B) HCT116 $p53^{-/-}$ cells were transfected with TAp73α and either wild-type or p53 R175H or the deletion mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

Both unfolded mutants p53 R175H and p53 I254R strongly bound TAp73α and wild-type p53 only interacted with TAp73α very weakly (Fig 5-10 B). The p53 I254R Δ151-200 construct was expressed at much lower levels in the input and levels were also lower after immunoprecipitation, consequently the decreased amount of TAp73α pulled down in this lane is difficult to interpret (Fig 5-10 B). However, p53 R175H Δ201-250 and p53 R175H Δ251-312 were expressed to similar levels and the latter co-immunoprecipitated lower levels of TAp73α (Fig 5-10 B).

Although the reduction of TAp73α binding to p53 R175H deleted for amino acids 251-312 (Fig 5-10 B) was not as prominent as the effect of the deletion on TAp63α binding (Fig 5-7 A), the same region in mutant p53 was identified to be important for interacting with both p63 and p73. Thus, the DNA binding domain of p53, in particular the region towards the C-terminus, is critical for the formation of the mutant p53-p63 and mutant p53-p73 complexes, while the aggregation domain, also located in this region, is dispensable for either complex formation.

5.3 p63 and p73 interact differentially with mutant p53.

The interaction of mutant p53 with p63 and p73 is thought to involve the DNA binding domain of p53 and to take place via the core domains of p63 and p73 as well[576,579]. While mutant p53 interacted with TAp63α, ΔNp63α, TAp73α and ΔNp73α to the same extent (Fig 5-5), deletion of only the last 62 amino acids of the DNA binding domain (p53 R175H Δ251-312) had a more disruptive effect on the mutant p53-p63 complex (Fig 5-7 A) than on the mutant p53-p73 complex (Fig 5-10 B). It is therefore possible, that p63 and p73 interact with mutant p53 in slightly different ways. Hence, the contribution of the functional domains of p73 and p63 in contacting mutant p53's core domain was studied.

5.3.1 p73 interacts with mutant p53 via the DNA binding domain

In order to identify the domain responsible for interaction of TAp73α with mutant p53, a series of p73 deletion mutants was created, deleting the proline-rich domain (Δ47-130), the DNA binding domain (Δ131-307), the central nuclear localisation signal, tetramerisation domain and second transactivation domain (Δ308-386) and a C-terminal region up to the SAM domain (Δ387-500) (Fig 5-11).

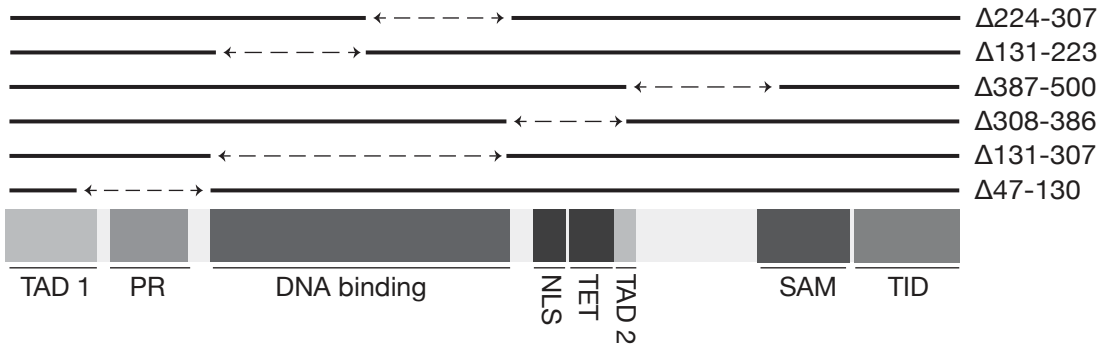

Figure 5-11: Representation of TAp73α deletion mutants.
Domain structure of TAp73α with indication of deletion mutants. TAD: transactivation domain, NLS: nuclear localisation, TET: tetramerisation, SAM: sterile α motif, TID: transcription inhibitory domain.

A mutant deleted for the extreme C-terminus (1-500) failed to express (not shown) and the effect of deleting the SAM domain and transcriptional inhibitory domain could therefore not be studied.

While most p73 deletion mutants bound p53 R175H, deletion of the DNA binding domain in TAp73α Δ131-307 reduced the interaction markedly (Fig 5-12 A). This finding is consistent with a previous report showing that a p73 protein containing only the DNA binding domain and oligomerisation domain bind mutant p53[579]. Since this deletion mutant lacks almost 200 amino acids, attempts were made to narrow the interacting region down further and two TAp73α mutants with smaller deletions of either the N-terminal half (Δ131-223) or the C-terminal half (Δ224-307) of the DNA binding domain were created (Fig 5-11).

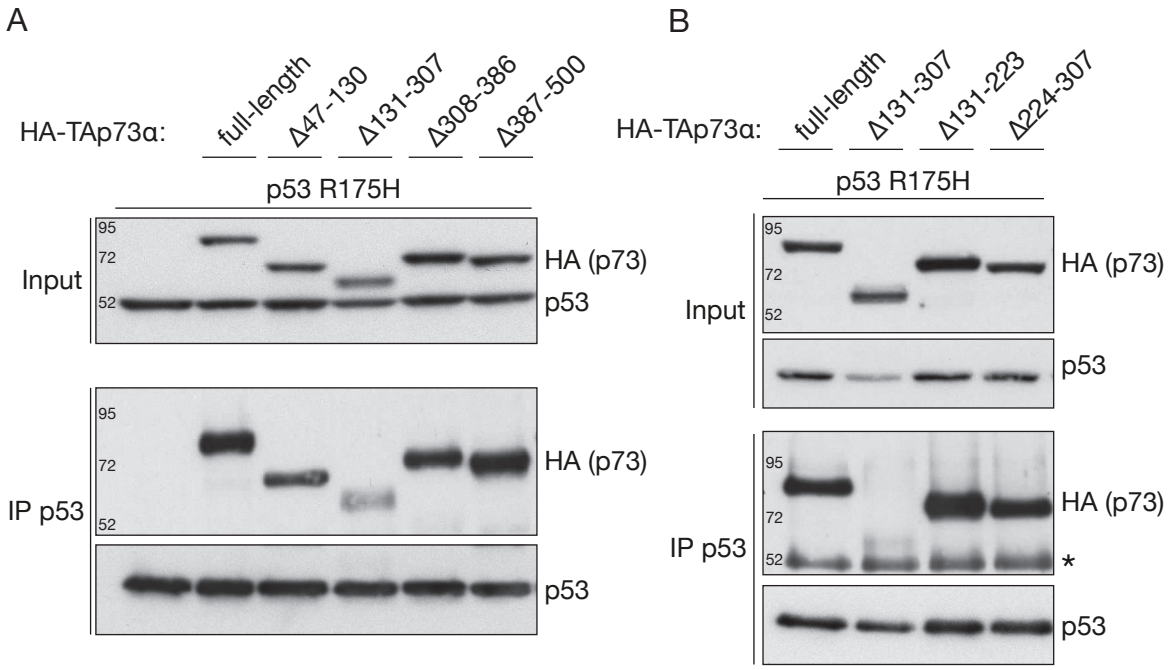

Figure 5-12: TAp73α interacts with p53 R175H via the DNA binding domain.
(A) and (B) HCT116 $p53^{-/-}$ cells were transfected with p53 R175H and either empty vector, full-length TAp73α or the deletion mutants indicated. Cells were lysed under native conditions and p53 was immunoprecipitated with the DO-1 antibody (IP p53). * indicates the IgG heavy chain.

Surprisingly, while TAp73α deleted for the whole DNA binding domain (Δ131-307) hardly bound any p53 R175H as observed before (although p53 is expressed at a lower level in this lane), both smaller deletion mutants TAp73α Δ131-223 and TAp73α Δ224-307 bound p53 R175H very strongly. It can be concluded that these deletion mutants both contain regions interacting with mutant p53, suggesting that at least two areas of the DNA-binding domain are involved in the binding, with either of them being sufficient for complex formation.

5.3.2 p63 does not require the DNA binding domain to bind mutant p53.

Since TAp73's DNA-binding domain was crucial for the interaction with mutant p53, the experiment was repeated with a ΔNp73 mutant lacking the DNA binding domain. It was furthermore studied whether the DNA-binding domains of TAp63 and ΔNp63 were also important for their interaction with mutant p53.

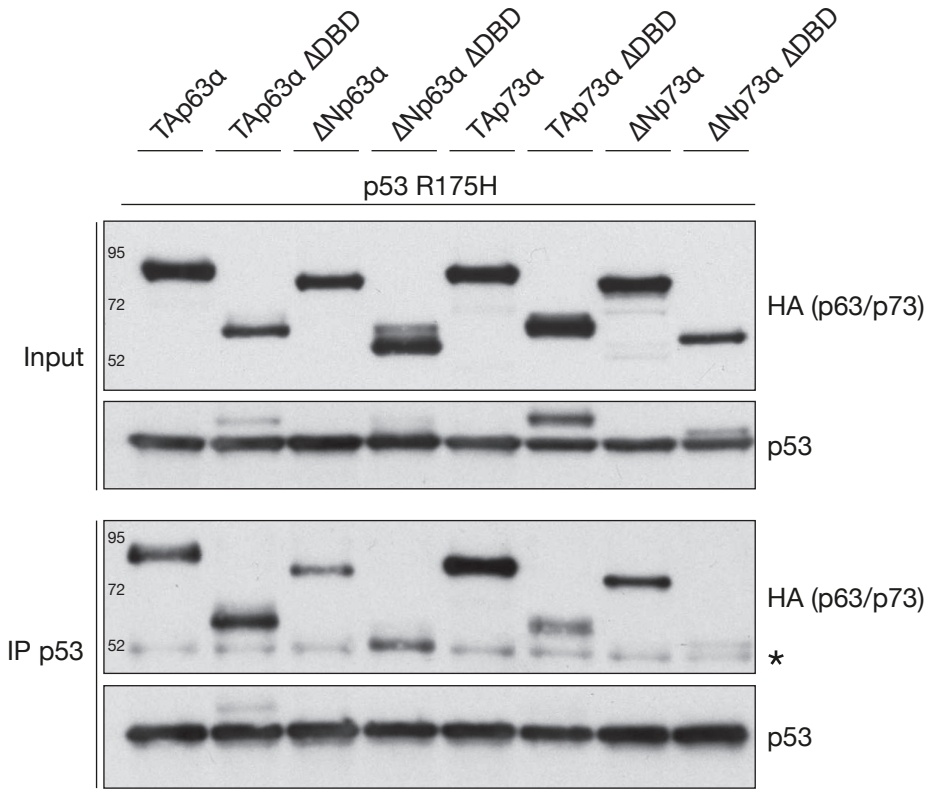

Figure 5-13: p63's DNA binding domain is dispensable for binding to p53 R175H.
HCT116 $p53^{-/-}$ cells were transfected with p53 R175H and either full-length TAp63α, ΔNp63α, TAp73α, ΔNp73α or deletions of the DNA binding domain (DBD): TAp63α Δ138-319, ΔNp63α Δ84-265, TAp73α Δ131-307, ΔNp73α Δ82-258. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody. * indicates the IgG heavy chain.

Deletion of the DNA-binding domain of TAp73α once again largely reduced interaction with p53 R175H and interaction of ΔNp73α with p53 R175H was equally compromised by deletion of the DNA-binding domain (Fig 5-13). By contrast, deletion of the DNA binding domain did not affect the interaction of TAp63α or ΔNp63α with p53 R175H (Fig 5-13). This finding is in contrast to a study that found the DNA-binding domain of p63 sufficient to bind to mutant p53[576]. It is possible that the core domain of TAp63 contacts mutant p53, while another part TAp63 outside

this region provides a secondary interaction, which is sufficient for co-immunoprecipitation.

In order to determine which region outside TAp63's DNA binding domain serves as contact point for mutant p53, differences in the amino acid sequence between TAp63 and TAp73 were analysed. In light of the recent report linking aggregation of mutant p53 to interaction with TAp63 and TAp73[577], the poly-glutamine region in the C-terminal region of TAp63 was considered a possible point of interaction. Poly-glutamine stretches of up to hundreds of glutamines tend to aggregate and multiplication of glutamine stretches have been linked to neurodegenerative diseases[776]. TAp63's sequence contains six glutamines in a row with 3 further glutamine residues in close proximity, but TAp73's sequence harbours only four sequential glutamines and one proximal glutamine (Fig 5-14 A).

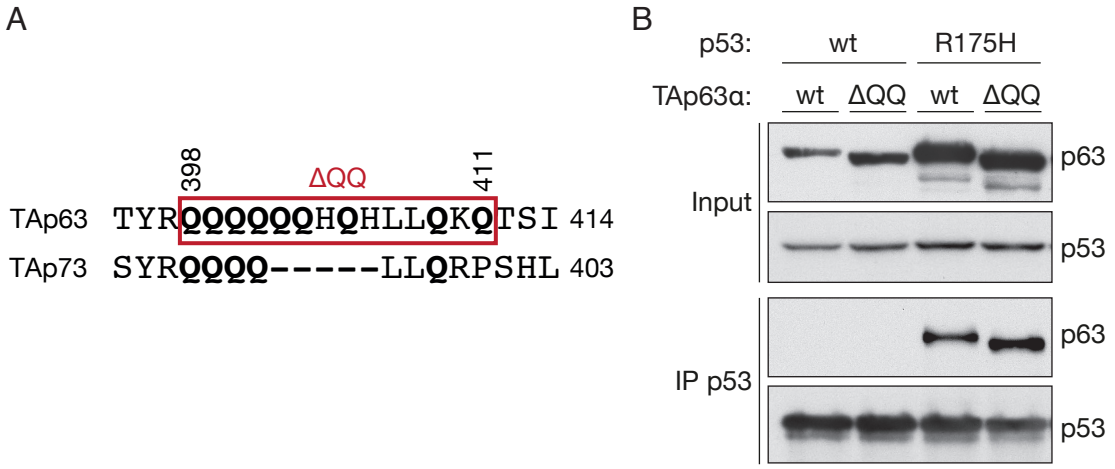

Figure 5-14: Deletion of a glutamine stretch does not affect TAp63α's binding to p53 R175H.
(A) Alignment of TAp63α and TAp73α sequences with indication of ΔQQ deletion. Accession numbers: TAp63α: AAF43487, TAp73α: O15350.
(B) HCT116 $p53^{-/-}$ cells were transfected with either wild-type p53 or p53 and either wild-type or ΔQQ TAp63α. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

Neither full-length TAp63α nor TAp63α deleted for the glutamine-rich region (TAp63α ΔQQ) bound to wild-type p53, however both full-length and deletion mutant bound strongly to p53 R175H (Fig 5-14 B). Therefore this glutamine-rich region does not appear to be crucial for the TAp63α-mutant p53 interaction. However, in case two interactions - one within the DNA binding domain and outside the DNA binding domain - are responsible for the TAp63α-mutant p53 binding, it would be informative

to repeat this experiment with a double deletion mutant, lacking both DNA-binding domain and poly-glutamine stretch in order to rule out that the glutamine stretch does not contribute to the binding.

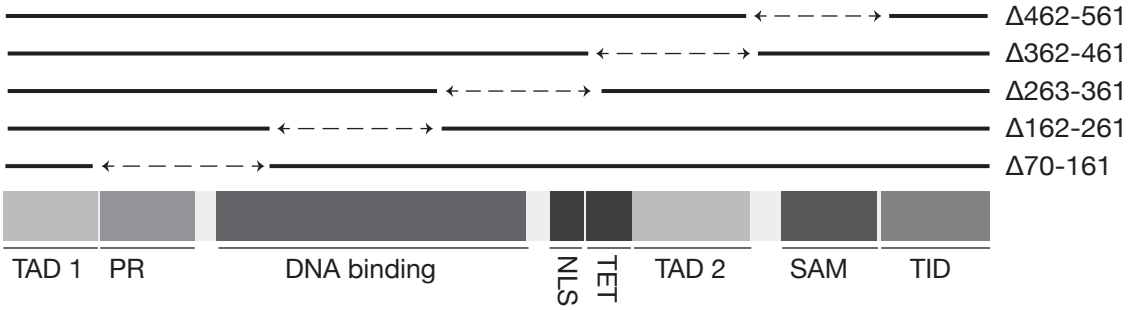

Figure 5-15: Representation of TAp63α deletion mutants.
Domain structure of TAp63α with indication of deletion mutants. TAD: transactivation domain, NLS: nuclear localisation, TET: tetramerisation, SAM: sterile α motif, TID: transcription inhibitory domain.

In order to identify the region important for the mutant p53-TAp63α interaction, a series of deletion mutants, lacking 100 amino acids each (Fig 5-15), was created.

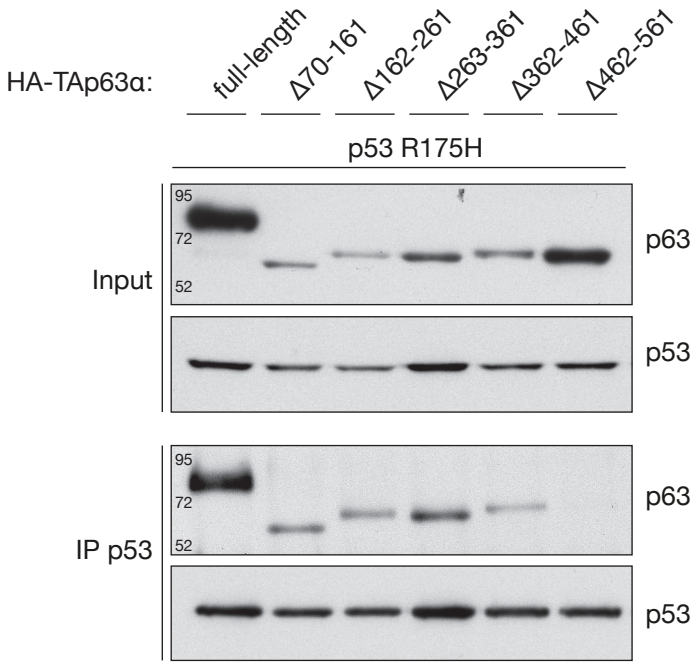

Figure 5-16: Deletion of amino acids 462-561 abrogates TAp63's interaction with p53 R175H.
HCT116 $p53^{-/-}$ cells were transfected with p53 R175H and either full-length TAp63α or the deletion mutants indicated. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

The deletion mutants expressed to slightly different levels. The TAp63α Δ462-561 mutant, which showed the strongest expression out of all mutants, failed to interact with p53 R175H, while full-length TAp63α and all other deletion mutants readily co-immunoprecipitated with p53 R175H (Fig 5-16). The Δ462-561 deletion comprises the entire SAM domain. Interestingly, the α-helices of the sterile α motif (SAM) are generally thought to act as platform for protein-protein interactions[441]. Since deletion of this C-terminal region completely abrogated the complex formation with mutant p53, it is unlikely that the DNA binding domain of TAp63α plays a major role in binding to mutant p53.

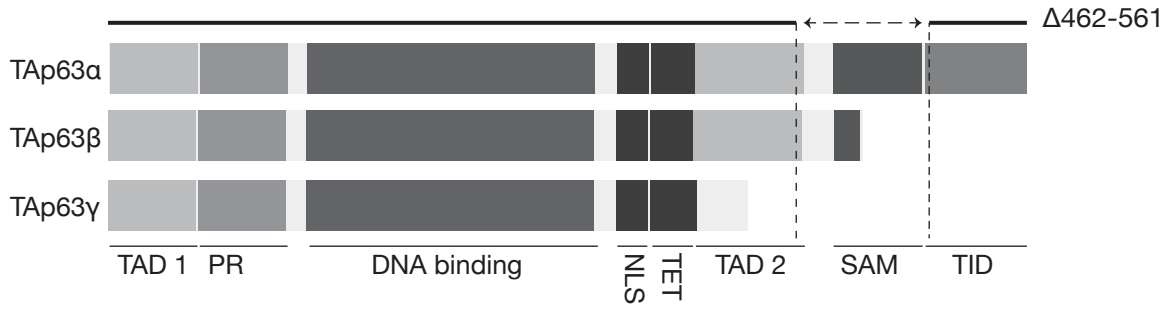

Figure 5-17: Amino acids 462 to 561 are not present in TAp63γ.
Domain structure of TAp63α, TAp63β and TAp63γ with indication of the region deleted in the TAp63α Δ462-561 mutant. TAD: transactivation domain, NLS: nuclear localisation, TET: tetramerisation, SAM: sterile α motif, TID: transcription inhibitory domain.

Strikingly, only the α-isoforms of p63 contain the full SAM domain, while the p63β proteins retain a small part of it and the p63γ proteins lack it entirely (Fig 5-17). Testing the TAp63β protein and its interaction with mutant p53 could give further information as to which part of the region deleted in the TAp63α Δ462-561 protein is responsible for contacting mutant p53. Following from the observations that the TAp63α Δ462-561 protein does not interact with p53 R175H, the TAp63γ protein would be expected not to bind to mutant p53 less well than TAp63α. This hypothesis could be investigated with further co-immunoprecipitation experiments.

In summary, these experiments clearly show that mutant p53 interacts with the DNA binding domains of TAp73α and ΔNp73α, while the core domains of TAp63α and ΔNp63α are dispensable for mutant p53 binding. This is a striking observation since the DNA binding domains of p63 and p73 are highly homologous with 86% identical amino acids. It would be interesting to determine which regions in p73's core domain

are responsible for the interaction and to analyse whether these regions are not present in the p63 protein.

5.4 MDM2 interplay with p63, p73 and mutant p53.

One of p53's strongest interacting proteins is its negative regulator MDM2. In addition to ubiquitinating and targeting p53 for degradation[107,108], MDM2 also shields the N-terminal transactivation domain of p53[114,115] and modulates its transcriptional activity by SUMOylation[777]. TAp63 and TAp73 show 26% and 30% homology to the p53 N-terminus, respectively[470]. It was therefore investigated whether MDM2 could interact with and SUMOylate the p53-family transcription factors.

5.4.1 p63 and p73 are SUMOylated

A SUMO-site homologous to that of p53 is not present in the p63 or p73 sequences. However, a SUMO consensus can be found in the extreme C-termini of p63α and p73α (Fig 5-18 A). Consistent with this observation, only the α isoforms of p63 and p73 were reported to be SUMOylated[450,734,778-781]. The lysine residue K627 within the SUMO consensus in TAp73's transcription inhibitory domain is to date the only lysine residue in p73 shown to be modified by SUMO-1[734,781]. By contrast, lysine residue K549, residing in a non-classical SUMO motif, was described as a second SUMO site in TAp63α[778], although the majority of studies only reports SUMOylation of lysine 637 within the classical SUMO consensus[450,778-780]. This second non-classical motif is also present in p73α and was predicted as potential SUMO site by the SUMOsp2.0 SUMO consensus analysis software for both p63α and p73α (Fig 5-18 B). Since some SUMO-1 modification of TAp73α K627R, disrupted for the classical SUMO site, could still be observed[734], it is possible that a lysine 532 in TAp73α is also SUMOylated. Since MDM2 ΔAD strongly induced SUMO-2/3 modification of p53, SUMO-2/3 modification of p63α and p73α in the presence of MDM2 ΔAD were tested.

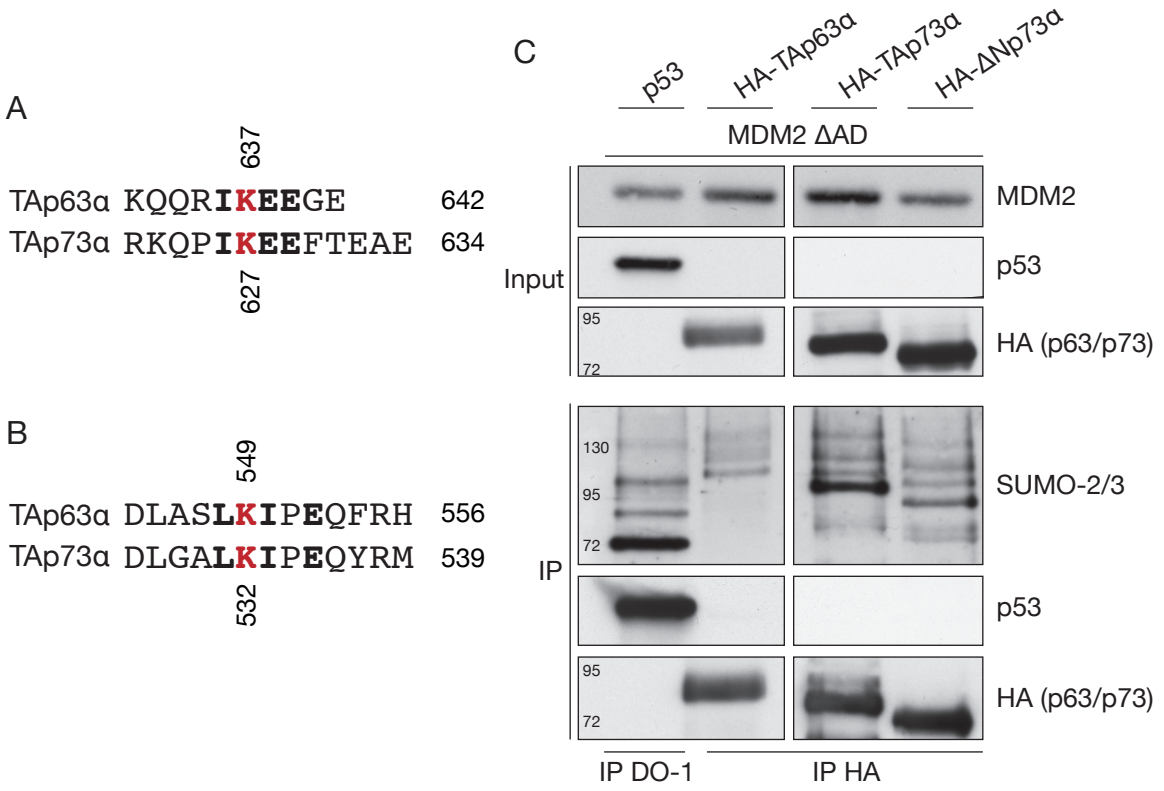

Figure 5-18: p63α and p73α are SUMO-2/3 modified.
(A) Alignment of the extreme C-termini of TAp63α and TAp73α. The SUMO consensus motif is highlighted in bold with SUMO-accepting lysine in red. Accession numbers: TAp63α: AAF43487, TAp73α: O15350.
(B) Alignment of non-classical SUMO motif in SAM domains of TAp63α and TAp73α. amino acids matching the classical consensus highlighted in bold, SUMO-accepting lysine in red.
(C) U2OS cells were transfected with p53, TAp63α, TAp73α or ΔNp73α and MDM2 ΔAD. Cells were lysed under denaturing conditions. p53 was immunoprecipitated with the DO-1 antibody (IP DO-1) and TAp63α, TAp73α and ΔNp73α were immunoprecipitated with the HA antibody (IP HA). Samples were part of one experiment and were run on the same gel, a sample in the middle has been removed as indicated for this figure.

SUMOylation of p53 was strongly induced by MDM2 ΔAD as previously observed (see chapter 3) and served as a positive control. TAp63α showed a faint ladder of bands with the lowest band running at the height corresponding of TAp63α plus one SUMO molecule (+20kD). Both TAp73α and ΔNp73α showed stronger bands corresponding to SUMO-modified p73, with TAp73α being most strongly SUMO-2/3 modified. This is the first time that SUMO-2/3 modification of TAp63α, TAp73α and ΔNp73α has been observed, since previous studies had only investigated SUMO-1 modification of TAp73α[734,781] and TAp63α[450,778,779]. Since no samples without co-expression of MDM2 ΔAD were included in this experiment, it cannot formally be excluded that the SUMOylation of p63 and p73 is promoted independent of MDM2 ΔAD. MDM2 needs to bind p53's N-terminus in order to promote SUMOylation, thus MDM2's ability to interact with p63 and p73 was investigated next.

5.4.2 MDM2 interacts with p73, but not p63.

MDM2 binds p53 at its N-terminal transactivation domain[163]. The amino acid sequence of the p53 family of transcription factors is well conserved, particularly in the DNA binding domain, where p63 and p73 share about 60% of amino acids with p53. The N-termini are less well conserved with TAp73's sequencing containing 18% amino acids and TAp63 containing 6% amino acids identical with p53. Regardless of this relatively low homology, the three amino acids in p53's N-terminus (F19, W23 and L26), which are contacted by MDM2 for the p53-MDM2 interaction[163], are present in both TAp63 and TAp73 (Fig 5-19). A number of reports suggest that MDM2 interacts only with TAp73[520-522], however some studies also found MDM2 binding to TAp63[518,519].

```
                                19     23   26
p53     MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAM 40
TAp63   M--SQS-TQTNEFLSPEVFQHIWDFLEQPICSVQPIDLNF 37
TAp73   M--AQS-TATSPDGG-TTFEHLWSSLEPDSTYFDLPQSSR 36
ΔNp63   ---------------------------------------- 0
ΔNp73   ---------------------------------------- 0
```

Figure 5-19: Alignment of the N-termini of p53 family members.
Both TAp63 and TAp73 contain the amino acids, which have been shown to be crucial for p53 binding to MDM2 (highlighted in red). ΔNp63 and ΔNp73 lack this N-terminal region entirely. Accession numbers: p53: P04637, TAp63α: AAF43487, TAp73α: O15350.

In order to study the interaction of MDM2 with p63 and p73, cells were transfected with the MDM2 C464A mutant, which is incapable of ubiquitinating, so that no protein degradation could take place. As expected, MDM2 C464A strongly interacted with p53 (Fig 5-20). A very subtle interaction with TAp63α and ΔNp63α was observed and MDM2 C464A bound much more strongly to both TAp73α and ΔNp73α (Fig 5-19).

The binding of ΔNp73 to MDM2 had not been assessed previously, but several reports suggested that MDM2 and TAp73 interact in a manner homologous to p53, with the p53-binding pocket of MDM2 contacting the N-terminus of TAp73[482,483,510]. While the amino acids crucial for p53's interaction with MDM2 (F19, W23 and L26)[163] are present in TAp73's N-terminus, they are deleted in the ΔNp73 isoform (Fig 5-19).

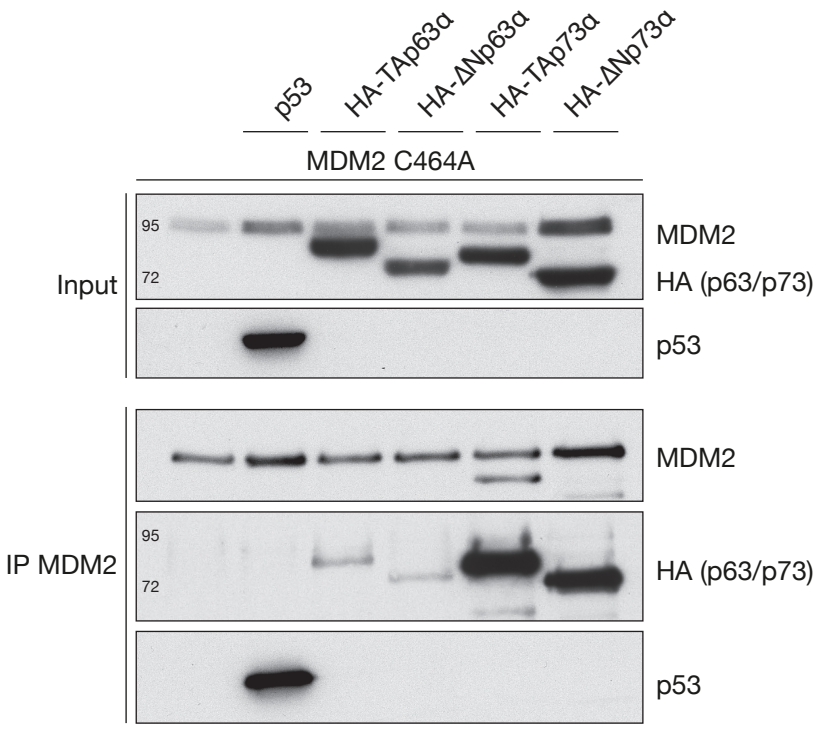

Figure 5-20: MDM2 strongly interacts with p73, but not with p63.
HCT116 $p53^{-/-}$ cells were transfected with MDM2 C464A and either p53 or HA-tagged TAp63α, ΔNp63α, TAp73α (Simian) or ΔNp73α constructs. Cells were lysed under native conditions and MDM2 immunoprecipitated with the Ab-1 antibody (IP MDM2).

Strikingly, the interaction between MDM2 and ΔNp73α was as strong as the interaction with TAp73α (Fig 5-20), suggesting that a domain other than the amino acids homologous to p53's MDM2-binding domain is also involved in the interaction with MDM2. Hence, it was investigated whether interaction of p73 and MDM2 exclusively required the p53-binding domain in MDM2.

In order to study the role of the N-terminal interaction in the MDM2-p73 complex formation, an MDM2 mutant deleted for the p53-binding domain (Δ58-89) was co-expressed with p73. Consistent with the previous experiment, MDM2 C464A interacted strongly with p53, TAp73α and ΔNp73α, but only weakly with TAp63α or ΔNp63α (bands visible after longer exposure). An MDM2 mutant lacking the p53-binding domain, MDM2 Δ58-89 C464A, interacted strongly with p73, particularly with ΔNp73α, suggesting that this interaction takes place via a domain outside the p53-binding region in MDM2 (Fig 5-21).

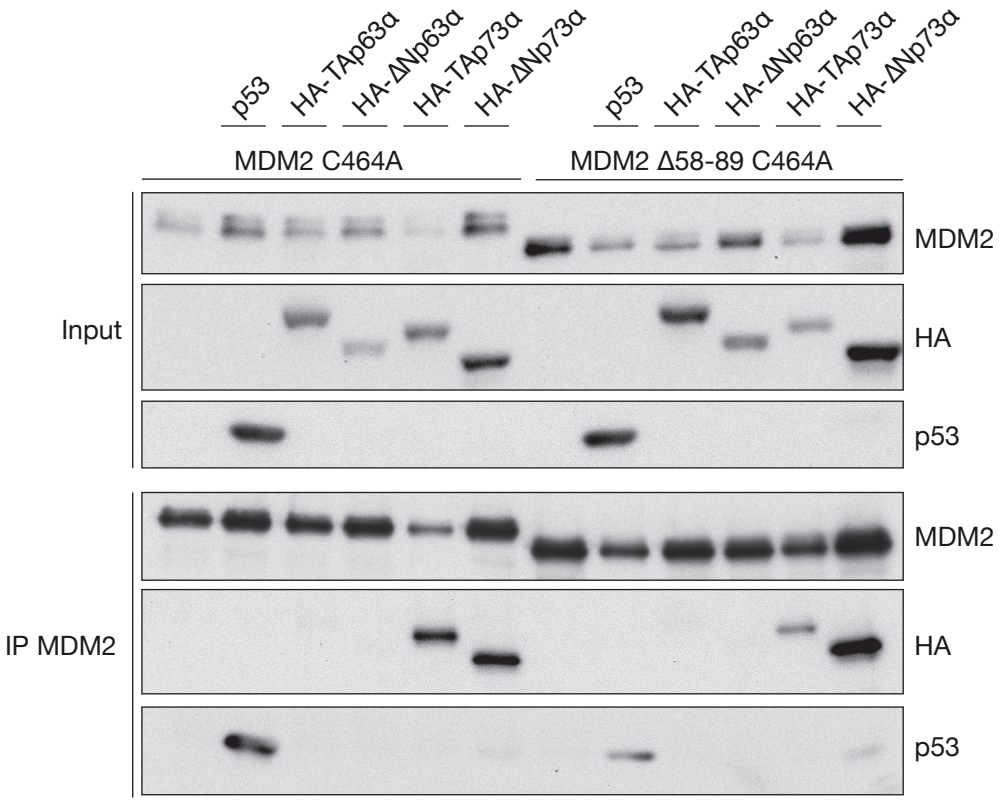

Figure 5-21: MDM2's p53-binding domain is dispensable for binding p73.
HCT116 $p53^{-/-}$ cells were transfected with either MDM2 C464A or MDM2 Δ58-89 C464A MDM2 and empty vector, p53, ΔNp63α, TAp63α, ΔNp73α or TAp73α. Cells were lysed under native conditions and MDM2 immunoprecipitated wtih the Ab-1 antibody (IP MDM2).

The interaction of MDM2 Δ58-89 C464A with TAp73α was slightly reduced compared to the binding of MDM2 C464A, raising the possibility that the p53-binding region of MDM2 contacts the N-terminal amino acids in TAp73, as previously suggested by others[482,483,510], although a second interaction appears to take place outside the p53-binding domain. Surprisingly, deletion of the p53-binding region in MDM2 reduced the co-immunoprecipitation of p53, but did not completely abrogate the interaction (Fig 5-21). While the N-terminal p53-MDM2 interaction serves as the primary docking site, a secondary binding mechanism with the central acidic domain of MDM2 contacting a region around box V in p53's DNA binding domain was reported[171,172,782,783]. Since the MDM2 Δ58-89 C464A mutant contains a functional acidic domain, this secondary interaction between box V in p53 and the acidic domain in MDM2 could explain the residual interaction observed here.

Taken together, the data suggests that MDM2 only poorly interacts with p63, but binds well to both TAp73α and ΔNp73α and that this binding takes place outside the N-termini of p73 and MDM2.

Unlike p53, TAp73 was found not to be targeted for ubiquitination by MDM2[482,483,510]. By contrast, some studies reported an increase in TAp73 levels in the presence of MDM2[483,516]. However, without affecting TAp73 levels, co-expression of MDM2 with TAp73 was found to dampen its transcriptional activity in most studies[482,483,510], while one group reported an increase in transcriptional activity[516].

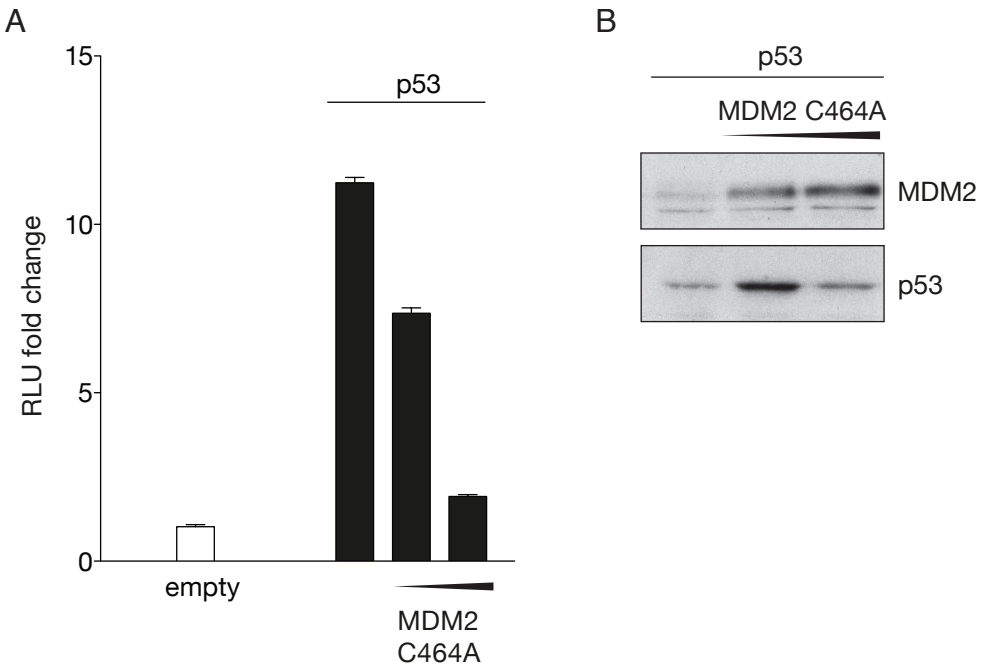

Figure 5-22: p53-induced PG13 Luciferase is repressed by MDM2 C464A. HCT116 $p53^{-/-}$ cells were transfected with PG13 Luciferase, TK Renilla and either empty vector or p53 together with increasing amounts of MDM2 C464A (25 and 100ng). (A) Activation of the promoters was assayed using the Promega Luciferase system. Data is plotted as Relative Luciferase Units (RLU) (Firefly Luciferase readings divided by Renilla Luciferase readings) fold change relative to p53-null control. The diagram represents the mean of triplicates with error bars as standard error of the mean. (B) Equal transfection was confirmed by western blot.

In the case of p53, MDM2 binds to p53's transactivation domain and interaction alone affects the transcriptional activity of p53[114,115]. Consequently, co-expression of the RING finger mutant MDM2 C464A, which is incapable of ubiquitinating p53, prevented p53-induced activation of PG13 Luciferase (Fig 5-22 A).

In contrast to previous studies that had addressed the MDM2-p73 interaction as homologous to the MDM2-p53 interaction and proposed a model in which MDM2 shields the TAp73 transactivation domain[482,483,510], a strong MDM2-p73 interaction outside the N-terminus was observed here. Therefore the ability of MDM2 C464A to interfere with p63 and p73 transcriptional activity was assessed.

Induction of BPAG1-Luciferase by TAp73α was strongly reduced by addition of MDM2 C464A, supporting the theory that MDM2 interacts with the p73's transactivation domain homologous to p53 (Figure 5-23 A). Since BPAG1-Luciferase was not induced by ΔNp73α no conclusions could be drawn about the repression of ΔNp73α's transcriptional activity.

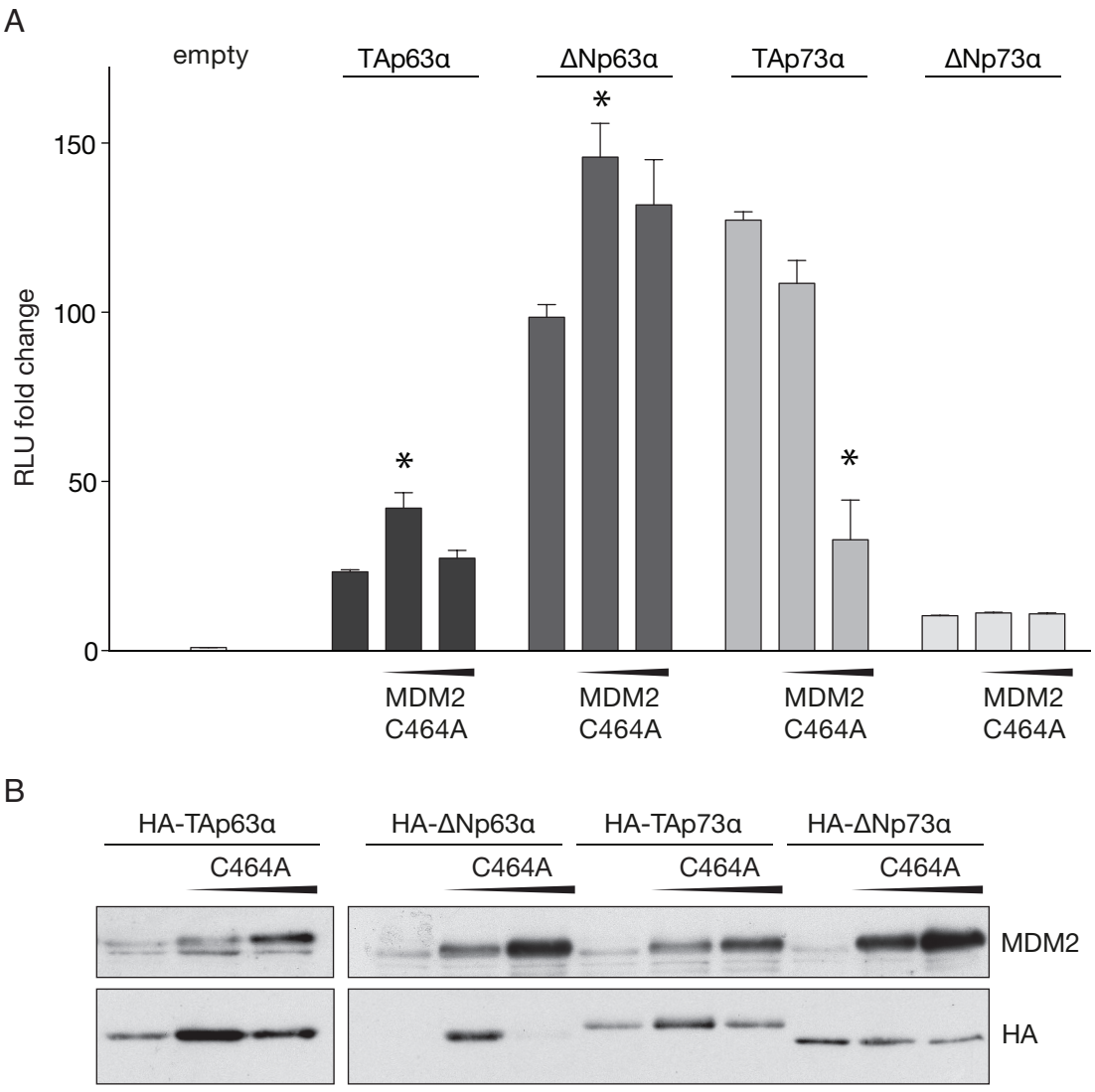

Figure 5-23: TAp73α induced BPAG1 Luciferase is repressed by MDM2 C464A.
HCT116 $p53^{-/-}$ cells were transfected with BPAG1-Luciferase, TK Renilla and either empty vector, TAp63α, ΔNp63α, TAp73α or ΔNp73α plus increasing amounts of MDM2 C464A (0, 25 or 100ng) (A) Activation of the promoters was assayed using the Promega Luciferase system. Data is plotted as Relative Luciferase Units (RLU) (Firefly Luciferase readings divided by Renilla Luciferase readings) fold change relative to empty vector control. The diagram represents the mean of two independent sets of triplicates with error bars as standard error of the mean. * indicates a p-value < 0.02 as results of an unpaired two-tailed Student t-test compared to readings from cells not transfected with MDM2 C464A. (B) Transfection efficacy was determined by western blot.

In line with the finding that MDM2 C464A only interacted well with p73, but not p63, induction of BPAG1-Luciferase by TAp63α and ΔNp63α was not reduced by co-

expression of MDM2 C464A (Fig 5-23 A). Surprisingly, addition of small amounts of MDM2 C464A significantly increased induction of BPAG1 Luciferase by both TAp63α and ΔNp63α (Fig 5-23 A). Indeed, it had previously been reported that MDM2 induced the transcriptional activity of p63[518]. However, in this experiment, the samples co-expressing low MDM2 levels express higher levels of TAp63α or ΔNp63α as compared to the samples not co-transfected with MDM2 C464A, which could be the reason for the higher Luciferase activity observed here (Fig 5-23 B).

It is a striking observation that MDM2 binds to p53 and p73, but not to p63. In order to investigate, whether these differences in MDM2 binding would translate into effects on mutant p53-p63 or mutant p53-p73 complexes, MDM2 was co-expressed with mutant p53 and p63 or p73 and complex formation was studied by co-immunoprecipitation.

5.4.3 MDM2 affects mutant p53 binding to p63 and p73.

While the interaction of MDM2 and mutant p53, and the interaction of MDM2 and p63 and p73 were studied previously, the consequence of MDM2 addition to the mutant p53-p63 or mutant p53-p73 complexes had not been investigated before.

TAp63α did not interact strongly with wild-type p53 or the DNA contact mutants p53 R273H and p53 C277Y, as observed earlier, and addition of MDM2 C464A did not alter the p53-p63 interaction (Fig 5-24). By contrast, the unfolded p53 mutants p53 R175H and p53 I254R interacted strongly with TAp63α and strikingly, addition of MDM2 C464A markedly reduced the amount of TAp63α co-precipitated with p53 (Fig 5-24). Those two mutants also co-immunoprecipitated a considerable amount of MDM2 C464A and it is possible that MDM2 competes with TAp63α for its interaction with mutant p53. Since MDM2 bound much more strongly to TAp73 than to TAp63, the effect of MDM2 C464A co-expression on the mutant p53-p73 interaction was also analysed.

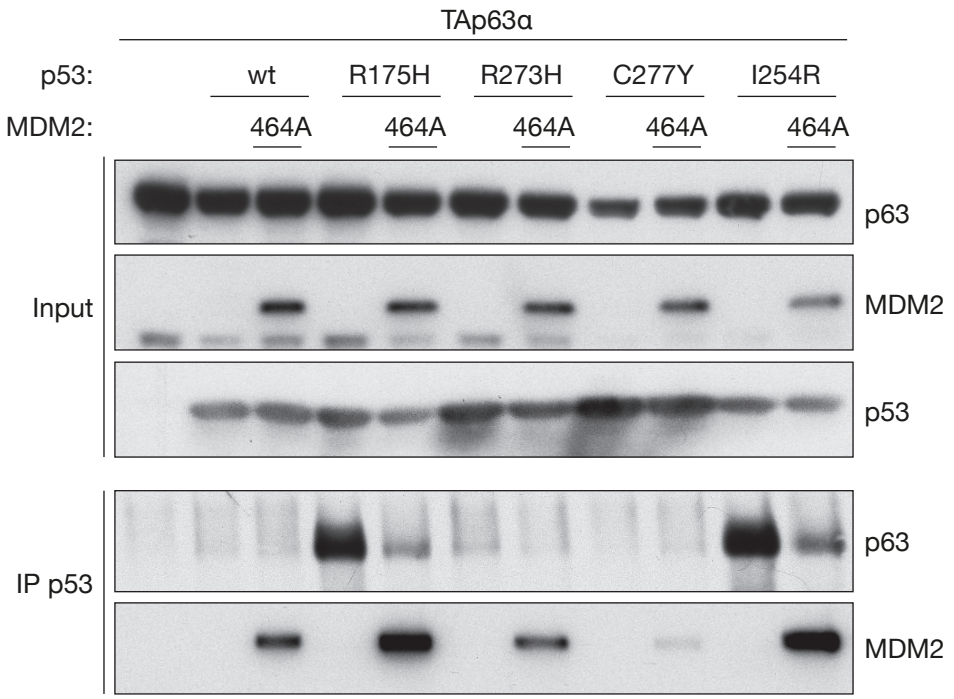

Figure 5-24: MDM2 C464A disrupts the mutant p53 - TAp63α complex.
HCT116 p53$^{-/-}$ cells were transfected with TAp63α, the p53 constructs indicated and either empty vector or C464A MDM2. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

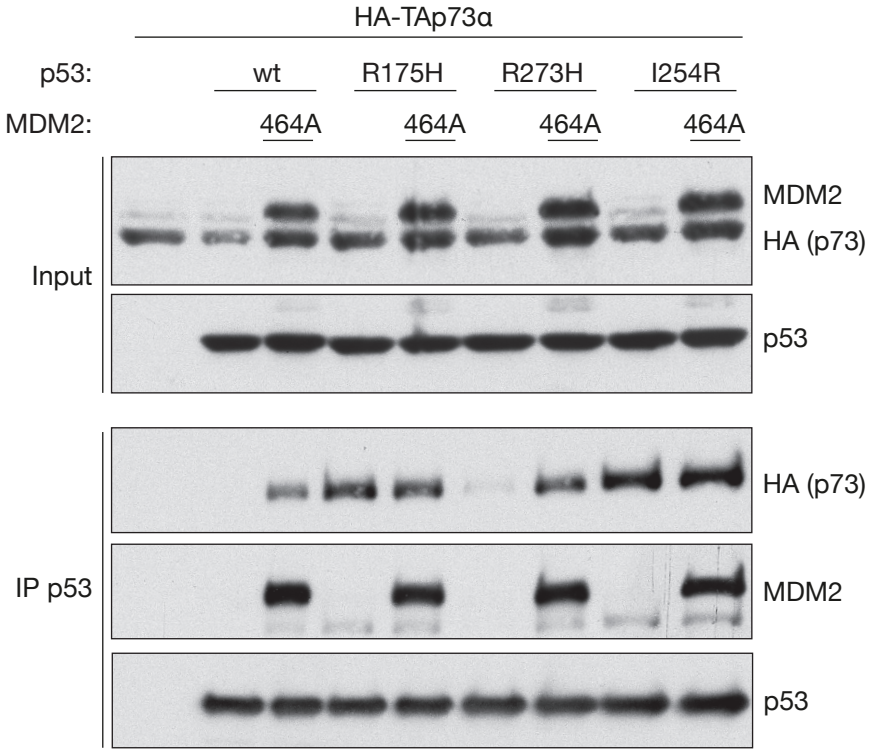

Figure 5-25: MDM2 C464A induces the interaction of folded p53 with TAp73α.
HCT116 p53$^{-/-}$ cells were transfected with TAp73α, the p53 constructs indicated and either empty vector or C464A MDM2. Cells were lysed under native conditions and p53 immunoprecipitated with the DO-1 antibody (IP p53).

As seen before, wild-type p53 and the contact mutant p53 R273H hardly interacted with TAp73α. Remarkably, upon co-expression of MDM2 C464A, TAp73α was co-immunoprecipitated with p53 in both cases (Fig 5-25). In contrast to the reduction of interaction observed when MDM2 C464A was added to the mutant p53-p63 complex, MDM2 did not have any effect on the interaction of the conformational mutants p53 R175H and p53 I254R with TAp73α (Fig 5-25).

These observations suggest the following model of complex formation: Conformational mutants of p53, such as p53 R175H bind strongly to both TAp63α and TAp73α (Fig 5-26 A and C). Addition of MDM2 displaces p63 and p73 in the complex, possibly due to higher affinity for a similar region in p53 (Fig 5-26 B and D). However, since MDM2 binds p73 but not p63, this results in different complexes: In the case of TAp63α, MDM2 forms a dimeric complex with mutant p53, releasing TAp63α, while in the case of TAp73α, a trimeric complex of mutant p53, MDM2 and TAp73α is formed.

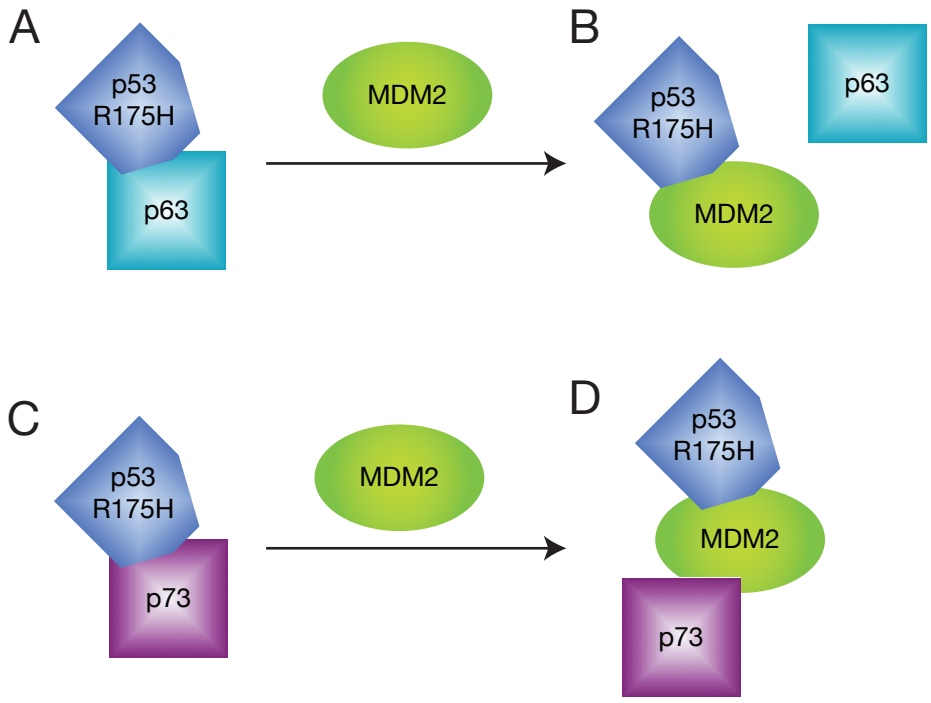

Figure 5-26: Model of the MDM2/mutantp53/p63/p73 complexes.
While MDM2 replaces TAp63 in the dimeric complex with mutant p53, TAp73 forms a trimeric complex with MDM2 and p53.

For conformational p53 mutants, no alteration of the mutant p53-p73 interaction was observed despite this change of complex, since TAp73 is still co-immunoprecipitated

with mutant p53, albeit now via MDM2. However, due to this sandwich-structure with MDM2 contacting p73, p53 in native conformation, which does not interact with TAp73 directly, but does bind to MDM2, starts to co-immunoprecipitate p73 via MDM2 as well.

ΔNp73α potentially interacts with MDM2 in a manner distinct from TAp73α, since there is no N-terminal component contributing to the binding. Therefore, the effect of MDM2 on the p53-ΔNp73 complex formation was also analysed.

The same pattern as previously observed for TAp73α, could be reproduced with ΔNp73α. Co-expression of MDM2 C464A induced co-immunoprecipitation of ΔNp73α with both wild-type p53 and DNA contact mutant p53 R273H, while the interaction of conformational mutants p53 R175H and p53 I254R with ΔNp73α was not affected (Fig 5-27).

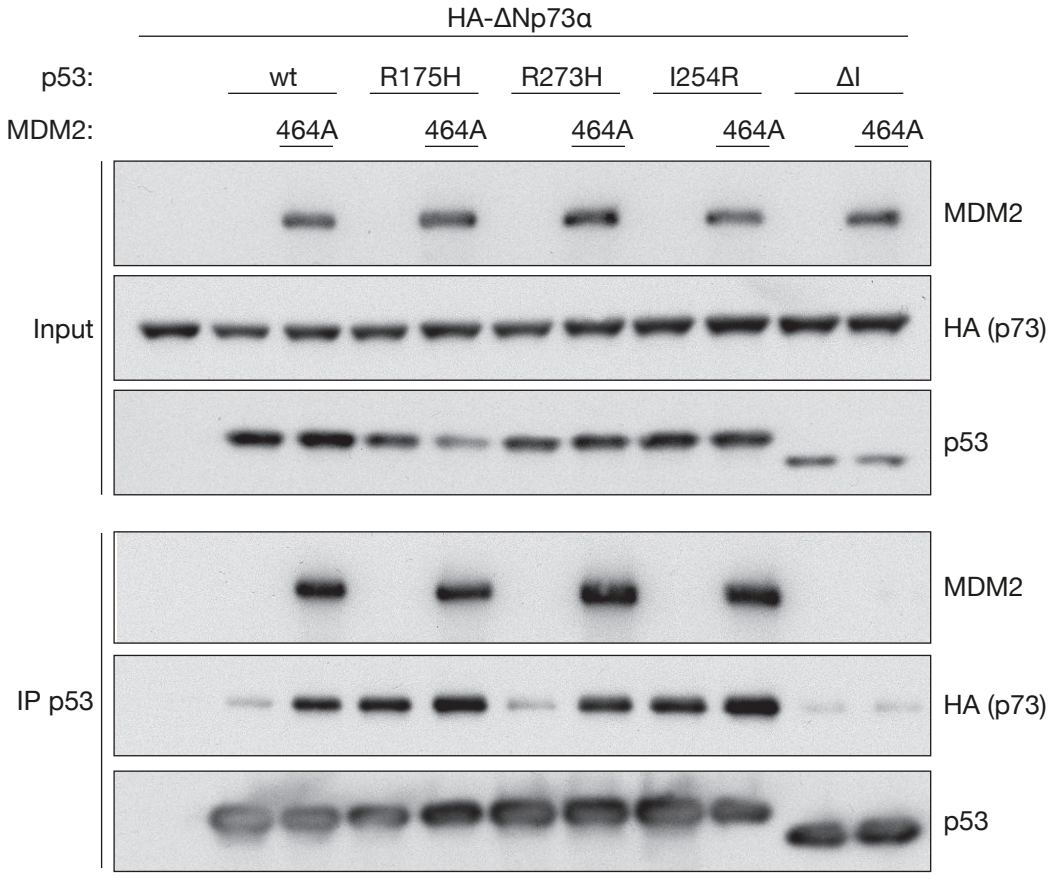

Figure 5-27: p53 ΔI does not interact with ΔNp73α via MDM2.
HCT116 $p53^{-/-}$ cells were transfected with TAp73α, the p53 constructs indicated and either empty vector or C464A MDM2. Cells were lysed under native conditions and p53 immunoprecipitated with the 1801 antibody (IP p53).

In order to test the proposed model that the induced interaction of wild-type p53 and ΔNp73 is mediated by trimeric complex formation via MDM2, p53 ΔI, which lacks the amino acids critical for interaction with MDM2, was co-expressed with ΔNp73α with and without MDM2 C464A. Immunoprecipitation of ΔI p53 did not pull down any TA-p73α and due to the lack of MDM2-binding domain, no MDM2 was co-immunoprecipitated either. Co-expression of MDM2 C464A did not induce co-immunoprecipitation of ΔNp73α with ΔI p53 (Fig 5-27), supporting the model of a trimeric p53-MDM2-p73 complex. MDM2 has strikingly contrasting effects on the mutant p53-p73 and mutant p53-p63 complexes and it would be important to determine whether releasing TAp63 from its complex with mutant p53 by MDM2 also has functional consequences for TAp63's activity as a transcription factor.

Mutant p53's interaction with p63 and p73 was shown to repress the ability to induce their transcriptional targets in many studies[134,575-581]. However, big differences in mutant p53 binding to p63 and p73, depending on the type of mutation, were observed here. The repression of TAp63α was shown to be crucial for stimulating an invasive phenotype by stimulating integrin- EGF-receptor recycling and Met-signalling[586,594]. Hence, the correlation between mutant p53-TAp63α binding, repression of TAp63α transcriptional activity and translation into an invasive phenotype was investigated.

5.5 The extent of mutant p53 and p63 interaction does not reflect the gain of invasive phenotype.

Since mutant p53 exerts some of its gain of function activity via the repression of p63 and p73, the interaction between the proteins was investigated in more detail. Only conformational mutants p53 R175H and p53 I254R were found to interact strongly with both p63 and p73, while DNA contact mutants such as p53 R273H and p53 C277Y bound rather weakly, comparable to residual levels sometimes observed after overexpression of wild-type p53 (Fig 5-4). According to the distribution spectrum of somatic mutations in tumours (see Fig 1-24), contact mutants do not seem to pose a disadvantage for tumours compared to conformational mutants. Remarkably, the mutations of the 273 codon is even more frequent than mutations of the 175 codon[524]. A previous report suggested that the degree of mutant p53 interaction with

TAp63 or TAp73 translated into the extent of repression of p63 or p73 target genes[575] and therefore the panel of p53 mutants used in this study were analysed for their ability to repress TAp63α -induced K14-Luciferase.

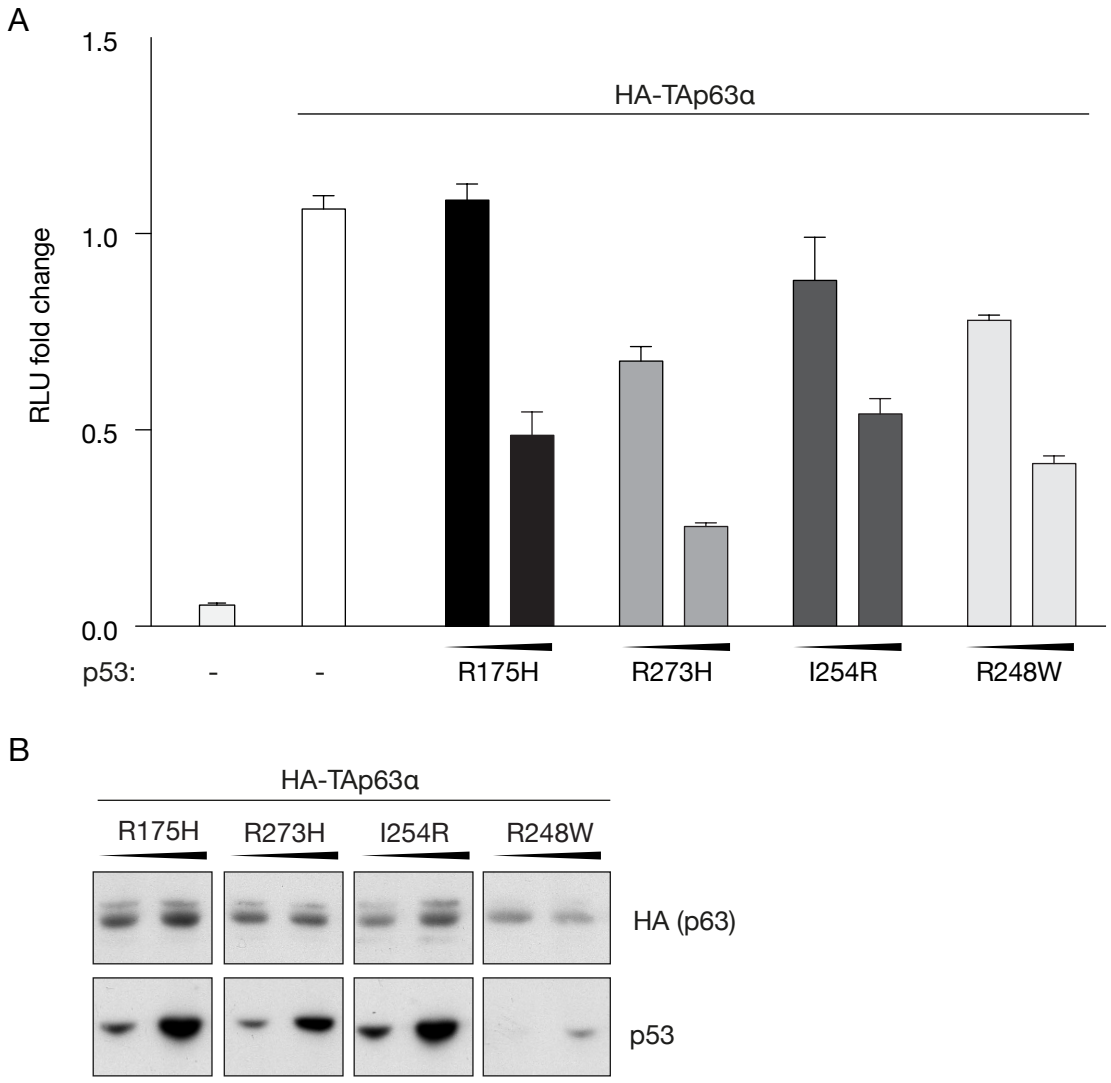

Figure 5-28: All p53 mutants reduce TAp63α-induced K14 Luciferase.
HCT116 p53$^{-/-}$ cells were transfected with K14 Luciferase, TK Renilla and either empty vector or TAp63α together with increasing amount (25 and 100ng) of the p53 mutants indicated. (A) Activation of the promoters was assayed using the Promega Luciferase system. Data is plotted as Relative Luciferase Units (RLU) (Firefly Luciferase readings divided by Renilla Luciferase readings) fold change relative to p53-null control. The diagram represents the mean of triplicates with error bars as standard error of the mean. (B) Transfection efficacy was determined by western blot.

Expression of TAp63α induced K14 Luciferase activity and co-expression of increasing amounts of mutant p53 inhibited Luciferase activation in all cases (Fig 5-28 A). If the strength of interaction were a predictor for strength of repression, the conformational mutants p53 R175H and p53 I254R would have been expected to be stronger repressors. However, the DNA contact mutants p53 R273H and R248W

repressed TAp63α at least as well, if not even better (Fig 5-28 A). In particular p53 R248W, which is expressed at lower levels in this experiment (Fig 5-28 B), still markedly inhibited the Luciferase activity (Fig 5-28 A). It would be informative to repeat this experiment on ΔNp63α and TAp73α induced Luciferase in order to investigate whether this mechanism is specific to TAp63α.

Our lab has previously shown that an important gain of function activity of mutant p53 is its stimulation of invasion towards EGF and HGF via the repression of TAp63[586,594]. Therefore, the ability of the conformational and contact mutants to drive invasion in an inverted transwell invasion assay towards the hepatocyte growth factor (HGF) was investigated.

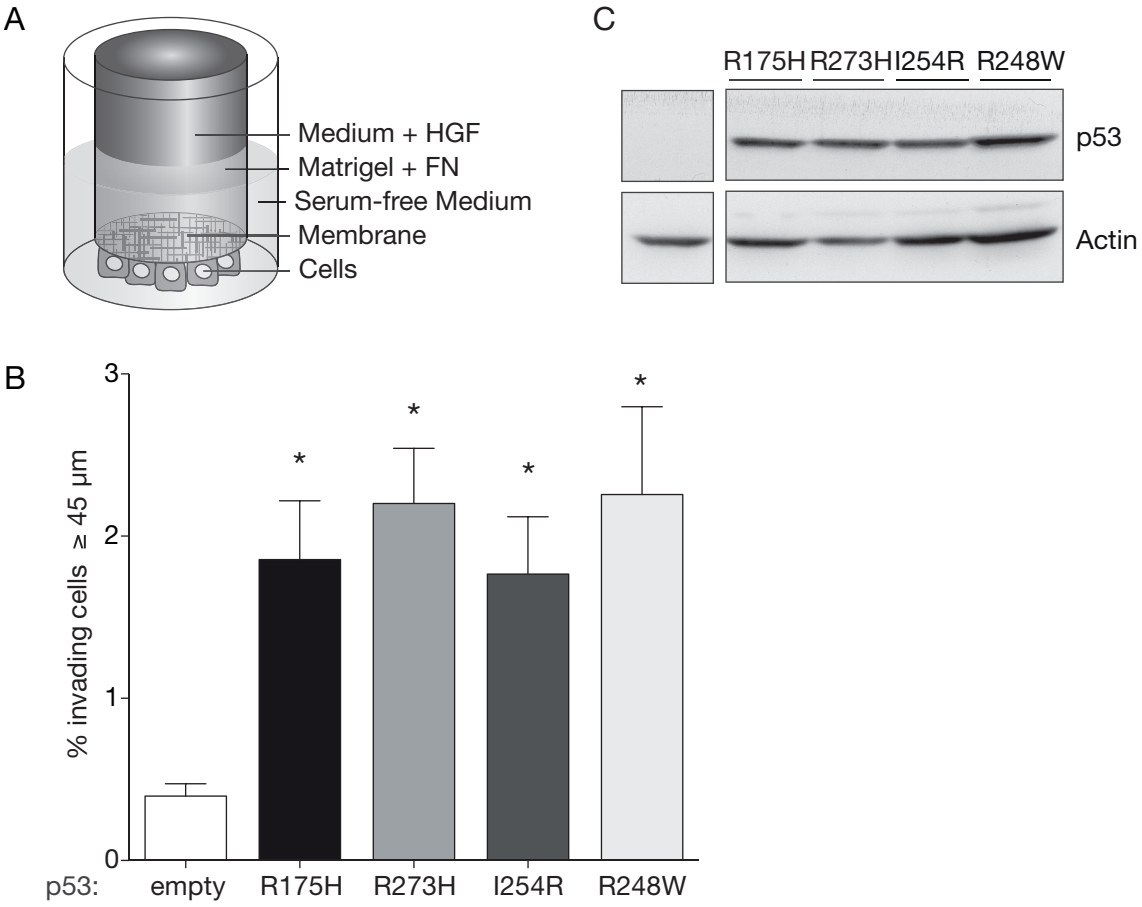

Figure 5-29: All p53 mutants induce invasion of H1299 cells towards HGF.
(A) Representation of an inverted transwell invasion assay setup. Cells are seeded on the membrane of the transwell insert, invading upwards into a matrigel-fibronectin (FN) plug towards HGF.
(B)+(C) H1299 were retrovirally infected with an empty vector or the p53 mutants (in a pWZL Blast vector). Blasticidin-resistant cells were selected and their invasion towards HGF over 72 hours measured in inverted transwell invasion assays. (B) Bars represent the mean of invasion beyond 45µm in 4 sets of triplicates. Error bars as standard error of the mean. * indicates a p-value <0.02 compared to the empty vector expressing cells. (C) Equal expression was confirmed by western blot.

In line with the observation that both contact and conformational mutants were able to inhibit TAp63-induced Luciferase, all p53 mutants tested significantly induced invasion of H1299 cells towards HGF (Fig 5-29). This finding raises the question, how the contact mutants inhibit p63 and drive the gain of function. While it is possible that a very weak interaction of mutant p53 with p63 and p73 is sufficient to achieve the gain of function effect, mechanisms other than protein-protein interaction with p63 and p73 are likely to also play a part. It could be informative to test the mutant p53 deletion mutants, which were identified to bind less well to p63 and p73 mutational analysis (p53 R175H Δ251-312), in order to investigate whether the reduced binding translates into a less effective repression of p63 and p73 and reduced invasion. However, in order to determine whether the mutant p53 interaction with p63 and p73 is required for mutant p53 to repress p63 and p73 and exert its gain of function, a p53 mutant without residual weak binding to p63 and p73 would be required.

5.6 Summary and Discussion

Taken together the results presented here show that, despite their high level of homology, p63 and p73 interact differently with mutant p53 and MDM2. Three critical amino acids phenylalanine 19, tryptophan 23 and leucine 26 in p53 were shown to interact with MDM2 (Fig 5-19)[163,563]. Since both TAp63α and TAp73α contain the same three amino acids, it is surprising that MDM2 only interacts with p63 but not with p73 (Fig 5-20). Three further amino acids, leucine 14, aspartate 21 and leucine 22, in p53 were shown to play a minor role in interacting with MDM2's N-terminus[163,563]. Aspartate 21, mutation of which to alanine has been thought to break the helical structure[163], is present in neither p63 nor p73. However, leucine 14 is conserved only in p63, but not p73 and leucine 22 only in p73, but not in p63 (see Fig 5-20). Although all three crucial amino acid residues are present in the N-termini of both TAp63 and TAp73, in vitro studies with N-terminal peptides of TAp63 and TAp73 suggest that MDM2 has a much lower affinity towards the transactivation domain of TAp63 compared to the transactivation domain of TAp73[523]. Furthermore, there clearly are differences between TAp63α and TAp73α from a structural point of view: The TAp63α protein was reported to adopt a closed inactive dimeric structure with a stretch of amino acids located within the transcriptional domain (amino acids 604 to 613) contacting the N-terminal transactivation domain[450]. This intra-molecular binding involves the conserved N-terminal three amino acids, which serve as contact points for MDM2 in p53[784]. Therefore, despite their presence in TAp63α, these critical amino acids might be shielded in this closed conformation, preventing MDM2 from interacting with TAp63α. Since the TAp63β and TAp63γ isoforms do not contain this inhibitory C-terminal domain, it would be very interesting to assess their binding to MDM2 as well. However, one study showed that TAp63γ could also not interact with MDM2[521], similar to TAp63α, making it unlikely that this closed folded structure is the reason why MDM2 cannot interact with TAp63α.

Furthermore, MDM2 also binds to ΔNp73α, which lacks the region homologous to the MDM2-binding domain in p53. Since an MDM2 mutant lacking the p53-binding domain can still interact with both TAp73 and ΔNp73 (Fig 5-20), another interaction outside the N-terminal regions of MDM2 and TAp73 must take place. The conserved box V in p53 can contact the acidic domain of MDM2 in a secondary

interaction[171,172,782,783]. This region of p53 is highly conserved in p63 and p73 and it is possible that MDM2 contacts p73 in a box V homologous domain.

Figure 5-30: Alignment of box V in p53, TAp63 and TAp73.
Amino acids in red are crucial for the interaction with MDM2, while amino acids in bold are thought to have a subtle influence. Accession numbers: p53: P04637, TAp63α: AAF43487, TAp73α: O15350.

Alignment of the box V regions of p53, TAp63 and TAp73 reveals that two amino acids that are crucial for interaction with p53 (serine 269 and phenylalanine 270)[172] are conserved in TAp73, while only one is present in TAp63 (Fig 5-30). Two of the four amino acids, which have been found to only weakly contribute to the interaction, are present in both TAp63 and TAp73, while the other two are not present in either protein (Fig 5-30). The lack of the crucial serine residue in TAp63 could serve as an explanation for the much weaker affinity of MDM2 to TAp63. It would therefore be informative to test the interaction of TAp73 lacking the box V or TAp63-TAp73 chimeras with swapped box V domains to study the influence of this region on MDM2 binding.

Importantly, the MDM2 inhibitor Nutlin-3a, which binds to MDM2's N-terminal p53 binding pocket, was shown to also induce cell cycle arrest and apoptosis in settings without wild-type p53. The suggested mechanism of action was that Nutlin-3a released TAp73 from MDM2's inhibition[642-645]. It could be informative to repeat the MDM2-TAp73 co-immunoprecipitation experiments in the presence of Nutlin-3a. However, since an MDM2 mutant lacking the N-terminal p53-binding domain Δ58-89 still bound TAp73 (Fig 5-21), Nutlin-3a would not be expected to disrupt the binding of MDM2 and TAp73 under the conditions used in this study. Since Nutlin-3a also disrupts the binding of MDM2 to E2F1, which is a transcriptional activator for TAp73[493], it is also possible that the observed TAp73 activation is at least partially due to E2F1-induced TAp73[640].

Strikingly, the much lower affinity of MDM2 towards TAp63 resulted in a very different effect on the mutant p53-p63 and mutant p53-p73 complexes. MDM2 separated the complex of conformational p53 mutants and TAp63 but induced formation of a trimeric complex of wild-type p53 or DNA contact mutant p53 with TAp73α or ΔNp73α (Fig 5-26). It would be important to assess the consequence of MDM2 binding mutant p53 and releasing TAp63α. Presumably, the free TAp63α would be transcriptionally active and thereby high levels of MDM2 in the presence of mutant p53-p63 complexes could rescue TAp63α activity. This hypothesis could be examined further experimentally, for example by determining TAp63α's transcriptional activity in presence of mutant p53 alone and mutant p53 with additional MDM2 in a Luciferase assay.

Understanding the mechanism through which MDM2 regulates p63 and p73 could prove very important in respect to tumour therapy, since both TAp63 and TAp73 can activate a number of p53's cell cycle arrest and apoptotic target genes[785] and contribute to the activation of apoptosis[582]. Furthermore, loss of p63 activity in particular has been associated with an enhanced invasive phenotype[469,586,595,596], leading to metastasis and poor prognosis for cancer patients[771,772]. While high expression of MDM2 is more common in tumours expressing wild-type p53 (since mutant p53 cannot induce the MDM2 target gene), treatment with an MDM2 E3-inhibitor would lead to accumulation of MDM2 protein, because MDM2 is also a Ubiquitin-ligase for itself. The MDM2 protein would then displace TAp63α bound to mutant p53 and release TAp63α from its inhibition. Reactivation of TAp63α could be beneficial to constrain tumour growth and metastasis. TAp73 would not be reactivated by this mechanism, since MDM2 forms a trimeric complex with mutant p53 and p73.

With the long-term therapeutic goal of interrupting the repressing interaction of mutant p53 with p63 and p73, it is important to understand how these proteins bind each other. TAp73α interacted with mutant p53 via its core domain, while TAp63α's interaction with mutant p53 still occured even without the DNA binding domain (Fig 5-13). The region identified to contribute to TAp63α's binding to p53 R175H comprises the sterile α motif in the C-terminus (Fig 5-16), which is only entirely present in the p63α isoforms (Fig 5-17). Despite its presence in only one of the

isoforms, the SAM domain is crucial to TAp63α's function: Multiple germ-line mutations associated with severe developmental effects cluster in this region, for example point-mutations at L518, G532, T537 and Q540 were identified in patients with AEC syndrome[456]. It would be of interest to test the other C-terminal splice-variants and assess their ability to interact with mutant p53. Based on the result that Δ462-561 TAp63α did not bind p53 R175H, TAp63γ would not be predicted to interact with p53 R175H, however one study showed a weak interaction of TAp63γ with p53 R175H[576]. This study identified the DNA binding domain as point of interaction, raising the possibility that two points of contact contribute to the mutant p53-p63 complex formation. TAp73α also seems to contact mutant p53 via two regions: While deletion of the entire DNA binding domain severely impaired the interaction, TAp73α deleted of either the N-terminal or the C-terminal half of the DNA binding domain strongly interacted with mutant p53 (Fig 5-12).

Since the level of mutant p53 interaction with p63 and p73 does not appear to correlate with their ability to repress p63 and p73 activity, a mutant p53, which was completely negative for interaction with p63 and p73 would be a very useful tool to determine whether interaction of the proteins is required for the inhibitory function. While deletion of the entire DNA binding domain abrogated all interaction (Fig 5-6), the remaining protein lacks more than half of all amino acids (216 out of the 393 amino acids). A shorter deletion or point mutation, resulting in a protein more closely resembling the full-length p53 protein, would be suited better to study its functional impact on p63 and p73 activity and gain of function, such as invasion.

The C-terminal region of the DNA binding domain (amino acids 251-312) was identified to be required for the interaction of mutant p53 with TAp63α (Fig 5-20) and it would be interesting to study whether the observed reduction in binding translates into an effect on p53 R175H's ability to repress TAp63α and drive invasion. The recently published aggregation region around isoleucine 254 falls into this region. Mutation of isoleucine 254 to arginine was reported to inhibit co-immunoprecipitation of p63 and p73 with p53 R175H by preventing their coaggregation[577]. Surprisingly, in my hands, introduction of the I254R mutation failed to stop interaction of p53 R175H with TAp63, ΔNp63 or TAp73 (Fig 5-8, 5-9, 5-10). Following discussion with the authors of the original paper, the experiments performed here were repeated with

exactly the same plasmids, buffers and cells as in the original study and the discrepancies are therefore unexplainable. Importantly, mutation of isoleucine 254 in the wild-type p53 background completely unfolded the p53 DNA binding domain (Fig 5-3). Somatic mutation of codon 254 is documented in the p53 database at a rate of 0.2% compared to 6.1% for the very frequent mutation of codon 175[524], suggesting that mutation of the I254 codon in the p53 protein occurs in tumours and thus possibly confers some oncogenic activity. This would be consistent with the classification of the p53 I254R mutant as an unfolded mutant (Fig 5-3), which inhibits TAp63α 's transcriptional activity (Fig 5-28) and promotes invasion (Fig 5-29).

Despite the fact that the level of co-immunoprecipitation of p63 and p73 with mutant p53 does not directly correlate with repression of p63 and p73 (Fig 5-28, 5-29), targeting the mutant p53-p73 interaction with small molecule inhibitors may be a useful strategy to reduce tumour growth. A study with small interfering peptides of the p73 DNA binding domain (between amino acids 190 and 210) rendered mutant p53 cells more susceptible to Adriamycin and Cisplatin treatment in SK-BR-3 cells expressing p53 R175H[786]. However, no such effect could be observed in MDA-MB-468 cells which express the p53 R273H mutant[786], suggesting that in this cell-line the contact mutant p53 exhibited its oncogenic gain of function by a mechanism distinct from p73 binding.

By contrast, the small molecule RETRA has recently been shown to reduce the number and size of tumours in a xenograft model of the p53 R273H-expressing cell line A431[662]. Although the p53 R273H DNA contact mutant does not bind strongly to p73, treatment of RETRA was shown to reduce the interaction by two to three-fold while inducing the p73 levels[662]. The release of mutant p53-mediated inhibition was measured by the activation of p53 target genes and only treatment with p73-specific shRNA, but not p63-specific shRNA prevented the induction of p53 target genes[662]. The conclusion drawn from this finding was that RETRA functioned exclusively by releasing p73 from its complex with mutant p53. Considering the different interaction mechanisms of p63 and p73 with mutant p53, it is conceivable that RETRA targets only the mutant p53-p73 interaction. However, it is important to note that the A431 cell line expresses extremely high amounts of ΔNp63α and hardly any TAp63. Since the TA-isoforms of p63 are thought to be the stronger inducers of the classic p53

target genes[445], it is not surprising that the observed induction of p53 target genes in this cell line was predominantly mediated by p73. It would therefore be interesting to investigate in a different cell line, whether RETRA also reactivates TAp63. Furthermore, it would be important to study whether the observed effect can be reproduced or even augmented in cell lines expressing a conformational mutant of p53.

6 Final Summary and Discussion

In brief, the following main conclusions can be drawn from the data presented here: MDM2 drives conjugation of the tumour suppressor p53 with SUMO-2/3 at lysine 386, independently of its ability to promote ubiquitination. SUMO-2/3 modification modulates both transcriptional activation and repression by p53. While mutant p53 can also be SUMOylated, this does not affect its interaction with p63 and p73. MDM2 on the other hand alters mutant p53's interaction with p63 and p73, displacing p63 in the complex with mutant p53 and forming a trimeric complex with mutant p53 and p73.

Although MDM2's activity in ubiquitinating p53 is undoubtedly crucial to control p53's activity, many more ubiquitination-independent roles for MDM2 in controlling the p53-family have been uncovered in this study.

Interestingly, conditions that inhibit ubiquitination, such as deletion of the RING domain and the central acidic domain (Figure 3-6), but also expression of the ubiquitination-inhibitory proteins $p14^{ARF}$ and L11, did not dampen but rather enhanced MDM2's ability to SUMOylate p53 (Figure 3-7). This suggests that ubiquitination and SUMOylation may be two competing activities of MDM2. However, the levels of SUMOylation- and ubiquitination activity do not match a perfect inverse correlation, since the MDM2 ΔAD protein retained some residual ubiquitination activity, but promoted SUMOylation more strongly than the MDM2 RING mutants, which had lost all ubiquitination activity (Figure 3-7). Nevertheless, overall conditions that block the ubiquitination activity induced MDM2's SUMOylation activity and the particularly efficient SUMOylation induced by the MDM2 ΔAD mutant could be due to its strong interaction with the SUMO-E2 Ubc9 (Figure 3-24).

Although the particular MDM2 deletion mutants used in this study were intended purely as molecular tools, MDM2 isoforms deleted for the N-terminal, central and C-terminal domains also occur in cells. The full-length MDM2 transcript encodes for a 90 kD protein, but shorter isoforms of around 75 kD and 60 kD were also observed[787]. The 60 kD MDM2 isoform does not arise from alternative splicing or translation initiation and was shown by a number of groups to be the product of cleavage by caspase-3 related proteases[788-791] or caspase-2 in complex with the p53 target PIDD[792]. The p60 MDM2 protein lacks the entire RING domain, since cleavage

takes place after aspartate 361 within the Caspase cleavage motif DVPD. Therefore, this protein does not ubiquitinate p53 and was found to stabilise p53 by preventing its interaction with full-length MDM2[792]. However, MDM2 deletion mutants lacking the RING domain were able to promote SUMOylation of p53 efficiently and thus p60 MDM2 could influence p53's transcriptional activity by SUMO-2/3 modification. While MDM2 cleavage is induced during apoptosis as Caspases become activated, the p60 product is also present without apoptosis[791] and has been observed in breast[793] and lung tumours[794]. Notably, the HCT116 colon carcinoma cell line used throughout this study expresses multiple MDM2 isoforms, with a band at 60 kD being the most prominent band on western blots, suggesting that this cell line might express the cleaved MDM2 isoform. The 75 kD MDM2 isoforms arises from alternative translation initiates at internal ATG codons 61 or 101[795]. The resulting proteins lack the N-termini and are unable to bind p53 and would neither ubiquitinate nor SUMOylate p53.

Many more MDM2 splice variants have been reported, some of which were exclusively found in tumour cells. Strikingly, the majority of variants retain the N-terminal p53-binding site, but show large deletions in the central region of the protein, including the acidic domain and zinc finger[796,797], similar to the strongly SUMOylating MDM2 ΔAD deletion mutant, which lacks amino acids 222 to 437. It would be very interesting to investigate the SUMOylation potential of these natural MDM2 splice variants. While most of them will have lost their ubiquitination activity, the isoforms that retain the p53-binding motif will probably be able to promote SUMOylation of p53, possibly to higher levels than full-length MDM2.

SUMO-2/3 modification of p53 modulates its transcriptional activity and can activate, repress or alleviate repression depending on the particular target gene analysed (Fig 4-11). p53 mutants that could not be SUMOylated were more efficient inducers of a PG13-Luciferase, but weaker inducers of a Bax-Luciferase construct compared to wild-type p53 (Fig 4-9). While the p53-target gene p21 was also induced to higher levels on mRNA (Fig 4-11) and protein level (Fig 4-13) by the SUMO consensus mutants p53 K386R and p53 E388A, no significant differences of Bax mRNA regulation could be observed (Fig 4-11). However, cell cycle profiling of cells by FACS suggested that cells expressing p53 E388A and K386R underwent less apoptosis, since the sub-G_1 population was slightly reduced in these cells (Fig 4-14).

Furthermore, cell cycle genes repressed by p53, were repressed to an even greater extent by p53, which could not be SUMOylated (Fig 4-11). Overall, the data suggest that SUMO-2/3 modification channels p53 activity towards apoptosis rather than G_1 or G_2 arrest.

In light of the attractive therapeutic option to inhibit p53-ubiquitination by MDM2, effects of MDM2 inhibitors on SUMOylation need to be taken into consideration. For example, the MDM2-inhibitor Nutlin-3a disrupts the binding of MDM2 and p53[604] and thereby inhibits not only ubiquitination, but also SUMOylation of p53. By contrast, E3-ligase inhibitors such as HLI373 only affect ubiquitination of p53[641], but do not disrupt SUMOylation of p53 (Figure 3-8). It is still unclear, whether p53 SUMOylation would pose a general advantage or disadvantage to tumour growth. The data presented here suggest that disruption of p53 SUMOylation could induce cell cycle arrest at G_1 via induction of p21 or G_2 via stronger repression of G_2/M genes. Despite no significant difference of G_1 phase population in the FACS analysis of cells expressing SUMO-site mutant p53 compared to wild-type p53, it is possible that a slight alteration of p21 levels could translate into a cell cycle arrest in vivo, where tumour cells are subject to many other stresses simultaneously. Furthermore, since a slight reduction of sub-G_1 population for cells expressing the SUMO-site mutant p53 K386R or E388A was observed, disrupting SUMOylation could also be detrimental to therapy outcome by lowering the apoptotic potential. The functional consequences of SUMO-modification might depend on other posttranslational modifications present. It was recently suggested that the SUMO-E3 PIAS4 induces SUMO-modification of p53 and Tip60 simultaneously, thereby activating Tip60, which in turn acetylates p53 at lysine 120. This particular combination of posttranslational modifications, K120 acetylation and K386 SUMOylation served as a binary death signal, inducing cytoplasmic accumulation of p53 and triggering autophagy[420]. It could be very informative to study the role of p53 SUMOylation in tumourigenesis and cancer treatment in vivo and it would be important to consider the lack of SUMO consensus in the mouse p53 protein when choosing an animal model. Even a knock-in mouse with human p53 with the K386R mutation would potentially not recapitulate the situation in human cells, since mouse cells obviously do not require their p53 protein to be SUMOylated.

Interestingly, the SUMO pathway is often deregulated in cancer and many tumours express high levels of Ubc9. Consequently, p53 might also be highly SUMOylated in tumours with overexpression of the SUMO conjugating enzyme. Overall, high levels of SUMOylation confer an advantage to tumours. Indeed, in the majority of studies high expression of the SUMO-E2 Ubc9 was correlated with poor prognosis[374,798-800]. Ubc9 promotes cell growth and prevents apoptosis in breast cancer cells, possibly by regulating Bcl-2 since MCF7 cells expressing a dominant negative Ubc9 showed reduced mRNA levels of the anti-apoptotic Bcl-2 mRNA[374]. Furthermore, Ubc9 overexpression confers chemoresistance in breast cancer[376]. Although targeting the SUMO-conjugating function of Ubc9 has been suggested as a promising strategy for cancer therapy[801], some functions of the E2 conjugating enzyme seem to be independent of SUMOylation such as its promotion of breast cancer cell invasion, which is also induced by the dominant negative Ubc9 C93A, possibly via down-regulating miR-224[802].

Other members of the SUMO-pathway are also involved in tumourigenesis: It has recently been reported that Myc-driven tumourigenesis depends on the presence of SUMO-E1 SAE1/2[803] and Myc itself has been shown to be a transcriptional activator of SAE1[804]. Contrasting to the situation in breast cancer, melanoma, multiple myeloma and colon cancer, where high levels of SUMOylation were observed, deSUMOylation (indicated by high SENP1 expression) correlates with tumour aggressiveness and recurrence in prostate cancer[805]. Indeed, mouse models showed that SENP1 overexpression in the prostate induced development of neoplasias[806]. In cervical cancer the human papillomavirus E6 protein was reported to target the SUMO-E2 Ubc9 for degradation, leading to a global drop in SUMO levels as part of the malignant transformation[807]. These data raise the possibility that the status of p53 SUMOylation could also be different depending on tumour type and it would be informative to study SUMO-2/3 modification of p53 on tissue microarray. This would be greatly aided by the development of a SUMOylated p53-specific antibody raised against a p53-SUMO branched peptide, similarly to an antibody successfully raised against ubiquitinated Histone H2B[808].

Despite high levels of SUMOylation conferring resistance to cytotoxic therapy, SUMOylation of certain proteins was shown to be crucial for the response of some

drugs. For example, treatment of acute promyeloytic leukaemia patients with arsenic relies on the PML-RARα fusion protein to become SUMOylated and form nuclear bodies in order to then recruit the SUMO-targeted Ubiquitin ligase RNF4, which targets the PML protein for degradation[366,675]. SUMO-1 modification of Topoisomerase I was found to be key to the success of Irinotecan treatment in non-small lung cancer patients, where therapy response was associated with SUMO-1 and Ubc9 expression[809]. It would be very interesting to investigate whether the SUMO status influenced therapy response, particularly since SUMO-2/3 modified p53 seemed to activate apoptosis more readily than cell cycle arrest.

The C-terminus of mutant p53, which contains the C-terminal SUMO site, had been shown to be important for invasion via suppression of p63[586], hence SUMOylation of mutant p53 was also studied. While mutant p53 was readily SUMOylated independent of conformation (Fig 5-1), deletion of the area comprising the SUMO site did not impair mutant p53 interaction with TAp63α and mutation of the SUMO site in p53 R273H also did not affect its ability to promote invasion (experiment performed by P. Muller, data not shown). Thus, SUMOylation does not obviously influence mutant p53 gain of function via p63. Strikingly, SUMO-2/3 modified TAp63α, TAp73α and ΔNp73α could be observed in the presence of MDM2 ΔAD (Fig 5-18). While TAp73α, ΔNp73α and TAp63α had not previously been shown to be modified with SUMO-2/3, a recent study identified $p14^{ARF}$ as inducer of SUMO-2-modification of ΔNp63α, resulting in degradation of ΔNp63α[780]. SUMO-1 modification of TAp63α and TAp73α similarly destabilised these proteins[450,780,781], suggesting that SUMOylation of p63 and p73 could lead to the recruitment of SUMO-targeted Ubiquitin ligases. Further experiments could be conducted to assess whether the SUMO-2/3 modification of p63 and p73 is indeed induced by MDM2 and whether protein interaction with MDM2 is required for this activity. Importantly, a larger fraction of TAp73α and ΔNp73α was SUMO-2/3 modified compared to TAp63α (although it was expressed to slightly lower levels) and this could be due to MDM2 interacting much better with p73 than p63 (Fig 5-19).

Since MDM2 interacts differently with p73 and p63, MDM2 also influences the mutant p53-p63 differently than the mutant p53-p73 complex. While MDM2 induced interaction of wild-type p53 and DNA contact mutant p53 with TAp73α (Fig 5-25),

possibly by formation of a trimeric complex, it disrupted the interaction of conformational p53 with TAp63α (Fig 5-24). This observation is intriguing, since MDM2 is thought to bind the N-terminus of p53, while TAp63 interacts with the core domain of mutant p53. However, our lab recently published evidence that MDM2 interacts differently with mutant p53 than with wild-type p53, since p53 R175H lacking the MDM2-binding region (p53 R175H ΔI) can still bind MDM2, probably via its RING domain[544]. It is unclear which region in mutant p53 serves as point of contact for the RING domain and it is possible that a region of the mutant p53 core domain that is required for binding to p63, binds to MDM2 with higher affinity, leading to the displacement of p63 in the presence of MDM2. It would be important to follow up these findings and investigate whether the release of TAp63α from the complex coincides with a release of functional inhibition by mutant p53, which could have implications on the treatment of tumours expressing mutant p53.

Similar to the differences of p63 and p73 in binding to MDM2, the interactions of the p53-family transcription factors with mutant p53 were also not homologous. While TAp73α bound mutant p53 via at least two regions in the DNA binding domain (Fig 5-12), the core domains of TAp63α and ΔNp63α were dispensable for mutant p53 binding (Fig 5-13). Instead, deletion of a C-terminal region of TAp63α (Δ462-561), which contains the SAM domain, severely impaired the interaction with mutant p53 (Fig 5-14). Many protein interactions take place in this region and an extended PPxY motif located between amino acids 501 and 510 in the SAM domain was recently shown to be required for recognition of p63α by its E3-ligase Itch[810]. This motif is conserved in p73, which is also recognised by Itch[484], possibly via the same region. The SAM domains of p63 and p73 are fairly similar with 53% identical amino acids and it is intriguing that only p63's SAM domain was identified to be important for interaction with mutant p53 (Figure 5-14). However, the influence of deletion of the SAM domain in p73 was not assessed, because a TAp73α mutant containing a stop codon at codon 500 did not express. Since TAp73α Δ131-307, which lacks the DNA binding domain, showed some residual interaction with mutant p53, it is possible that another interaction takes place at the TAp73α SAM domain, similar to TAp63α. However, in contrast to p73, deletion of the DNA binding domain in p63 did not weaken the interaction with mutant p53, clearly demonstrating that the p63 core domain is not required for mutant p53-p63 complex formation.

Strikingly, while both p63α and p73α contain a C-terminal transactivation inhibitory domain, they seem to function by different mechanisms. Only the p63 inhibitory domain folds back to bind and shield the N-terminal transactivation domain[449], while the p73α inhibitory domain does not contact its N-terminus[481]. It is possible that due to this closed conformation of TAp63α, the DNA-binding domain is not accessible to bind to mutant p53 and the interaction therefore has to take part via a different region, exposed in the structure. However, since ΔNp63α, which cannot form this closed conformation, also does not require the DNA-binding domain for binding mutant p53, the unusual conformation of TAp63α is unlikely to be the reason that the core domain of p63 does not to bind mutant p53. Strikingly, p63 and p73 contain 86% identical amino acids in their DNA binding domain and no areas of clear differences between the two sequences could be identified in the sequence alignment. The DNA binding domains of p63 and p73 are also highly homologous to p53, with roughly 60% identical amino acids, but both p63 and p73 contain a divergent L2 loop, the result of an insertion of two extra amino acids, lacking in p53[811]. As a consequence of this slightly different structural arrangement, p63 and p73 are more thermodynamically stable than p53[812] and are resistant to inhibition by SV40 large T[811]. While p63 and p73 retain their ability to bind ASPP and iASPP, one report suggests that the iASPP and ASPP2 C-terminal domains, which bind to the p63 and p73 core domains, bind three-fold more strongly to p63 over p73[813]. Thus, preferences of proteins to either interact with the p63 or p73 DNA binding domain clearly exist and it is conceivable that mutant p53 to the p73 core domain with higher affinity than to the p63 core domain.

Regardless of the exact interaction mechanism, mutant p53 was reported by many groups to inhibit the transcriptional activity of both p63 and p73[134,575,576,579]. Surprisingly, the DNA contact p53 mutants, which only bound weakly to both p63 and p73, were able to suppress TAp63α -induced K14 Luciferase at least as well as the strongly interacting conformational p53 mutants (Fig 5-28). It would be interesting to repeat this experiment on TAp73-induced Luciferase in order to determine whether the same effect can be observed with the inhibition of p73-activated target genes, since one previous report demonstrated that p53 R273H did not inhibit TAp73-mediated activation of a Luciferase-reporter[134].

The finding that strength of interaction does not correlate with the extent of repression of p63 raises questions about the exact mechanism transcriptional repression by mutant p53. Clearly, DNA contact mutants are among the most frequently observed p53 mutations and many studies have shown evidence for gain of oncogenic function by mutants such as p53 R273H. It is possible that the weak interaction observed with the DNA contact mutants and p63 is sufficient to disrupt p63's transcriptional function. Mutations of many DNA-contacting residues such as R248 or R280 can indeed marginally impair the folding of the DNA binding domain[774]. Strikingly, even the DNA binding domain of the wild-type p53 protein is thermodynamically unstable and the wild-type protein can adopt an unfolded conformation, for example under hypoxic conditions[814]. While unfolded wild-type p53 has been demonstrated to bind and inhibit p73 in vitro[581], in endogenous settings, wild-type p53 levels are probably too low to impact on p63 and p73 activity under unstressed conditions. By contrast, mutant p53 often accumulates to high levels in neoplastic lesions and thus even if only a small percentage of the contact mutants were to be unfolded, this fraction could be sufficient to bind and repress p63 and p73. It is possible that the interaction with the DNA stabilises the conformation of the wild-type p53 DNA binding domain, but since the DNA contact mutants have lost this ability, even the DNA binding domain of p53 R273H, which was not found to be thermodynamically destabilised in vitro[653], could be less stable in a cellular context. Furthermore, the ratio of unfolded to folded p53 protein is affected by many factors: The conformation of the DNA binding domain can be stabilised by posttranslational modifications and binding partners such as heat shock proteins[815,816]. Other proteins destabilise p53 conformation and binding to MDM2 was reported to induce unfolding of p53[817,818].

Three recent reports suggest that additional proteins might be involved in stabilising the mutant p53-p63 complex: Upon TGFβ signalling Smads were shown to act as a scaffold for mutant p53-p63 interaction[595]. Furthermore, the scaffold protein Topoisomerase βII binding protein (TopBP1) was shown to facilitate complex formation of mutant p53 with p63 and p73 and in the absence of TopBP1, p53 R273C was unable to form a complex with p63[819]. Similarly, the Prolyl-isomerase Pin1 alters the conformation of proline residues in p53 and thereby promotes binding of p53 R280 and p63[668]. It might be informative to test, whether more p63 would be

co-immunoprecipitated with p53 R273H in the presence of Pin1, TopBP1 or Smad2 under the conditions used in this study and whether strengthening of the interaction would translate into stronger p63 repression.

Mutant p53 expression is often associated with more aggressive and invasive tumours. The p53 mutants used throughout this study were found to induce invasion in an inverted transwell invasion assay and this effect was independent of the class of p53 mutation (Fig 5-29). A number of different mechanisms were reported to contribute to mutant p53's gain of oncogenic function, some of them limited to certain types of p53 mutation: Both p53 R175H and p53 R273H were shown to enhance Rab-coupling protein-mediated recycling of the EGF-receptor and integrin α5β1, inducing invasion towards EGF and this effect was mediated by repression of p63[586]. Similarly, different kinds of mutant p53 (p53 R175H, p53 R273H, p53 R280K) induced invasion upon TGFβ signalling via repression of p63, mediated by Smads[595]. Recently, p53 R248Q and p53 R282W were shown to alleviate repression of p63's induction of miR-155 expression, driving invasion and metastasis of breast tumours[820]. p53 R175H also prevented induction of miR-205 by p63 in prostate cancer[596]. miR-155 and miR-205 both target ZEB-1, which regulates epithelial-to-mesenchymal transition. Furthermore, p53 R175H and p53 R273H were shown to induce scattering and invasion in response to HGF-signalling, mediated by Met recycling[594]. While this effect was p63-dependent in cell lines expressing TAp63, it was p63-independent in other cell lines[594] and it would be interesting to investigate, whether the mechanism could be instead mediated by p73 repression. Repression of p63-target genes had been proposed to be a consequence of mutant p53 preventing p63 to bind to the promoters of its target genes[819], nonetheless mutant p53 was recently shown to recruit p63 to promoters of genes usually not regulated by p63[821]. Strikingly, an expression microarray comparing the transcriptional changes induced by 5 different hotspot mutants including p53 R175H, p53 R273H and p53 R248W demonstrated that all mutants, no matter whether conformational or contact, induced a very similar change in target mRNA levels, regulating the same 59 genes[822]. Induction of these genes was mediated by a mutant p53-p63 complex with p63 binding to promoters, which are not induced by p63 alone[822]. These studies suggest that the low binding of p53 R273H is sufficient to recruit p63 to new promoters.

A number of p53 mutants, including p53 R273H were recently shown to disrupt the structured tissue organisation of breast epithelial cells by upregulating the mevalonate pathway[597] and to induce CXCL5 and CXCL8, leading to migration[823]. Induction of the mevalonate pathway required the SREBP transcription factors[597] and expression of the chemokines was dependent on NF-κB2[823]. Analysis of the role of p63 in these pathways would be very informative. Additional mechanisms, which were shown to be independent of p63, also contribute to mutant p53-induced invasion: Mutant p53 was reported to induce epithelial to mesenchymal transition by inhibiting transcription of miR-130b, which is a negative regulator of ZEB-1[824]. Strikingly, although p63 had been shown to activate miR-130b[770], the authors found that the repression of miR-130b by mutant p53 was independent of p63 and both p53 R175H and p53 R273H bound directly to the miR-130b promoter[824]. Furthermore, mutant p53 could induce the expression of proteins such as Twist1, promoting epithelial-mesenchymal transformation, and this was not mediated by p63[825]. While p53 mutants can retain some DNA-binding activity, their sequence-specific selectivity is largely lost and even the residual binding of p53 R273H was shown to be too weak to activate p53 target genes[826]. Instead of binding in a sequence-dependent manner, mutant p53 was reported to bind DNA depending on structural elements, showing a preference for G-rich regions, which form G-quadruplex structures[565] and AT-rich regions, which lead to regional unwinding of DNA[564]. Mutant p53 also interacts with other transcription factors (such as p63 as described above), which take over the role of contacting the DNA. Interestingly, a recent study found that different mutant p53 proteins induced a cancer-related gene signature by distinct mechanisms: While conformational mutants such as p53 R175H were shown to induce H-Ras signalling, DNA contact mutants such as R273H and R248Q did not affect Ras signalling, but cooperated with NF-κB instead[827]. Some of the gain of function, however is mediated completely independent of transcription factors and instead involves complex formation with other proteins: The p53 R273H mutant, but not p53 R280K, was shown to interact with Nardisylin and thereby induce invasion towards hepatocyte growth factor, independent of p63[598], suggesting that particular p53 mutants can utilise separate mechanisms - potentially completely independent of transcriptional regulation - to complement their gain of function.

While many different mechanisms contribute to mutant p53's ability to promote invasion and metastasis, a large fraction of studies found that the invasive phenotype was mediated by p63. On the one hand, weak mutant p53-p63 binding seems to be sufficient for mutant p53 to repress p63-induced target genes and also to recruit p63 to new target genes, on the other hand, reduction of mutant p53-p63 complex formation was recently shown to revert invasive behaviour induced by mutant p53: The Ankyrin repeat domain protein 11 (ANKRD11) was found to restore DNA-binding of p53 R273H[828] and furthermore to refold conformational p53 mutants, leading to 50% reduction of p53 R175H complex formation with p63 and p73, which was sufficient to revert the mesenchymal phenotype induced by mutant p53[829].

Taken together, mutant p53 can drive an aggressive and metastatic phenotype by different mechanisms, many of which involve repression of the p53-family transcription factor p63. p73 is less studied in this context, however, the finding that similarly to $p53^{+/-}$;$p63^{+/-}$ mice, half of all $p53^{+/-}$;$p73^{+/-}$ mice developed metastatic tumours[468], while hardly any of the $p53^{+/-}$ mice did, suggests an important role for p73 in counteracting invasion and metastasis as well. Importantly, p73 has been linked to repressing anchorage independent growth by inducing anoikis[760], which could pose an effective barrier to the development of metastasis.

The findings presented here suggest that mutant p53 interacts differently with p63 and p73. Clearly, understanding the inhibitory interaction with p63 and p73 better, will aid the development of therapies targeting mutant p53's gain of function via p63 and p73. While conformation of p53 mutants interacted much more strongly, DNA contact mutants were still able to repress p63 and p73. Our data together with results from studies conducted by other groups suggest that the weak interaction of the DNA contact mutants is sufficient to repress p63 and promote an invasive phenotype. Importantly, it was identified here that MDM2 releases p63 from its interaction with mutant p53, which could serve as a mechanism to reactivate p63. Exemplified by the small molecule RETRA, which disrupts the mutant p53-p73 complex and is thereby able to suppress tumour growth[662], targeting mutant p53's interaction with p63 and p73 seems to be a promising therapy strategy for treating the 50% of all tumours that contain mutant p53.

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