Contribution of the DNA binding domain of p53 to regulation of its stability

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Abstract.

Tumour suppressor p53 is frequently mutated in cancers. While wild type p53 is normally a rapidly degraded protein, mutant forms of p53 are stabilised and accumulate to high levels in tumour cells. Several studies have shown that mutant p53 acquires oncogenic properties and actively contributes to tumourigenesis. It is therefore important to understand how the stability of mutant p53 is regulated. This thesis shows that mutant and wild type p53 are ubiquitinated and degraded through overlapping but distinct pathways. While Mdm2 can drive the degradation of both mutant and wild type p53, this study suggests that the ability of Mdm2 to function as a ubiquitin ligase is less important in the degradation of mutant p53, which is heavily ubiquitinated in an Mdm2-independent manner. The contribution of Mdm2 to the degradation of mutant p53 may reflect an ability of Mdm2 to deliver the ubiquitinated mutant p53 to the proteasome. Ubiquitination does not efficiently target mutant p53 for the proteasomal degradation, however ubiquitinated p53 mutants localize to the cytoplasm. This thesis suggests the role for the chaperone-associated ubiquitin ligase CHIP in ubiquitination of mutant p53, although other unidentified ubiquitin ligases appear to contribute. Interaction of mutant p53 with its family member p73 decreases ubiquitination, suggesting p73 can play a role in regulation of stability of mutant p53.

Acknowledgements.

Abbreviations.

AA	*Amino acids*
APS	*ammonium persulfate*
ATCC	*American Type Culture Collection*
ATP	*Adenosine 5'-triphosphate*
BSA	*Bovine serum albumin*
Cdk	*cyclin dependent kinase*
cDNA	*DNA complementary to mRNA*
CHIP	Carboxyl terminus of Hsc70-interacting protein
DAPI	*4',6-Diamidino-2-phenylindole*
DBD	*DNA binding domain*
DDW	*Double distilled water*
DMEM	*Dulbecco's Modified Eagles Medium*
DMSO	*Dimethyl sulphoxide*
DNA	*Deoxyribonucleic acid*
DTT	*Dithiothreitol*
ECL	*Enhanced chemiluminescence*
EDTA	*Ethylene diamine triacetic acid*
EtBr	*Ethidium bromide*
FCS	*Foetal calf serum*
GAPDH	*Glyceraldehyde-3-Phosphate Dehydrogenase*
h	*hours*
HEPES	*4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid*
HRP	*Horseradish peroxidase*
IARC	*International Agency for Research on Cancer*
kDa	*kilodalton*
LB	*Luria-Bertani medium*
min	*minutes*
mRNA	*messenger RNA*
NLS	*Nuclear localisation signal*
NES	*Nuclear export signal*
NP-40	*Nonidet P40*
OD	*Oligomerization domain*

PBS	*Phosphate buffered saline*
PCR	*Polymerase chain reaction*
PD	*Proline rich domain*
PFA	*Paraformaldehyde*
PMSF	*phenylmethylsulphonyl fluoride*
RD	*Regulatory domain*
RNA	*Ribonucleic acid*
RNase	*Ribonuclease*
RPE	*Retinal pigment epithelia*
Rpm	*Revolutions per minute*
rRNA	*Ribosomal RNA*
RT-PCR	*Reverse transcription polymerase chain reaction*
SDS	*Sodium dodecyl sulphate*
siRNA	*Short interfering RNA*
T	*Thymine*
TA	*Transactivation domain*
TBS	*Tris buffered saline*
TBST	*TBS-Tween*
TE	*Tris-EDTA buffer*
TEMED	*N,N,N',N'-Tetramethylethylenediamine*

Table of Contents.

List of figures.

List of tables.

Accompanying material.

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

Lukashchuk N, Vousden KH. Ubiquitination and degradation of mutant p53. Mol Cell Biol. 2007 Dec;27(23):8284-95.

Declaration

I declare that all of the work in this thesis was performed personally. No part of this work has been submitted for consideration as part of any other degree or award.

Chapter 1. Introduction.

1 Introduction.

1.1 Cancer.

Cancer is a complex disease evolving as a result of a multi-step deregulation of normal processes controlling cell growth and proliferation. Different cellular stresses, such as DNA damage and oncogene activation, can lead to accumulation of mutations in cells, which can deregulate crucial cell regulatory pathways. The current model suggests that several genetic alterations conferring growth advantage to the cell are necessary for oncogenic transformation (1, 2). These hallmarks of cancer have been originally described as self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis and limitless replication potential, sustained angiogenesis and tissue invasion (3). Most of these changes occur as cell-autonomous events or can target cell microenvironment. Later the avoidance of immunosurveillance has been proposed as a seventh hallmark of cancer, representing a more complex cell-extrinsic view of cancer development (4). The current update of this model also describes the alteration in tumour metabolism as an important cancer-associated feature (5), which is linked to most of the mentioned molecular changes and becomes the eighth hallmark of cancer.

Most of the genes altered in cancer have been classified as oncogenes and tumour suppressors (6). Under normal conditions oncogenes stimulate cell growth, subject to a tight regulatory mechanism. Oncogenes are mutated in a way that makes the gene consitutively active under the conditions when the wild type gene is inactive. This can result from chromosomal translocations, gene amplifications or from single amino acid substitutions affecting residues crucial for the activity of the protein. An activating mutation in an oncogene confers growth advantage on the cell. For example, Ras proteins are a family of small GTPases, and are frequently mutated in cancer. The GTPase activity shuttles the protein from active to inactive state. Oncogenic mutation in Ras (for example Gln61Leu in H-Ras) leads to structural alteration of the protein, which impairs the GTPase activity, "locking" Ras in active state. Activated Ras leads to aberrant growth via activation of MAPK pathway (7). Ras oncogenes play a key role in malignant transformation of primary cells (8-10). Other examples of oncogenes include B-Raf, c-Myc, EGFR, met, and many others (11). In contrast to oncogenes, genetic alterations in tumour-suppressor genes lead to inactivation of their function. Such alterations usually represent missense mutations at residues essential for the activity, mutations leading to a truncated protein, deletions or insertions or epigenetic silencing of the gene. Though inactivation of only one allele of some of the tumour suppressor genes can be enough to

confer growth advantage upon the cells, in most other cases mutations in both alleles are required to confer growth advantage (6). The loss of heterozygosity commonly occurs through the deletion of the second allele via a large chromosomal event, such as loss of chromosomal arm (12). The best characterized examples of tumour-suppressor genes are p53, Rb and APC, which are often inactivated in cancers (13, 14). One of the examples is the frequently inactivated in retinoblastomas Rb (retinoblastoma) tumour suppressor, which encodes the Rb protein, which blocks proliferation by sequestering E2F (15). Another tumour suppressor frequently inactivated in cancers is p16Ink4A, which inhibits cyclin dependent kinases (16). The subgroup of the tumor suppressor genes important in cancer is defined by the stability genes. This group involves genes whose products are involved in DNA repair during normal processes, such as replication, mitotic recombination and chromosomal segregation, or upon exposure to mutagens. These genes are exemplified by BRCA1, ATM, BLM and many others. Inactivation of the stability genes leads to higher rate of accumulation of mutations, which can accelerate cancer progression (17). Many hereditary cancer predisposition syndromes occur by mutations of the stability genes (6).

Mutations can be of either germline or somatic origin. Germline mutations lead to increased frequency of occurrence of cancer. Some of the examples of the hereditary types of cancers include familial adenomatous polyposis (mutations in APC), familial breast cancer (mutations in BRCA1 or BRCA2) or Li-Fraumeni syndrome (LFS, caused by mutations in p53). Somatic mutations are acquired in somatic tissue during the lifetime and predominantly give rise to cancer with mutations restricted to cancer cells (12).

Genetic changes in tumours have a different weight in contributing to malignant progression. Some of them are crucial for sustaining tumour growth, such as oncogenic changes in Myc and Ras. Elimination of such oncogenes from tumours leads to tumour regression and has been termed "oncogene addiction" effect (18). Other changes that were selected in early stages of tumour development may not be required for growth of advanced aggressive tumour or could occur as secondary alterations due to genetic instability of tumours.

The concept of cancer being a heterogeneous disease, which arises through a number of several mutations, is supported by recent evidence of cancer-genome studies. One such study, which analyzed 13000 genes in 11 breast and 11 colorectal cancers, revealed that on average there are about 11 mutated genes per tumour, which contribute to tumour progression. Noteworthy, each tumour specimen had its own distinct gene signature with maximum of 6 genes overlap. Though this could be due to the limited number of samples, it seems to reflect the high heterogeneity of tumours even from the

same tissue origin. More recent studies focused on pancreatic cancers and glioblastomas and carried out more extensive analysis (19-21). These studies have found that there are on average 63 genetic alterations in pancreatic cancer and about 47 mutations in glioblastoma with significant variation between the samples. It remains to be determined which of these genes are the "driving" force of tumour progression and which were accumulated on the way. Mutations in pancreatic cancer affected only 12 pathways and 3 pathways in glioblastoma, which implies that several pathways are disrupted in tumour development and several genetic alterations are required to deregulate the pathway.

In addition to mutations, discussed above, there are many other genes, involved in tumourigenesis, which are not mutated, however their expression levels are altered. Changes in expression are generally associated with the epigenetic changes, such as methylation of DNA, which silences the gene. These changes can be found in normal cells at some stage of development, suggesting their reversibility. In many cancers the promoters of some tumour-suppressor genes, such as Rb, VHL and BRCA1, are hypermethylated, which results in inactivation of these genes (22, 23).

One of the most commonly found alterations in cancer is inactivation of the p53 pathway (24) (IARC TP53 database http://www-p53.iarc.fr/). p53 gene is mutated in about 50% of most human cancers (25, 26) and large proportion of the cancers that retain wild type p53 have mutations in pathways directly regulating p53. The germline mutation in p53 causes the cancer predisposition syndrome Li-Fraumeni (27). The key role of p53 in tumourigenesis is also reflected in the literature with over 40000 publications since its discovery. p53 has been well described as a tumour suppressor, which is involved in numerous sometimes opposing biological processes preventing the rise of malignant cells (28).

1.2 p53 is a tumour suppressor gene: history of discovery.

Already during the first years after its discovery the unique character of p53 was revealed, which distinguishes it among other tumour suppressors. p53 was identified in 1979 as a protein in complex with large T-antigen oncoprotein of the SV40 DNA tumour virus (29, 30). Another study reported high levels of p53 in transformed, but not normal cells, with no history of viral infection, suggesting it was coded by cellular genes (31). p53 gene was cloned (32-34) and originally described as an oncogene, due to its ability to transform cells in cooperation with other H-Ras oncogene (35, 36). In support of this notion, expression of p53 then was shown to immortalize the cells (37) and enhance tumourigenic potential of cells injected in mice (38, 39). Later it was realized that the originally studied p53 protein was the product of a mutated p53 gene, which indeed

promoted tumourigenesis. However, after the wild type p53 gene was cloned it became evident that wild type p53 protein blocked the ability of oncogenes to transform cells (40-42). It also became clear that SV40 needed to inactivate wild type p53 in order to transform cells (43). Wild type p53 was then reclassified as a tumour suppressor gene and numerous studies since then have demonstrated its key role in protecting cells from cancer (24). The fact that p53 is mutated in at least half of all human cancers indicates a strong selection for its loss during tumour progression (25, 26). Additional support for its crucial role in tumourigenesis came from the study of Li-Fraumeni patients, who inherit one allele of mutant p53 gene and are extremely predisposed to cancer (44). Most convincing evidence for the tumour suppressive role of p53 has come from the study of p53 knock-out mice (45, 46). Those mice can develop without gross abnormalities, though some problems with development have been reported, however they all spontaneously develop tumours by the age of 6 months. Recognition of p53's prominent role in protection from cancer has boosted a huge amount of scientific reports (around 20000) describing the function of p53 as a tumour suppressor. Quite an unusual feature of a tumour suppressor was noted – p53 is point mutated rather than inactivated in cancers and is highly expressed in tumours. Mutation of p53 gene confers novel oncogenic properties on p53 protein.

1.3 Function of p53.

p53 is activated in response to oncogene activation, DNA damage and spindle damage, which can potentially increase the mutation occurrence in cells and increase the risk of becoming cancerous. p53 is also induced in response to other types of cellular stresses such as hypoxia, rNTP depletion and nutrient deprivation which can predispose cells to malignant transformation (Fig.1.1). Activated p53 can induce cell-cycle arrest, allowing DNA repair, or cause senescence, or promote apoptosis, eliminating the damaged cells (24, 28). Numerous studies have demonstrated that p53 can influence many other biological processes, such as invasion and motility, angiogenesis, differentiation, cell survival and more recently discovered glycolysis (47, 48) and autophagy (49) (Fig.1.1).

1.3.1 p53 is a transcription factor.

The p53 gene encodes a transcription factor and mediates much of its biological activities by regulating the expression of numerous p53 target genes (Fig.1.1). p53 binds to the specific sequences – p53 responsive elements - in the regulatory region of its target genes and more than hundred different p53 target genes have been described with various biological functions and the list is likely to grow (50). p53 activates transcription of most

of its targets by recruiting general transcription factors (TATA-binding protein-associated factors) and histone acetyltransferases (HAT) CBP, p300 and PCAF to the promoter
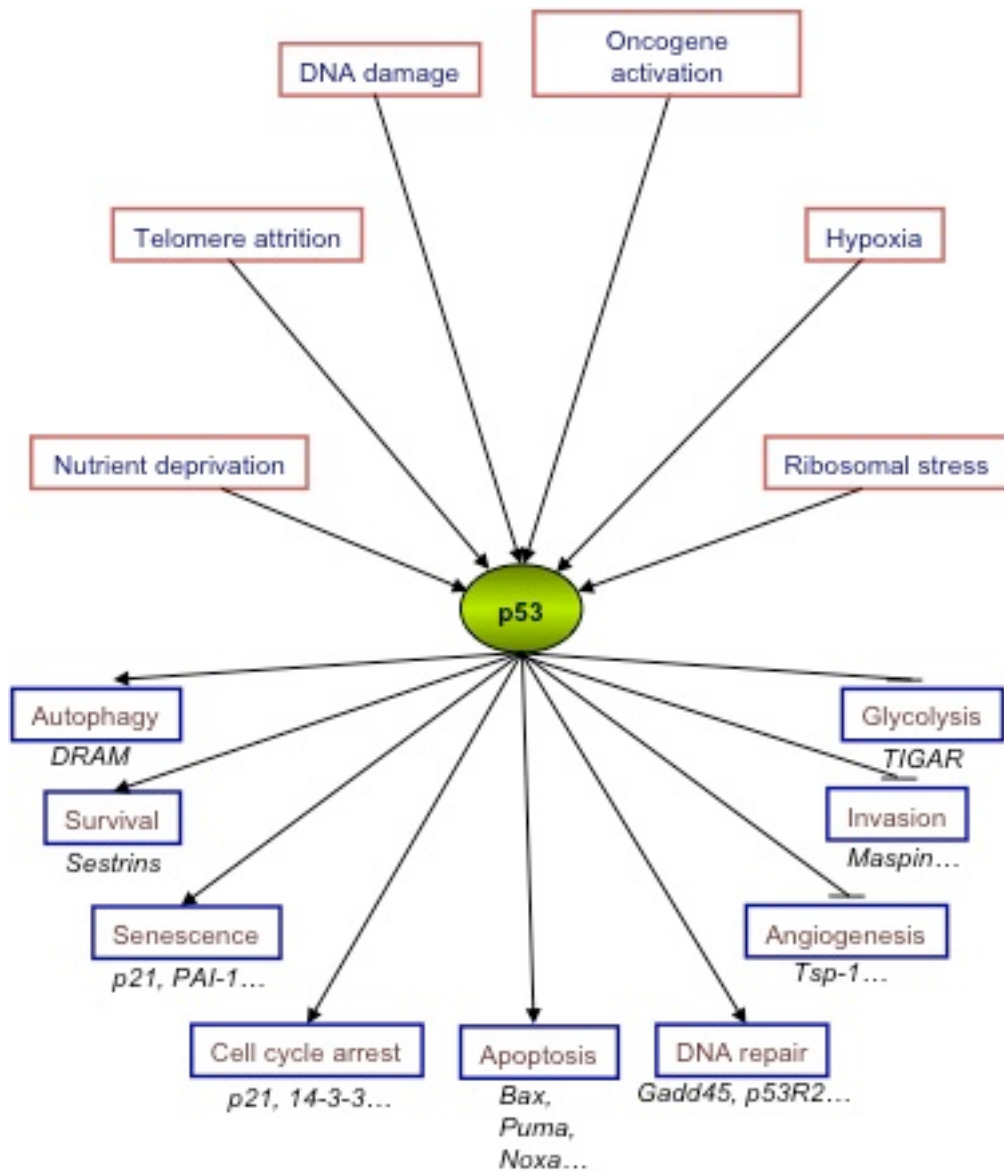
FIGURE 1.1 Scheme of p53 response.
p53 is activated by a number of cellular stresses (blue boxes) and regulates different biological processes (red boxes) via transcriptional activation of its target genes (marked in black).

(51-54). One of the first discovered p53 target genes was the cyclin-dependent kinase inhibitor (CDK) p21, which induces a cell cycle arrest (55). Other p53-regulated genes mediating cell cycle arrest include 14-3-3σ (56), GADD45 (57) and Reprimo (58). p53 induces apoptosis by activating genes mediating extrinsic and intrinsic apoptotic pathways (59). Such targets include genes encoding death receptors, Fas/CD95/Apo-1 (60) and Killer/R5 (61), and mitochondrial proteins Bax (62), Noxa (63) and PUMA (64). The mitochondrial proteins function by inducing the loss of mitochondrial membrane potential, leading to release of cytochrome C and activating caspases leading to apoptosis. Out of

these proteins PUMA is the key mediator of apoptosis, as it is required for most of the apoptotic activity of p53 (65). In addition to its well-established role as a transcription factor, p53 can induce apoptosis independent of transcriptional activity by direct interaction with members of the BCL2 family proteins at the mitochondria (66). PUMA also mediates induction of apoptosis by cytoplasmic p53 (67). Activation of autophagy via induction of novel gene DRAM by p53 also contributes to cell death (49, 68). Recent studies have identified microRNA miR-34 as a p53 target gene, adding a new twist on regulation of p53 gene network (69-72). miRNAs are a class of small regulatory RNAs that mediate post-transcriptional silencing of specific target mRNAs (73). The miR-34 family is directly induced by p53 in response to DNA damage and oncogenic stress, which can lead to induction of growth arrest and apoptosis through inhibiting gene expression of proliferative and anti-apoptotic genes (69, 72). p53 can contribute to cell survival by allowing DNA repair by activating genes such as Gadd45 (57), p53R2 (74). p53 has also been suggested to play a direct role in mediating DNA repair by interacting with components of the repair machinery (75). In addition, p53 plays a survival role by protecting the genome from damage by reactive oxygen species (ROS). This activity of p53 is mediated by activation of TIGAR (47), sestrins (76), aldehyde dehydrogenase-4 (77) and Sco2 (78), which can decrease the levels of intracellular ROS. TIGAR, one of the most recently described p53 target genes, is involved in regulation of glycolysis, and plays anti-oxidant role by decreasing ROS, which attenuates cell death (47). The survival function of p53 is suggested to be important under conditions of basal physiological stress in normally growing cells to prevent accumulation of DNA damage (79). In the absence of severe stress, low levels of p53 activate the expression of antioxidant genes. This has been demonstrated by removal of p53 in unstressed cells, which leads to down-regulation of levels of sestrins and Sco2. The current model suggests that at low levels of stress p53 plays a survival role and helps the cell to cope with stress, by decreasing ROS and allowing DNA repair. When stress is severe and/or DNA damage is irrepairable, p53 triggers irreversible growth arrest or apoptosis, to eliminate the damaged cells from the healthy pool (28). In light of the current data, the role of p53 therefore emerges as a master regulator of cells well-being, which prevents cancer development.

Several p53 target genes inhibit p53 activity in a negative feedback loop. p53 transcriptionally activates its major negative regulator Mdm2 (mouse double minute) (80, 81), a ubiquitin ligase, which inactivates p53 mainly by targeting p53 it for proteasomal degradation and promoting p53 nuclear export (82). Similarly to Mdm2, p53 target genes Cop1 (83) and Pirh2 encode ubiquitin ligases which can degrade p53 (84).

Though binding of p53 to the response element of most of its target genes results in activation of their transcription, some genes are repressed by p53. p53 can prevent the activation of the promoters of the genes in a number of different ways. p53 can prevent the binding of more potent transcription factors, for example NF-Y and CEBP, due to overlapping binding sites in the promoter. By this mechanism, p53 can repress transcription of BRCA1 and some other genes (85). Similarly, p53 represses anti-apoptotic gene Bcl-2 by preventing its activation by POU4F1 family transcription factor (86). In addition, p53 can directly interact with the transcription factors, such as Sp1 and AP1 and others, preventing their binding to the target genes. By this mechanism p53 leads to repression of genes such as cyclin B1 (87) and TERT (88). p53 also recruits histone deacetylases (HDACs) to the promoters which is mediated by the interaction with SIN3A (89). HDAC2 deacetylates lysine residues of histones in chromatin, repressing transcription of genes (90). By this mechanism, p53 represses transcription of genes such as MAP4 and stathmin (89).

One of the novel target genes CD44 is inhibited by p53 under conditions of basal stress (91). CD44 plays a key role in mediating tumour progression in cells lacking p53. CD44 encodes a cell-surface molecule and can block p53-dependent stress-induced apoptotic signals. Inactivation of p53 function in tumour cells derepresses CD44 expression, which is essential for tumour-initiating ability of transformed mammary epithelial cells (91).

The repertoire of p53 target genes is extremely broad and in addition to genes mentioned above also includes secreted proteins regulating migration (92) and angiogenesis (93, 94). Though some of these biological responses have sometimes opposing roles, they all seem to contribute to the tumour suppressive function of p53.

The choice of p53 response depends on the type of the particular stress and cellular context and is the active area of research (50), which has mostly focused on the choice between the fundamental p53 responses – cell cycle arrest and apoptosis. Posttranslational modifications are involved in dictating the choice of transcriptional target genes by p53. Upon UV and DNA damage HIPK2 (95, 96) and DYRK2 (97) phosphorylate p53 on S46. This promotes induction of apoptosis by p53 via activation of pro-apoptotic p53AIP1 gene (95, 98). Acetylation of p53 on lysine 120 by MOF and TIP60 also promotes p53-dependent apoptosis in response to DNA damage, via recruitment of p53 to pro-apoptotic target genes, PUMA and Bax (99, 100). Ubiquitination of p53 on Lys320 by E3 ligase E4F1 promotes cell cycle arrest function of p53 via activation of p21, Gadd45 and cyclin G1, while not affecting the pro-apoptotic target genes (101). p53 family members p63 and

p73 can also selectively enhance the apoptotic activity of p53 in some cell types, by promoting transactivation of PERP and BAX but not p21(102).

p53 interacting partners play an important role in the outcome of p53 response. The members of the ASPP (ankyrin-repeat-SH3-domain- and proline-rich-region-containing) family play an important role in regulating the apoptotic function of p53. The family of ASPPs, which also stands for apoptosis-stimulating protein, includes three members: pro-apoptotic - ASPP1, ASPP2 and anti-apoptotic iASPP (inhibitory) and their function is evolutionary conserved. The ASPP family members contain highly conserved ankyrin repeats, an SH3 domain and proline rich region in their C-terminus (103). Unlike many other proteins, ASPP family members interact with the DNA binding domain of p53 (104). ASPP1 and ASPP2 specifically stimulate the induction of apoptosis by p53, but not cell cycle arrest (105). ASPPs act by selectively enhancing the p53 binding and transactivating promoters of pro-apoptotic target genes such as Bax, PIG3 (p53-induced gene 3) and PUMA, while not affecting the promoters of the CDKN1A and mdm2 genes. The mechanism of the promoter selectivity is currently unknown, but could involve the change of conformation of DBD of p53 or the recruitment of other chromatin-remodelling factors (105). iASPP specifically inhibits p53-induced apoptosis by inhibiting the transactivation of p53 of pro-apoptotic BAX and PIG3 genes, but not of Mdm2 and CDKN1A. It has been suggested that iASPP acts by displacing ASPP2 from p53 (106). ASPP1 and ASPP2 can inhibit the transforming activity of the oncogenes RAS and E1A in cells, suggesting they can act as tumour suppressors. On the other hand iASPP can enhance the transforming activity of RAS and E1A in the same system, suggesting its potential proto-oncogenic role (105, 106). Interestingly, ASPP2+/- mice develop spontaneous tumours, which is accelerated in p53+/- background (107). Downregulation of ASPP1 and ASPP2 expression has been reported in several types of cancer, reflecting their important role in tumourigenesis. Consistent with its anti-apoptotic role, iASPP has been found overexpressed in breast carcinomas (103).

1.3.2 Activation of p53.

Cellular stresses engage different, sometimes overlapping, pathways to activate p53. The most well understood signals that activate the p53 protein in cells are DNA damage and oncogene activation (Fig.1.2). There are different types of DNA damage resulting from gamma or UV irradiation, alkylation of bases, depurination of DNA and reactive oxygen species (108). The major sensors of DNA damage are ATM and ATR

(109-112) and their downstream kinases Chk1 and Chk2 (113-115), respectively, which primarily act by phosphorylation of p53 leading to its stabilization and activation.
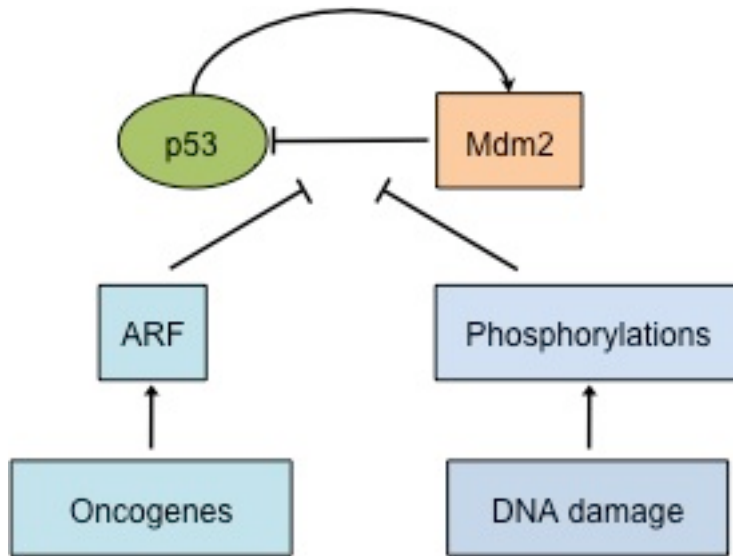
FIGURE 1.2. Regulation of p53.
Core regulatory pathway of p53 is shown. Mdm2 is the major negative regulator of p53, whereas p53 transcriptionally activates Mdm2. Stresses, such as DNA damage and oncogenes activation, via ARF, activate p53 by inhibiting its negative regulation by Mdm2.

Oncogene activation leads to accumulation of p53 by a mechanism, which is mostly mediated by the tumour suppressor protein ARF (p19ARF in mouse and p14ARF in human) (14, 116). The ARF protein (Alternative Reading Frame) is a product of the INK4a locus, which also encodes the cyclin-dependent kinase inhibitor p16INK4a (117). Deregulated oncogenes, such as overexpressed Myc, oncogenic Ras or deregulated E2F, induce ARF expression, resulting in activation of p53 response (118-120). ARF leads to p53 activation, by inhibiting its major negative regulator Mdm2 (121-124). Mice lacking ARF are tumour-prone, although the phenotype is not as severe as in p53-null mice (125, 126). Interestingly, tumour cell lines retaining wild type p53 almost always lose ARF, suggesting the importance of signalling through this pathway in tumour development (16). Loss of ARF does not prevent the activation of p53 in response to DNA damage, suggesting oncogene activation and DNA damage are independent signals (127). However, the situation is more complex as oncogenes activation has been shown to induce DNA damage response in precancerous lesions (128, 129), suggesting the cross-talk between the two pathways. Indeed, loss of ARF can attenuate the DNA damage response and loss of ATM can impair the ARF response. The contribution of these two fundamental pathways

of p53 activation to tumour-suppression by p53 remains quite controversial. On one hand it has been shown that DNA damage pathway is an early event and is constitutively activated in tumours, and activation of p53 response has been shown to protect from tumour formation (130, 131). On the other hand, a study in mice generated to have switchable p53 has shown that p53 is important as a tumour suppressor only after most of the DNA damage has been resolved (132). Consistent with this, another group has shown that signalling through the ARF pathway accounts for almost all tumour suppression activity by p53 (133) . This suggests that p53 response to oncogene activation is the most important in tumour suppression. These studies question the importance of one of the most fundamental signals activating p53 function, which remains an issue of debate.

Ribosomal stress, such as treatment with the RNA polymeraseII inhibitor actinomycin D, is a well known activator of p53. This is mediated via a number of ribosomal proteins, such as L5, L11 and L23 which bind and inhibit Mdm2, leading to stabilization and activation of p53 (134-138).

Metabolic stress due to lack of nutrients can activate p53 through a pathway involving AMP kinase, where p53 induces reversible growth arrest playing a survival role during starvation (139). Loss of p53 can contribute to continued proliferation of tumour cells in the conditions when nutrients are scarce and therefore confer a growth advantage. Other stresses that activate p53 pathway include hypoxia, which can lead to growth arrest, autophagy or apoptosis, depending on the severity of hypoxic stress. Interestingly, hypoxia activates a different transcriptional program than DNA damage (140).

1.3.3 Structure of p53.

The p53 gene contains eleven exons with two alternative translation start sites in exon 2 and 4 (GenBank Accession Number: NC_000077) (141). The p53 protein contains three major functional domains: N-terminal transcriptional activation domain (TA), the central sequence-specific DNA-binding domain (DBD) and the oligomerization domain (OD) in the C-terminus (Fig.1.3). There is also an N-terminal proline rich domain involved in protein interactions and regulatory domain in the C-terminus (Fig.1.3). p53 also contains several nuclear localization (NLS) and nuclear export signals (NES) (Fig.1.3) (142). There are five highly evolutionary conserved regions in p53 protein boxes I to V (Fig.1.4). Box I (13-18aa) lies in the TA domain of p53 and encompasses the region interacting with Mdm2, whereas boxes II, III, IV and V reside in the DBD of p53 and are regions most frequently mutated in cancer (143).
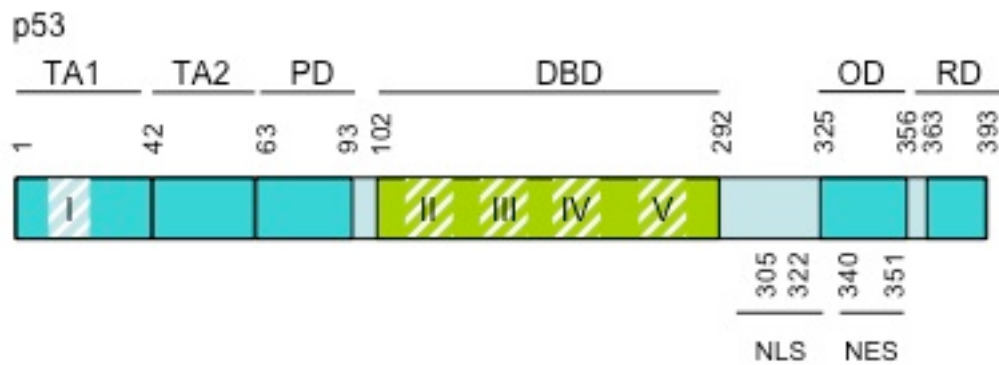
FIGURE 1.3. Structure of p53 protein.
The main domains of p53, nuclear export (NES), nuclear localization (NLS) signals and the location of the conserved boxes I, II, III, IV and V are shown.
TA - transactivation domain, PD - proline-rich domain, DBD - DNA binding domain, OD - oligomerization domain, RD – regulatory domain.

The transcriptional activation domain of p53 is crucial for its function. Two TA domains are defined in the N-terminus – TA1 (1-42aa) and TA2 (43-92aa) (144, 145) (Fig.1.3). Both TA1 and TA2 interact with the basal transcriptional machinery and independently are sufficient to activate transcription when fused to heterologous DBD (144). TA1 and TA2 are regulated by associated proteins such as p300, Mdm2 and Pin1. Structural studies reveal that N-terminal domain is natively unfolded and fully folds upon binding to its interaction partners (146, 147). A fragment containing 15-29aa adopts an alpha-helical conformation upon binding to N-terminus of Mdm2 (148). N-terminus of p53 is also subject to multiple phosphorylations by different protein kinases. Next to the TA lies the proline-rich domain (64-92aa) (PD), containing five PXXP motifs, which is involved in multiple protein interactions (149). The function of the PD is not entirely understood and has been suggested to contribute to proapoptotic function of p53 (150).

The DNA binding domain (DBD) of p53 is essential for its sequence-specific transcriptional activation. The p53 DBD spans amino acids 102-292 and contains four of the five highly evolutionary conserved regions defined in p53 – boxes II (117-142aa), III (171-181aa), IV (234-256aa) and V (270-286aa) (143) (Fig.1.4). The crystal structure of p53-DBD bound to DNA has been solved (151). Several other structures of p53 DBD have been reported (147) including structures of p53-DBD in solution in its DNA-free form (152) and in complex with interacting patners (104, 153). The structural studies show that the conserved regions are crucial for the p53-DNA interaction. The larger part of the DBD forms an anti-parallel β-sandwich. This sandwich serves as a scaffold for structures interacting with DNA. The DNA-binding surface consists of loop-sheet-helix and two large loops interacting with the minor and major grooves of DNA respectively. The loop-

sheet-helix motif spans conserved region V (151, 152). Two large loops are stabilized by a zinc ion, which is coordinated by residues C176 and H179, located in box III and C238 and C242, located in box IV (154). The p53 consensus site contains two half-sites RRRCWWGYYY, separated by a spacer of 0-13bp, where R=purine, C=cytosine, W=adenine or thymidine, G=guanine and Y=pyrimidine. Residues S241, R248, K120, R273, A276, R283, C277 and R280 directly contact DNA (151, 155). The DBD of p53 is only marginally stable, which is likely to have evolved to allow high flexibility between folded and unfolded states. This can provide the structural basis for diversity of p53-mediated responses (151, 152). Most of the p53 mutations found in cancers affect the DNA binding domain and occur as point mutations of either amino acids contacting DNA or residues important for the structure of the DBD, implying the crucial role of the DBD for tumour suppression (147).
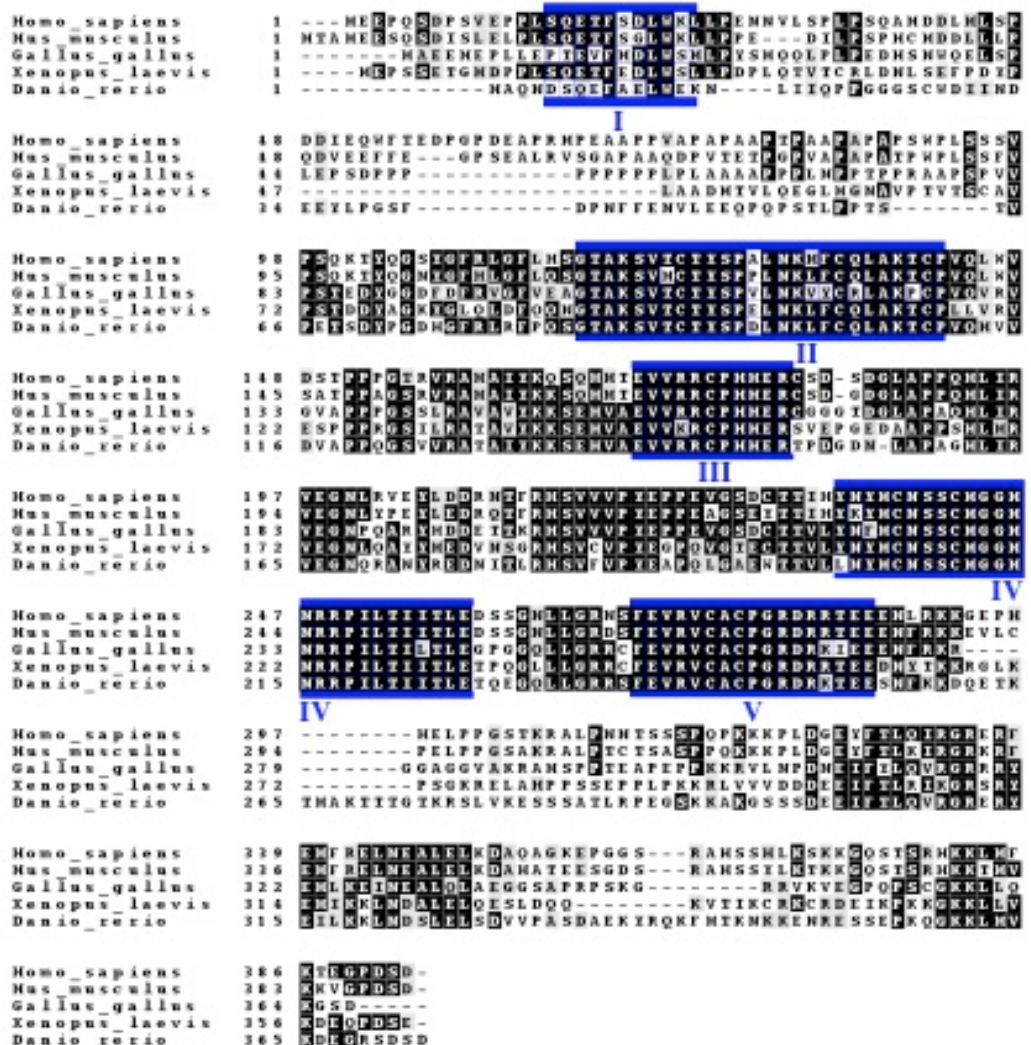
Figure 1.4. Comparison of p53 protein sequence across species.
Alignment of human p53 protein sequence with p53 from other species: Mus musculus, Gallus gallus, Xenopus laevis and Danio rerio was done using the ClustalX program. Evolutionary conserved residues are highlighted in black. Divergent amino acids belonging to the same class are highlighted in gray. Highly conserved boxes I, II, III, IV, V are marked in blue.

Full-length p53 functions as a tetramer, which is mediated by the oligomerization domain in the C-terminus of the protein (325-356aa) (Fig.1.3). Structural studies describe the tetrameric structure as a dimer of primary dimers (156). Tetramerisation of p53 is required for high-affinity DNA binding and transcriptional activation. The model suggests that one DBD dimer binds to one half of the consensus DNA binding site, and second dimer to the adjacent half of the site (155). Oligomerization domain also regulates the binding to Mdm2 and nuclear export of p53 (157, 158).

The extreme C-terminus of p53 (364-393aa) is considered to be a regulatory domain and is subjected to extensive posttranslational modifications, including phosphorylation, acetylation, ubiquitination, sumoylation, neddylation and methylation, which regulate p53 activity and stability (Fig.1.3). Structure reveals it is intrinsically unstructured but can fold upon binding to other proteins or nonspecific DNA (159). The C-terminus has been shown to be required for activation of many p53 target genes (160).

1.4 Regulation of p53.

Upon cellular stresses, the p53 protein rapidly accumulates and activates transcription of its target genes. The p53 protein has a very short half-life in normal cells and is primarily regulated at the level of protein stability, which provides the means to rapidly induce p53 when needed. In unstressed cells p53 protein levels are kept low due to continuous ubiquitination which targets p53 to proteasomes for degradation. This is primarily mediated by the ubiquitin ligase Mdm2, a key negative regulator of p53. In addition to proteasomal degradation, ubiquitination by Mdm2 can also lead to nuclear export of p53. Several other ubiquitin ligases have also been reported to contribute to regulation of p53 stability (82, 161) and will be discussed later.

1.4.1 Ubiquitination.

Ubiquitin is a highly conserved 76-amino acid polypeptide, which is encoded on multiple genes. The genes encode oligomers of ubiquitin, which are processed to active monomers in cells. Ubiquitins are then covalently attached to lysines on target proteins in a specific and tightly regulated reactions cascade called ubiquitination (Fig.1.5) (162-165). Briefly, ubiquitination is a sequential reaction which is mediated by coordinated action of the E1 - ubiquitin activating enzyme, the E2 - ubiquitin conjugating enzyme, and E3 or ubiquitin protein ligase, which determines the substrate specificity. First E1 forms a thiol-ester bond between its active site cysteine and the C-terminal glycine of ubiquitin through

ATP-dependent process. The activated ubiquitin is then transferred from E1 to a conserved cysteine on E2 in a reaction of transthiolation. Next, E3 interacts with ubiquitin-bound E2 and the substrate and facilitates formation of an isopeptide linkage between C-terminal glycine on ubiquitin and the ε-amino group of an internal lysine on the substrate (Fig.1.5). Polyubiquitin chains are formed by attachment of ubiquitin to the lysine on the ubiquitin already attached to the substrate (165). In some cases ubiquitin can be attached to the free α-amino group of the substrate (166, 167).
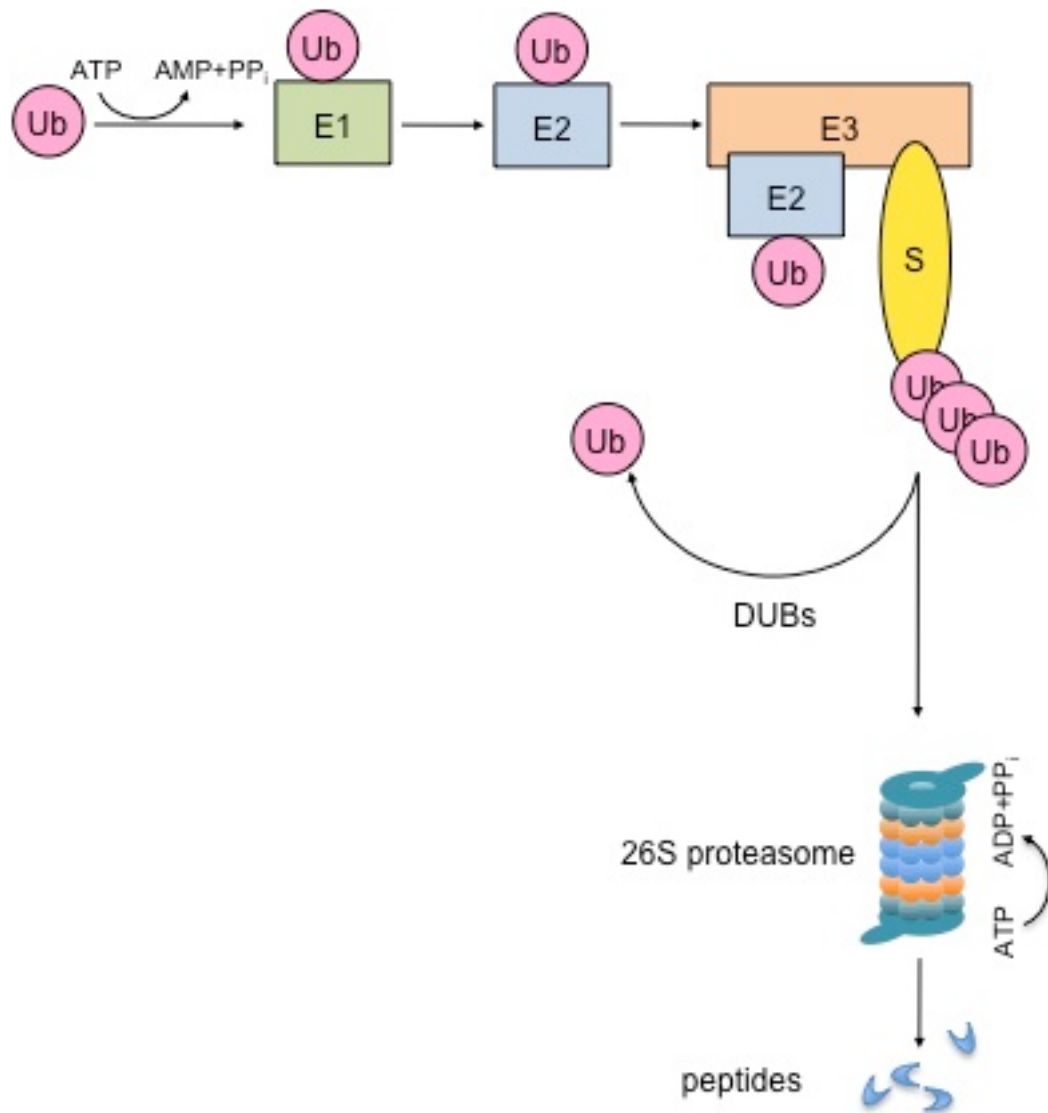
FIGURE 1.5. Ubiquitin-proteasome system.
Ubiquitin is activated by a ubiquitin-activating enzyme E1 and is transferred to a ubiquitin-conjugating enzyme E2. The ubiquitin ligase E3 mediates the transfer from the E2 to the protein substrate. Polyubiquitinated substrates are targeted to the 26S proteasome for degradation. The ubiquitin can be cleaved off the by deubiquitinating enzymes (DUBs). P_i, inorganic phosphate; PP_i, pyroposphate; Ub, ubiquitin. (Modified from Weissman, A. M. (2001) Nat Rev Mol Cell Biol 2(3): 169-78.).

E1

It is considered there is one E1 in mammals, which is essential for ubiquitination. There are two isoforms E1a and E1b, which result from alternative translation initiation (168). The reaction of activation of ubiquitin usually involves two steps: the ATP-dependent formation of ubiquitin-adenylate intermediate, which is followed by the formation of the E1-ubiquitin thiol ester (169).

E2

There are about 13 genes encoding E2-like proteins in yeast and more than 30 E2 in mammalian genomes. Not all of them can conjugate ubiquitin, for example Ubc9 conjugates ubiquitin-like protein SUMO. They all have a characteristic domain (UBC) of ~ 150 amino acids that contains cysteine that accepts ubiquitin from E1 and is conserved ~35% between the family members. Many E2s also have N- or C-terminal extensions, which are involved in the interactions with E3s (163) and can facilitate the interaction between E3s and the substrate by serving as membrane anchors (170). Importantly, the ubiquitin binding site on the E2 does not overlap with the E3-binding site (171, 172). Another significant finding is the discovery that E1 and E3 binding sites on the E2 overlap, and their binding to E2 is mutually exclusive (173). This implies that for polyubiquitination multiple cycles of E2-E3 binding and release need to occur. There are many more E3s described than E2s. The structural similarity between different E2s suggests redundancy in their function. Indeed, UbcH5 and UbcH7 for example can function with HECT domain (174) and RING domain E3 ligases (171, 175). The structures of UbcH7 bound to HECT domain of E6-AP (172) and RING domain of c-Cbl (171) have been solved. Interestingly, these different E2s interact with the same region on UbcH7. The conserved Phe63 on UbcH7 is crucial for the interaction between UbcH7 and the HECT of E6-AP and RING of c-Cbl and appears to be present in other E2s, suggesting its importance in mediating E2-E3 binding (176). In vitro studies suggest that E3s can also function with several E2s. For example, in the screen for Mdm2-specific ubiquitin-conjugating enzymes it has been found that that UbcH5A, -B, -C and E2-25K support Mdm2-mediated ubiquitination of p53 in vitro (177). Another report suggests that RING ubiquitin ligase Topors functions with UbcH5a, UbcH5c and UbcH6, but not with UbcH7, CDC34 or UbcH2b (178). It is possible that the E2 step might provide an additional point for specifying and diversifying the signal of ubiquitination and by this dictate the biological function of the ubiquitin conjugation.

E3.

E3s, ubiquitin ligases, largely determine the substrate specificity (163, 179). There are more than 200 different ubiquitin ligases currently characterized and several hundreds are predicted by bioinformatics approach, based on the search for signature domain (180).

Three large families of E3s are defined based on the presence of the functional domain: the HECT (Homologous to E6AP Carboxy Terminus), RING (Really Interesting New Gene) and U-box (UFD2) homology proteins. The RING family contains also a PHD (Plant HomeoDomain)-containing subfamily. Each of the functional domains directly interacts with the corresponding E2.

HECT family.

This family is defined by the presence of the HECT domain and was founded by the first discovered and recognized mammalian ubiquitin ligase E6-AP (E6-Associated Protein). Upon binding to the E6 protein of the human papillomavirus (HPV) E6-AP ubiquitinates and degrades p53 (181). A highly conserved C-terminal domain of ~350 amino acids is a characteristic feature of the members of the HECT family (182). The N-terminus mediates the substrate recognition. The HECT domain binds the E2-ubiquitin intermediate and accepts ubiquitin at a conserved cysteine residue which is usually ~35 aa upstream the C-terminus of E3. The formation of the E3~ubiquitin intermediate is a unique feature of this family, whereas other E3s transfer ubiquitin directly to the substrate. Other family members involve HUWE1/ARF-BP1/Mule which ubiquitinates a number of substrates, such as: Mcl-1 (183), c-Myc (184) and p53 (185)

RING family.

The largest family of the E3s is defined by the presence of RING finger domain usually in the range of 40-100 amino acids (186). The RING finger consists of eight conserved cysteines and histidines [CX2CX(9-39)CX(1-3)HX(2-3)C/HX2CX(4-48)CX2C] that together coordinate two zinc ions in a cross-braced arrangement. The RING domain directly binds E2 and is essential for the ubiquitin ligase activity (171). RING domain E3 functions as an adaptor that positions the substrate lysine in close proximity to E2-ubiquitin intermediate (186).

RING domain ubiquitin ligases can consist of single- or multiple subunits. Mdm2 is the single subunit ubiquitin ligase and contains amino-terminal p53-binding domain and the RING finger domain in its carboxyl terminus. Together with E1 and E2 enzymes Mdm2 is sufficient to ubiquitinate p53 *in vitro* (175). Multisubunit ubiquitin ligase complexes usually contain a RING finger subunit, a member of the cullin family and a F-

box containing structural adaptor (187). The RING finger subunit provides ubiquitin ligase activity, cullins play a role of scaffold, and F-box proteins link the cullin to the substrate. For example, SCF (Skp1-Cul-F-box) complex consists of RING finger protein (Rbx1), cullin1 and Skp1 as an adaptor. The known SCF E3s play an important role in regulation of the G1/S cell cycle transition. The subunits of the complex are in such structural organization, which positions the core ubiquitin ligase activity with multiple substrates. The substrates of the SCF E3 ligases include oncogenes b-catenin, cyclin D, E2F1, as well as proteins involved in growth arrest such as p21, p27 (188).

A small subfamily of RING E3s is defined by the presence of a PHD domain. Structural studies show that PHD domain resembles the RING domain in folding which also relies on the coordination of two zinc ions in a cross-brace arrangement. It usually includes a cysteine rather than a histidine in the fourth position and an invariant tryptophan before the seventh zinc-binding residue. This family includes viral proteins MIRs (modulator of immune recognition), which downregulate MHC class I (189).

U-box.

U-box domain is distantly related to the RING finger, but has no conserved zinc coordinating residues. Sequence analysis demonstrated that the U-box proteins share conserved charged and polar residues and the structure prediction suggests similarity to the RING. Several U-box proteins have been shown to ubiquitinate *in vitro* in a manner similar to the RING finger (163). U-box domain was first identified in yeast Ufd2 ubiquitin ligase. Interestingly, Ufd2 lacks its own substrate and instead promotes the polyubiquitination of another E3's substrate, which led to its classification as an "E4" (190). Another U-box protein, C-terminus of Hsc70 Interacting Protein (CHIP) also displays E4-like activity (191, 192). CHIP is known to ubiquitinates several substrates, most of which are misfolded proteins associated with Hsp70 or Hsp90 chaperones (193). It can also associate with Parkin, belonging to the RING family, and promotes Parkin-mediated ubiquitination of substrate protein Pael-R (194).

Lysines on the substrates can be mono- and polyubiquitinated. A conjugation of single ubiquitin can also occur on several lysines and is termed multiubiquitination. The best studied role of monoubiquitination is regulation of endocytosis and DNA repair and has been shown to affect subcellular localization, conformation, activity and protein interactions of the substrates (195-197). Polyubiquitin chains are formed by conjugation of G76 carboxyl group of the next ubiquitin to the ε-amino group of a lysine within preceding ubiquitin (198, 199). Ubiquitin has seven internal lysine residues K6, K11, K27, K29, K33, K48 and K63, which are able to serve for conjugation of ubiquitin (200). The best characterized polyubiquitin chains are those linked through K48 and K63 (201). K48-

linked ubiquitin chains of four ubiquitins or longer target the substrate to the proteasomes for degradation (202). However, K48 chains can have proteolysis-independent functions (101, 203). For example, K48- linked ubiquitination of p53 by E4F1 plays a novel role and has been reported to modulate p53 transcriptional activity without affecting proteolysis of p53 (101). K63-ubiquitination requires the action of distinct conjugating enzymes E2s and does not promote degradation, leading to different biological outcomes. For example, IKK is activated through the K63-linked polyubiquitination, mediated by TRAF6 (204). The K63-linked polyubiquitination of PCNA is essential for error-free repair of the damaged DNA (205). There is much less known about the role of the linkages of ubiquitin via K6, K11 and K29 (206-208). Chains that catalyze formation of K6 and K11 have been shown to bind the proteasomal subunit S5α, however, it is not clear whether they can promote the degradation of the substrates (207). K6-linked ubiquitination of BRCA1 seems to stabilize BRCA1 and direct it to the DNA repair foci (208). The biological significance of other ubiquitin chains remains to be determined. The mechanisms regulating the choice of the lysine on the ubiquitin during ubiquitination are largely unknown. It is clear that the same E3 can conjugate K48 and K63 linkages. The possible step of specificity could be provided by E2, as distinct E2s are involved in K63 chain formation (209). It is also possible that the interaction partners of the ubiquitin ligases may direct which ubiquitin chain is formed. The same substrate can be modified by different types of ubiquitination, which leads to various biological outputs. For example, K48-linked polyubiquitination of the C-terminus of p53 leads to its proteasomal degradation, whereas K63-linked ubiquitination of lysine K320 modulates its transcriptional activity directing p53 to target genes mediating cell cycle arrest (101).

The ubiquitination can be reversed by the action of deubiquitinating enzymes (DUBs): ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (USPs), both of which are cysteine hydrolases (210). In general, UCHs are capable of cleaving ubiquitin precursors to generate active ubiquitin. UBPs are a large and diverse group of enzymes which can cleave and disassemble ubiquitin chains. DUBs play several biological roles in generation of ubiquitin, recycling of ubiquitin, editing polyubiquitin chains and aiding in proteasome-dependent degradation (211). Various DUBs are associated with the 26S proteasome and regulate its function. For example, UCH37 is an intrinsic subunit of the 19S proteasome and is involved in 'editing' of the ubiquitinated substrates according to the length of polyubiquitinated chains (212). Rpn11 also represents proteasome-associated DUB and releases the polyubiquitin chain from the substrate, allowing full translocation of the substrate protein to the proteolytic core of the proteasome (213). Some DUBs have been shown to have substrate specificity. HAUSP

plays an important role in regulating p53 protein levels by deubiquitinating both p53 and Mdm2 (214, 215). CYLD deubiquitinates TRAF2 leading to downregulation of NFkB signaling (216, 217). DUBs are currently intensively investigated and have been implicated in regulation of various processes (218).

Since the discovery of the ubiquitin an entire family of ubiquitin-like molecules has been discovered which currently counts more than ten members including Nedd8, SUMO, ISG15 and Atg8 (219, 220). Though not having high sequence similarity, ubiquitin-like molecules are structurally similar to ubiquitin and all have characteristic ubiquitin, or β-grasp, structural fold. Ubiquitin-like molecules are attached to the lysine on the substrate in a reaction similar to ubiquitination. Distinct E1 and E2 enzymes participate for each type of the ubiquitin-like modification. The best characterized so far SUMO and Nedd8 are the closest to ubiquitin with 20 and 60% sequence homology respectively. Several SUMO-specific ligases have been identified: for example PIAS family (221), TOPORS (222). Interestingly, some of the ubiquitin ligases, including Mdm2, can also function as NEDD8 E3 ligases (223).

Conjugation of ubiquitin-like molecules leads to different physiological outputs. SUMOylation is involved in regulation of subcellular localization (224), DNA repair (205), chromatin remodelling (225) and regulation of transcription (226). Neddylation often targets ubiquitin ligases, such as cullins, and can activate their function (227, 228). In addition, neddylation can inhibit the transcriptional activity of the target protein, as has been shown for p53 (223). Isgylation plays a role in regulation of immune response (229). The function of other ubiquitin-like molecules remains largely unknown.

1.4.2 Proteasome.

The fundamental role of the polyubiquitination chains is to target the substrates to the proteasomes for degradation (179, 230). Chains of four and more ubiquitins linked through K48 is the signal for proteasomal recognition (202). The 26S proteasome is a ~2.5 MDa complex made up of two copies of at least 32 subunits which are highly conserved in eukaryotes (Fig.1.6). Proteasomes are highly abundant in cells and degrade proteins in the cytoplasm, the nucleus or the endoplasmic reticulum (ER). The proteasome can be divided by biochemical methods into two major subcomplexes: the 20S core particle (CP) and two 19S regulatory particles (RP) (Fig.1.6). Structural studies in yeast reveal that CP is a 670 kDA barrel-shaped complex consisting of four stacked rings, each composed of seven proteins (231). The two outer rings α and the two inner rings β are identical. The

proteolytic active sites are within a cavity formed by the two β rings at the center of the CP
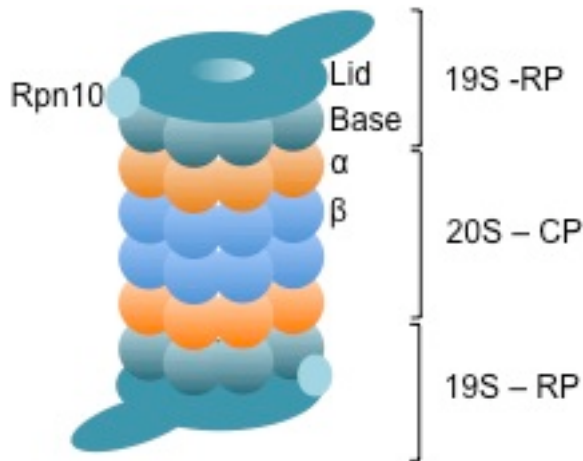
FIGURE 1.6. Structure of the proteasome.
The schematic organization of 26S proteasome consisting of 20S core particle (CP) and of two 19S regulatory particles (RP) is shown. The 20S subunit is composed of four rings: two outer α-rings and two inner β-rings. Each ring is made of 7 homologous subunits. The RP is subdivided into the lid and the base. RPN10 is found outside of the proteasome. (Modified from Hanna, J. and D. Finley (2007). FEBS Lett 581(15): 2854-61).

(Fig.1.6). Substrates gain access to the CP only through the narrow pores formed by the alpha ring subunit and a mechanism of gate opening is required to facilitate substrate degradation (232). This function is mediated by the 19S RP. The RP is nearly a 1MDa complex and each one associates with the axial end of the CP. The RP is further divided into the base and the lid (233). The base is proximal to the CP and contains six AAA-type ATPases (Rpt1-6) and four non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13). The six ATPases form a ring and function in substrate unfolding, which is thought to be a prerequisite for the degradation (234). Rpt2 plays a role in opening the pore, whereas Rpt5 is involved in recognition of the substrate-bound ubiquitin chain (235). Non-ATPase Rpn10/S5α functions as a ubiquitin receptor (236). Several proteins involved in degradation are associated with the proteasomes – Rad23, Dsk2, Ddi1, which share a common ubiquitin-like domain, recognized by Rpn1 (237). These proteins also have a C-terminal UbA domain which binds to the polyubiquitinated chains. Binding of the UbA domain can protect polyubiquinated chains from cleavage by deubiquitinating enzymes (DUBs) (238). It is not clear whether the function of these proteins is to shuttle the substrates to the proteasome or tether the polyubiquitinated substrate to the proteasome (239). The lid of the RP is distal to the base and is an eight-subunit (Rpn3-Rpn11) complex highly homologous to the COP9 signalosome and translation initiation factor eIF3, suggesting the functional homology between these different regulatory pathways (233). Rpn11 has been shown to possess deubiquitinating activity, which removes the ubiquitin molecules from the substrates (213). There are other DUBs that associate with the proteasome. For example, UCH37 trims polyubiquitin chains from substrates attached to the proteasome (212). In addition to deubiquitinating enzymes, ubiquitin ligase Hul6 has

recently been reported to associate with the proteasome (240), which can extend the polyubiquitin chain of the substrate at the proteasome.

Therefore the proteasome represents a very complex subunit, with a number of activities. In summary, ubiquitinated substrates are first recognized and bound by the ubiquitin receptors in the RP. Ubiquitins are then removed from the substrate by deubiquitinases and recycled. Substrates are unfolded and then enter the pore of CP through the gate. Some proteins can undergo degradation directly by 20S CP in a ubiquitin-independent manner, such as p21 (241), p53 (242) and Rb (243). This suggests, that ubiquitination is indeed required for recognition of the proteins by the RP of the proteasome.

1.4.3 Ubiquitination and proteasomal degradation of p53 by Mdm2.

1.4.3.1 Ubiquitin ligase Mdm2.

The major mechanism, that keeps p53 inactive in cells, is mediated by proto-oncoprotein Mdm2 (244), which targets p53 for proteasomal degradation (245, 246). p53 transcriptionally activates Mdm2 in an autoregulatory negative feedback loop (80, 81). Mdm2 was identified as the product of one of the three genes located on extrachromosomal amplifications in a spontaneously transformed murine cell line (3T3-DM) (247, 248). Its key role in negatively regulating p53 is best illustrated by the studies of the knock-out mice. Mdm2-null mice die early in embryogenesis (E5.5-6) as a result of p53-dependent apoptosis due to accumulation of p53. Simultaneous inactivation of p53 completely rescues the lethality of Mdm2-null mice, suggesting the key role of Mdm2 in negatively regulating p53 activity (249, 250).

Mdm2 is a RING finger ubiquitin ligase and ubiquitinates p53 and itself in a RING-dependent manner (175). Though several E2s can support Mdm2-mediated ubiquitination of p53 in vitro, in cells the most important are UbcH5B and C (177). At low levels Mdm2 can promote monoubiquitination of p53, which leads to the nuclear export, whereas at high levels Mdm2 efficiently polyubiquitinates p53 leading to its proteasomal degradation (245, 246, 251). In addition Mdm2 can inhibit the transcriptional activity of p53 by binding directly to its N-terminal transactivation domain (252, 253). The contribution of these roles of Mdm2 has been addressed in a recent study of mice with knock-in of Mdm2 mutant lacking the E3 activity (254). This has been done by substituting a zinc-coordinating cysteine 462 (464 in human) with alanine, which inactivates the ubiquitin ligase activity of Mdm2 but does not prevent the binding to p53.

Mice homozygous for the RING mutation died early in embryogenesis (E7.5), and the lethality was rescued by the simultaneous deletion of p53. This demonstrates that though the Mdm2 RING mutant is capable of binding to p53, it cannot fully suppress p53 activity, suggesting that the ubiquitin ligase activity of Mdm2 plays the key role in inhibiting p53 (254). Many mechanisms of p53 activation converge onto disrupting the interaction between p53 and Mdm2, leading to accumulation of p53 in the nucleus and transactivation of its target genes. Such mechanisms involve posttranslational modifications of both p53 and Mdm2, and their binding partners.

Mdm2 gene consists of 12 exons and has two different promoters P1 and P2, the second of which is p53-responsive. Alternative translation initiation generates two proteins, the full-length p90 and a shorter p76. p76 does not bind p53 and can act as a dominant-negative inhibitor of p90 leading to activation of p53 (255-258). Other alternative splice variants of Mdm2 lacking the p53-binding domain have been described and some of them are overexpressed in tumours (259). One of them Mdm2-ALT1 sequesters full-length Mdm2 in the cytoplasm and inhibits the interaction of Mdm2 with p53, enhancing p53 activity (260). It is not yet clear why these alternative splice forms are overexpressed in cancers.

Full-length human Mdm2 is a 491-aa protein and contains several functional domains (Fig.1.7). The p53 binding domain is in the N-terminal portion of Mdm2 and interacts with a highly conserved region on the N-terminus of p53 (Fig.1.7) (148, 256). Mdm2 also contains a central acidic domain, which is important for its E3 activity (261, 262). Acidic domain is involved in interaction with several binding partners, such as ARF (121, 122), ribosomal proteins L5, L11 and L23 (134-138, 263) and also mediates the interaction with the central domain on p53 (264-266). Mdm2 also contains a zinc finger domain, which has been recently suggested to be involved in interaction with ribosomal proteins L5 and L11 (267). However, the exact function of this domain remains unclear. As already mentioned, RING finger domain is required for ubiquitin ligase activity of Mdm2 (Fig.1.7). The solution structure of the C2H2C4 RING domain of human Mdm2 reveals a symmetrical dimer with a unique cross-brace zinc-binding scheme (268). Recently it has been shown that C-terminal tail of Mdm2 is also critical for efficient E3 activity (269, 270).

RING domain of Mdm2 also contains a conserved Walker A or P loop motif, found in nucleotide binding proteins. Mdm2 has been reported to interact with adenine-containing nucleotides, which leads to conformational change at the C-terminus of Mdm2

(271). Nucleotide binding is not required for the ubiquitin ligase activity of Mdm2, and is involved in regulating nucleolar localization of Mdm2. Other important structural elements of Mdm2 involve nuclear localization signal (NLS), which lies close to nuclear export signal (NES) (272) and a nucleolar localization signal (273).
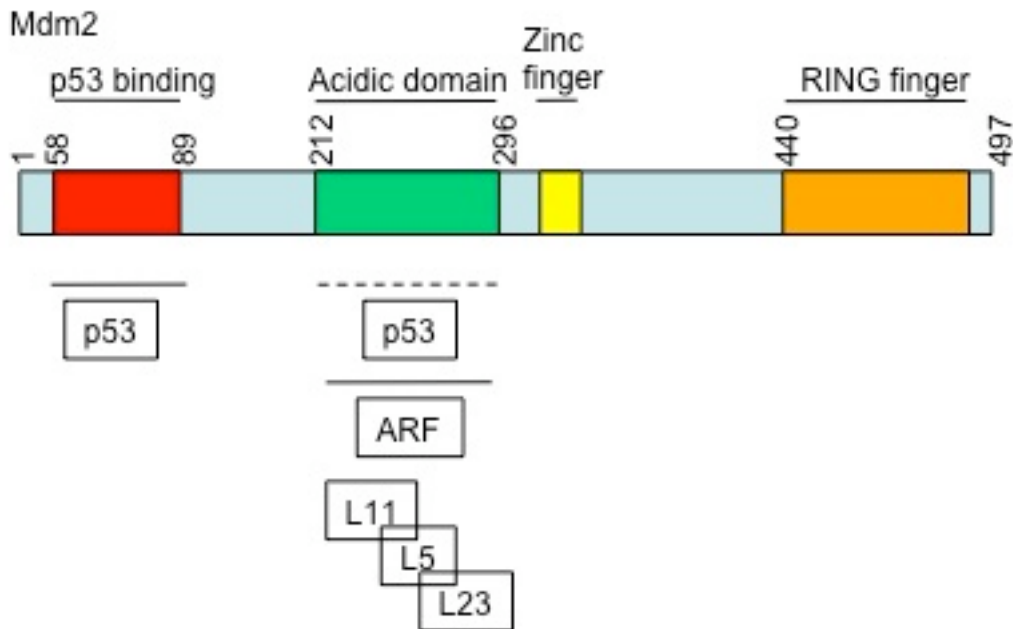
FIGURE. 1.7. Structure of Mdm2 protein.
The main domains of Mdm2 - p53-binding domain, acidic domain, RING domain and zinc finger domain are depicted. The major p53 binding site (full line) is in the N-terminus and second p53 binding site (dashed line) is in the acidic domain of Mdm2. Other interacting partners ARF, L11, L5, L23 interact with the acidic domain of Mdm2.

1.4.3.2 Interaction between p53 and Mdm2.

The interaction between p53 and Mdm2 is necessary for the ubiquitination and degradation of p53 by Mdm2 and is tightly regulated. A conserved region in the N-terminus of p53 binds to a deep hydrophobic binding cleft in the N-terminal domain of Mdm2 (25-109aa), as shown by crystal structure of the p53-Mdm2 complex (148). On Mdm2 the cleft is formed by the residues 26-108 and residues G58, G68, V75 and C77 are the most important, as shown by mutagenesis analysis (274). The Mdm2 interaction region on p53 has been mapped to 18-26aa and residues L14, F19, L22, W23 and Leu26 have been found to be important for the interaction by mutational analysis (256, 275-277). The crucial role of the residues F19, W23 and L26 has been also demonstrated by the structural study (148). The neighbouring residues also appear to contribute, probably by modulating the conformation of this region. Conserved box I (13-18aa) on p53 overlaps the Mdm2-binding site on p53 and is required for interaction and degradation by Mdm2 (158).

Mutation of residues L22 and W23 on p53 inhibits the interaction with Mdm2 and also reduces p53 transcriptional activity (278). Mouse models with p53 with equivalent mutations of L25 and W26 have shown that p53 is very stable due to decreased binding to Mdm2 (279-281), confirming the importance of binding of Mdm2 to regulation of p53 stability. Small-molecule inhibitors named nutlins bind to the p53-binding pocket on Mdm2 and effectively prevent the interaction, resulting in stabilization and activation of the p53 pathway (282).

The C-terminus of p53 also plays an important role in regulating the interaction. Tetramerization of p53 is necessary for the efficient Mdm2 binding and degradation, as has been demonstrated by deletion studies (158).

In addition to the N-terminus and C-terminus, the DNA binding domain of p53 provides a secondary binding site for Mdm2, involving its acidic domain (264, 265). In agreement with this, structural study has shown that the acidic domain and part of the zinc finger domain of Mdm2 are involved in the interaction with the core domain of p53 (266). This second interaction has been shown to contribute to efficient ubiquitination. The current model suggests that the N-terminal interaction between p53 and Mdm2 induces a conformational change in Mdm2 and leads to the interaction of the acidic domain of Mdm2 with the core domain on p53 (265).

In response to different types of stress p53 is stabilized and activated due to several mechanisms, including posttranslational modifications and interacting partners. Most of these mechanisms affect Mdm2-mediated proteasomal degradation of p53 (161).

1.4.3.3 Posttranslational modifications involved in regulation of p53 stability.

In response to stresses p53 is phosphorylated and acetylated predominantly at its N-terminus and C-terminus, which lead to accumulation and activation of p53 (283). The most characterized modifications of p53, which can regulate p53 stability, are summarized on Figure 1.8. p53 is phosphorylated in response to DNA damage and other stresses on numerous sites: S6, S9, S15, T18, S20, S33, S37, S46, T81 in the N-terminal domain of p53; S315 and S392 in C-terminus; and T150, T155 and S149 in the DBD. In addition, residues T55, S376 and S378 are constitutively phosphorylated in unstressed cells (283). Mdm2 has also been reported to undergo phosphorylations in response to DNA damage, such as Ser395 by ATM kinase (284) and Y394 by c-Abl (285), which impair its ability to degrade p53. Akt phosphorylates Mdm2 on residues 166 and 186, which promote nuclear import of Mdm2 resulting in increase in ubiquitination of p53 (286).
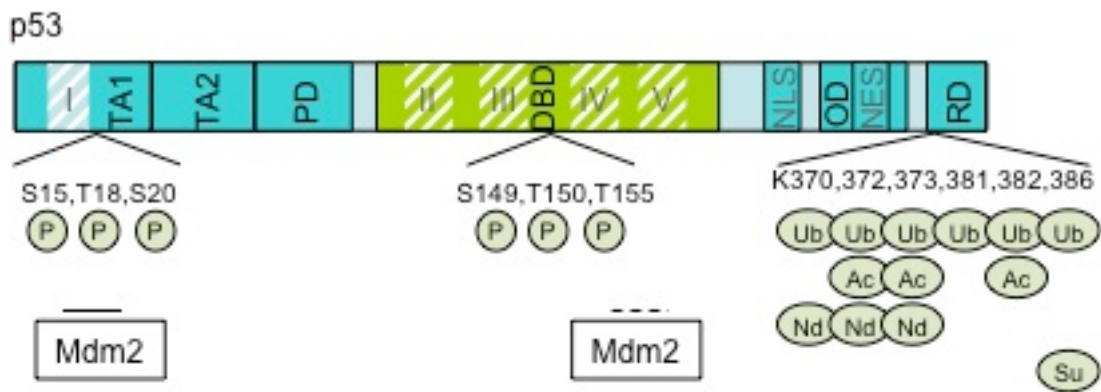
FIGURE. 1.8. Modifications of p53 regulating its stability.
The schematic organization of p53 and the Mdm2 binding sites are shown. The best described phosphorylations of the N-terminus and of the DBD of p53 and modifications of the C-terminal lysines indicated.
p-phosphorylation; ac – acetylation; ub – ubiquitination; nd – neddylation; su – sumoylation.

N-terminal phosphorylations have been suggested to stabilize p53 by inhibiting the interaction with Mdm2 (111, 112). Upon DNA damage, kinases ATM and Chk2 phosphorylate p53 on S15 and S20 respectively (110, 287, 288) (113-115), whereas ATR and Chk1 phosphorylate p53 at S15 and S37 (109, 115, 289). The biological significance of N-terminal phosphorylations sites on p53, which have been best characterized in cells systems, has been addressed in mouse models. p53 knock-in mice with mutation S18A and S23A (equivalent to S15 and S20 in human) have been generated which would be predicted to have increase in binding of Mdm2 to p53 and impaired stabilization and transactivation if p53. However, the knock-in mice with single mutations do not have a gross phenotype and p53 stability and response are not largely affected (290, 291). Knock-in mice with mutations in both residues have mild alterations in p53 stability and transactivation capability (292). They also develop tumours, however after long latency (>1 year). These observations suggest that although S15 and S20 partially contribute to regulating the interaction, other mechanisms might be involved. There is a significant redundancy in that some sites are phosphorylated by different kinases, and one kinase can phosphorylate several residues on p53. This could potentially explain the lack of pronounced phenotypes in mice. For example, several kinases can phosphorylate Ser15, and Chk2 can phosphorylate several different residues (283). In support of the functional redundancy, mutations of these sites are very rare in cancers. The variety of phosphorylation sites and kinases suggests that it could be the way of regulating the choice

of p53 response to different stimuli. Other phosphorylations in the N-terminus lead to accumulation of p53 possibly by modulating the interaction with local alterations of the conformation. Their physiological relevance remains to be elucidated.

Three phosphorylation sites (S33, T81 and S315) are followed by proline sites, which are subject to regulation by Pin1 (293, 294). Phosphorylation of these sites in response to certain types of stress promotes interaction with Pin1, which isomerises prolines, and is required for full activation of p53. Loss of Pin1 impairs p53 response to DNA damage. The mechanism is not entirely clear but it is proposed that conformational change on p53 induced by Pin1 reduces the interaction with Mdm2, leading to stabilization of p53 and also increases transcriptional activity of p53 (293, 294). In agreement with this, p53 lacking proline-rich domain (PD), which removes one Pin1-binding site, is hypersensitive to Mdm2-mediated degradation (295). The study in which mice lacking murine PD were generated, has confirmed the role of PD in regulation of stability of p53. Contradictory to *in vitro* data, however, p53ΔP fails to induce cell cycle arrest, but can promote apoptosis (296).

Modifications of p53 in the DBD are much less well characterized but also have been shown to affect the stability of p53. In contrast to N-terminal phosphorylations, which stabilize p53, phosphorylation of the DBD of p53 (S149, T150 and T155) targets it for degradation (297). This is mediated by COP9 signalosome kinase and occurs in unstressed cells, providing another way to restrain p53 activity. Some of the recently reported modifications also involve glycosylation of p53 on S149 in the DBD of p53, which leads to stabilization of p53 by blocking its ubiquitination and degradation. This modification prevents T155 phosphorylation (298).

Mdm2 binds to the N-terminus of p53 and ubiquitinates its C-terminal lysines (K370, K372, K373, K381, K382 and K386) (Fig.1.8), targeting p53 for proteasomal degradation. Mutation of these lysines (6KR) impairs the degradation of p53 by Mdm2 (299, 300) (301). However, some amount of Mdm2-dependent ubiquitination of p53-6KR has been noted, suggesting that other lysines on p53 can be targeted by Mdm2. In addition to ubiquitination C-terminal lysines on p53 are modified with ubiquitin-like molecules – Nedd8 and SUMO-1. Neddylation of p53 is promoted by Mdm2 on lysines K370, K372 and K373 (Fig.1.8) (223). In addition to Mdm2, a member of F-box family FBXO11 has been identified as a new p53-interacting protein, which can neddylate p53 on K320 and K321 (302). Neddylation inhibits transcriptional activity of p53, probably by preventing acetylation of these residues. Modification of p53 with another ubiquitin-like molecule SUMOylation of p53 occurs on K386, however its biological significance remains unclear as it has been shown to both positively and negatively regulate p53 function (226, 303-

305). Since these modifications affect the same lysines it is possible that they can inhibit or potentiate each other.

p53 stability and activity is also regulated by the acetylation on C-terminal lysines by p300/CBP and PCAF which activate p53-mediated transcription PCAF acetylates p53 at K320 and p300/CBP heterodimers are recruited to the N-terminus of p53 and acetylates p53 on C-terminal lysines (K370, K372, K373, K381, K382, K386) (54, 306-308). Acetylation of p53 is regulated by deacetylaton by HDAC-1 (histone deacetylase1)-containing complex or by Sir2a (silent information regulator 2α) (309, 310). The C-terminal lysines are also used for ubiquitination (299) and acetylation of these lysines has been proposed to compete with ubiquitination (311). Acetylation inhibits ubiquitination and stabilizes p53, whereas deacetylation of p53 results in its ubiquitination and degradation. Similarly to acetylation, methylation of lysine 370 by SET9 also leads to stabilization of p53 and upregulation of its target genes (312).

The physiological significance of the C-terminal lysines has recently been addressed in two studies which generated the knock-in mice with substitutions of 6 or 7 C-terminal lysines of p53 with arginines. Surprisingly, p53-7KR or p53-6KR mice develop normally and only have a mild phenotype, which proposes their role in fine-tuning the response of p53 (313, 314). This suggests the C-terminal lysines are not required for regulation of the stability of p53 and other lysines can be the targets of ubiquitination. Consistent with this, significant amount of Mdm2-dependent ubiquitination of p53-6KR has been detected (299), suggesting that other lysines can be used when the C-terminal lysines are not available. It is possible to envision that ubiquitination is quite promiscuous and if the primary lysines are removed, ubiquitin ligase moves to the other lysines. In fact, p53 protein contains 20 different lysines, which can potentially be used for the ubiquitin conjugation.

1.4.3.4 Regulation of p53 stability by proteins interacting with p53 and Mdm2.

In addition to modifications, p53 and Mdm2 interacting partners can regulate Mdm2-mediated ubiquitination and degradation of p53 and are summarized on Table 1.1. Several factors appear to contribute to efficient ubiquitination and degradation of p53 by Mdm2. p300, a transcriptional co-activator, has been suggested to act as E4 in Mdm2-mediated ubiquitination of p53. In *in vitro* system, when Mdm2 can only monoubiquitinate p53, p300 acting together with Mdm2 can further polyubiquitinate p53. In cells, p300 has been shown to play an important role in p53 turnover (315). Several other proteins promote ubiquitination by binding to Mdm2 and/or p53, such as: YY1 (316, 317), gankyrin (318) and MTBP (319) (Table 1.1A). Gankyrin, an ankyrin repeat protein, interacts with Mdm2

and enhances the p53-mediated ubiquitination and degradation (316, 317). The transcription factor YY1 binds to both p53 and Mdm2 and facilitates their interaction, promoting the ubiquitination of p53 (316, 317). Consistent with their role as negative regulators of p53, gankyrin is often overexpressed in hepatocellular carcinomas (320), whereas YY1 has been found to be overexpressed in several types of tumours (321). These studies suggest that factors such as YY1 and gankyrin promote Mdm2-mediated ubiquitination by facilitating the binding of p53 and Mdm2. Another co-factor of ubiquitination MTBP was identified in a yeast-two-hybrid screen as an Mdm2-binding protein and promotes ubiquitination of p53 by Mdm2 in a different way. It interacts with Mdm2 and inhibits autoubiquitination of Mdm2, resulting in stabilization of Mdm2. This results in increased ubiquitination and degradation of p53 (319). However, the early embryonic lethality phenotype of inactivation of MTBP is not rescued by the simultaneous loss of p53 leaving the physiological relevance of the MTBP unclear (322). It is possible that these co-factors play an important role when Mdm2 levels are less abundant, and their physiological relevance remains to be elucidated.

Table 1.1 Proteins interacting with Mdm2/p53 and affecting ubiquitination.

A. Binding partners which promote ubiquitination.

Protein	Description	Binding	effect	ref
gankyrin	Ankyrin repeat protein	Binds to Mdm2 and facilitates p53/Mdm2 binding	Enhances ubiquitination and degradation of p53	(318)
YY1 (Yin Yang1)	Transcription factor	Binds to Mdm2 and p53	Enhances ubiquitination and degradation of p53	(316, 317)
P300	Transcriptional coactivator	Binds Mdm2 and p53	E4, polyubiquitinates p53	(315)
MTBP	Mdm2 binding protein	Binds to Mdm2	Enhances ubiquitination and degradation of p53, but not Mdm2	(319)

B. Binding partners which inhibit ubiquitination.

Protein	Description	Binding	effect	ref
ARF	Alternative reading frame product of ink4a locus	Interacts with Mdm2	Inhibits E3 activity of Mdm2, leads to stabilization of p53	(121)
L11, L5, L23	Ribosomal proteins	Interact with Mdm2	Inhibits E3 activity of Mdm2, leads to stabilization of p53	(137, 263, 323)
Numb	Notch antagonizer	Binds Mdm2	Inhibits	(324, 325)
TSG101	tumour susceptibility gene	Binds Mdm2 and p53	Inhibits autoubiquitination, downregulates p53	(326)

In addition to ARF and ribosomal proteins mentioned above, other proteins inhibit Mdm2-mediated ubiquitination and lead to stabilization and activation of p53 (Table 1.1B). Recent report shows that the Numb protein, Notch antagonizer, which plays a role in cell fate and differentiation, can enter a tricomplex with p53 and Mdm2 and inhibit ubiquitination of p53. Numb seems to play an important role in tumour development, as it is frequently lost in breast cancers leading to inactivation of p53 pathway (324). It is worth mentioning another regulator of Mdm2 - TSG101 (tumour susceptibility gene). TSG101 physically interacts with both p53 and Mdm2 and leads to inhibition of autoubiquitination of Mdm2, elevating its protein levels. This is associated with the downregulation of p53 protein (326), suggesting that TSG101 does not inhibit E3 activity of Mdm2 towards p53. tsg101 knock-out mice embryos die at day 6.5 which is partly due to accumulation of p53 (327). However, inactivation of TSG101 results in transformation of NIH 3T3 cells and their ability to generate metastatic tumours in nude mice (326), which is inconsistent with the negative role of TSG101 in regulation of p53 and the reason is not clear. The existence of multiple positive and negative regulators might provide the way to differentially regulate p53/Mdm2 pathway in response to various stresses and cellular context.

In addition to its ubiquitin ligase activity, Mdm2 has been suggested to play a role at the post-ubiquitination step. Acidic domain of Mdm2 might play a role in this function, as some acidic domain mutants of Mdm2 retain the ubiquitin ligase activity, but fail to degrade p53 (328-330). The details of such function of Mdm2 are not clear and could involve the interaction with hHR23A (Rad23) protein. However, there is a certain discrepancy in the role of hHR23 in p53 degradation, with one report suggesting that hHR23A blocks the entry of p53 to the proteasome (330) and the other showing that hRH23 delivers p53 to the proteasome and promotes its degradation (331).

The important role of Mdm2 in antagonizing p53 is reflected in its frequent overexpression in human cancers. Mdm2 gene is amplified in about 7% of human tumours, most frequently in sarcomas (30-40%) and is overexpressed in leukemias (332-336). In a subset of tumours the overexpression of Mdm2 is mutually exclusive to inactivation of p53, suggesting it can functionally substitute for loss of p53 function.

A more recent study suggests that single nucleotide polymorphism within the Mdm2 promoter can lead to increased susceptibility to cancer in population. This study shows that SNP309 in Mdm2 promoter can lead to increased affinity for the transcription factor SP1, which results in higher levels of Mdm2 mRNA and protein. This can result in attenuation of p53 pathway and is associated with an increased cancer incidence (337, 338).

p53/Mdm2 pathway can also be regulated by deubiquitination. The deubiquitinase HAUSP (Herpes virus-associated ubiquitin-specific protease) was originally shown to deubiquitinate p53 *in vitro* and *in vivo* and stabilize p53 protein (215). However, another report has demonstrated that disruption of HAUSP leads to p53 accumulation (214, 339). Later it has been realized that the interplay between p53, Mdm2 and HAUSP is complex, as the primary target of HAUSP is Mdm2, and HAUSP deubiquitinates Mdm2 *in vitro* and *in vivo* (340). This seems to be required for maintaining a sufficient level of Mdm2 to act as a ubiquitin ligase on p53. In addition to HAUSP, another deubiquitinase of Mdm2 Usp2a play an important role in regulation of stability of p53. Usp2a has been identified in bacterial two-hybrid screen. Usp2a selectively deubiquitinates Mdm2 in cells, but does not have deubiquitinating activity towards p53. Downregulation of Usp2a destabilises Mdm2 and leads to accumulation of p53, whereas overexpression of Usp2a stabilises Mdm2, which degrades p53 (341). Deubiquitinating p53 can provide a quick way for stabilizing p53 in response to stress, however a p53-specific deubiquitinase is yet to be found.

1.4.4 Other ubiquitin ligases.

Mdmx is another important regulator of p53, structurally related to Mdm2, and has been identified as a product of extrachromosomal amplification similarly to Mdm2 (342). Mdmx shows significant homology to Mdm2, including the N-terminal p53-binding domain and C-terminal RING finger domain (343). Despite similarity between RING domains Mdmx does not possess the intrinsic ubiquitin ligase activity. Mdmx interacts with the N-terminus of p53 similarly to Mdm2 (344), however fails to ubiquitinate p53 and does not target p53 for degradation (345, 346). Mdm2 and Mdmx heterodimerize with each other (347) and Mdmx can cooperate with Mdm2, contributing to its ubiquitin ligase activity (270) when overexpressed in cells. At physiological levels Mdmx has been shown to play an important role in negative regulation of p53 activity. This has been demonstrated by the rescue of embryonic lethal phenotype of Mdmx-/- mice by simultaneous inactivation of p53 (348-350). These studies have suggested that Mdm2 can not compensate for the loss of Mdmx and they might have distinct roles in regulation of p53. The recent study of roles of Mdm2 and Mdmx done with mice with conditional p53 expression suggests that Mdmx inhibits the transcriptional activity of p53 independently of Mdm2, whereas Mdm2 is required for regulation p53 protein levels (351, 352). Loss of Mdmx does no lead to accumulation of p53, suggesting that Mdmx does not regulate p53 stability (351, 352). However, the effect of Mdmx on downregulation of p53 protein can be

masked by Mdm2-mediated degradation due to an increase in transcriptional activity of p53. Further analysis is needed to resolve the role of Mdmx in regulation of p53 stability.

Other E3 ligases which can ubiquitinate p53 independently of Mdm2 have been reported. Their number is constantly growing and the currently known p53-specific ubiquitin ligases are summarized in Table 1.2. The first discovered ubiquitin ligase acting on p53 was E6-AP (E6-associated protein). E6-AP ubiquitinates and degrades p53 in cells infected with oncogenic HPV (human papilloma virus) where it forms complex with the viral protein E6 (353). Other ubiquitin ligases have also been discovered (Table 1.2). These include Pirh2 (84), Cop1 (83), TOPORS (178, 354), ARF-BP1 (185),

Table 1.2. p53 ubiquitin ligases.

Ubiquitin ligase	Class	function	reference
Mdm2	RING	Mono and polyubiquitination, degradation, nuclear export	(175)
Mdmx	RING	Does not ubiquitinate p53, inhibits transcriptional activity of p53	(345, 346)
Pirh2	RING	Ubiquitination and degradation	(84)
Cop1	RING	Ubiquitination and degradation	(83)
ARF-BP1/ HectH9/ MULE	HECT	Ubiquitination and degradation.	(185)
E6-AP	HECT	Ubiquitination and degradation	(181)
CHIP	U-box	Ubiquitination and degradation	(355)
Cullin7	RING	Mono- and di-ubiquitination; reduces transactivation of p53	(356)
E4F1	atypical	K48 ubiquitination, modulates p53 activity to induce growth arrest	(101)
Synoviolin	RING	Ubiquitination and degradation, sequesters in the cytoplasm	(357)
WWP1	HECT	Ubiquitination, accumulation of p53 in the cytoplasm	(358)
Topors	RING	Ubiquitination and degradation	(178)
Carps	RING	Ubiquitination and degradation	(359)

Synoviolin (360), Carps (361) and CHIP (C-terminal of Hsp70-interacting protein) (355), WWP1 (358), E4F1 (101) and Cullin 7 (356). They belong to different classes of ubiquitin ligases and all can ubiquitinate p53 independently of Mdm2. Similarly to Mdm2, Pirh2 and Cop1 are p53 target genes and both belong to the RING family of ubiquitin ligases (83, 84). ARF-BP1/HectH9/Mule, a HECT-domain ubiquitin ligase, was purified as a major ARF-binding protein from p53-null cells. However, ARF-BP1 has been shown to bind and ubiquitinate p53 (185). ARF interacts with ARF-BP1 and inhibits its ubiquitin ligase

activity, suggesting it could control p53 in response to oncogenes activation. Synoviolin and CHIP are ER-associated ubiquitin ligases. Synoviolin ubiquitinates p53 and sequesters it in the cytoplasm and could be involved in response to ER stress (357). CHIP can ubiquitinate and degrade p53 bound by the chaperones Hsp70 and Hsp90, suggesting it degrades the misfolded p53 protein (355, 362). Most of these ubiquitin ligases affect the stability of p53, with the exception of E4F1, which changes the transcriptional activity of p53 without affecting its proteolysis (101). TOPORS has been reported to conjugate both ubiquitin and SUMO to p53, however, the biological output of this is not clear (222). Some of the ubiquitin ligases as CHIP, Synoviolin, Cullin-7 reside in the cytoplasm, suggesting they contribute more to regulation of cytoplasmic p53. p53 has also been shown to undergo K63-linked ubiquitination mediated by an E2 ubiquitin-conjugating enzyme Ubc13, which increases p53 stability and leads to its cytoplasmic localization (363). Other ubiquitin ligases and their function are much less well characterized and are listed in the table. A variety of ubiquitin ligases targetting p53 may be necessary to specifically regulate p53 in response to different types of stresses, however their biological significance needs to be validated.

1.4.5 Ubiquitin-independent mechanism of p53 degradation.

In addition to ubiquitination-mediated degradation of p53 in 26S proteasome, p53 can also be degraded directly by 20S subunit of the proteasome, which does not require its prior ubiquitination (242). Several other proteins have been shown to be degraded via the the ubiquitin-independent pathway (241, 243, 364). This mechanism is regulated by NQO1 (NAD(P)H quinone oxidoreductase 1) which is associated with 20S proteasome, where it is proposed to act as a gate-keeper. NQO1 has been shown to interact with p53, which prevents the degradation of p53 by 20S. DNA damage signals increase the association of p53 with NQO1, which can contribute to stabilization of p53 (242, 365). However, it seems that ubiquitin-independent mechanisms plays a minor role in p53 stabilization in cells, as p53 is stabilized in the absence of Mdm2 during embryogenesis and in cells, when binding of Mdm2 to p53 is prevented.

1.4.6 Regulation of p53 localization.

In addition to degradation, p53 activity can be modulated by regulation of its localization (366). It appears that p53 shuttles during the cell cycle locating to the nucleus during G1 and G1/S and to the cytoplasm in the S phase (367). p53 is known to be

regulated by nuclear import and nuclear export mechanisms (368). There are three NLSs in the C-terminus of p53, of which the most active one is the bipartite NLS, which consists of two basic motifs K305-306 and K316-322 and a spacer between them (369). p53 contains two CRM-1 dependent nuclear export signals (NES) in the N-terminus and in the C-terminus (11-27aa, 340-351aa respectively) (157, 370). Several studies suggest that nuclear export of p53 is mediated mainly via c-NES (157, 224, 371). c-NES resides within the OD of p53 and is masked when p53 is a tetramer. Ubiquitination of C-terminus of p53 results in exposure of c-NES of p53, leading to its relocation to the cytoplasm (157, 224, 371). When Mdm2 levels are low, Mdm2 monoubiquitinates p53 and leads to its nuclear export (251, 372-374). The current model suggests that ubiquitination of p53 by Mdm2 promotes further modifications of p53 by other E3, such as PIASγ, which leads to release of Mdm2 and nuclear export of p53 (224). In addition, p53 can be exported in Mdm2-independent manner as demonstrated by a p53 mutant, which cannot interact with Mdm2 but undergoes nucleocytoplasmic shuttling (157).

p53 has been also reported to be sequestered in the cytoplasm by interaction with other proteins. Parc – Parkin-like ubiquitin ligase – forms a stable 1 MDa complex with p53 in the cytoplasm (375). Parc has an intrinsic ubiquitin ligase activity, however it fails to ubiquitinate p53. Parc binds to the C-terminus of p53 and sequesters p53 in the cytoplasm, by masking p53 NLS and therefore blocking p53 nuclear import (375). Another ubiquitin ligase WWP1 has been reported to ubiquitinate p53 and increase its cytoplasmic localization, leading to inactivation of p53 (358). Another report suggests that E2-conjugating enzyme Ubc13 can promote ubiquitination and cytoplasmic localization of p53 (363). Interaction with an Hsp70 family member, Mot2, can also tether p53 in the cytoplasm and inhibit its nuclear import (376). Relocation of p53 to the cytoplasm can represent the means of inhibiting transcriptional activity of p53 by keeping it away from its target genes or by degrading in cytoplasmic proteasomes. It may also promote the cytoplasmic function of p53 in inducing apoptosis, which is still a subject of extensive investigation (377-379).

Many neoplasms display cytoplasmic wild type p53 suggesting it is the way of inactivation of p53 function (380-384). In neuroblastomas p53 is virtually exclusively wild type and is sequestered in the cytoplasm (382). It has been found that p53 translocates to the cytoplasm due to enhanced nuclear export and is sequestered in the cytoplasm by binding to Parc in cell lines derived from neuroblastomas. Inactivation of Parc can restore nuclear localization of p53 and cause p53-dependent apoptosis in neuroblastoma cell lines (375).

1.5 p53-like proteins.

1.5.1 p53 isoforms.

The human p53 gene is composed of 19, 200 bp, spanning over 11 exons on chromosome 17p13.1 (NC_000017). Until recently only 3 mRNA splice variants of p53 have been known, which encode full-length p53, p53i9 (385) and p53Δ40 (386, 387). p53i9 results from alternative splicing at exon 9 and encodes a protein truncated of the last 60 amino acids, which is defective in transcriptional activity. p53Δ40 (other names p47 and ΔNp53) protein is truncated of the first 40 amino acids and can be generated by two mechanisms: either by an alternative splicing of the intron 2 (387) or by alternative initiation of translation (386). p53Δ40 contains the second transactivation domain and is capable of activating some of the p53 target genes. Interestingly, it can also inhibit
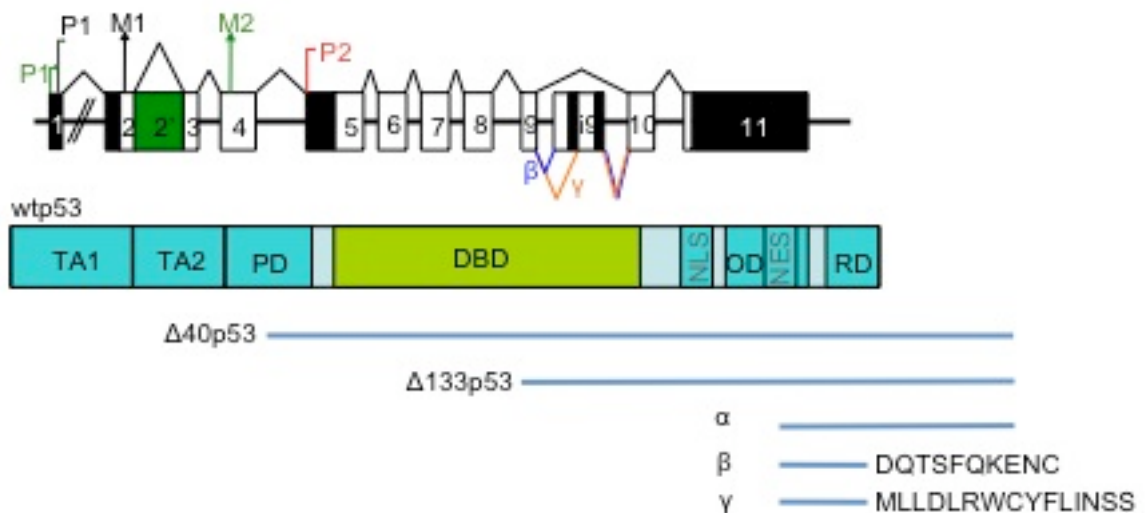
FIGURE 1.9. Human p53 gene.
The structure of p53 gene and the alternatively spliced p53 isoforms are depicted. (Adopted from Murray-Zmijewski, F., D. P. Lane, et al. (2006). Cell Death Differ 13(6): 962-72).

transcriptional activity of the full-length p53 in a dominant-negative way (387). A recent study reports that the structure of the p53 gene is much more complex than previously thought and many more p53 isoforms exist (388). The structure of p53 gene and the currently known p53 isoforms are summarized on Figure 1.9. The p53 gene is transcribed from two distinct sites upstream of exon 1 and from an internal promoter located in intron 4. The alternative promoter leads to the expression of an N-terminally truncated p53 (Δ133p53), which lacks the entire TA domain and part of the DNA binding domain. Usage of alternative promoter in intron 4 gives rise to Δ40p53 with truncation of N-terminal

transactivation domain. In addition alternative splicing at intron 9 gives rise to α, β and γ isoforms. Therefore at least 9 different isoforms of p53 can be generated. These p53 isoforms have been shown to be expressed at both mRNA and protein levels. It was found that p53 isoforms have different subcellular localisations, suggesting the possibility of differential regulation of their activities. p53 isoforms, like p63 or p73 isoforms, have distinct transactivation functions. p53β binds preferentially to the p53-responsive promoters p21 and Bax, rather than Mdm2, and can specifically enhance the transcriptional activity of full-length p53 at Bax promoter in response to cellular stress. This leads to an interesting interplay between p53 isoforms where p53-mediated apoptosis is increased by p53β, which can be inhibited by the action of Δ133p53 (388). Many of the p53 isoforms are differentially expressed in normal human tissues, which explain the mechanism of the tissue-specific regulation of p53 transcriptional activity. This suggests that deregulation of p53 isoforms could be another way to down-regulate p53 function in cancers. Indeed, head and neck and some breast tumours show abnormal expression of p53 isoforms (389). Transactivation-capable isoform p53β is lost, whereas dominant-negative Δ133p53 isoform is overexpressed frequently in breast tumours, consistent with their tumour-suppressive and oncogenic functions respectively. The differential expression of p53 isoforms can also alter drug sensitivity of some human cancers which retain wild type p53 (141, 389).

1.5.2   p53 family members.

p53 family members p63 (390) and p73 (391) show significant structural and sequential homology to p53, and are even more similar to each other. Sequence comparison suggests that p63 and p73 are evolutionary more ancient, as the p53-like protein in lower species is more similar to p63 and p73. Both proteins retain some of the p53 functions, but also play distinct roles during development. p63 and p73 contain three functional domains with significant amino acid sequence homology to those of p53: DNA binding domain (65% homology) with most of the structural and contact residues conserved, N-terminal domain (25%), and oligomerization domain (35%). p63 and p73 also have a unique sterile alpha motif (SAM) domain within their C-termini, which is involved in protein-protein interactions (392-394). Similarly to p53, p63 and p73 can be upregulated by oncogenes and DNA damage and both function as transcription factors and can upregulate many of the p53 target genes and induce growth arrest and apoptosis (102, 395-399). There are some target genes, which are differentially regulated by p53 family members (400, 401).

However, unlike p53, both p63 and p73 are necessary for normal development. Original studies have shown that p53-null mice are viable and do not have any gross developmental abnormalities (45, 46), although small developmental defects have been noticed. A fraction of female p53-null embryos show exencephaly due to the failure of the closure of the neural tube (402) and some p53-null animals have a reduced fertility (403). p73-null mice suffer from different neurological abnormalities, such as hydrocephalus and hippocampus dysgenesis, and inflammatory defects and die within the first two months (404). p63 knock-out mice have the most severe phenotype: they are born alive but do not survive beyond a few days postnatally due to severe abnormalities in development (390). Their limbs are truncated or absent and they also display craniofacial malformations. The skin of p63-null mice does not progress past an early developmental stage and lacks stratification. This severe phenotype suggests p63 plays an essential role in ectodermal differentiation during early embryogenesis (390).

In all studied adult tissues p73 is expressed at very low levels with differential expression of some isoforms. The expression of p63 is restricted to the nuclei of basal cells of normal epithelia (skin, prostate, esophagus) and also to some populations of basal cells of glandular structure (prostate, breast, bronchi) (394).

Both p63 and p73 are expressed as multiple isoforms due to usage of different promoters and alternative splicing (391, 405). The two promoters give rise to full-length transcriptionally active TA isoforms and N-terminally truncated ΔN isoforms, which act as dominant negative inhibitors of all full-length members. Both p63 and p73 undergo additional splicing at the C-terminus, which generates at least 9 forms of p73 (α, β, γ, δ, ε, ζ, η, η₁, φ) and 3 for p63 (α, β, γ). Structurally, γ isoforms are the most similar to p53, harbouring a small C-terminal extension beyond the last 30 amino acids stretch of p53. ΔNp73 seems to be the most highly expressed isoform in the brain, suggesting differential expression of isoforms in tissues (394, 406).

N-terminally truncated (ΔN) isoforms of p63 and p73, which are also present in all possible C-terminal variations, lack the TA domain and are incapable of inducing gene expression (406, 407). However, ΔN isoforms oligomerize with TA isoforms of p63 and p73, and inhibit their transactivation ability in a dominant-negative effect, promoting cell survival. In addition, ΔNp73 inhibits p53 transactivation ability. This seems to occur via competition for the same DNA binding sites (408). Interestingly, p53 and TAp73 can induce the expression of ΔNp73 through p53-responsive element in its promoter, in a negative feedback loop (409, 410).

Despite having functional similarities to p53, p63 and p73 are rarely mutated in cancers (<1%) (392, 393). Their role in cancers appears to be more complicated due to existence of TA and ΔN isoforms which can have opposing effects on tumour progression. The current knowledge suggests that TA isoforms can play a tumour-suppressive role, whereas ΔN isoforms can be oncogenic by inhibiting TA isoforms (411, 412). p63 gene maps to chromosome 3q27-28 region, which is frequently amplified in squamous cell carcinomas (413), though it is not clear whether it is the targeted gene driving the amplification in this locus. Numerous studies have shown that p63 is overexpressed in nearly 80% of primary head and neck squamous carcinomas and in some other types of epithelial cancers due to genomic amplification or other mechanisms (392). The early studies of p63 expression did not discriminate between the different isoforms. Recent approach using the isoform-specific RT-PCR has shown that TAp63 mRNA overexpression is a rare event and ΔNp63 is the predominant form in squamous cell carcinomas. However, at later stages of tumour development the p63 expression seems to be unfavourable and is often lost with progression to invasion and metastasis, which correlates with poor prognosis (414).

Like p63, p73 has been supposed to play a tumour suppressor role, based on its relation to p53 and the fact that its cytogenetic locus 1p36.33 is commonly deleted in a variety of cancers (391). Overexpression of p73 mRNA and/or protein has been shown in a large variety of tumour types, suggesting oncogenic role for p73. The only exception is lymphoid malignancies, where p73 is often silenced due to hypermethylation (392). Recent studies, which analyzed isoform-specific expression of p73, have found that both ΔN and TA-isoforms are upregulated in tumours compared to normal tissues (412, 415). However, the exact functions of overexpressed p63 and p73 isoforms in cancers and the extent of their contribution to tumour development are still unclear.

In addition to their regulation at transcriptional level, p63 and p73 are subjected to regulation at the level of protein stability. Like p53, p73 protein turnover is mediated by proteasomal degradation, as shown by stabilization of p73 isoforms by the proteasome inhibitors (416). All three p53 residues essential for binding to Mdm2 (F19, W23 and L26) are conserved on both TAp63 and TAp73. Mdm2 binds to N-terminus of TAp73, however it fails to ubiquitinate or degrade it (416-418). This may be due to the absence of C-terminal lysines on p73, which are preferentially targeted by Mdm2 on p53. Motif-swapping experiments have also shown that region of 93-112aa of p53 which is not conserved in p73 could be the reason for resistance to degradation by Mdm2 (419). However, the interaction with Mdm2 inhibits the transcriptional activity of p73 (416-418).

Other ubiquitin ligases have been found to interact with p63 and p73. NEDL2 (NEDD4-related protein), a HECT-domain ubiquitin ligase, has been identified as a protein interacting with the C-terminal proline-rich region of p73. NEDL2 efficiently ubiquitinates p73, although this leads to stabilization of p73, rather than proteolysis, and enhances the p73-dependent transcriptional activation (420). Another NEDD4-related HECT-domain ubiquitin ligase Itch has been shown to regulate the protein degradation of both p63 and p73 (421, 422). Itch interacts with the proline-rich domains of TA and ΔN isoforms of p63 and p73, ubiquitinates and targets them for degradation, however does not degrade p53. Itch regulates p63 and p73 protein levels both in normal and in stress conditions. p73α but not p73β can be sumoylated on lysine 627 and this has been shown to potentiate its proteasomal degradation (423). This modification seems to be specific for p73α and not other splice variants, suggesting possible differential regulation of the isoforms.

It is emerging that p63 and p73 have an important role in cancer development, however their contribution is complex due to existence of TA and ΔN isoforms with opposing functions. Generation of isoforms-specific knock-out mice would help to understand the functions of p63 and p73 in cancer. Recently the mice knock-out for TA isoforms of p73 have been generated (424). The mice have an intermediate phenotype between the p73-/- and p53-/- mice. Similarly to p73-/- mice TAp73-/- mice are infertile. In addition, TAp73-/- mice have mild defects in brain morphology. Interestingly, 30% of TAp73+/- and 70% of TAp73-/- mice spontaneously develop tumors, suggesting that TAp73 isoforms play tumor-suppressive role. Interestingly, TAp73-/- mice develop different spectra of tumors compared to p53-/-mice, suggesting differences in the roles of p73 and p53 in tumor suppression (424).

1.6 Mutant p53.

1.6.1 Mutations of p53 in cancer.

1.6.1.1 Li-Fraumeni syndrome.

Li-Fraumeni syndrome (LFS) is a rare inherited cancer predisposition syndrome, affecting individuals before the age of 45 years. Unlike other inherited cancer syndromes, LFS is characterized by a variety of different cancers, predominantly sarcomas, breast cancers, brain tumours and adrenocortical carcinomas, though other cancers have also been reported. LFS is dominantly-inherited and is associated with high mortality. Analysis of the LFS families has shown that around 70% of these families have a germline mutation in

the p53 gene. Li-Fraumeni-like syndrome (LFL) describes a similar syndrome, which does not have all features of the classical LFS and similarly has been found to have germ-line mutations in p53 gene (44, 425, 426). In a few cases where no germline mutation in p53 has been described, the mutation in Chk2 has been reported (427, 428). From the database information it is revealed that most of the p53 mutations are missense mutations (72%) and some are deletions (10%). About 46% of the mutations were located at the codons 175, 213, 245, 248, 273 and 282 in the DBD of p53, which correspond to hotspot mutations in sporadic cancers (26). It should be noted however that most studies analyzed the mutations in exons 5-8 and therefore were biased to identify the mutations in the DNA binding domain. There is some correlation between the type of the mutation and the type of tumour. Moreover, the mutations within the DNA binding domain of p53 have generally more cancers and at younger ages than families with null mutations, suggesting a functional importance of type of the mutation in tumour progression in humans (27).

1.6.1.2 p53 in sporadic cancers.

As already mentioned, p53 gene is found mutated in nearly half of all human cancers analyzed. In many other types of cancers p53 pathway is inactivated by other ways, such as inactivation of ARF or overexpression of Mdm2. Unlike most of the tumour-suppressor genes, more than 80% of the p53 alterations are missense mutations which lead to generation of full-length p53 with single amino acid substitution (26, 429, 430). The initial observations, which showed that p53 mutations are a frequent event in many tumour types, were made some twenty years ago (431-433). Those studies demonstrated that most of the mutations are localized in the exons 5-8, which lead to a single amino acid substitution of the DNA binding domain. Therefore most of the later studies (40% of all) have focused on the characterization of these mutations, which mostly affect the DNA binding domain. The database of p53 mutations has been updated and includes the analysis of some of the recent studies have found that mutations also occur outside exons 5-8 (about 10%) (Fig.1.10) (26, 434). The biochemical and functional consequences of the mutations can be described as heterogeneous, but most of the frequently found mutations inactivate the tumour-suppressive p53 function and confer novel oncogenic properties upon mutant p53.

1.6.1.3 Structure of p53 mutations.

The current version of the TP53 mutation database reports about 24000 different mutations most of which occur as single amino acid substitutions in the DNA binding domain of p53 (http://www-p53.iarc.fr). Most of the mutations locate within the highly

evolutionary conserved regions of the DBD of p53 (boxes II-V). The hotspots of mutations are codons 175, 245, 248, 249, 273 and 282 and account for about 30% of all p53 mutations (Fig.1.10) (26, 429). The core domain of p53 is highly flexible and rapidly

CODON DISTRIBUTION / 20828 single base substitutions

(C) IARC TP53 Mutation Database, R12 release, October 2006
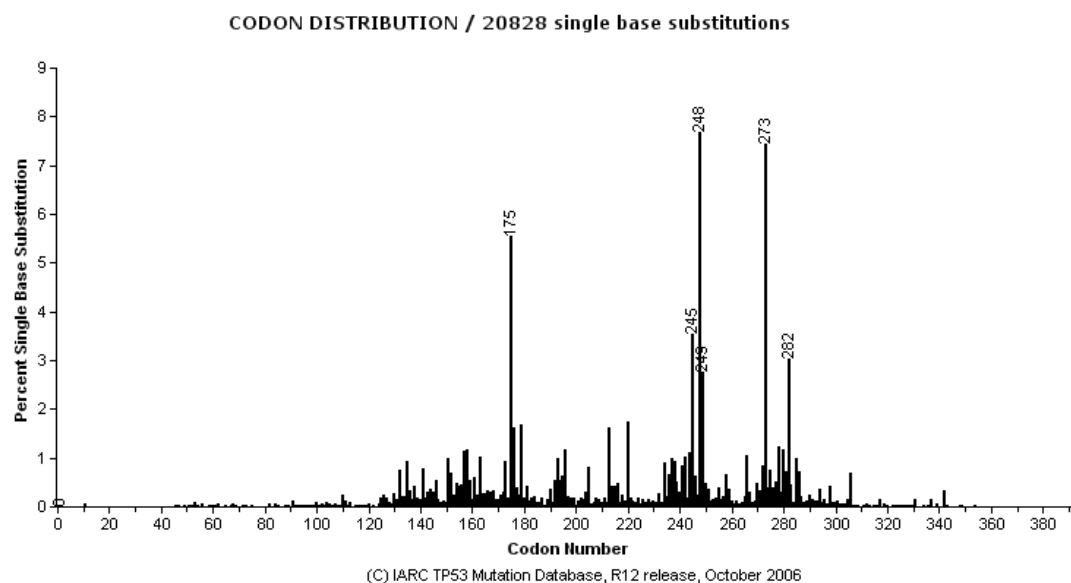
FIGURE 1.10. Mutational frequency of p53.
The frequency of the point mutations of each codon of p53 found in tumors (IARC database, R12 release, 2006, www-p53.iarc.fr).

changes between folded and unfolded states, which can explain such high concentration of the mutations found in this domain (152, 435). Each residue in the DBD has been found mutated, with frequency ranging from two to one thousand times. There are several structural changes conferred by mutations on the DBD of p53. Many mutations alter the conformation of the DBD to a various degree and can lead to its unfolding (147, 435, 436). The change was originally described based on the reactivity with the wild type and mutant conformation-specific antibodies, pAb1620 and pAb240 respectively. The pAb1620 antibody is specific for wild type conformation and does not bind to mutant p53 with altered structure of the DBD (437). This antibody recognizes a specific structural motif on the surface of the core domain, which involves residues R156, Leu206, R209 and Asn210 (438). The pAb240 antibody is directed against the cryptic epitope on the β-strand S7 (213-217aa) which becomes accessible only upon unfolding of the region and recognizes many of p53 mutants, which originally suggested the change in the conformation (439). However, some of the commonly found p53 mutants retain wild type conformation and are not recognized by the unfolded-conformation specific antibody. Structural studies have characterized most of the hot spot p53 mutants (154, 440-443). These structural studies have shown that mutations can either affect the residue which directly contacts the DNA without the effect on the conformation of DBD or can alter the overall structure of the

DBD (441, 442). Based on the reactivity with the conformation-specific antibodies and structural data, p53 mutants have been classified as either contact or structural mutants. Contact mutants usually have substitutions at residues R248 (to W or Q) and R273 (to C or H). These changes result in the loss of contact with the DNA and have only a minor effect on the thermodynamic stability of the protein. The structural changes are very localized and do not perturb the overall structure of the DNA binding domain (441). However, in some cases contact mutants can undergo some structural alterations and can exhibit the mutant-conformation specific epitope, and therefore are described as flexible (436). The structural mutations reduce thermodynamic stability of p53, causing it to unfold at body temperature both *in vitro* and *in vivo*. The L3 loop in the minor-groove-binding region is the site of the hotspot mutations G245S and R249S. G245S induces small conformational changes that weaken DNA binding. The R249S has more severe effect on conformation and results in substantial impairment of DNA binding and loss of stability (441). R175, located in the L2 loop is involved in the coordination of the zinc binding and is one of the most frequent sites of mutation, R175H. Although there is no structural data for this mutant yet, it has been proposed that mutation R175H directly affects the zinc binding and is highly destabilizing, based on data for other mutants (147, 444). Mutation R282W has a destabilizing effect, though causing only local changes in conformation, which allows R282W to bind some promoters at a subphysiological temperature (441). All these mutations of p53 result in significant loss of binding to its consensus site on DNA, which leads to an impaired transactivation ability.

The discovery of temperature-sensitive p53 mutants has suggested that p53 cancer mutants can be pharmacologically rescued by conformational alterations. For example, V143A mutant is in mutant conformation and therefore inactive at 37°C, however is in wild type conformation and exhibits transactivation ability at 32°C (445, 446). Many other temperature-sensitive p53 mutants with similar properties have been isolated (447), that show transactivation activity at 30°C. The majority of these mutations cluster in the β-sandwich region of DBD. This suggests that the conformation of the DBD of p53 mutants retains flexibility and can be folded as wild type.

1.6.1.4 p53 polymorphism.

In addition to mutations, a common polymorphism is found in the proline-rich domain of p53 (R72 or P72). The frequency of the p53P72 allele varies between populations and is higher in populations living near the equator, which suggests that P72 is beneficial in the environment with higher UV exposure (448). The study in cells

demonstrate that in fact R72 is more active in p53 tumour suppressor functions as assessed by the ability to induce apoptosis (449). In addition to DBD, the proline-rich region of p53 contributes to binding to the ASPP family members. Recent study, showing that the anti-apoptotic iASPP preferentially binds to and inhibits activity of p53P72 more efficiently than that of p53R72, which provides the mechanism for more potent induction of apoptosis by p53R72 (450).

1.6.2 Properties of mutant p53.

1.6.2.1 Interaction partners of p53 mutants.

Mutations in the DNA binding domain can affect the interactions with proteins binding to p53. It has been long noted that many conformational p53 mutants can associate with the molecular chaperones Hsc70, Hsp70 and Hsp90 (41, 439, 451-454). The binding sites to the Hsp70 have been mapped and involve the β-sheets of the hydrophobic core of the central DNA binding domain, where the majority of the mutations are found (452). Mutant p53 bound to heat shock proteins Hsp70 and Hsp90 can be recognized by the ubiquitin ligase CHIP, which leads to its degradation (355, 455). Wild type p53 has also been reported to interact with the molecular chaperones though only transiently, and can also be degraded by CHIP, suggesting that wild type p53 can be unfolded in cells (355).

ASPP family members bind to the core domain of p53 (103, 456). The crystal structure of the DNA binding domain of p53 and C-terminus of ASPP2 (53BP2) has been reported (104) and has shown that the binding site for ASPP2 overlaps the DNA-binding site. All six p53 hot spot mutants (175H, 245S, 248W, 248S, 273H, 282W) fail to bind to ASPP2 (104). The residues on p53 involved in direct contact with ASPP2 are R178, R181, M243 and N247 and are found mutated in cancer. Interestingly, p53 with mutation of the R181 (to L or K), found in cervical and breast tumours, retains the ability to induce cell-cycle arrest, but fails to promote apoptosis (457). These mutations also impair the binding of p53 to ASPP1 and ASPP2 (105).

Interestingly, p53 family members p63 and p73 can associate with hot spot p53 mutants both *in vitro* and *in vivo*, but not with wild type p53 (458-464). This interaction is direct and involves the corresponding core domains of each protein (460, 461, 464). There is a correlation between the unfolded conformation of the DBD of p53 mutant, and the ability to interact with p63/p73, based on the reactivity of p53 with the "mutant" conformation specific antibody (461). However, both conformational (R175H and G245W) and contact (R248H) p53 mutants have been shown to interact with p63/p73. The

ability of contact p53 mutants to interact with p63/p73 might reflect some structural distortions of DBD by contact mutations (436). Apart from the unfolded conformation of DBD, there are other structural requirements on mutant p53 for interaction. The polymorphism of mutant p53 at residue 72 P/R can affect the affinity of binding to p73 (459, 463). Mutant p53 with 72R associates with p73 more efficiently, than p53 72P variant. Though there is not much known yet, several studies suggest the importance of the interaction of mutant p53 with p63 and p73 in malignancy. Mutant p53 inhibits the transcriptional activity of p63/p73 and their ability to induce growth arrest and apoptosis, which has been proposed to contribute to its gain-of-function properties (458-464). This could be related to the direct binding of mutant p53 to the core domain of p63/p73, which might prevent their binding to DNA. Inhibition of p63/p73 by exogenous or endogenous mutant p53 has been suggested to confer chemoresistance in tumour cell lines, which correlates with the ability of mutant p53 to bind p63/p73 (462). In further support of the biological significance of the interaction, there is a correlation between the codon 72 polymorphism in p53 mutants and the response of tumours to the chemotherapy, as shown by study of the head and neck cancers (463). The polymorphism 72R in mutant p53, which is associated with better binding to p63/p73, in many cases correlates with the worse response to chemoradiotherapy.

1.6.2.2 Loss-of-function.

Biochemical studies have shown that p53 mutants exhibit certain heterogeneity in terms of structural alterations and loss of DNA-binding activity. The DNA-binding site recognized by p53 is highly degenerated and the affinity of p53 for target sites varies (465). Though many p53 mutants exhibit total loss-of-function, some p53 mutants retain partial transactivation ability. Tumour-derived point mutants p53175P and p53181L retain the ability to activate p21 and induce cell cycle arrest, however fail to induce other target genes, which impairs their ability to induce apoptosis (457). Mice homozygous for R172P mutation (equivalent to R175P in humans) are defective in p53-dependent apoptosis, but retain a partial cell cycle checkpoint function. Importantly, these mice have a delayed tumour onset compared to p53-/- mice indicating that cell cycle arrest partially contributes to tumour suppression, however the ability to induce apoptosis is needed to prevent tumour development (466). A large-scale study of over 2000 p53 mutants with an attempt to correlate structure and transcriptional activity of p53 was conducted in a yeast-based functional assay (467). This study has analyzed all possible amino acid substitutions caused by a point mutation throughout the protein and indicates a strong correlation of p53 structure and transactivation function and with frequency of the tumour-derived mutations.

Most of the DNA binding mutations impair the ability of p53 to transactivate to a various extent. All p53 mutants most frequently found in cancers (such as R175H, R273H, R248H) exhibit a complete loss of the transactivation ability.

In addition to loss-of-function, p53 mutants acquire cancer promoting properties (38, 39, 41, 468), which have been attributed to the ability of mutant p53 to inhibit wild type p53 in a dominant-negative manner and by gain-of-function effect.

1.6.2.3 Dominant-negative effect.

Overexpression studies in cells have shown that mutant p53 inhibits the function of wild type p53 acting in a dominant-negative manner (469-473). This results in interference with several p53-mediated biological processes, such as: apoptosis (474), growth arrest (475), differentiation (476), genetic stability (477) and transformation suppression (478, 479). One of the explanations was that mutant p53 can induce a conformational change in wild type p53 (480). However, structural studies suggest that contact mutants do not have a gross change to their structure, though are capable of inhibiting wild type p53 when overexpressed (470, 472). The current mechanism of the dominant-negative effect suggests the formation of mixed tetramers of mutant and wild type p53 proteins, which reduces the level of fully active homotetramers of wild type p53 (469, 470, 472, 473, 481, 482). One report suggests that at least three mutant molecules are required per tetramer to inactivate the transactivation ability of p53 (483). This suggests that dominant-negative effects of mutant p53 can be biologically relevant only when the levels of mutant p53 are high. Indeed, recent studies of mutant p53 knock-in mice allele suggest that in normal cells expressing one wild type and one mutant allele derived from these animals, wild type p53 retains its transactivation ability, suggesting that mutant p53 does not inhibit wild type p53 at low levels (484, 485). It is possible that in tumour cells, where mutant p53 accumulates to high levels, it might lead to inhibition of the wild type p53. In the course of tumour progression the wild type allele is often lost (431, 484-488). This might imply that wild type p53 retains its function to some extent in the presence of mutant p53, as there is a selective pressure to lose it.

1.6.2.4 Gain of function.

Experimental systems on a p53-null background have demonstrated novel tumour-promoting properties of mutant p53, which is known as "gain-of-function" effect. One of the early studies showed that mutant p53 expression in cells lacking p53 enhanced their tumourigenic potential (39). Mutant p53 can enhance the transformation potential of p53-null cells as assessed by colony formation assay and leads to enhanced growth of the cells.

Several studies have shown that exogenously expressed mutant p53 confers tumourigenic potential in several p53-null cell types : murine fibroblasts, murine L-12 pre-B cells and human osteosarcoma cell line (39, 489, 490). The increase in tumourigenic potential could be mediated via the increase in genomic instability. Several reports indicate that mutant p53 increases genomic instability, which could underlie the ability to increase tumourigenic potential. This is reflected by impaired mitotic spindle checkpoint in Li-fraumeni-derived fibroblasts expressing mutant p53 (491). Expression of mutant p53 in mouse fibroblasts following DNA damage leads to aberrant centrosome number (492) and in Saos-2 cells results in gene amplification (493). Mutant p53 also enhances colony formation upon overexpression in p53-null cells (492, 494-496) and increases proliferation of these cells (496). Another gain-of-function property of mutant p53 is the ability to interfere with the induction of apoptosis in response to various stress signals, such as DNA damage and growth factor deprivation when overexpressed in cells (497-502). However, most of these studies describe the role of the exogenous mutant p53. Studies showing that LFS-fibroblasts exhibit increased resistance to apoptosis in response to UV and IR, compared to p53-/- cells, indicate that endogenous mutant p53 behaves similarly (491). Mutant p53 actively contributes to cancer cells proliferation, chemoresistance and the ability to form tumours in mice, as evidenced by down-regulation of the expression of the endogenous mutant p53 in various cancer cell lines (503-505). However, the most convincing evidence for the gain-of-function effect is provided by the study of knock-in mice with "hot-spot" mutations in p53. p53 mutant mice with mutation at either R172H (equivalent to 175 in humans) or R270H (equivalent to 273 in humans), belonging to structural and contact class of hot-spot mutants respectively, have been generated (484, 485). Both mutant p53 knock-in and p53-null mice develop tumours, however mutant p53 knock-in mice exhibit different spectra of tumour spectrum, with predisposition to carcinomas and endothelial tumours. Tumours in mutant p53 knock-in mice display more aggressive phenotypes and metastasize with higher frequency. Further analysis of MEFs derived from mice expressing mutant p53 has shown that mutant p53 confers higher growth rate and increases transformation potential by Ras in MEFs derived from these mice compared to p53-null counterparts (484). Interestingly, there are some clear differences in tumour spectra between 172/+ and 270/+ mice. 172/+ mice also have increased frequency of metastatic osteosarcomas compared to 270/+ mice (485). This provides evidence for functional difference between contact and structural classes of mutants in tumour progression. These findings provide the most physiologically relevant evidence for the gain-of-function effect of certain p53 mutants (484, 485). The mechanism of the gain-of-function effect of p53 mutants has been proposed to be mediated via their

interaction with p63/p73. In support of this, mutant p53 interacts with both p63 and p73 in the metastatic tumour cell line established from p53172/+ mice. Mutant p53 inactivates transcriptional activity of p63 and p73 in MEFs derived from these mice (484). Some of the phenotypes of the mutant p53 mice, such as increased metastasis, can be recapitulated in mice with only one copy of p53 and one copy of p63 and p73 (506), suggesting that the gain-of-function is mediated via inactivation of p63/p73. However, the exact mechanism of gain-of-function of mutant p53 is still unknown.

Although mouse p53 gene is similar to human p53 gene, with 91% homology in the DNA binding domain, the existing differences prompted the researchers to develop a humanized p53 knock-in (HUPKI) mouse model (507). HUPKI is composed mostly of the human sequence of p53 (amino acids 33-332) and has the murine p53 N- and C-termini. It is considered to be functionally similar to mouse p53 (507, 508). Recent study has addressed the gain-of-function effects of p53 hot spot mutations (R248W and R273H) by introducing them into the HUPKI allele (509). In agreement with the studies described above, mice with R248W develop different tumour spectra compared to tumours in p53-/- mice, providing further support for the gain-of-function effect. Mutant p53-expressing cells also exhibit impaired G2-M checkpoint after DNA damage. The gain-of-function effect of mutant p53 in this system has been attributed to the active disruption of the DNA damage-response pathway. This seems to occur via physical interaction of p53 mutants with the nuclease Mre11 in p53 mutant knock-in mouse cells and human cancer cells (509). The interaction inhibits the binding of the MRN (Mre11-Rad50-NBS1) complex to DNA double-stranded breaks, leading to the impaired activation of the ATM.

Another mechanism of the gain-of-function of mutant p53 involves regulation of the expression of a specific set of genes. One of the first genes shown to be upregulated by mutant p53 was MDR-1, which was suggested as a mechanism underlying chemoresistance promoted by mutant p53 (510). Mutation of L22 and W23, required for transcriptional activity of p53, abrogated the ability of mutant p53 to transactivate MDR-1 and enhancement of tumourigenic potential of the cells by mutant p53 (511). This study has provided the evidence for transcriptional regulation mechanism of the gain-of-function of mutant p53. Several other studies have show that p53 mutants can upregulate the expression of genes involved in growth regulation, such as EGFR (512), PCNA (490) and c-Myc (501). Microarray analyses have shown that several p53 mutants can regulate the expression of genes involved in various processes, such as growth regulation, angiogenesis and genomic stability, which can promote tumour development (494, 497, 513-515). Another study suggests that CD95/Fas/Apo1 gene, encoding a death receptor is negatively regulated by mutant p53, which can contribute to its antiapoptotic activity (498). These

studies have also compared different p53 mutants and show that they can share some transcriptional targets, however they may possess distinct gain-of-function phenotypes. Interestingly, mutant p53 can also increase NF-kB activity, which can play an important antiapoptotic role in many human cancers (514). The mechanism of how mutant p53 regulates gene expression is not known. Mutant p53 is found at the promoters of its target genes (494, 497, 498), however, it is not known whether it can directly bind DNA. The mutant p53 consensus site has not been found so far. It is likely that mutant p53 is engaged in protein complexes, which target it to specific promoters. A recent report has demonstrated that mutant p53 associates with the transcription factor NF-Y and is recruited to the NF-Y target genes involved in regulation of cell cycle, such as cyclin A, cyclin B1 and cdk1. Upon DNA damage, mutant p53 recruits p300 and upregulates the expression of these genes, leading to cell cycle progression (503). Though it is possible, that mutant p53 inhibits transcriptional function of p63 and p73, most of the target genes of mutant p53 do not have the p53 consensus sequence. The contribution of these gain-of-function mechanisms to the tumour promotion by mutant p53 remains to be elucidated.

1.6.3 Accumulation of mutant p53 protein.

In the first studies describing p53 it was noted that p53 protein is highly expressed in many transformed cell lines, whereas in normal cells its expression is low (29-31, 516). Though wild type p53 is a short-lived protein in normal cells, mutant p53 protein has increased half-life in transformed cells (41, 517, 518). High levels of mutant p53 protein in cancer cells has become a characteristic feature of cancers with mutant p53 (430, 432, 488). This suggests the selection for overexpression of mutant p53 in the process of tumour development. Though many potential explanations have been suggested, it is still not clear why mutant p53 is stable in tumours. First, it was thought that mutant p53 is not degraded via normal mechanisms. However after Mdm2 was shown to bind and degrade mutant p53 when overexpressed in cells (246, 519), the inability to transactivate Mdm2 was proposed to underlie the stability of mutant p53 proteins. Though this mechanism can account for stability of mutant p53 in some cases, the situation seems to be more complex. Recent studies of mouse models expressing only mutant p53 show that while mutant p53 accumulates in most tumours, it remains unstable in normal cells. In agreement with other reports, accumulation of mutant p53 does not correlate with the loss of heterozygocity (484, 485, 488). This suggests that mutant p53 protein is normally degraded in normal cells and therefore the failure to transactivate Mdm2 is not the underlying cause of mutant p53 stability in tumours. Further supporting this, it has been reported that mutant p53

accumulates to high levels in normal cells when Mdm2 is simultaneously inactivated in mice expressing mutant p53 (520). This suggests that Mdm2 can degrade mutant p53 in normal cells, but not in tumour cells. Indeed, high levels of Mdm2 are found in many tumour cell lines that express high levels of mutant p53 (518). Another study shows that though Mdm2 interacts with mutant p53, it still fails to degrade the protein in some tumour cell lines (518). It is possible that secondary events might occur during tumour development, which contribute to stabilization of mutant p53. Given the gain-of-function effect, mutant p53 can be favorable for tumours, however particularly adverse for cancer patients. High levels of mutant p53 protein are often correlated with the worse clinical prognosis (521-529). Therefore studying how the stability of mutant p53 is regulated can help to understand why it is stable in tumours, which can have important therapeutic implications.

1.7 p53 and cancer therapy.

Recent studies have shown that similarly to "oncogenes addiction", tumours depend on having p53 inactivated for their continued growth. Three recent mouse tumour models studies have shown that restoration of p53 function in established tumours causes regression of tumour growth and can be a potent way to treat cancers (530-532). One study has generated mice with switchable p53 in all tissues (531). In this system when p53 is inactivated mice develop lymphomas and sarcomas. Restoration of p53 leads to a pronounced regression of both types of tumours, though by different mechanisms – apoptosis in lymphomas and cellular senescence in sarcomas. Interestingly, re-expression of p53 does not induce toxicity in normal tissues, suggesting that oncogenic environment of the tumour is needed for full activation of p53 function (531). Another group has developed a mouse model of hepatocellular carcinoma with activated Hras, in which p53 expression can be downregulated by switchable short hairpin RNA against p53 (530). The injection of Hras-expressing p53-inactivated cells into immunodeficient mice results in formation of invasive hepatocarcinomas. When p53 function is restored, even if transiently, tumours completely regress. Similarly to sarcomas, activation of p53 in hepatocellular carcinomas leads to induction of growth arrest with signs of cellular senescence (530). Another study used the p53 knock-in mouse model where one gene is replaced by a switchable p53 gene on the background of Eµ-myc lymphoma mouse model (532). In this system E-myc mice with one wild type p53 allele and one inducible p53 allele develop B-cell lymphomas as they lose the wild type p53 allele. Switching on the remaining p53 allele causes tumour cell death and prolongs survival of the mice. However,

the mice eventually succumb to tumours, which evade p53 action by loss of p19ARF or by deletion of the remaining p53 allele (532). Similarly to two other models this study shows that though p53 activation in tumours can have an important therapeutic benefit in lymphomas, it can be quite short term, suggesting that efficiency of p53 activation might vary between tumour types. All in all these three studies strongly suggest the potential therapeutic benefit for p53 activation in tumours with inactivated p53. These studies have modelled the tumours with loss of p53, however the interesting question of whether tumours are arising with mutant p53 can regress upon inactivation of mutant p53, has yet to be investigated.

One of the promising approaches for cancer therapy using p53 pathway has been gene therapy. The delivery of p53 gene in an adenovirus vector has been already implemented into clinical trials (533). However, specific targeting of the tumours might be quite challenging using this approach.

Several other approaches have been used to activate wild type p53 in tumours (534, 535). The most successful strategy that has been implemented is by blocking the interaction with Mdm2 (536). In about half of all cancers p53 remains wild type and many of these tumours overexpress Mdm2, which inactivates p53. Using synthetic peptides and protein aptamers it has been shown that inhibition of the interaction between p53 and Mdm2 can be used to activate p53 in cells which results in cell cycle arrest and apoptosis (276, 277, 344). This suggests that blocking the interaction between p53 and Mdm2 could be implemented for cancer therapy. Targeting protein-protein interactions by small molecules has been considered difficult, however in the case of p53/Mdm2 only three amino acids of p53 F19, W23 and L26 are directly involved in interaction with deep hydrophobic cleft on Mdm2, which allowed design of inhibitors mimicking the interaction (148). Recently highly specific small-molecule inhibitors of the interaction – nutlins - have been described which lead to efficient stabilization and activation of p53 in cells, resulting in cell cycle arrest and apoptosis (282). Nutlins were identified from a class of cis-imidazoline compounds and can potently inhibit interaction of p53 and Mdm2 *in vitro* and in cells (282, 537). Structural studies have demonstrated that nutlins bind to the p53 pocket of Mdm2 and mimic the molecular interaction with p53. Interestingly, though binding region of Mdm2 is well conserved in Mdmx, nutlin does not potently inhibit the p53/Mdmx interaction (538).

Nutlins activate p53 pathway with high specificity and only cells with wild type p53 are sensitive to these compounds (282). Importantly, treatment of normal fibroblasts with nutlin-3 induces cell cycle arrest, whereas cancer cells undergo apoptosis when treated at similar doses with nutlin-3a, suggesting nutlins can be promising candidates for

cancer therapy (539). Treatment of human xenografts with nutlins at non-toxic doses results in tumour shrinkage, suggesting promising cancer therapy (537).

Several other inhibitors have been discovered. A class of benzodiazepine Mdm2 antagonists have been reported to prevent the interaction of Mdm2 with p53 and have been shown to be able to suppress growth of cells with wild type p53, however they are much less potent than nutlins (540). A structure-based design study has identified several compounds with a spiro-oxinadole core structure, which can potently inhibit the p53-Mdm2 interaction *in vitro* (541). These compounds have been shown to have anti-proliferative activity in cells with wild type p53, however their biological activity is not well characterized yet.

Another compound that inhibits p53/Mdm2 interaction - RITA (reactivation of p53 and induction of tumour cell apoptosis) - has been discovered based on cell-based screening with chemical library (542). RITA activates p53 and induces p53-dependent cell cycle arrest and apoptosis. In SCID mice RITA inhibits the growth of HCT116 human tumour xenografts. It has been proposed that unlike nutlins, RITA inhibits p53/Mdm2 interaction by binding to p53. However, the mechanism of action of RITA remains controversial, as NMR structural studies reported that RITA does not inhibit the p53/Mdm2 interaction (543).

Other routes to stabilize p53 exploit the ways to inactivate Mdm2. Several studies have inhibited Mdm2 expression by antisense oligonucleotides and have shown p53 stabilization and p53 activation in cancer cells and in tumour xenografts in nude mice (544-546). However, this approach has not been well characterized yet. Inhibiting ubiquitin ligase activity of Mdm2 is another important strategy to activate p53. This approach emerges as particularly important since ubiquitin ligase activity is a crucial determinant for inactivation of p53 by Mdm2. Recently small-molecule inhibitors that specifically target the E3 ligase activity of Mdm2 have been identified in a high-throughput screen. Some of these compounds, named HLI98, potently inhibit p53 ubiquitination *in vitro* and activate p53 in cells, resulting in p53-dependent apoptosis (547, 548). However, some p53-independent toxicity of these compounds in cells has been observed. Further steps of optimization of these compounds might be required for this approach to be implemented into cancer therapy.

The success of the therapeutic approach of reactivating wild type p53 in tumours will depend on the functionality of the p53 pathway. For example, melanomas have low frequency of p53 mutation, however are resistant to chemotherapy. The deregulation of downstream of p53 signalling pro-apoptotic APAF-1 and BAX can account for the

chemoresistance (549). Overexpression of Mdmx, an inhibitor of p53 transcriptional activity can attenuate the p53 response to nutlin treatment (538).

In many tumours where p53 is inactivated by mutation, stabilization of mutant p53 would be undesirable approach as it can have adverse effects due to gain-of-function of mutant p53. The restoration of the activity of mutant p53 has been suggested as a strategy for development of therapy for such tumours. Though many p53 mutants are unable to bind DNA in p53 target promoters due to conformational changes in the DBD, several evidence have suggested that DBD of p53 mutants is flexible, as exemplified by the finding of the temperature-sensitive p53 mutations (550-552). In further support, second site mutations in DBD of p53 can restore the structure and activity of some of the p53 mutants, suggesting the possibility to reactivate mutant p53 (553-555). Several compounds, which activate mutant p53 in cell-based assays, have been described, including CP-31398 (556), PRIMA-1 (p53 reactivation and induction of massive apoptosis) (557) and MIRA-1 (558). CP-31398 acts by modifying the mutant conformation of p53 and can rescue p53 function in some tumour-derived cell lines and xenografts. It has been suggested that CP-31398 stabilize wild type conformation by binding to newly synthesized protein, however there is no evidence of direct interaction and the mechanism of action remains unclear. CP-31398 also has some p53-independent effects and needs to be further characterized and optimized (556). PRIMA and MIRA represent another class of compounds, which have been suggested to restore tumour-suppressive p53 activity of mutant p53 (557-559). These compounds have been identified in a cell-based screen for ability to induce apoptosis in a cell line expressing inducible mutant p53 273H. PRIMA can induce apoptosis in cells expressing wild type p53, however seems to be more specific for cells with mutant p53. PRIMA also has some p53-independent effects and the mechanism of its action is largely unknown (557). MIRA is able to suppress growth of mutant-p53 expressing cells but not p53-null cells. MIRA has been shown to inhibit tumour growth of xenografts in SCID mice (558). Both PRIMA and MIRA have been suggested to promote wild type conformation of mutant p53 *in vitro*, however there is no data for direct binding of these compounds to p53. These compounds have not been fully characterized and optimized yet. The discovery of such compounds suggests potentially promising approach for refolding conformation of mutant p53 by small molecules. This approach is particularly attractive as it will target specifically tumour cells which bear mutant p53 and not cells with wild type p53.

Clearly the strategy of the therapies should be completely different dependent on whether tumours have wild type or mutant p53. In the first case the approach would be to

activate p53, whereas cancer therapy of tumour cells with mutant p53 should aim at downregulating mutant p53 expression.

1.8 Aims.

The aim of this study has been to analyze the contribution of the DNA binding domain (DBD) of p53 to regulation of its stability. Specifically, the contribution of the conserved boxes of DBD of p53 and of point mutations of p53 found in tumours has been addressed. Chapter 3 describes the contribution of DBD of p53 to regulation of p53 stability by Mdm2. In chapter 4 the contribution of Mdm2-independent pathway to ubiquitination of p53 has been investigated. In chapter 5, the role of DBD of p53 in regulation of subcellular localization has been analyzed. Involvement of DBD of p53 to interaction with its family members has been studied in Chapter 6.

Chapter 2. Materials and methods.

2 Materials and Methods.

2.1 *Materials.*

All general laboratory reagents used in this study are listed in table 2.1.

Table 2.1. General chemicals and reagents.

Reagent	Source
Agarose	Sigma
Acrylamide 29:1	National diagnostics
SDS (sodium dodecyl sulphate)	Fisher
APS	Sigma
TritonX-100	Sigma
BSA (bovine serum albumin)	Sigma
DTT (dithiothreitol)	Sigma
Na_2HPO_4	Fisher
KH_2PO_4	Fisher
NaCl	Fisher
KCl	Fisher
PFA (paraformaldehyde)	TAAB labs
Tris-HCl	Sigma
$MgCl_2$	Sigma
Methanol	Fisher
Ethanol	Fisher
ATP (Adenosine-5'-triphosphate)	Roche
Agar	Fluka
β-Mercaptoethanol	Sigma
NP-40	Roche
DMSO (dimethyl sulfoxide)	Sigma
TEMED (Tetramethylethylenediamine)	Sigma
Tween-20	Sigma
Protease inhibitors cocktail	Roche
Nutlin-3A	Cayman chemicals
Cycloheximide	Sigma
DAPI (4',6-diamidino-2-phenylindole)	Sigma
ECL (enhanced chemiluminescence) reagent	Perbio
Nitrocellulose membranes	VWR
Leptomycin B	Sigma
Kanamycin	Sigma
Ampicillin	Sigma

All general buffers and solutions used in this study are listed in Table 2.2.

Table 2.2. Solutions and buffers.

Solution	Composition
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 7.4 137 mM NaCl 5 mM KCl
TBS-T (TBS-Tween)	TBS+0.1%Tween-20
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
Tris-EDTA (TE)	10 mM Tris-HCl pH 8.0 1 mM EDTA
2x SDS-PAGE sample buffer	125 mM Tris pH 6.8 4% SDS 10% ß- mercaptoethanol 15% glycerol 0.01% bromophenol blue
SDS-PAGE running buffer	0.1% SDS 192 mM glycine 25 mM TrisH-Cl pH8.3
Electroblotting buffer	192 mM glycine 25 mM Tris 20% methanol
NP-40 buffer	20 mM Tris-HCl pH 8 120 mM NaCl 1mM EDTA 0.5% NP-40
Lysis buffer (in vitro ubiquitination)	50mM Tris-HCl pH7.5 100mM NaCl 1% Triton 0.8 mg/ml DTT supplemented with PMSF
Reaction buffer (in vitro ubiquitination)	50mM Tris-HCl pH8 2mM DTT 5mM $MgCl_2$ 2mM ATP
Blocking solution (western blotting)	5% milk powder in TBS-T
Blocking solution (immunostaining)	1% BSA in PBS
Permeabilizing solution (immunostaining)	0.2% TritonX-100 in PBS
Fixing solution	4% PFA in PBS
Resolving gel	8-10% acrylamide 375mM Tris pH8.8 0.1% SDS 0.1% APS 50mM TEMED
Stripping buffer	0.2M Glycine 1% SDS pH 2.5

2.2 Methods.

2.2.1 DNA preparation.

DNA preparations were carried out as described previously (560). E. Coli (strains DH5α or TOP-10) competent cells (Molecular Biology services, Beatson Institute, Glasgow, UK) were thawn on ice. The DNA plasmid was added to 30-100 μl of competent cells, mixed and incubated on ice for 20 min. After a heat shock of 45 seconds at 42 °C the cells were grown in 0.5 ml LB for 30 min at 37°C with shaking at 450rpm. Cells were seeded on agar plates with ampicillin or kanamycin and grown upside down at 37°C overnight. The next day the colonies were inoculated in LB with ampicillin or kanamycin and grown overnight at 37°C whilst shaking. Small scale or large scale plasmid DNA preparations were performed using Qiagen kits following manufacturer's instructions (Qiagen, UK).

2.2.2 Site-directed mutagenesis.

Point mutations were introduced by site-directed mutagenesis Quick Change kit (Stratagene) following manufacturer's instructions. Briefly, oligonucleotide primers were designed by web-based design program (Stratagene) containing the desired mutations (Table 2.3). Primers were synthesized and purified (MWG).

The reaction was set up in 50 μl containing 30ng plasmid DNA, 1μM primer, 1 μl of 0.2mM dNTP mix, 5μl of 10xreaction buffer (Stratagene) and 1μl PfuTurbo DNA polymerase (Stratagene) (2.5U/μl). The reaction was cycled using the following parameters.

Cycles	Temperature	Time
1	95°C	30''
17	95°C	30''
	55°C	1'
	68°C	8'

After brief incubation on ice 1 μl of DpnI restriction enzyme (10U/μl) (New England biolabs) was added and reaction was incubated for 3h at 37°C. Then 10μl of the reaction was transformed into E.Coli Top10 competent cells. All constructs were verified by sequencing (Research services, Beatson Institute).

2.2.3 RNA extraction and RT-PCR.

Cells were washed twice in PBS and total RNA was isolated with RNeasy extraction kit following manufacturer's instructions (Qiagen). To synthesize cDNA reverse transcription reactions were set up. First RNA (1.4μg) and 200ng random hexamers and 1mM dNTP were mixed in 10μl and denatured at 65°C for 5 min. Next the 10xreaction buffer, 10mM MgCl$_2$ and 20mM DTT and 1μl RNASe OUT recombinant ribonuclease inhibitor (40u/μl) were added and primers were annealed at 25°C for 2 min without reverse transcriptase (RT) and for 10 min with 1μl SuperScript II RT (GIBCO). The cDNA synthesis was carried out at 42°C for 50 min. Then reaction was stopped by heat inactivation at 70°C for 15 minutes. To remove RNA the reaction mixes were incubated with 1 μl RNase H (2u/μl) for 37°C for 20 min and cDNAs were stored at -20°C.

For polymerase chain reactions 2μl cDNA was mixed with 0.2μM corresponding forward and reverse primers, 1U AmpliTaq DNA polymerase, 10x PCR buffer, 1.5 mM MgCl$_2$ and 0.2mM NTP in 50 μl volume. For analysis of CHIP and GAPDH expression the following primers were used (Table 2.3).

Table 2.3 RT-PCR primers.

GAPDH-fw	GCAGAGATGATGACCCTTTTGGCT
GAPDH-rv	TGAAGGTCGGATCAACGGATTTGGT
CHIP-fw	TCATTTTGACCCCGTGACC
CHIP-rv	ACCTCAGTAGTCCTCCACC

Reactions were cycled for 25 cycles in a Peltier Thermal Cycler (MJ research, Helena Bioscience), using the following parameters and transferred to 4°C.

Cycles	Temperature	Time
1	95°C	30''
25	95°C	30''
	60°C	40''
	72°C	1'

Reaction products were resolved by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis.

1% Agarose (Sigma) in TAE buffer was boiled, mixed with 0.5μg Ethidium bromide and allowed to solidified in gel chamber. DNA samples diluted in 5x loading buffer (30$glycerol, bromphenol blue) were electrophoresed at 120V in 1xTAE running buffer and visualized by UV transilluminator.

2.2.5 Plasmids.

All p53, Mdm2 and other expression plasmids with deletions and mutations of the indicated amino acids used in this study are shown on table 2.4.

Table 2.4. Plasmids.

A. **p53 constructs.**

name	mutation	vector	source
wt	Wild type p53	Pcb6+; pGEM	(561)
ΔI	Δ13-19aa	Pcb6+; pGEM	(561)
ΔII	Δ117-142aa	Pcb6+; pGEM	(561)
ΔIII	Δ171-181aa	Pcb6+; pGEM	(561)
ΔIV	Δ234-258aa	Pcb6+; pGEM	(561)
ΔV	Δ270-286aa	Pcb6+; pGEM	(561)
ΔNES	L348A,L350A	Pcb6+	(224)
ΔNLS	K305A, R306A, K319A, K320A, K321A	Pcb6+	(224)
175H	R175H	Pcb6+	Site directed mutagenesis
273H	R273H	Pcb6+	Site directed mutagenesis
175/ΔI	R175H; Δ13-19aa	Pcb6+	Site directed mutagenesis
ΔV/ΔI	Δ270-286aa; Δ13-19aa	Pcb6+	(561)
ΔII/ΔI	Δ117-142aa; Δ13-19aa	Pcb6+	(561)
175/ΔNLS	R175H; K305A, R306A, K319A, K320A, K321A	Pcb6+	Site directed mutagenesis
175/ΔNES	R175H; L348A,L350A	Pcb6+	Site directed mutagenesis

B. **Mdm2 constructs**.

wt	Wild type Mdm2	pCHDM1A	(562)
ΔR	Δ440-497aa	pCHDM1A	(562)
464	C464A	pCHDM1A	(563)
ΔN	Δ58-89aa	pCHDM1A	(256)
ΔA	Δ212-296aa	pCHDM1A	Provided by Dr. Uldrijan
ΔC	Δ483-497aa	pCHDM1A	Provided by Dr. Uldrijan
ZF	C305A, C308A	pCHDM1A	Provided by Dr. Uldrijan
GFP-RING	GFP-384-491aa	pEGFP-C1	(270)
GST-Mdm2	GST-tagged wild type Mdm2	pGEX	(564)

C. Other constructs.

V5-S4	pcDNA3	Provided by prof. Blattner
V5-S5α		
V5-S6α		
V5-S6β		
HA-p73α	pcDNA3	Provided by prof. G Melino
FLAG-p73α	pcDNA3	Provided by prof. G Melino
HA-Ub	pMT123	Provided by prof. R.Hay
FLAG-Parc	pcDNA3	Provided by prof. W.Gu
pEGFP-N1	pEFGP	Clontech

Constructs encoding human wild type p53 and p53 deletion mutants (ΔI, ΔII, ΔIII, ΔIV, ΔV, ΔV/ΔI, ΔII/ΔI) in mammalian expression vector pcb6+ were made in the laboratory by Dr. N Marston and were characterized previously. p53 ΔNLS, p53-C6 and p53 ΔNES in vector pcb6+ were kindly provided by Dr. Stephanie Carter and are described previously (224). p53175H, p53273H, p53175H/ΔI, p53273H/ΔI, p53175H/ΔNLS, p53175H/ΔNES and p53175H-C6 in pcb6+ vector were made by introducing point mutations R175H and R273H by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) with the corresponding p53 primers (Table 2.4). The polymorphic codon 72 in all p53 constructs was mutated from P to R if needed, using corresponding primers (Table 2.5). For *in vitro* translation p53ΔV, ΔIV, ΔIII, ΔII in pGEM vector, previously described, (561) and p53175H in pSp65 vector, made by site-directed mutagenesis, were used. All p53 contsructs were verified by sequencing (Research services, The Beatson Institute) with p53-specific primers (Table 2.6).

Table 2.5. Primers used for site-directed mutagenesis of p53.

p53R175Hfw	ACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCT
p53R175Hrv	TGCCTCCAACACTCCGTGACGGGGGTGGTACTCGCGACGA
p53R273Hfw	ACTGGGACGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG
p53R273Hrv	TGACCCTGCCTTGTCGAAACTCCACGTACAAACACGGACAGGACCC
p53P72Rfw	CCAGAGGCTGCTCCCCGCGTGGCCCCTGCACC
p53P72Rrv	GGTCTCCGACGAGGGGCGCACCGGGGACGTGG

74

Table 2.6. Primers used for sequencing of p53.

146-fw	ATATTGAACAATGGTTC
189-rv	TGCTTCATCTGGACCTGG
282-fw	ATCTTCTGTCCCTTC
361-fw	TCTGTGACTTGCACGTAC
661-fw	GAGCCGCCTGAGGTTGGC
887-rv	ACTCCGAGGGGAAAGAACGCCTC
971-fw	ATGGAGAATATTTCACCCTT

Plasmids expressing wild type Mdm2 (562), C464A Mdm2 (563), GST-Mdm2 (564), Mdm2Δ58-89 (256), Mdm2 ΔR (562) were described previously (Table 2.3). Plasmids encoding Mdm2ΔA and Mdm2ZF were kindly provided by Dr. Stjepan Uldrijan (Table 2.3).

Plasmid encoding HA-tagged ubiquitin was kindly provided by Prof. R. Hay. pEGFP-N1 encoding GFP was obtained from Clontech (Palo Alto, CA). FLAG-Parc was kindly provided by Prof. W. Gu. Constructs encoding HA-p73α and FLAG-p73α were generously provided by Prof. G. Melino.

2.2.6 Cells.

H1299 is a human non-small cell lung adenocarcinoma with a homozygous deletion of p53 (565).

U2OS is a human osteosarcoma expressing wild type p53 (566) (ATCC).

MCF7-p53ΔII human breast cancer cells expressing wild type p53 and stably transfected with p53ΔII mutant were previously made in the laboratory (567).

MCF7-p53ΔII/ΔI human breast cancer cells expressing wild type p53 and stably transfected with p53ΔII mutant were previously made in the laboratory (567).

p53-/-mdm2-/- mouse embryonic fibroblasts (DKO) cells were described previously (249).

2.2.7 Growth conditions.

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) (Autogen bioclear), 2mM glutamine and 60 μg/ml penicillin, 200μg/ml streptomycin (Invitrogen) at subconfluent conditions. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 and split every 3-4 days at a 1/5-1/10 ratios. Medium was aspirated and cells were

washed once with TE+0.25% trypsin, followed by incubation with trypsin for 2-3 minutes. Cells were collected into fresh media and seeded for maintenance or transfections.

For transfections cells were seeded at 30-50% confluency and allowed to adhere overnight unless otherwise stated.

For long-term storage cell lines were cryo-frozen. Cells were trypsinized and pelleted. After one wash with fresh medium, cells were resuspended in 90%FBS and 10%DMSO, frozen in cryotubes at -70°C overnight and stored in liquid nitrogen.

2.2.8 Transfections.

Transfections of cells with plasmids were performed with Effectene transfection reagent (Qiagen) or lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. DKO were transfected with Effectene reagent. For immunoprecipitation experiments U2OS and H1299 were transfected with lipofectamine reagent. Transfection of U2OS cells for *in vivo* ubiquitination was carried out with effectene, for immunofluorescence and subcellular fractionation experiments U2OS cells were transfected by calcium phosphate precipitation similarly to as described previously. For 6-well plate 2-4 μg total amount of appropriate DNA plasmids were diluted in 110 μl of sterile water and 15 μl of 2M $CaCl_2$ was added and mixed. The mix was added dropwise to 125 μl of 2xHBS (pH 7) and vortexed. The precipitates were allowed to form for 30 minutes at room temperature and added to the cells dropwise. Approximately 5-6 hours later the medium was changed and the cells were harvested 36-48 hours after transfection. For transfections of larger plates the amounts were scaled according to the surface area.

Short interfering RNA (siRNA) oligonucleotides were transfected into cells with lipofectamine 2000 (Invitrogen) or Hiperfect (Qiagen). For down-regulation of Cop1, ARF-BP1 and Mdm2 expression, cells were transfected twice with siRNA with 24 hours between transfections and analyzed 48 hours later. For down-regulation of CHIP expression cells were transfected twice with siRNA with 72 hours between transfections and analyzed 72 hours later. For down-regulation of Cop1, ARF-BP1 and CHIP expression, predesigned pools of four siRNA oligonucleotides were used (SMARTpool, Dharmacon). In the control experiments non-targetting siRNA was used (Dharmacon). Mdm2-specific siRNA oligonucleotides were described previously (568). The following siRNA oligonucleotides were used (Table 2.7).

Table 2.7 siRNA oligonucleotides.

Itch1	AAGUGCUUCUCAGAAUGAUGA	(422)
Itch2	AACCACAACACACGAAUUACA	(422)
Stub1	CGCUGGUGGCCGUGUAUUAUU	On-target Smartpool (Dharmacon)
Mdm2	AAGGAAUAAGCCCUGCCCA	(568)
ARF-BP1	Sequence not provided	On-target Smartpool (Dharmacon)
Hsp70	GAAGGACGAGUUUGAGCACAA	Designed with Invitrogen RNAi designer software
CHIP3	CAGCUGGAGAUGGAGAGCUAU	Designed with Invitrogen RNAi designer software
CHIP4	CCAACUUGGCUAUGAAGGAGG	Designed with Invitrogen RNAi designer software
GFP	GGCUACGUCCAGGAGCGCACC	(569)
Cop1	Sequence not provided	On-target Smartpool (Dharmacon)

Cells were treated for indicated time periods with 10-20 µM proteasome inhibitor MG132 (Sigma), Nutlin-3a (Cayman chemical), cycloheximide (Sigma) and leptomycin B (Sigma) at indicated concentrations.

2.2.9 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

Protein samples were subject to SDS-PAGE electrophoresis as described previously (570). Protein samples were boiled in 2x sample buffer for 10 min at 95°C and loaded into SDS-polyacrylamide gels consisting of 5% stacking gel and 8-10% resolving gel. Prestained protein markers were used (Benchmark, Invitrogen). Small and large gels were electrophoresed at 40-50mA in 1x SDS-PAGE running buffer on vertical tanks (Amersham Biosciences, SE400).

Gels with [^{35}S]-labelled proteins were fixed in isopropanol:water:acetic acid (25:65:10) for 30 min, soaked in Amplify reagent (Amersham, UK) for 15 min, dried and exposed to X-ray film.

2.2.10 Western blotting.

Western blot analysis was carried out similarly to described previously (563). Proteins were transferred from the gel to the nitrocellulose membrane as described previously (571) in 1xtrasfer buffer in Hoefer TE42 Protein Transfer tanks. Small gels were transferred in at 200mA for 2h or 10V overnight. Large gels were transferred at 500mA for 3 h or 30V overnight. The membranes were blocked in 5% milk/TBS-T for 30min-60min and incubated with the primary antibodies usually at a dilution of 1:1000-1:2000 in blocking solution for 2-3h at room temperature or overnight at 4°C. Following 3

washes in TBS-T the blots were incubated with the horseradish peroxidase conjugated anti-rabbit, anti-mouse or anti-goat HRP-conjugated secondary antibodies (GE Healthcare) and visualized with Enchanced Chemiluminescence (ECL, Amersham) detection kit.

The primary antibodies used in this study are detailed in table 2.8.

Table 2.8. Primary antibodies

A. Human p53-specific antibodies.

	antibody	epitope	Source
p53	DO1	20-25aa (572)	Beatson Institute
	1801	46-55aa (573)	Calbiochem
	421	371-380aa (574)	Calbiochem
	1620	106 to 114aa 146 to 156aa (575) (detects for wild type conformation)	Calbiochem
	240	213-217aa (576) detects mutant conformation	Calbiochem
	CM1	recombinant human p53 (577)	Novocastra

B. Other antibodies.

protein	name	Source
Actin		Chemicon
ARF-BP1		Provided by Prof. M. Eilers
Cdk4		Santa Cruz
CHIP		Abcam
Cop1		Provided by Prof. V. Dixit
FLAG (M2)	M2	Sigma
GFP		Roche
HA	F7 or Y11	Santa Cruz
Hsp70		Calbiochem
LaminA/C		Calbiochem
Mdm2	Ab-1 Ab-2 SMP-14	Calbiochem Calbiochem Santa cruz
p73	sc-20	Santa Cruz
V5		Calbiochem

2.2.11 Immunoprecipitation under native conditions.

Immunoprecipitation was carried out similarly to described previously (563). 24h before transfection cells were seeded in 6-cm plates. Cells were transfected with 0.6mg p53 and 1.2mg Mdm2 using Lipofectamine 2000 reagent (Invitrogen). After 20-24h cells were treated with 10μM MG132 for 4h. Cells were washed in cold PBS and lysed in cold NP-40 buffer containing proteinase inhibitors (Complete, Roche). Immunoprecipitations were performed with Mdm2 or p53-specific antibodies bound to 30ml protein G-Sepharose for 1 or 2h at 4°C. Mdm2 was immunoprecipitated with 0.3-0.6 μg of anti-Mdm2 antibodies Ab-2 (Calbiochem) and SMP-14 (Santa Cruz), p53 was immunoprecipitated

with 0.3-0.6 μg of 1801 and 421 antibodies (Calbiochem). Immunoprecipitated proteins were washed with NP-40 buffer and resuspended in 2xSDS sample buffer. Proteins from whole-cell extracts and immunoprecipitations were resolved by SDS–PAGE and analyzed by Western blotting with anti-p53 polyclonal antibody CM1 (Novocastra) and anti-Mdm2 Ab-1 and Ab-2 antibodies (Calbiochem).

2.2.12 *In vivo* ubiquitination of p53.

Cells were seeded at 60%-80% confluency in 6-cm plates the day before transfection. Cells were transfected with 0.3mg p53, 0.6mg hemagglutinin (HA)-ubiquitin and with 0.15mg Mdm2 or pcDNA3 where indicated, using Effectene transfection reagent (Qiagen). After 20-30h cells were treated with 10-20 mM MG132 for 4-5h. Cells were washed twice in cold PBS and lysed in 350ml 0.5% SDS in TBS. After boiling and vigorous vortexing, extracts were supplemented with 1ml 1.5% Triton X-100 in TBS. Protein G sepharose beads were washed in Np-40 buffer and incubated with DO1 antidoby for 1h at 4°C rotating. Lysates were added to 30ml protein G sepharose beads preconjugated to p53-specific DO1 antibody and rotated for 2h at 4°C. The beads were washed 3 times in NP-40 buffer and the proteins were extracted by adding 40μl 2x SB and boiling for 5 min. Proteins were resolved by SDS-PAGE and analyzed by analyzed by Western blotting with monoclonal anti-HA antibody followed by anti-light chain secondary antibody. The blot was then reprobed with polyclonal CM1 antibody followed by anti-rabbit secondary antibody.

2.2.13 *In vitro* ubiquitination of p53.

In vitro ubiquitination of p53 was carried out similarly to described previously (270). DHL5a *E. coli* cells were transformed with pGEX-Mdm2 and grown at 37°C to approximately log phase. Protein expression was induced by addition of IPTG (300 mM) for 3h. Cells from 10 ml of night culture were lysed in 5 ml of lysis buffer and sonicated. GST-Mdm2 was purified on 100 μl glutathione-Sepharose beads (Amersham), mixed with 20 μl *in vitro*-translated p53 (TNT Quick Coupled Transcription/Translation System, Promega) and incubated at 4°C for 1 h. The beads were washed three times with 50mM Tris pH7.5 and incubated with 50ng mammalian E1 (Affiniti), 200ng human recombinant UbcH5B E2 (Affiniti) and 5μg ubiquitin or methylated ubiquitin (Sigma) in reaction buffer. The reaction was incubated at 37°C for 2h and then stopped by the addition of 2x

SDS sample buffer. Reaction products were resolved by SDS-PAGE and analyzed by Western blotting with anti-p53 DO-1 antibody.

2.2.14 Analysis of half-life of p53 by cycloheximide treatment.

The day before transfection 10^5 p53-/-mdm2-/- (DKO cells) were seeded in 6-well plates. Cells were transfected with 150ng of p53 with Effectene transfection reagent (Qiagen). After 24h cells were treated with 50mg/ml cycloheximide (Sigma) and collected at indicated time points. Cell lysates were subjected to Western blot analysis with p53-specific DO1 antibody.

2.2.15 Analysis of half-life of p53 by pulse-chase.

The day before transfection, 10^5 p53$^{-/-}$ mdm2$^{-/-}$ (DKO) cells were seeded into six-well plates. Cells were transfected with 400 ng of p53 with Effectene transfection reagent (QIAGEN). The pulse-chase experiment was performed 24 h later. Cells were incubated in methionine/cysteine-free DMEM with 5% dialyzed serum (GIBCO) for 30 min. The medium was then removed and replaced with DMEM with [^{35}S]methionine-cysteine (50 µCi/ml; Promix [Amersham]) for 2 h. Cells were washed twice with phosphate-buffered saline (PBS) and chased with DMEM supplemented with 15 mg of methionine/liter and 24 mg of cysteine/liter (both from GIBCO) for the times indicated below. Cells were washed in PBS and lysed in NP-40 buffer. Cell lysates were immunoprecipitated with p53-specific DO1 antibody and analyzed by SDS-PAGE electrophoresis.

2.2.16 Subcellular fractionation.

The day before transfection U2OS cells were seeded at 30-50% confluency. Cells were transfected with 7 µg of p53 plasmids by calcium phosphate precipitation method. After 48h fractions of cells were extracted using Subcellular fractionation kit () following manufacturer's instructions. The cell lysates were mixed with 2x Laemmli sample buffer and resolved by SDS-PAGE, followed by Western blot analysis.

2.2.17 Immunofluorescence labeling.

DKO or U2OS cells were seeded onto coverslips and transfected as described above for *in vivo* ubiquitination of p53. After 20-30h, cells were treated with 20 µM

MG132 for 5 h. Cells on coverslips were washed three times with PBS and then fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. After fixation, cells were washed three times in PBS and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. Cells were blocked in PBS containing 0.5% bovine serum albumin at room temperature for 30 min and then incubated for 2 h at room temperature with mouse anti-p53 DO1 or goat anti-p73 antibody at 1:150 dilution in blocking solution. Cells were washed three times with PBS and incubated for 2 h at room temperature with fluorescein isothiocyanate-conjugated anti-mouse or Alexa 494-conjugated anti-goat secondary antibodies at 1:150 dilution and in blocking solution containing DAPI (4′,6′-diamidino-2-phenylindole 1 μg/ml; Sigma). Cells were washed three times with PBS, and slides were mounted with Vectashield hard set (Vector Laboratories, Peterborough, United Kingdom). Cells were visualized with Fluoview 1000 Olympus confocal microscope by acquiring fluorescence for DAPI, FITC and Alexa 494 at the corresponding emission wavelength. To detect co-localization, the channes of red and green were merged and appearance of yellow color was analyzed. The cells with cytolasmic p53 were counted and the average from several experiments and standard deviation for at least three experiments was calculated and presented as a graph. Images representative of the majority of the cells were acquired.

2.2.18 Mdm2-mediated p53 degradation.

The day before transfection 10^5 DKO or H1299 cells were seeded in 6-well plates. Cells were transfected with 150ng plasmid encoding wild type p53 or indicated p53 mutants and 450ng (DKO cells) or 600ng (H1299 cells) plasmid encoding wild type Mdm2 or indicated Mdm2 mutants or same amount of pcDNA3.1 (Invitrogen). Each transfection mixture also contained 50ng pEGFP-N1 to control for transfection efficiency. Cells were collected 30-40h after transfection, washed with PBS and lysed with 200-250ml 2x SDS sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-p53 1801 antibody (Calbiochem), anti-Mdm2 Ab-1 and Ab-2 antibodies (Calbiochem) and anti-GFP antibody (Roche).

Chapter 3. Contribution of the DNA binding domain of p53 to regulation of its degradation by Mdm2.

3   Contribution of the DNA binding domain of p53 to regulation of its degradation by Mdm2.

Although previous studies have shown that tumour-derived p53 mutants can be targeted for degradation by Mdm2 when overexpressed in cells (246, 519), the recently identified contribution of the DNA binding domain of p53 to Mdm2 binding (265, 578) has suggested that mutant p53s may show some alterations in their sensitivity to Mdm2. Together, these observations suggest a more complex relationship between Mdm2 and p53, and that the degradation of mutant p53 might be selectively compromised in tumour cells.

Most p53 mutations found in tumours occur as point mutations in the DBD of p53 and localize to highly conserved regions of the DBD - boxes II, III, IV, V (143). Based on structural studies and reactivity with the antibodies, p53 mutants can be largely divided into structural and contact mutants, affecting the conformation of DBD or residues contacting DNA, respectively (147, 441). Structural p53 mutations alter the conformation of the DBD of p53 (436, 579), resulting in the exposure of an epitope recognized by the mutant p53-specific antibody (pAb240), which does not efficiently recognize native wild type p53 (576). This has been described as unfolded or "mutant" conformation and has been associated with many hot spot p53 mutants. Wild type p53 is preferentially recognized by wild type conformation antibody (pAb1620), which fails to recognize unfolded p53 mutants (437, 580). In our study we have utilized previously characterized p53 mutants with deletions of the conserved boxes of DBD - p53 ΔII, ΔIII, ΔIV and ΔV (561) (Fig. 3.1), as well as two hot spot tumour derived point mutants, structural - 175H, and contact - 273H. Similarly to tumour derived p53 mutants (581), each of the p53 deletion mutants fails to bind DNA and is therefore transcripitionally inactive (561). Each of the deletion mutants, like 175H, adopt the "mutant" conformation associated with structural tumour-derived p53 mutants (582). The 273H mutant is recognized by both wild-type and mutant p53 specific antibodies, and appears to show a flexible conformation (583, 584).

In this chapter the contribution of mutations and deletions in the DBD of p53 deletions to interaction, ubiquitination and degradation of p53 by Mdm2 was examined.

3.1 Analysis of the interaction of p53 DBD mutants with Mdm2.

3.1.1 Mutant conformation of DNA binding domain of p53 promotes binding of Mdm2 outside box I.

To analyze the contribution of the DBD previously characterized p53 constructs with deletions of conserved boxes of DBD p53 ΔII, ΔIII, ΔIV and ΔV and tumour-derived p53 point mutants p53175H and p53273H were used (Fig. 3.1). For analysis of the contribution of Mdm2 binding the following p53 mutants lacking N-terminal box I were used: previously described p53ΔI, p53ΔV/ΔI and p53ΔII/ΔI, and p53175H/ΔI and p53273H/ΔI, made by site-directed mutagenesis (Fig.3.1). All p53 mutants were sequenced and 72P/R polymorphism was checked. To avoid potential differences due to polymorphism, all mutants were made to have the same 72R polymorphism.
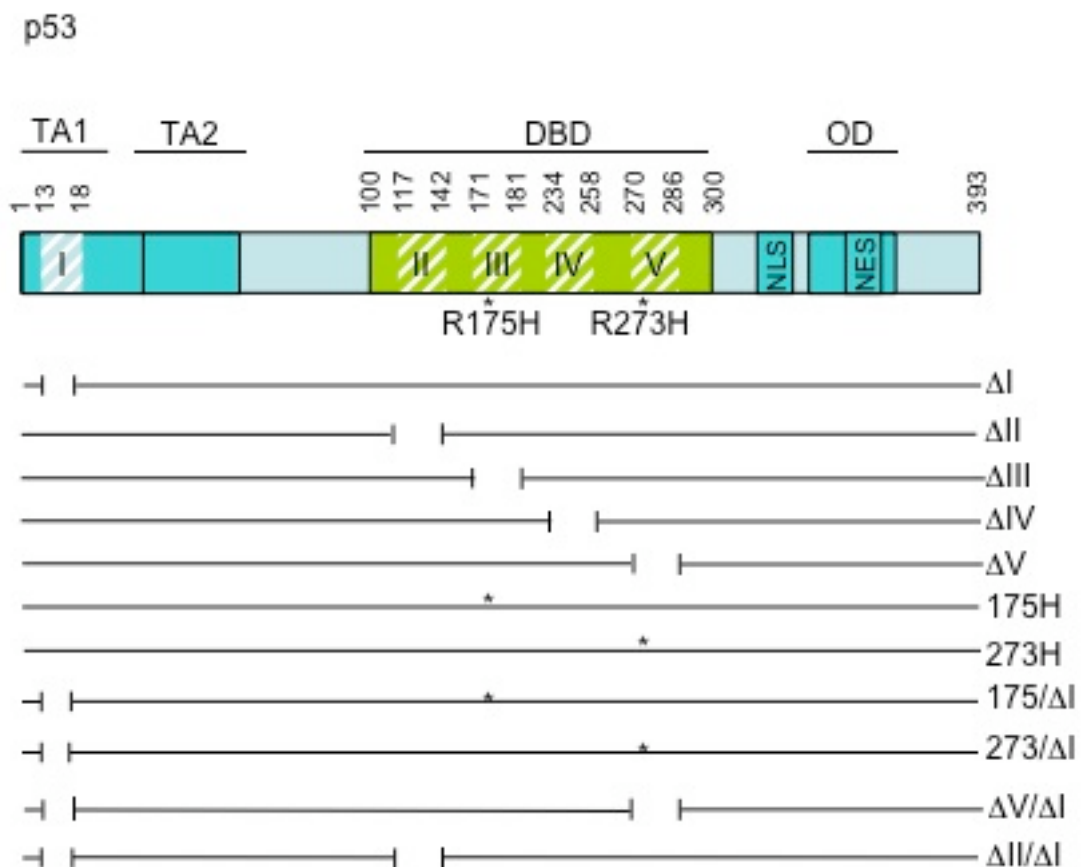
FIGURE. 3.1. Structural organization of wild type p53 and p53 mutants used in the study.
The main domains of p53, nuclear export (NES), nuclear localization (NLS) signals and the location of the conserved boxes I, II, II, IV and V and the corresponding deletions and point mutations 175H and 273H are shown.
TA - transactivation domain, DBD - DNA binding domain, PD – proline-rich domain, OD - oligomerization domain.

Previous studies have shown that the p53 mutants with deletion of each of the conserved boxes of DBD retain the ability to interact with Mdm2 *in vitro* (561). To assess their ability to interact *in vivo* co-immunoprecipitation analysis of each of the p53 DBD deletion mutants ΔII, ΔIII, ΔIV and ΔV (Fig.3.1) and wild type Mdm2 was carried out in H1299 cells. H1299 is a non-small cell lung carcinoma cell line, which is easily maintained and transfected. H1299 cells are p53-null and express low amounts of endogenous Mdm2, which makes it a convenient system to use for transient transfection of p53 constructs. As a negative control, p53 lacking box I – p53ΔI, which has been previously shown to lack the ability to interact with Mdm2, was also used in this experiment. H1299 cells were transfected with expression vectors encoding wild type p53, or p53ΔI or with p53 deletion mutants p53ΔII, ΔIII, ΔIV and ΔV along with wild type Mdm2 or empty vector (Fig.3.2). The p53 protein and its interacting partners were immunoprecipitated with p53-specific antibody 1801 and the Mdm2 protein was detected by Mdm2-specific antibodies (Ab-1, Ab-2). The 1801 antibody recognizes epitope 46-55 aa in the N-terminus of p53 and therefore should immunoprecipitate all p53 mutants used in this experiment. In this experiment wild type p53 was expressed less well than p53 mutants, though this was probably due to difference in the transfection efficiency of the wild type p53 construct as this was not observed in the subsequent experiments (Fig.3.2). p53 deletion mutants migrated slightly faster than wild type p53 on the gel, as expected. The levels of Mdm2 in the total cell lysates were not detected, however transfected Mdm2 protein was detected in the immunoprecipitates (Fig.3.2). Mdm2 protein is detected as a doublet, which migrates around 80 kD on the gel and represents full length p90 Mdm2 protein (337, 585) (Fig.3.2). It is not known what the double bands are and could be a modified form of Mdm2. All p53 mutants were efficiently immunoprecipitated with p53-specific antibody. Consistent with previous reports, wild type Mdm2 associated with wild type p53, but not with the p53ΔI lacking box I (Fig.3.2) (561), suggesting that Mdm2 binds to the N-terminus of wild type p53 and other regions on p53 do not appear to contribute. Though there have been reports of the interaction between the core domain of p53 and Mdm2 (264, 265), the interaction was not detected in this experiment. Similar amounts of Mdm2 were immunoprecipitated with all the p53 DBD deletion mutants (Fig.3.2). To confirm this observation, the immunoprecipitation was also carried out in reciprocal way with Mdm2 specific antibodies and p53 bound to Mdm2 was detected by Western blot with p53-specific antibodies. Some amount of endogenous Mdm2 was immunoprecipitated, however much more Mdm2 was detected when overexpressed (Fig.3.2). The expression of endogenous Mdm2 increased when p53ΔI was co-expressed,
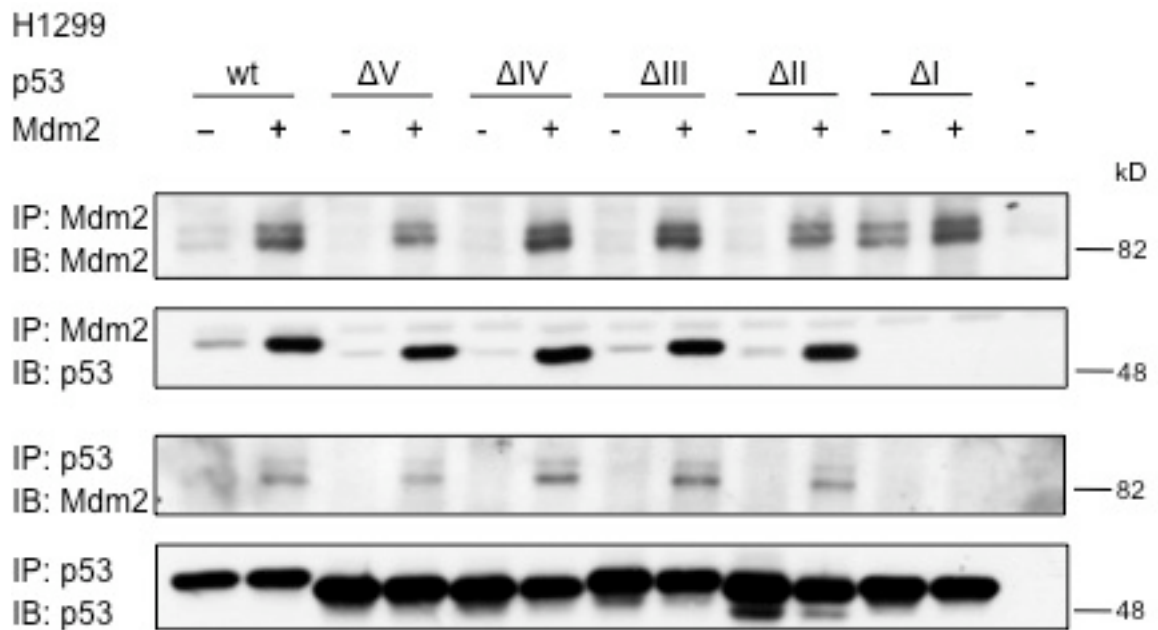
FIGURE 3.2 p53 mutants with deletions of the conserved boxes of DBD interact with Mdm2.
H1299 cells were transfected with indicated p53 constructs and with empty vector or with wild type Mdm2. Cell lysates were immunoprecipitated with Mdm2 or p53 specifc antibodies and analyzed by Western blotting.
IP: immunoprecipitation. IB: immunoblotting.

which may be due to transcriptional activation of Mdm2 by p53ΔI (Fig.3.2). Wild type p53 was readily co-immunoprecipitated with overexpressed Mdm2 (Fig.3.2). In agreement with previous reports, p53ΔI did not associate with transfected or endogenous Mdm2 (Fig.3.2). Weak p53 signal was detected in the lanes where no Mdm2 was overexpressed, which is probably due to interaction with endogenous Mdm2 (Fig.3.2). Each of the p53 DBD deletion mutants interacted with Mdm2 similarly to wild type p53 (Fig.3.2). The results of the reciprocal immunoprecipitation indicates that only part of p53 is engaged in complex with Mdm2 and not all Mdm2 is interacting with p53. Consistent with *in vitro* data (561), this experiment shows that the change in conformation of DBD of p53 does not affect the ability to interact with Mdm2 in cells.

Mdm2 is known to interact with both the N-terminus (256, 561) and the central DBD of p53 (578). To evaluate the contribution of the N-terminal binding site (box I) to the interaction between mutant p53 and Mdm2, the ability of Mdm2 to interact with p53 DBD mutants lacking conserved box I was tested - (Fig 3.1). To this end co-immunoprecipitation analysis of the p53 mutants with wild type Mdm2 was carried out in H1299 cells (Fig.3.3). This and all the following experiments were done in the presence of proteasome inhibitors to avoid effects of different degradation rates of transfected proteins, as shown in Figure 3.2. Mdm2 was efficiently immunoprecipitated with wild type p53, but not with p53 lacking box I (Fig.3.3). In addition to the p53 band, slower migrating form of

86

p53 was detected with p53 specific antibody (Fig.3.3). This modified form of p53 correlated with binding of Mdm2 to p53, suggesting it could be ubiquitinated form of p53. Slightly more Mdm2 was co-immunoprecipitated with p53175H and p53ΔV than with wild type p53 and similar amount of Mdm2 was immunoprecipitated with p53273H as with wild type p53 (Fig. 3.3). Interestingly, p53 DBD mutants lacking conserved box I - ΔV/ΔI, 175H/ΔI and 273H/ΔI clearly retained the ability to bind Mdm2, although the interaction was reduced compared to the DBD mutants retaining box I - 175H, 273H and ΔV (Fig. 3.3). These results suggest that mutation in the DBD of p53 promotes alternative interaction between Mdm2 and p53, which occurs outside box I of p53. This result was confirmed at least four times in independent experiments.
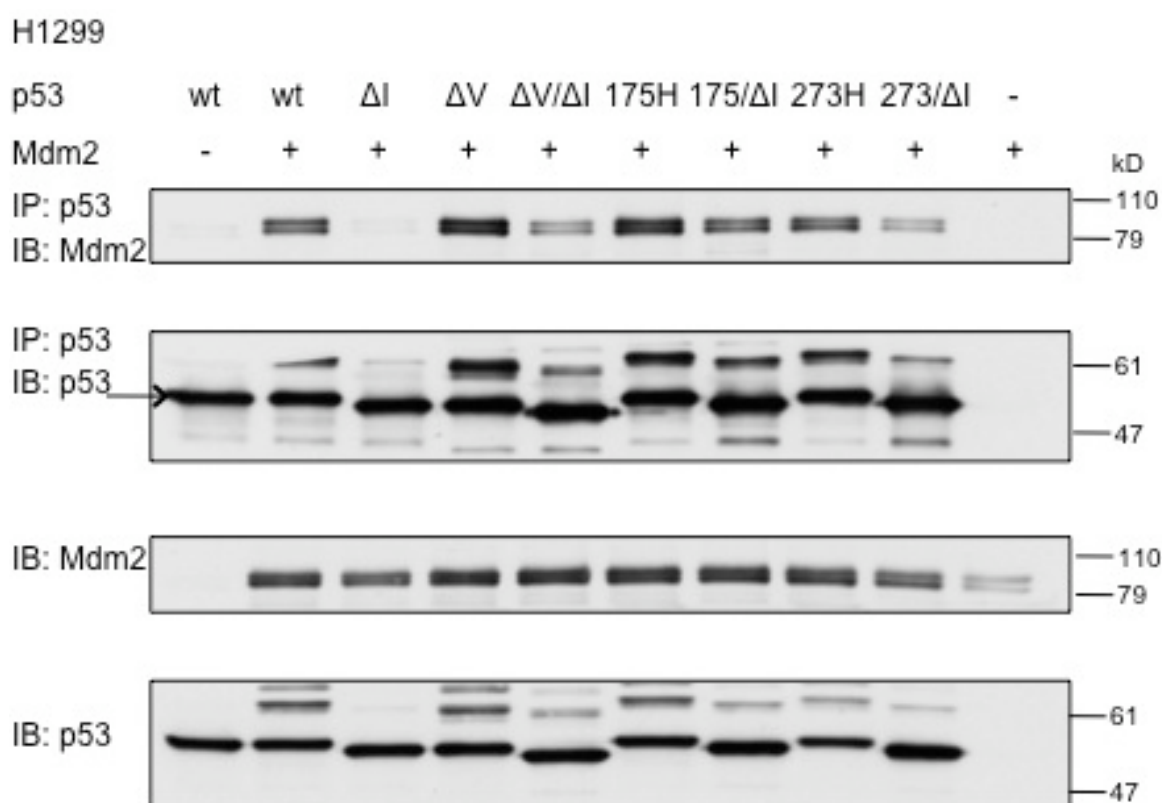
FIGURE 3.3. Mdm2 interacts with p53 DBD mutants lacking the N-terminal Mdm2 binding site.
H1299 cells were co-transfected with indicated p53 mutants and with empty vector or with indicated Mdm2 constructs. Cells were treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibodies and immunoblotted with p53- and Mdm2-specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

3.1.2 The RING domain of Mdm2 contributes to the interaction with p53 DBD mutant lacking box I.

Next, the analysis of the functional domains on Mdm2 involved in the interaction with mutant p53 was carried out. To this end Mdm2 mutants with deletions or mutations of functional domains, shown in Figure 3.4, were used.

The N-terminal domain on Mdm2 is involved in interaction with p53 and Mdm2 mutant lacking the region 58-89aa - Mdm2ΔN - fails to bind p53 and inactivate p53 (256) (Fig.3.4). Mdm2ΔA, has a deletion of a large part of the central acidic domain (212-296aa) which covers the region previously shown to bind ARF and involved in interaction with DBD of p53. Mdm2ZF mutant has two point mutations in the zinc finger domain substituting structurally important cysteins C305 and C308 with alanines (Fig.3.4). The previously characterized mutant Mdm2ΔR is truncated from the amino acid 440 in the C-terminus, which deletes the RING finger domain and the extreme C-terminus of Mdm2, and is deficient in the ubiquitin ligase activity, however Mdm2ΔR retains the ability to interact with p53 (256). Mdm2 C464A, previously characterized, has a substitution of cysteine 464 with alanine in the RING domain of Mdm2, which is involved in the

FIGURE 3.4. Structural organization of the wild type Mdm2 and Mdm2 mutants.
The main domains of Mdm2 - p53-binding domain, acidic domain, RING domain and zinc finger domain - and the corresponding Mdm2 deletion and point mutants are shown.
N – N-terminal p53 binding site, R – RING domain, A – acidic domain,
ZF – zinc finger, C – C-terminus.

coordination of zinc atom and abolishes ubiquitin ligase activity of Mdm2 (175). This mutation abrogates the structure of the RING domain of Mdm2 and this mutant also fails to oligomerize with wild type Mdm2. The Mdm2ΔC has a truncation of the 14 last C-terminal amino acids just after the RING domain (Fig.3.4) and has been made in our laboratory by Dr. Uldrijan. The C-terminus of Mdm2 has been shown to contribute to the ubiquitin ligase activity of Mdm2. GFP-tagged RING domain expresses the region of Mdm2 384–491aa, which encompasses the RING domain and a few adjacent residues and was described recently (270) (Fig.3.4).

FIGURE 3.5. The acidic domain of Mdm2 is not required for the interaction of Mdm2 with p53 DBD mutants lacking box I.
H1299 cells were co-transfected with indicated p53 and Mdm2 constructs. Cells were treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibodies and immunoblotted with p53- and Mdm2-specific antibodies.

The contribution of the domains of Mdm2 to interaction with p53 mutant outside the N-terminus was analyzed. To this end a co-immunoprecipitation analysis of Mdm2 mutants was carried out with the p53 DBD mutants lacking box I. Recent studies have shown that Mdm2 can interact with the DBD of p53 via its acidic domain (265, 266). To test whether the binding to p53 mutants outside box I involves the acidic domain of Mdm2, co-immunoprecipitation analysis of Mdm2 mutant ΔA lacking acidic domain (Δ212-296aa) with p53 DBD mutants lacking box I - ΔV/ΔI, ΔII/ΔI and 175/ΔI was carried out (Fig.3.5). Mdm2ΔA migrates lower than wild type Mdm2 around 65 kD and is detected as a single band (Fig.3.5). Similarly to the results shown in Fig.3.3, wild type Mdm2 did not interact with p53ΔI, however Mdm2 readily associated with all of the p53 DBD mutants lacking box I (Fig.3.5). However, deletion of the acidic domain of Mdm2 did not prevent the

interaction with wild type p53 or the DBD mutants lacking box I (Fig.3.5). The modified form of p53 was slightly reduced upon expression of Mdm2ΔA (Fig.3.5), suggesting that the acidic domain of Mdm2 is important for modifying p53, which could be ubiquitinated p53. This is consistent with previous results showing that acidic domain of Mdm2 is critical for ubiquitin ligase activity (261, 262). The modified form can also be neddylated p53, which is known to be mediated by Mdm2 (223).

Though previous reports have shown that acidic domain contributes to interaction with wild type p53 (264-266), it was not detected in this experiment. The reason for discrepancy is not clear, and is possibly due to difference in the cell types used.
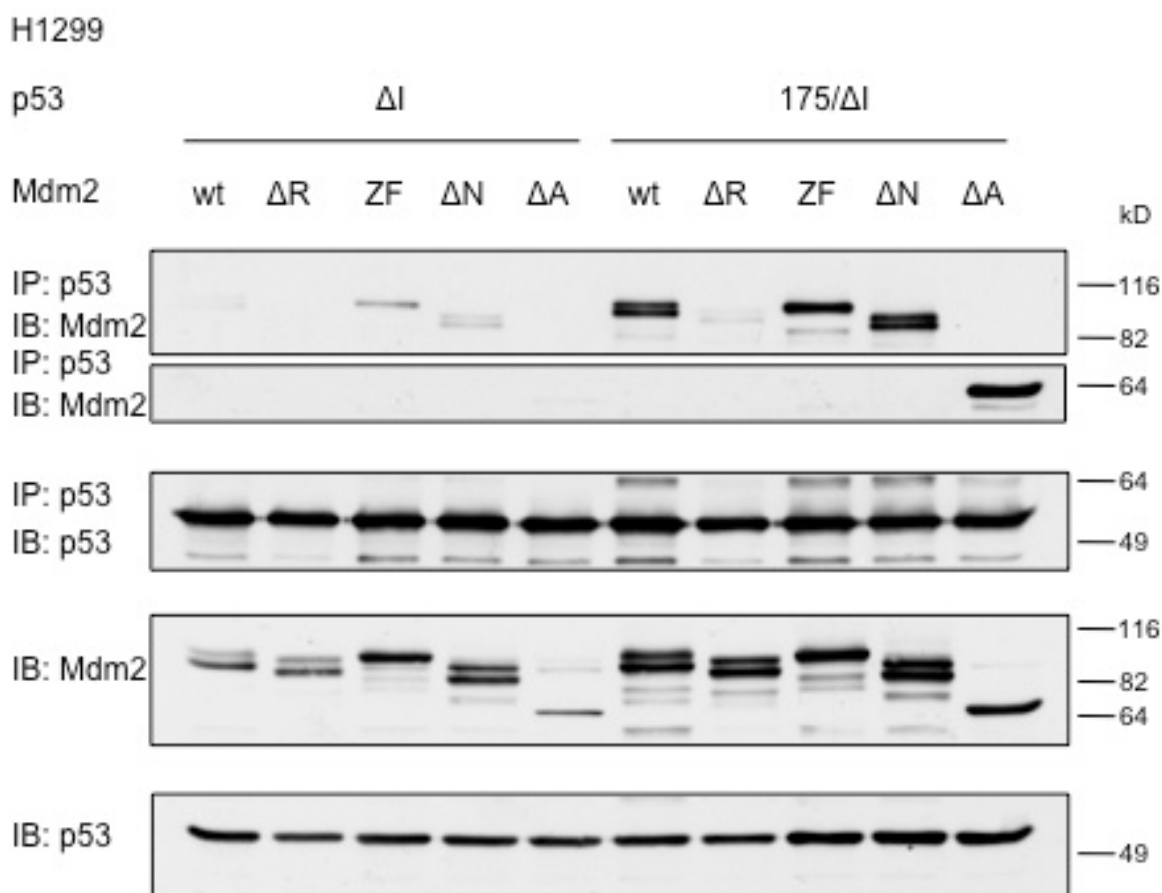
FIGURE 3.6. **Analysis of functional domains of Mdm2 involved in the interaction of Mdm2 with p53175/ΔI.**
H1299 cells were co-transfected with indicated p53 and Mdm2 constructs. Cells were treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibodies and immunoblotted with p53- and Mdm2-specific antibodies. After Western blotting with p53 the membrane was reblotted for Mdm2 to detect Mdm2ΔA in the immunoprecipitated samples.
IP: immunoprecipitation. IB: immunoblotting.

To determine which region on Mdm2 interacts with mutant p53 outside box I several additional Mdm2 mutants targeting functionally important regions were examined: N-terminus - ΔN, zinc finger domain - ZF and RING finger domain - ΔR (Fig.3.4) (484).

The Mdm2 ΔA mutant was also included again. Mdm2 ZF migrates slightly higher than the full-length Mdm2, probably due to change of the charge of the protein, and is detected as a single band, suggesting mutation of the cysteins prevents the modification of Mdm2 (Fig.3.6). As expected, Mdm2ΔN and Mdm2ΔR migrate on the gel slightly faster than wild type Mdm2 and appear as a doublet (Fig.3.6). Consistent with previous data, p53 lacking box I – p53ΔI - was deficient in the ability to bind wild type Mdm2 or Mdm2ΔRING (Fig.3.6). However weak interaction of p53ΔI with Mdm2ZF and Mdm2ΔN was detected (Fig.3.6), suggesting that other regions on p53 can contribute to the interaction with Mdm2. Confirming the results described above, wild type Mdm2 co-immunoprecipiated with p53175H/ΔI, and interaction was not affected by deletion of the acidic domain ΔA. Deletion of N-terminal p53 binding site on Mdm2 did not prevent the interaction (Fig.3.6), consistent with the structural studies suggesting that N-terminus of Mdm2 interacts onlwith N-terminus on p53, and not elsewhere on p53 (266). Mdm2ZF mutant also was able to interact with p53 175/ΔI with a similar affinity to wild type Mdm2 (Fig.3.6). However, deletion of RING almost completely abolished the ability of Mdm2 to interact with p53175/ΔI (Fig.3.6). This observation was made in three independent experiments. Next, to confirm this result and to test the contribution of the RING domain to interaction with full-length p53 proteins co-immunoprecipitation anaysis of wild type or p53175H, full length or lacking box I, with wild type Mdm2 or Mdm2ΔR, was carried out. Interestingly, deletion of the RING domain also decreased the interaction of wild type p53 with Mdm2 (Fig.3.7), suggesting that this region contributes to the interaction with wild type p53. Consistent with previously shown results, Mdm2 interacted with p53175H slightly better than with wild type p53, and retained the ability to interact with p53 175/ΔI though the interaction was reduced. Deletion of the C-terminus of Mdm2 significantly reduced the interaction with p53175H and with p53175/ΔI, suggesting that much of the interaction between Mdm2 and mutant p53 occurs via RING finger domain (Fig.3.7). RING is involved in binding to E2 and is required for ubiquitin ligase activity by Mdm2. A point mutation replacing cysteine residue 464 with alanine (C464A) abrogates zinc coordination and disrupts the structure of RING domain, inhibiting the ubiquitin ligase activity of Mdm2 (175). To examine whether the E3 activity of Mdm2 is required for the interaction with p53, Mdm2 mutant C464A was included in the co-immunoprecipitation analysis. Mdm2 C464A protein interacted with p53175/ΔI similarly to wild type Mdm2 (Fig.3.7), indicating that the ubiquitin ligase activity of Mdm2 is not important, but that the entire RING finger domain is required for the interaction of Mdm2 with mutant p53. These results also suggest that binding of p53 to Mdm2 is distinct from E2-binding, which is

abrogated by C464A mutation. Mdm2ΔR mutant lacks the RING domain and the extreme C-terminus, which also contributes to ubiquitin ligase activity of Mdm2 (270).
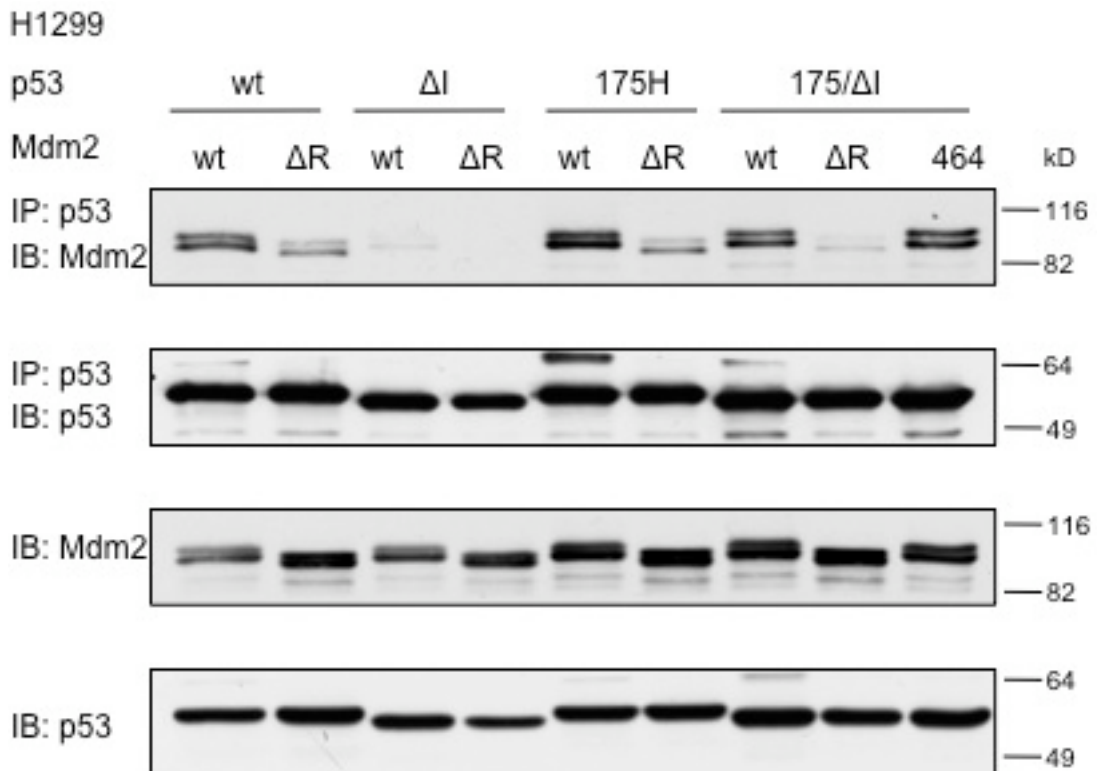
FIGURE 3.7. **Deletion of the RING domain on Mdm2 abrogates its ability to interact with p53175/ΔI.**
H1299 cells were co-transfected with indicated p53 and Mdm2 constructs. Cells were treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibodies and immunoblotted with p53- and Mdm2-specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

To test which part of the C-terminus of Mdm2 contributes to the interaction with mutant p53 Mdm2 ΔC lacking the last 14 amino acids, but retaining the RING domain and construct expressing GFP tagged RING domain was used (Fig.3.8). Co-immunoprecipitation assay of p53 175/ΔI with GFP-RING or Mdm2 mutant lacking the extreme C-terminus from 483aa - Mdm2ΔC - was carried out (Fig.3.8). RING has been also reported to interact with the acidic domain of Mdm2 (586), therefore in this experiment GFP-RING was also co-expressed with Mdm2ΔR to test if RING can rescue the ability of Mdm2ΔR to interact with mutant p53. GFP-RING is expressed in cells and is readily detected with the GFP-specific antibody as a single band (Fig.3.8). RING co-immunoprecipitated with p53175/ΔI, suggesting that RING binds to mutant p53, however it remains to be confirmed *in vitro*.
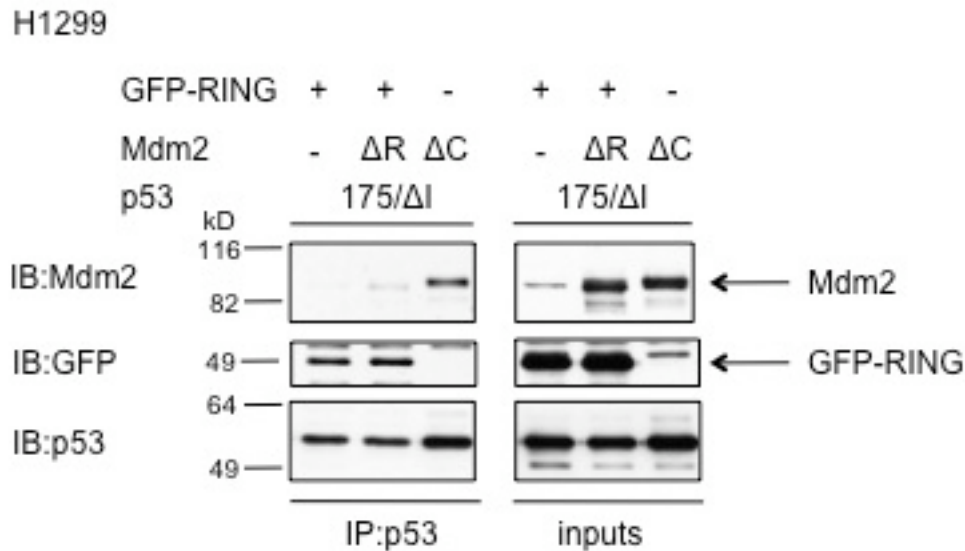
FIGURE 3.8. **RING domain of Mdm2 is involved in interaction with p53175/ΔI.**
H1299 cells were co-transfected with indicated p53 and Mdm2 constructs. Cells were treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibodies and immunoblotted with p53- and Mdm2-specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

whether the interaction is direct. Mdm2ΔC, retaining RING, associated with mutant p53 (Fig.3.8), implying that extreme C-terminus is not required for the interaction. Expression of RING did not rescue the ability of Mdm2ΔR to interact with p53 mutant. Taken together these results show that while both mutant and wild type p53 bind Mdm2, the mechanisms of binding are quite distinct. Whereas wild type p53 and Mdm2 associate via the corresponding N-termini of each protein, interaction of mutant p53 and Mdm2 can occur outside N-terminus on mutant p53. These results suggest, that in addition to the N-terminus, RING domain of Mdm2 is involved in the interaction with both wild type and mutant p53.

3.2   Analysis of the ubiquitination of p53 DBD mutants by Mdm2.

3.2.1   p53 mutants are less efficiently ubiquitinated by Mdm2 *in vitro*.

To test whether the difference in the interaction between Mdm2 and mutant p53 affects the ability of Mdm2 to ubiquitinate mutant p53, the efficiency of Mdm2-mediated ubiquitination of wild type p53 and p53 mutants *in vitro* was examined. *In vitro* translated wild type p53 or p53 DBD mutants were prebound to GST-purified Mdm2 and *in vitro* ubiquitination reactions were set up with E1 enzyme, ubiquitin and ATP in the presence or

absence of E2 enzyme as a negative control. Wild type p53 was extensively polyubiquitinated by Mdm2 when all components of ubiquitination reaction were present (Fig.3.9). It should be noted that in the negative control reactions, where E2 was omitted, some modified forms of p53 were present (Fig.3.9), suggesting that some of the E2 activity came from the reticulocyte lysate. Interestingly, each of the p53 deletion mutants was substantially less well ubiquitinated than wild type p53 (Fig. 3.9A). A similar reduction in the ability to be ubiquitinated by Mdm2 was also seen using the p53175H point mutant (Fig. 3.9B). This suggests that the wild type conformation of the DBD is required for the efficient ubiquitination of p53 by Mdm2. The difference in the interactions of wild type and mutant p53 with Mdm2 could be the reason. Binding of p53 to the N-terminus of Mdm2 is required for activation of the ubiquitin ligase activity of Mdm2. Interaction of mutant p53 with Mdm2 does not require the N-terminus, suggesting that the ubiquitin ligase activity might not be activated properly.
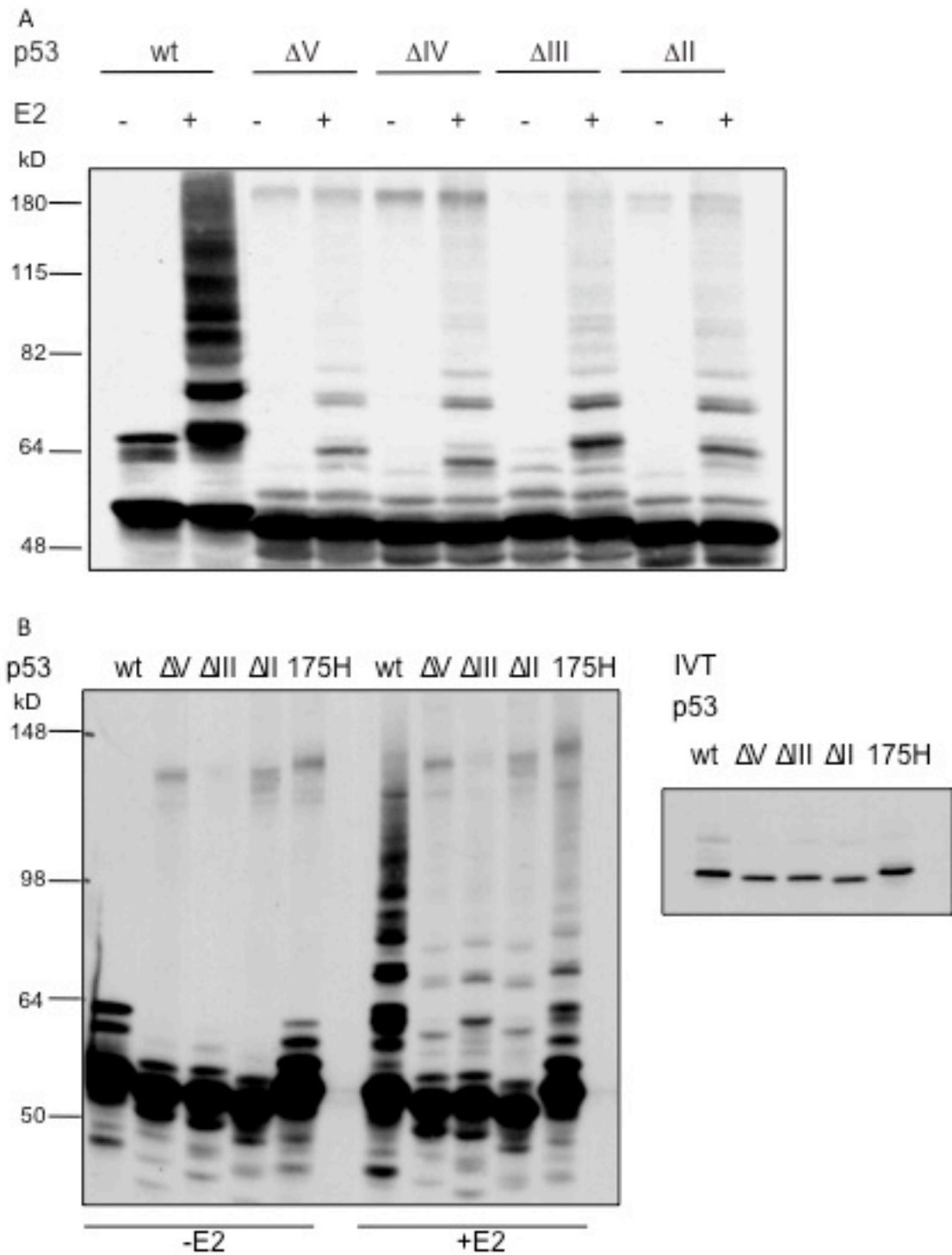
FIGURE 3.9. Analysis of ubiquitination of p53 DBD mutants *in vitro*.
A, B. Wild type p53 and p53 mutants with deletions of the indicated conserved and p53175H (B) were *in vitro* translated and bound to GST-Mdm2. *In vitro* ubiquitination reactions were carried out and analyzed by Western blot with a p53 specific antibody (DO1). **B** (right panel). *In vitro* translated reactions prior to *in vitro* ubiquitination reaction were subject to Western blot analysis

3.2.2 p53 mutants are more highly ubiquitinated *in vivo* independently of Mdm2.

The results presented so far showed that Mdm2 less efficiently ubiquitinates mutant p53 in *in vitro* reactions than wild type p53. To detect ubiquitinated p53 in cells, the *in vivo* ubiquitination assay was carried out in p53-/-mdm2-/- mouse embryonic fibroblasts (DKO), which provide a clean system to analyze the activity of transfected Mdm2. Wild type p53 was expressed with wild type Mdm2 or Mdm2C464A, a mutant that is inactive for E3 activity along with HA-tagged ubiquitin. Use of HA-tagged ubiquitin allows to specifically detect ubiquitinated p53, and not other ubiquitin-like modifications. To prevent degradation of ubiquitinated p53, cells were treated with proteasome inhibitor MG132. Cells were lysed under strong denaturing conditions in order to disrupt the interaction of p53 with its binding partners, so that only p53 would be immunoprecipitated with p53-specific antibodies. Following the lysis, p53 was immunoprecipitated with p53-specific antibody (DO1) and ubiquitinated p53 was detected with anti-HA antibody. Some weak residual ubiquitination was detected when p53 was expressed on its own in DKO cells (Fig. 3.10), possibly due to some contribution of other endogenous ubiquitin ligases.
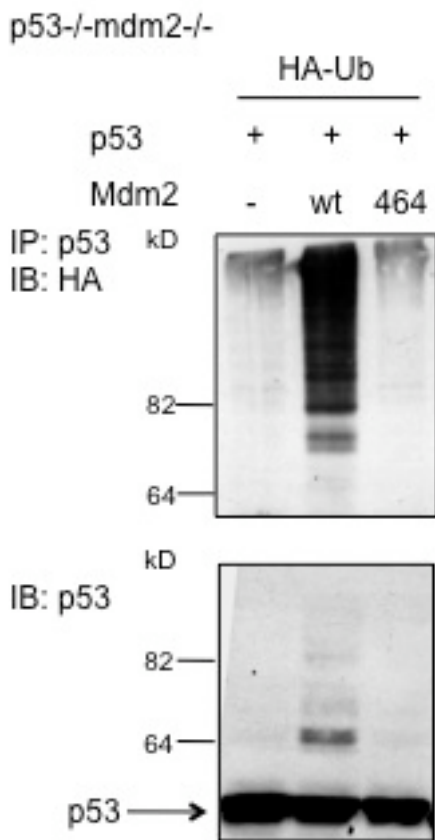
FIGURE 3.10. *In vivo ubiquitination assay.*
p53-/-mdm2-/- cells were transfected with wild type p53 and HA-tagged ubiquitin (HA-Ub) and with empty vector, wild type (wt) Mdm2 or Mdm2 C464A mutant (464). Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody.

Consistent with previous reports, when wild type Mdm2, but not E3-inactive Mdm-464, was co-expressed, heavily polyubiquitinated p53 was detected (Fig.3.10). Some modified

forms of p53 were detectable in the total cell lysate by direct Western blotting with the p53-specific antibodies, which may be monoubiquitinated p53. Polyubiquitinated p53 was not visible with p53-specific antibodies, possibly due to masking of p53 by ubiquitination. This assay was repeated in other cell types with similar results and was routinely used to analyze ubiquitination of p53.
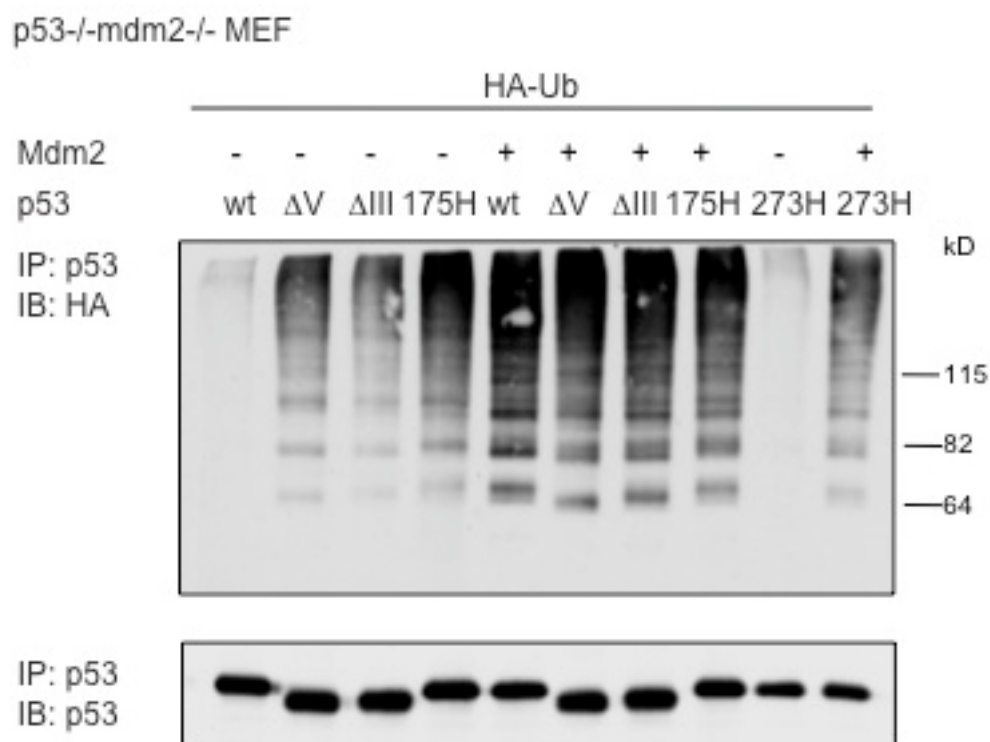
FIGURE 3.11. Analysis of ubiquitination of p53 DBD mutants in p53-/-mdm2-/- cells.
p53-/-mdm2-/- cells were transfected with wild type p53 or the indicated p53 mutants and HA-tagged ubiquitin (HA-Ub) and with empty vector or wild type Mdm2. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody.

Next, ubiquitination of p53 DBD mutants by Mdm2 *in vivo* was tested in DKO cells (3.11). While little ubiquitination of wild type p53 was detected in the absence of co-expressed Mdm2 (Fig.3.11), all of the p53 mutants that adopt an altered conformation (DBD deletions and p53175H) were already heavily ubiquitinated in the absence of Mdm2 (Fig.3.11). This may be due to the activity of the endogenous ubiquitin ligases, which selectively ubiquitinate mutant, but not wild type p53. This suggests that mutation in the DBD promotes Mdm2-independent ubiquitination of p53. To test if "mutant" conformation of DBD is required for Mdm2-independent ubiquitination, the contact p53 mutant 273H was included. The p53273H mutant behaved much more like wild type p53 in the *in vivo* ubiquitination assay. The p53273H was not ubiquitinated in the absence of Mdm2, and only when Mdm2 was co-expressed polyubiquitination was detected (Fig.3.11).

Expression of Mdm2 substantially increased the levels of ubiqutination of the wild type p53 protein, but had a less pronounced effect on enhancing the ubiquitination of the p53 mutants (Fig.3.11). This is consistent with the less efficient ubiquitination of p53 mutants by Mdm2 seen *in vitro* (Fig.3.9). In other words, though wild type p53 and p53 mutants are ubiquitinated to similar levels when Mdm2 is overexpressed, ubiquitination of wild type p53 is almost entirely mediated by Mdm2, whereas ubiquitination of p53 mutants results from Mdm2-independent and Mdm2-mediated ubiquitination. Given that Mdm2 is overexpressed, it seems that the contribution of endogenous ubiquitin ligase to ubiquitination of mutant p53 is quite pronounced. These results were reproduced several times. To confirm the results in other cell types, the ubiquitination assay was repeated in H1299 cells (Fig.3.12).
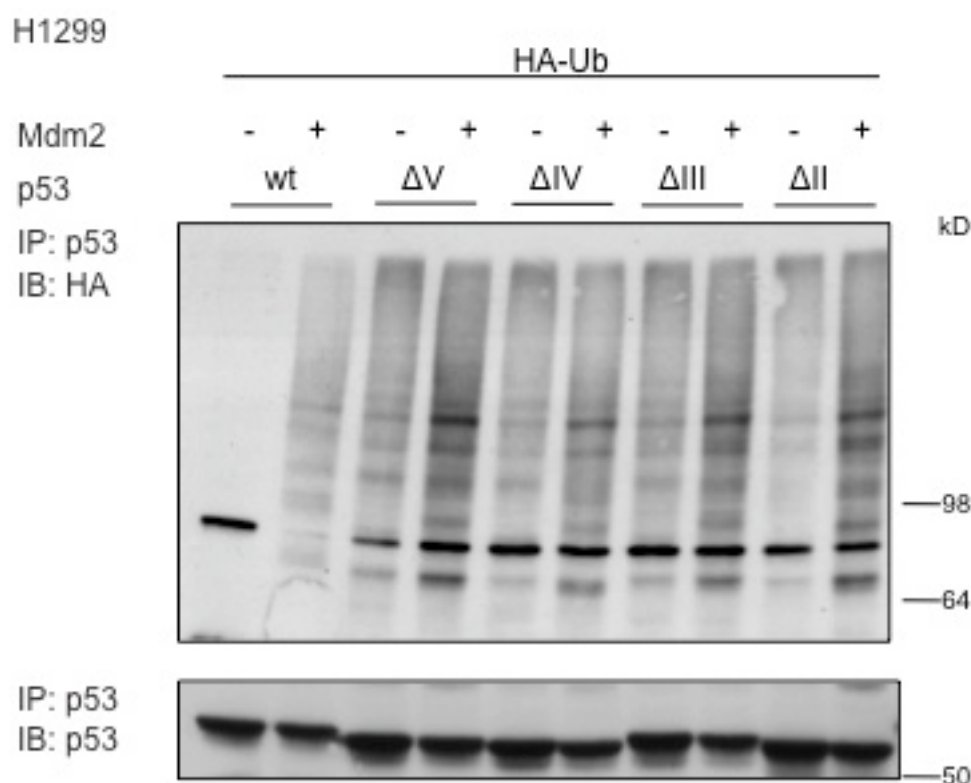
FIGURE 3.12. Analysis of ubiquitination of p53 DBD mutants in H1299 cells.
H1299 cells were transfected with wild type p53 or the indicated p53 mutants and HA-tagged ubiquitin (HA-Ub) and with empty vector or wild type Mdm2. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody.

To test if the endogenous Mdm2 can contribute to ubiquitination of mutant p53 in H1299 cells, the assay was carried out with wild type p53, p53ΔI, p53ΔV and p53ΔV/ΔI, a DBD mutant, deficient in N-terminal interaction with Mdm2 (Fig.3.13). The weak

polyubiquitination signal on wild type p53 was observed in this experiment (Fig.3.13), and p53 lacking box I, incapable of interacting with Mdm2, was ubiquitinated similarly to wild type p53, suggesting that residual ubiquitination of wild type p53 observed in these cells was not mediated by Mdm2 (Fig.3.13). Consistent with previous data, p53ΔV was hyperubiquitinated, and deletion of box I did not reduce ubiquitination (Fig.3.13). These data confirm the result in DKO and were reproduced in 3 independent experiments. The observation that box I is not required for this alternative ubiquitination also suggests there is no competition between Mdm2 and this other E3.
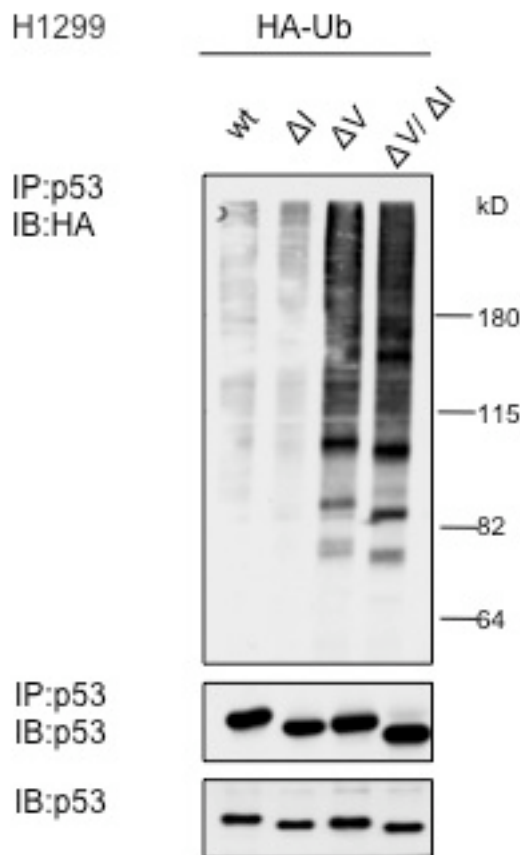
FIGURE 3.13. Deletion of Mdm2-binding site on p53 mutant does not reduce ubiquitination in H1299 cells.
H1299 cells were transfected with wt p53 or indicated p53 mutants with empty vector or wild type Mdm2 and with HA-ubiquitin and treated with MG132. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody.

In summary, Mdm2 contributes to ubiquitination of mutant p53, though to a lesser extent than to that of wild type p53. The conformational change in the DNA binding domain promotes the Mdm2-independent ubiquitination of p53 mutants. The Mdm2-independent ubiquitination is discussed in more details in chapter 4.

3.3 Analysis of degradation of p53 DBD mutants by Mdm2.

Despite the weak effect of Mdm2 on ubiquitination of the mutant p53s, previous reports have shown clearly that Mdm2 can contribute to the degradation of the p53 DBD mutants in cells (246, 519). The data of previous subchapter suggest that Mdm2 may play a role in the degradation of mutant p53s that is distinct from ubiquitination. Indeed, two

roles for Mdm2 in the degradation of wild type p53 have been suggested recently – one to ubiquitinate p53 and one to deliver it to the proteasomes (328-330). The ability of Mdm2 to degrade p53 DBD mutants and the contribution of its ubiquitin ligase activity were investigated in this subchapter.

First, the degradation ability of Mdm2 towards wild type p53 was verified in the DKO cells. In this experiment DKO cells were transfected with wild type p53 along with empty vector or wild type Mdm2. In order to analyze the degradation of p53 protein more accurately, GFP was co-transfected to control for the transfection efficiency. Cells were untreated or treated with proteasome inhibitor MG132 and protein levels of p53 and GFP were assessed with the p53- and GFP-specific antibodies by Western blotting (Fig.3.14). p53 protein levels were significantly decreased upon the co-expression of Mdm2.
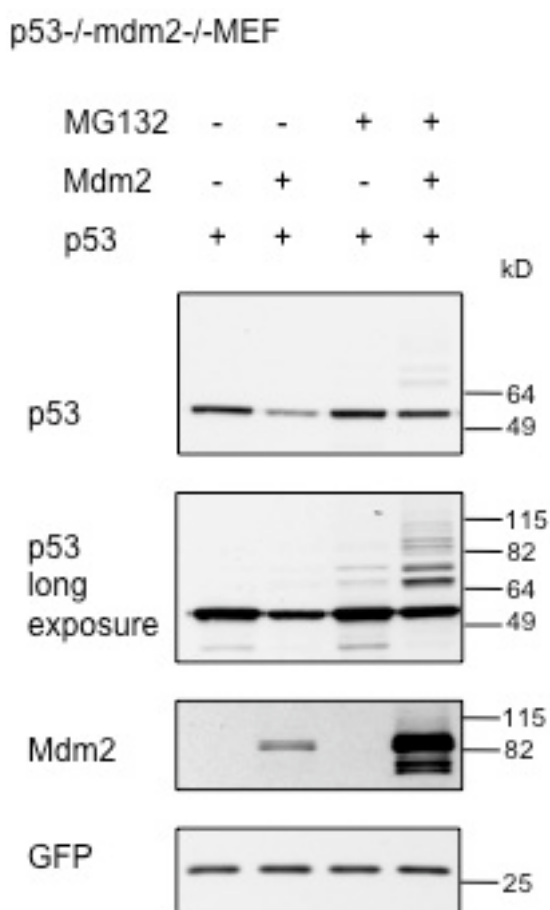
FIGURE 3.14. Mdm2-mediated proteasomal degradation of p53. p53-/-mdm2-/- MEFs were transfected with wild type p53 and with empty vector or wild type Mdm2 and were untreated or treated with proteasome inhibitor MG132 where indicated. GFP was co-transfected to control for transfection efficiency. Western blot analysis was done with p53 and GFP-specific antibodies.

(Fig.3.14). This was inhibited when cells were treated with proteasome inhibitors, confirming that Mdm2 degrades p53 by targeting it to the proteasomes. Appearance of higher modified forms of p53 was seen, when cells were treated with proteasome inhibitors, which may be ubiquitinated p53. It should be noted, that the efficiency of Mdm2-mediated degradation varied to a certain extent between the experiments, however usually the ratio 3:1 of Mdm2 to p53 plasmids was used for degradation effect.

3.3.1 p53 DBD mutants are degraded by Mdm2 *in vivo* independent of the N-terminal interaction.

After the system was set up, the degradation of p53 DBD mutants - p53175H, p53273H, p53ΔV and p53ΔIII - was tested in DKO cells. All experiments in this subchapter were done in the absence of proteasome inhibitors (Fig.3.15). Though mutant p53 protein accumulates to high levels in tumours and was shown to have an extended half life in some tumour cell lines (41, 517-519), p53 mutant proteins were expressed at levels similar to wild type p53 protein when transiently expressed in cells (Fig.3.15). This was observed in many experiments, though with some variation of the expression levels. Equal levels of GFP were expressed in this experiment, suggesting similar transfection
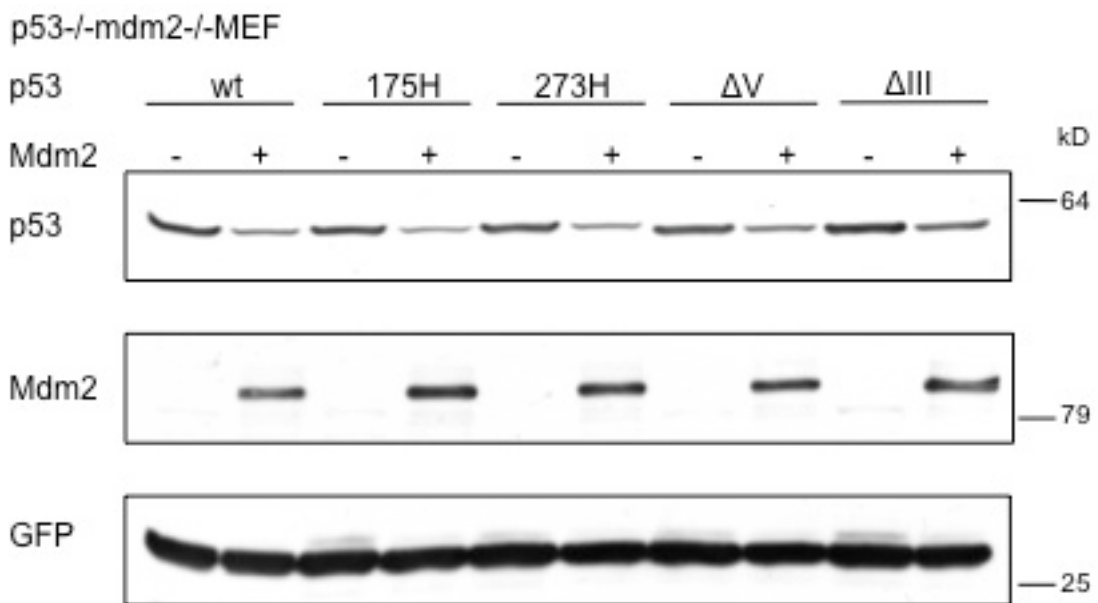
FIGURE 3.15. p53 DBD mutants are degraded by Mdm2.
p53-/-mdm2-/- cells were transfected with indicated p53 plasmids and with empty vector or wild type Mdm2. GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (DO1), Mdm2-specific and GFP-specific antibodies.

efficiency. Similarly to Fig. 3.14, wild type p53 was degraded by Mdm2 in this experiment (Fig.3.15), though the degradation appeared to be somewhat less efficient than in the result in Fig.3.14. However, there was no apparent difference in the ability of Mdm2 to degrade p53 DBD mutants (Fig.3.15), consistent with previous reports (158). Mdm2 degraded conformational 175H and contact 273H p53 mutants with similar efficiency to wild type p53 (Fig.3.15), suggesting the ability of Mdm2 to degrade p53 does not depend on conformation of p53 DBD. The result of this experiment was confirmed in several independent experiments, some of which are discussed later.

As shown above, Mdm2 can interact with mutant p53 lacking the N-terminal binding site (Fig.3.3). Therefore, the ability of Mdm2 to degrade p53 proteins lacking box I was tested in DKO cells (Fig. 3.16). Consistent with published reports (246), wild type p53 was efficiently degraded by Mdm2, whereas deletion of box I rendered p53 resistant to degradation (Fig.3.16). This is consistent with the crucial role of the N-terminal interaction between p53 and Mdm2 in the ability of Mdm2 to ubiquitinate and degrade wild type p53 (Fig.3.16). Interestingly, Mdm2 was also able to target the degradation of p53 DBD mutants lacking box I, although clearly to a lesser extent than p53 DBD mutants that retain box I (Fig.3.16). This is consistent with the ability of Mdm2 to interact with the p53 DBD mutants lacking box I. This result was confirmed in at least four independent experiments. In this experiment the levels of Mdm2 were slightly higher when co-transfected with mutant p53, which can be explained by higher transfection efficiency as evidenced by higher GFP levels. This result implies that the mechanism of Mdm2-mediated degradation of mutant p53 does not require the N-terminal interaction between p53 mutant and Mdm2, suggesting that the E3 activity of Mdm2 does not need to be activated. This suggests that Mdm2 can directly target p53 mutants that have been ubiquitinated by other E3 to the proteasomes.

To support these observations, the effect of Mdm2 mutant lacking the N-terminus Mdm2ΔN (Δ58-89) was examined. The N-terminus of p53 has been reported to interact only with the N-terminal p53 binding site on Mdm2 (266, 587). Therefore, if box I is not required for degradation of p53 mutants by Mdm2, Mdm2ΔN should still be able to degrade mutant p53. To test this, wild type p53, p53ΔV or p53175H mutants were expressed along with wild type Mdm2 or with Mdm2 ΔN (Fig.3.17). In agreement with the published data (563), Mdm2ΔN only weakly degraded wild type p53, confirming that the N-terminal p53 binding site on Mdm2 is required for efficient degradation of wild type p53. (Fig.3.17). Mdm2 (Δ58-89aa) retained some ability to degrade the p53 mutants (Fig.3.17), consistent with the ability of Mdm2 to degrade p53 mutants in the absence of
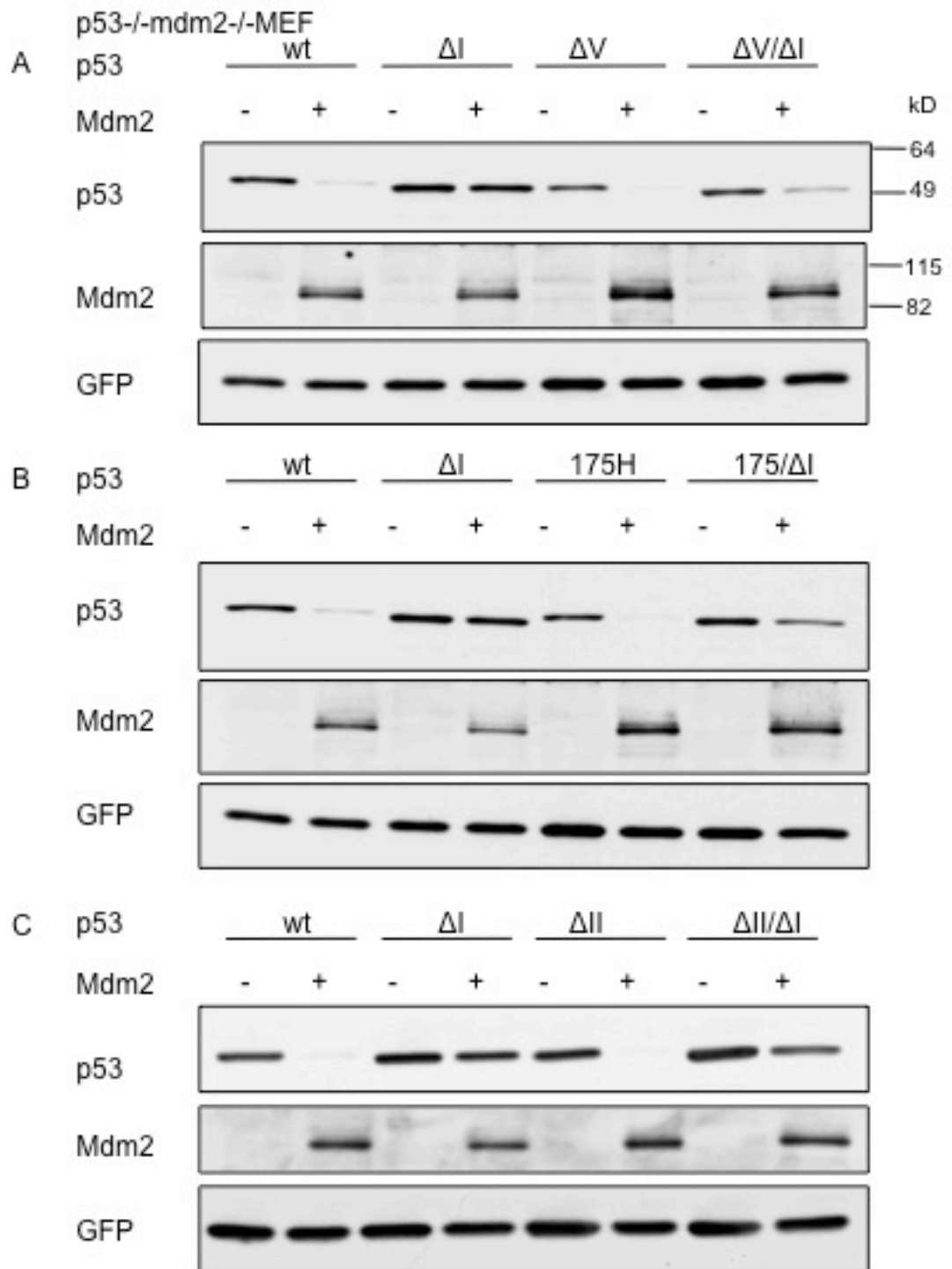
FIGURE 3.16. p53 DBD mutants lacking box I are degraded by Mdm2.
p53-/-mdm2-/- cells (A, B, C) were transfected with wild type p53 and ΔI and p53 DBD mutants: ΔV and ΔV/ΔI (A); 175H and 175H/ΔI (B); ΔII and ΔII/ΔI (C) along with empty vector or wild type Mdm2. GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (1801), Mdm2-specific and GFP-specific antibodies.

the N-terminal interaction. The result of this experiment was reproduced in two independent experiments.

These results are consistent with a model, in which mutant p53 has been ubiquitinated independently of Mdm2 and can be targeted to the proteasome by Mdm2. This activity of Mdm2 does not appear to require the interaction between N-terminal domains of Mdm2 and p53. However, it was consistently noticed that this degradation of mutant p53 was less efficient than that seen when the N-terminal interaction site was intact (Fig.3.16, 3.17), suggesting that the mechanisms that degrade wild type p53 still contribute to the degradation of the mutant p53s.
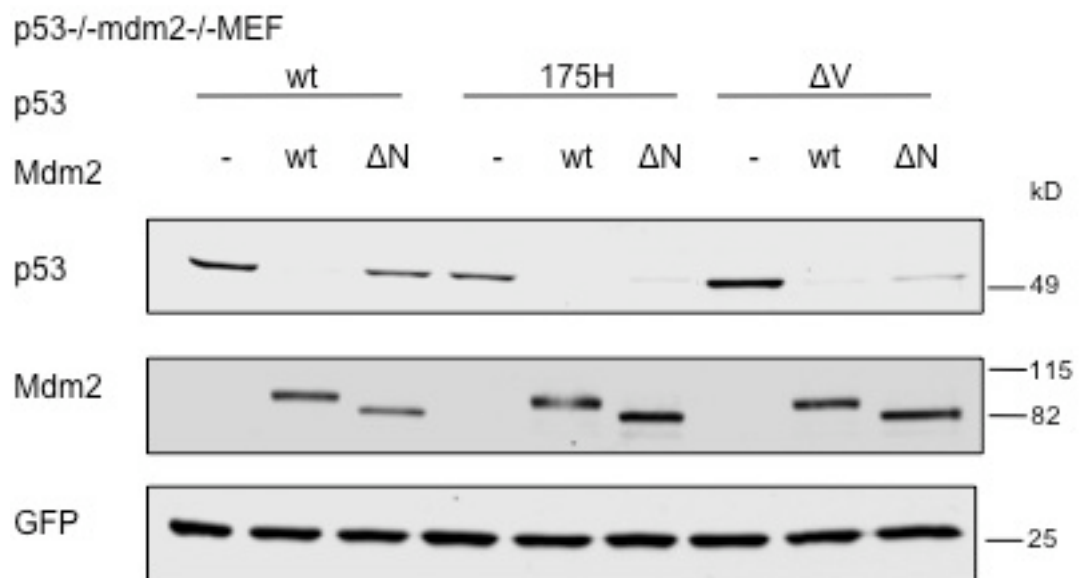
FIGURE 3.17. Mdm2 lacking N-terminus retains the ability to degrade p53 DBD mutants.
p53-/-mdm2-/- cells were transfected with wild type p53 or indicated p53 DBD mutants along with empty vector or wild type Mdm2 or Mdm2 with deletion of 58-89aa (ΔN). GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (1801), Mdm2-specific and GFP-specific antibodies.

To test the contribution of the N-terminal interaction to degradation of p53 mutants by Mdm2 in another cell type the experiment was repeated in H1299 cells (Fig.3.18). Wild type p53 or p53ΔI and p53175H or p53175/ΔI were co-transfected with empty vector or wild type Mdm2. In these cells wild type p53 was degraded by Mdm2 much less efficiently than in DKO (Fig.3.18). Similarly to the result in DKO cells, p53ΔI was resistant to degradation by Mdm2 (Fig.3.18). In this experiment, the expression levels of different p53 mutants were somewhat variable, although this was not seen in other experiments in H1299 (Fig.3.18). Both p53 DBD mutants lacking box I were resistant to degradation by Mdm2 (Fig.3.18), which is inconsistent with the observation seen in DKO cells (Fig.3.18). This result was observed in another independent experiment. It is not clear
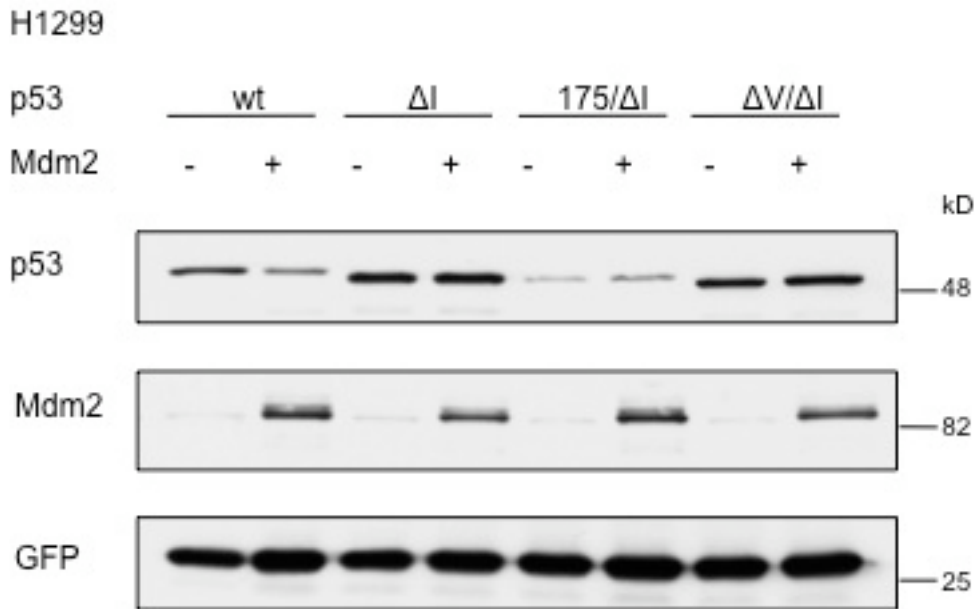
FIGURE 3.18. p53 DBD mutants lacking box I are resistant to degradation by Mdm2 in H1299 cells.
H1299 cells were transfected with wild type p53 or the indicated p53 mutants with empty vector or wild type Mdm2. GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (1801), Mdm2-specific and GFP-specific antibodies.

why Mdm2 fails to degrade mutant p53 lacking box I in this system and may be due to less potent ability of Mdm2 to degrade wild type p53 in this system. It is possible that in the process of tumourigenesis H1299 have lost one of the factors necessary for the efficient degradation of p53 in the proteasomes. It would therefore seem possible that the efficiency with which Mdm2 can target mutant p53 for degradation varies between cell types.

The binding studies showed that the interaction of mutant p53 that is independent of the N-terminal binding region (box I) requires the C-terminal RING domain (Fig.3.7). Therefore the ability of Mdm2 lacking this region to target the degradation of the DBD p53 mutants (ΔV and 175H) lacking box I in DKO cells was tested. Consistent with studies by others (270), ubiquitin ligase inactive Mdm2 mutants ΔR and 464 were expressed higher than wild type Mdm2 (Fig.3.19). This is probably due to inability of these mutants to autoubiquitinate and degrade themselves (175). As expected, deletion of box I in wild type p53 prevented degradation by all forms of Mdm2 (Fig.3.19). As shown above (Fig.3.13), wild type Mdm2 retained some ability to degrade p53 DBD mutants, lacking the box I (Fig.3.16). This activity was lost by deletion of the RING domain of Mdm2 (Mdm2ΔR) but was retained by the Mdm2-464 (Fig. 3.19), which has lost E3 activity but still binds to the p53 DBD mutant (Fig.3.7). These results therefore support the model in which Mdm2 can target degradation of the mutant p53s through a mechanism, which does not require the E3 activity of Mdm2.
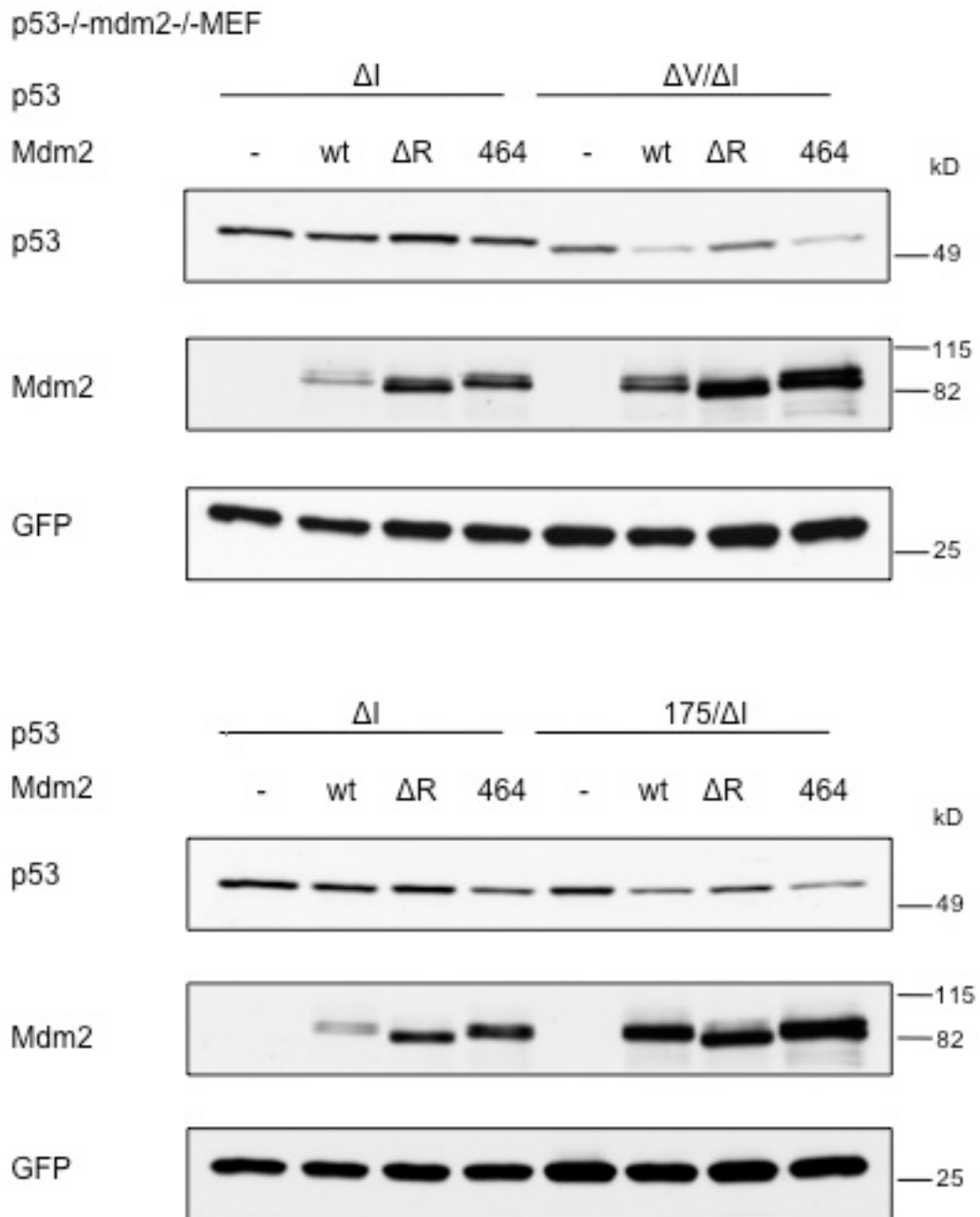
FIGURE 3.19. Mdm2C464A is able to degrade p53 DBD mutants lacking box I.
p53-/-mdm2-/- cells were transfected with indicated p53 mutants with empty vector or with wild type Mdm2, Mdm2 truncated from amino acid 440 (ΔR) or the Mdm2C464A mutant (464). GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (1801), Mdm2-specific (Ab-1, Ab-2) and GFP-specific antibodies.

Most of the studies described so far depend on the transient expression of p53 and Mdm2 mutants. Next the contribution of the endogenous Mdm2 to to the degradation of the endogenous wild type p53 and stably expressed mutant p53 was analysed. To this end MCF7 breast carcinoma cells, which express endogenous wild type p53 and stably express exogenous p53 DBD mutant (p53ΔII) were used (567). The p53 ΔII mutant has a deletion of the conserved box II in the DBD. These cells express endogenous Mdm2 to a level that can drive the degradation of both the wild type and mutant p53 - although somewhat higher basal levels of mutant p53 are maintained in these cells – and exposure of these cells to stress leads to the coordinate stabilization of both the mutant and wild type p53 proteins (567). In this system, p53 mutant lacking the N-terminal Mdm2 binding domain (ΔI) – p53ΔII/I is expressed at high levels and not further stabilized in response to stress (567).

The advantage of this system is that p53ΔII mutant migrates slightly slower than wild type p53 on the gel, which allows the analysis of both wild type and p53ΔII proteins in the same cell, under exactly the same experimental conditions. To examine the contribution of Mdm2 to the stability of wild type p53 and the p53ΔII mutant in this system, an siRNA-mediated approach to reduce endogenous Mdm2 was used. Cells were transfected with control or Mdm2-specific siRNA oligonucleotides and protein levels of p53 and Mdm2 were analyzed (Fig.3.20A). In this and the subsequent experiments to control for equal amount of total protein, cdk4 expression was monitored. In agreement with previous publications (567), p53ΔII was expressed at higher levels than wild type p53 (Fig.3.20A). This can be due to higher stability of p53ΔII in these cells or could be due to expression from the efficient CMV-driven promoter. Using previously published Mdm2-specific siRNA oligonucleotides, Mdm2 expression was significantly reduced (Fig.3.20A). Consistent with the ability of Mdm2 to degrade both wild type and mutant p53, wild type p53 and the p53ΔII mutant were stabilized to a similar extent when Mdm2 expression was down-regulated (Fig.3.20A). This result was confirmed with another pair of Mdm2-specific siRNA oligonucleotides in independent experiments. As shown above, degradation of wild type p53 is entirely dependent on the N-terminal interaction between p53 and Mdm2, whereas mutant p53 can be degraded by Mdm2 independently of N-terminal interaction (Fig.3.15, 3.16). Therefore it can be predicted that blocking N-terminal interaction between p53 and Mdm2 would have a stronger effect on stabilization of wild type p53 than on mutant p53. To examine the effect of disrupting the N-terminal p53/Mdm2 interaction a small-molecule inhibitor nutlin-3 was used. Nutlin-3 specifically binds to the p53-binding pocket in the N-terminus of Mdm2, preventing the interaction

through the N-terminal domains and stabilizing wild type p53 (282). The effect of treatment with nutlin-3 on stabilization of wild type and mutant p53 proteins was tested in MCF7 cells expressing p53ΔII (Fig.3.20B). For initial test of the effect of nutlin-3, cells were treated with 5μM nutlin-3 for 3h and 6h and with 15μM nutlin for 6h (Fig.3.20B). As expected wild type p53 was substantially stabilized upon treatment with nutlin-3, consistent with a major role of the N-terminal interaction between p53 and Mdm2 in regulation of wild type p53 stability (Fig.3.20B). The stabilization of wild type p53 was very pronounced already after 3h of treatment with 5μM of nutlin-3 and was further stabilized when cells were treated for 6h with 15μM of nutlin-3 (Fig.3.20B). Interestingly, the p53ΔII mutant was stabilised by nutlin-3 to a much lesser extent than wild type p53 (Fig.3.20B). And on the short exposure it was observed that wild type p53 was stabilized to higher levels than mutant p53, even though wild type p53 was expressed at lower levels than p53ΔII in untreated cells (Fig.3.20B). This result is consistent with the observation that degradation of mutant p53 does not completely depend on the N-terminal interaction between p53 and Mdm2. To test if accumulation of p53ΔII upon treatment with nutlin-3 was due to contribution of the N-terminal interaction to degradation of mutant p53, MCF7 cells expressing p53 mutant lacking box I - p53ΔII/ΔI – were treated with nutlin-3 (Fig.3.20C). In these cells wild type p53 was stabilized upon treatment with nutlin-3, however nutlin-3 did not lead to accumulation of p53ΔII/ΔI (Fig.3.20C). This could be explained by inability of p53ΔII/ΔI to interact with Mdm2 via N-termini. This suggests that nutlin-3 specifically inhibits the N-terminal binding of Mdm2 to p53, and therefore does not stabilize p53ΔII/ΔI. Therefore, weak accumulation of p53ΔII upon treatment with nutlin-3 (Fig.3.20B) is dependent on box I. This is consistent with some resistance of p53DBD mutants lacking box I to degradation by Mdm2 (Fig.3.16). In this experiment it was also noted that wild type p53 in MCF7p53ΔII/ΔI was stabilized less well than in MCF7p53ΔII cells, which suggests that p53ΔII/ΔI inhibits accumulation of wild type p53 (Fig.3.20C). The experiment was repeated in a different clone of the MCF7/p53ΔII cells. Since the most efficient stabilization of wild type p53 was observed at higher concentration, the experiment was repeated with 20μM of nutlin-3 and cells were treated for 4h and 7h (Fig.3.20D). Consistent with previous data, upon treatment with nutlin-3 wild type p53 protein accumulated, whereas the levels of p53ΔII only weakly increased (Fig.3.20D). The result discussed here was observed in at least four independent experiments. When the film was exposed for longer, modified forms of p53 were noted, which increase with longer treatment (Fig.3.20D). This could be ubiquitinated p53, which is inefficiently degraded. In this system it was not possible to distinguish between

ubiquitinated wild type and mutant p53 proteins. Nutlin-3 also leads to upregulation of Mdm2, which has been noted previously (282) and has been suggested to be due to
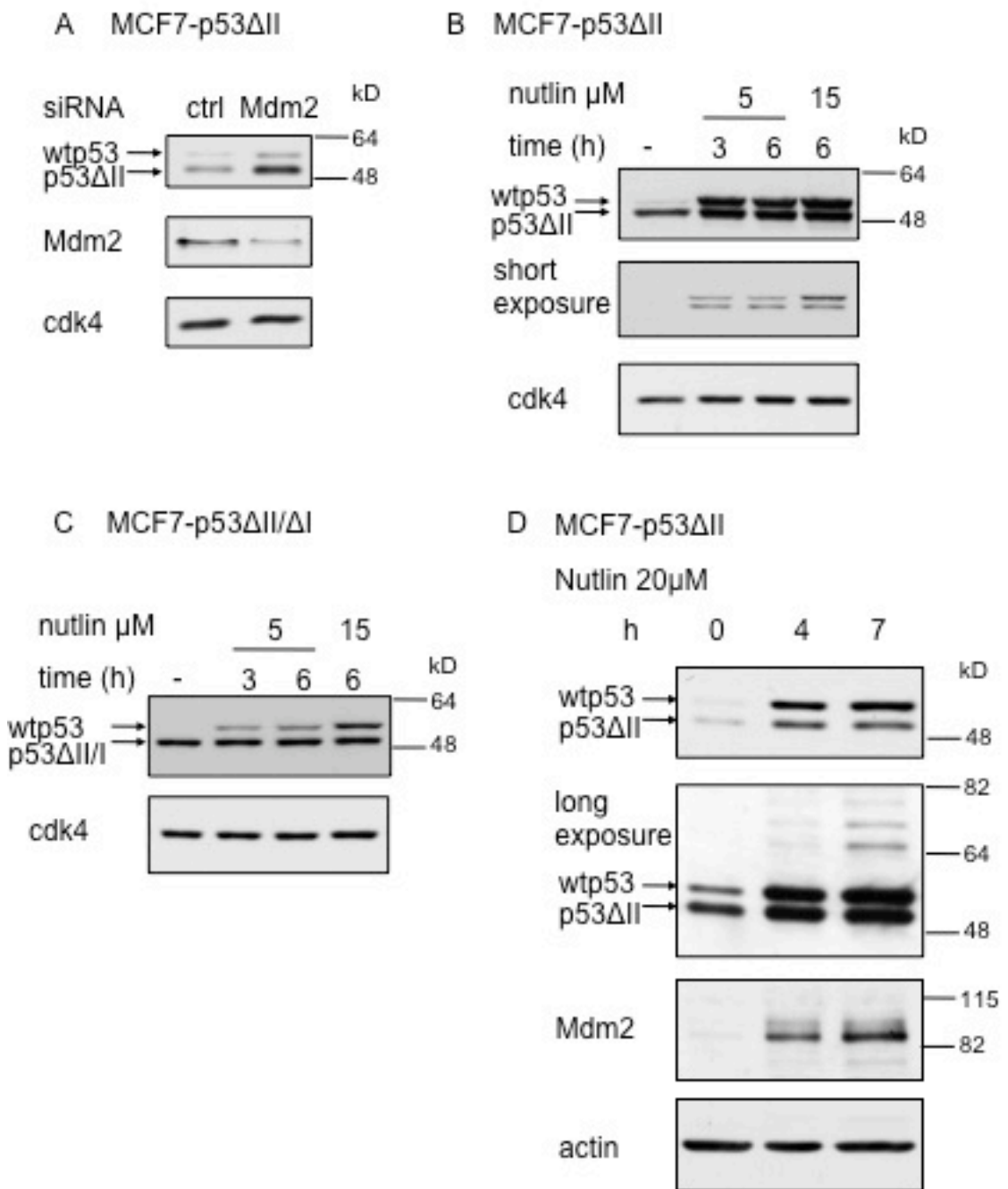
FIGURE 3.20 p53ΔII is stabilized by nutlin-3 treatment less than wild type p53.
A. MCF7 cells stably transfected with p53ΔII and expressing endogenous wild type p53 (MCF7-ΔII) were transfected twice with non-targetting (ctrl) or Mdm2-specific siRNA oligonucleotides. Cells were lysed 48 hours later and Western blot analysis was carried out with p53-specific (1801), Mdm2-specific or cdk4 specific antibodies. B, C. MCF7-ΔII (B) or MCF7-ΔII/ΔI (C) cells were treated with indicated concentrations of Nutlin-3 for 3h and 6 h. D. MCF7-ΔII cells were treated with 20μM nutlin-3 for 4h and 7h hours. B,C,D. Cell lysates were analysed by Western blot with p53-specific (1801) and cdk4 specific antibodies.

transactivation by p53 (Fig.3.20D). This can suggest that p53ΔII does not impair transcriptional activity of wild type p53. It would be interesting to check other target genes of p53 in this system and to compare MCF7/ΔII to parental MCF7 cells. However in this experiment the levels of Mdm2 protein are markedly increased already after four hours of nutlin-3 treatment, suggesting that protein stabilization mechanism can be involved.

These results are consistent with the observation that Mdm2 can degrade p53 mutants through a mechanism that does not depend on the N-terminal interaction (Fig.3.16). Degradation of wild type p53 is entirely dependent on the N-terminal interaction with Mdm2, therefore nutlin-3 has a much more pronounced effect on accumulation of wild type p53 than of p53 mutant. The fact that wild type p53 is stabilised more by treatment with nutlin-3 than mutant p53 might have an important implication for cancer treatments of tumours expressing mutant and wild type p53. It would be interesting to test the effect of nutlin-3 on endogenous mutant p53 in tumour-derived cell lines.

3.3.2 Mdm2 interacts with the proteasomal subunits.

The observation that Mdm2 can degrade p53 mutants independently of its ubiquitin ligase activity (Fig.3.19) suggests that Mdm2 can play a role in delivery of p53 to the proteasomes. Some evidence suggest that the acidic domain of Mdm2 can be involved in postubiquitination role of Mdm2 (328-330). Others have shown that Mdm2 can interact with the C8 subunit of the proteasome, which is necessary for degradation of Rb protein (243). The ability of Mdm2 to interact with subunits of the proteasome was therefore tested by co-immunoprecipitation analysis in H1299 cells. These cells were used due to the low levels of endogenous Mdm2 and their high transfection efficiency. As shown above, RING domain of Mdm2 was important for the interaction and the ability to degrade mutant p53. Wild type Mdm2 or Mdm2 lacking RING domain (ΔR) were co-expressed with V5-tagged proteasome subunits: S4, S5α, S6α and Sβ (Fig.3.21). The V5 tag allows to easily detect the transfected proteins in cells and to discriminate between transfected and endogenous proteasome subunits. S5α is the non-ATPase subunit, S4, S6α and S6β are ATPases, which are contained within the regulatory 19S part of proteasome. Cells were treated with proteasome inhibitors to prevent degradation of Mdm2. Mdm2 was immunoprecipitated with Mdm2-specific antibodies and the proteasome subunits in the immunoprecipitates were detected with antibody specific for V5. Direct western blotting analysis was carried out with Mdm2-specific and V5-specific antibodies to control for the levels of expression. Although cells were treated with proteasome inhibitors, somewhat higher levels of

Mdm2ΔR were observed, which could be due to more efficient transfection or expression of these constructs in this experiment (Fig.3.21). This resulted in larger ammounts of immunoprecipitated Mdm2 mutants compared to wild type (Fig.3.21). All proteasome subunits were expressed, as visualized by the detection with V5-specific antibody (Fig.3.21). Some background band was detected with V5 antibody in the immunoprecipitates where no Mdm2 was expressed (Fig.3.21). Interaction between all proteasome subunits with wild type Mdm2 was detected, which was quite strong with V5-S6β subunit, but interaction with other subunits - S4, S5α, and S6α - was only slightly above the background (Fig.3.21). However, all proteasome subunits interacted with the Mdm2ΔR (Fig.3.21). ATPase subunits appeared to interact with Mdm2 better than non-ATPase subunit S5α. The ability of Mdm2 to interact with several proteasomal subunits tested here suggests that Mdm2 might be engaged in complex with the entire proteasome. This would suggest that the RING domain is not involved in the interaction of Mdm2 with the proteasome, probably due to its binding to p53. To confirm the interaction of Mdm2 with some proteasome subunits and to test the involvement of other functional domains of Mdm2, the co-immunoprecipitation analysis was repeated with wild type Mdm2, Mdm2 lacking part of the acidic domain (245-295aa) - ΔA and Mdm2 lacking N-terminus ΔN with S4 and S6β subunits (Fig.3.22). Some studies have shown the involvement of this part of the acidic domain in post-ubiquitination function of Mdm2 (328, 329). The levels of wild type Mdm2 and Mdm2 mutant proteins were more similar in this experiment (Fig.3.22). In this experiment both S4 and S6β subunits interacted with Mdm2 more efficiently than in the previous experiment (Fig.3.20). Mdm2 lacking acidic domain (ΔA) or N-terminus (ΔN) interacted with S4 and S6β subunits similarly to wild type Mdm2 (Fig.3.22), suggesting that other regions on Mdm2 mediate the interaction. These suggest that Mdm2 can form complex with the proteasome, which can contribute to its degradation function. The region Mdm2 involved in interaction with proteasome remains to be determined in future experiments.
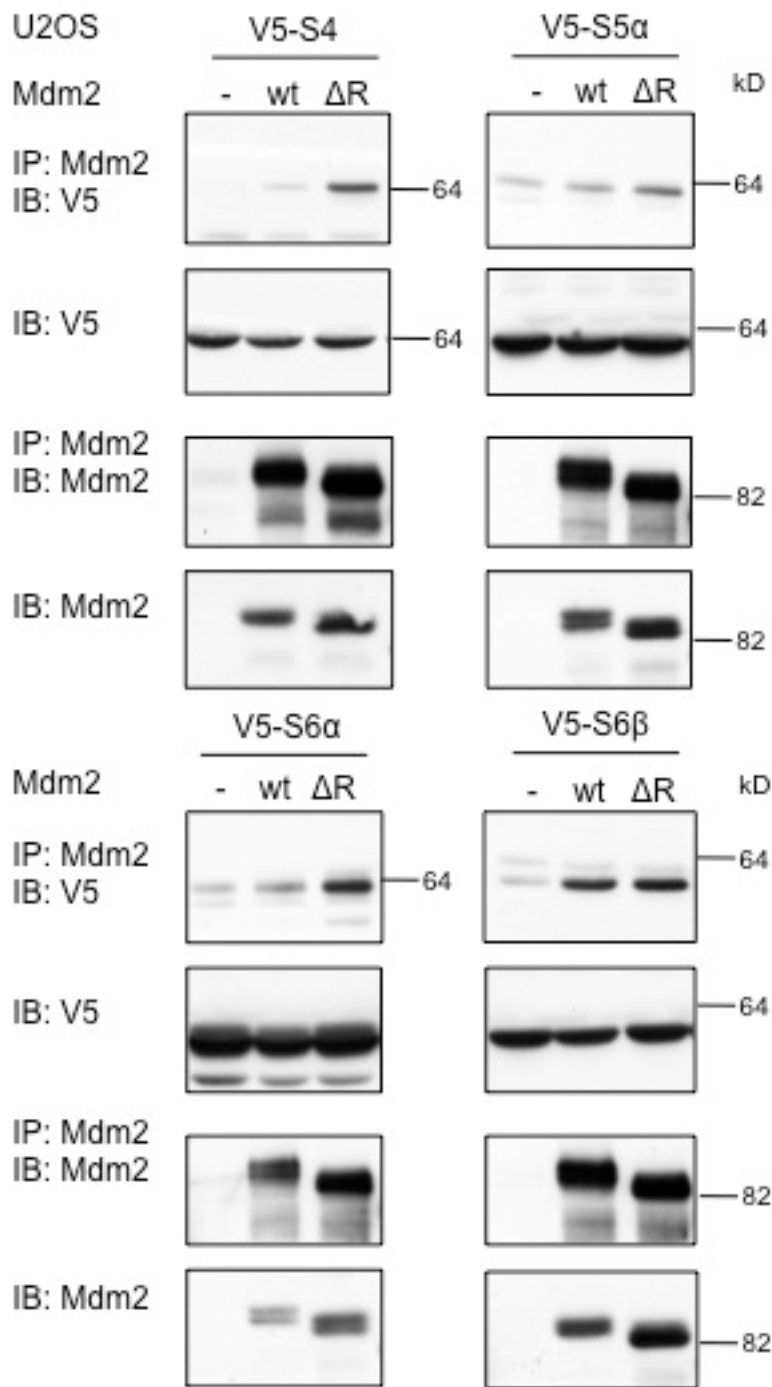
FIGURE 3.21. Interaction of wild type Mdm2 and Mdm2ΔR with the proteasomal subunits.
U2OS cells were tranfected with wild type Mdm2, Mdm2ΔN or Mdm2ΔR along with V5-tagged proteasomal subunits S4, S5α, S6α and Sβ. Cells were treated with MG132 and lysed 4h later. Cell lysates were immunoprecipitated with Mdm2-specific antibodies and immunoblotted with Mdm2 or V5.
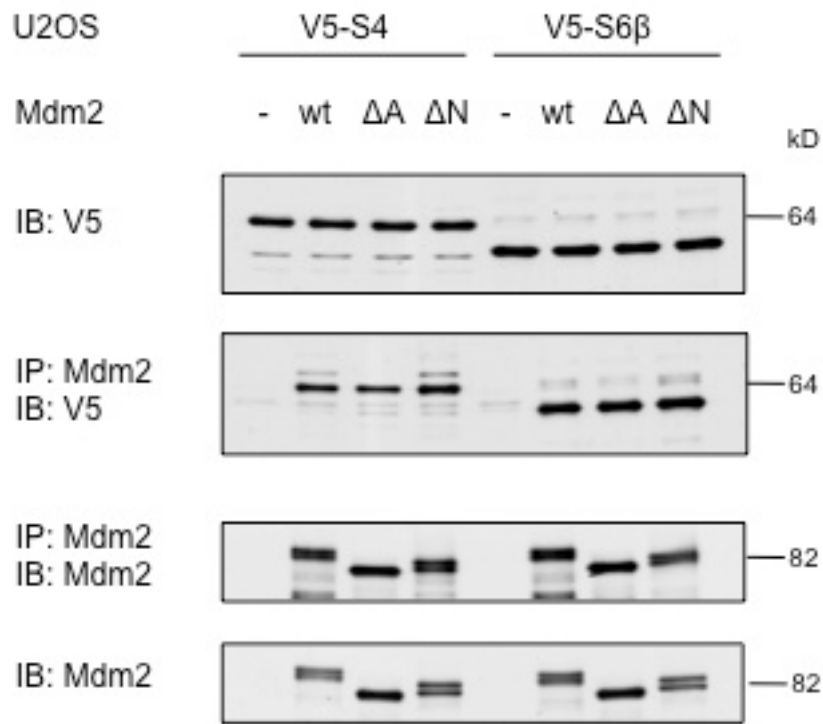
FIGURE 3.22. Analysis of the involvement of the N-terminus and the acidic domain of Mdm2 to the interaction with the proteasomal subunits.
U2OS cells were tranfected with wild type Mdm2, Mdm2ΔN (58-89aa) or Mdm2ΔA (245-295aa) along with V5-tagged proteasomal subunits S4 and S6β. Cells were treated with MG132 and lysed 4h later. Cell lysates were immunoprecipitated with Mdm2-specific antibodies and immunoblotted with Mdm2 or V5.

3.4   Summary and discussion.

In this chapter it has been shown that while Mdm2 retains the ability to interact with conformationally altered p53 DBD mutants, this interaction appears to be shifted from the N-terminal binding site that is predominantly used on wild type p53, to an alternative site on mutant p53. Whereas N-terminus of Mdm2 binds to N-terminus of with wild type p53, here it has been found that RING domain of Mdm2 is involved in binding to mutant p53. Several recent studies have shown that p53 can bind to Mdm2 through the DBD (265, 266, 578) and it seems likely that the conformational shift in mutant p53 reveals this binding site, thereby enhancing the interaction of mutant p53 with Mdm2 in a conserved box I-independent manner. The exact binding site on the DBD of p53 has not been mapped and one report suggests it can be between the boxes IV and V (578).

Mdm2 also contains several sites of interaction with p53 (256, 264-266). Recent studies have suggested that N-terminal interaction between Mdm2 and p53 triggers a conformational switch in Mdm2 that is required to promote a second interaction that involves the acidic domain of Mdm2 and DBD of p53 (265). Taken together with this study, it would appear that a conformational change in both p53 and Mdm2 contributes to their interaction through regions distinct from the N-terminus.

While other studies have shown an importance of the acidic domain of Mdm2 in alternative interactions with p53 (264-266), this study shows that the RING finger domain of Mdm2 is involved in binding to mutant p53. Although the RING finger domain of Mdm2 is required for ubiquitin ligase activity (564), this function is not necessary for the binding to mutant p53, as demonstrated by the E3 dead Mdm2 point mutant C464A. Furthermore, the interaction of Mdm2 with mutant p53 does not lead to the strong ubiquitination of p53 that is seen following interaction with the wild type p53 protein. This may reflect – to some extent – a lack of interaction with the N-terminus of Mdm2 that has been suggested to be required to activate E3 function (265). It is also possible that engagement of the RING domain in the interaction with mutant p53 impedes the binding of E2. Mdm2 can function with several E2s – UbcH5-A, -B, -C and E2-25K to ubiquitinate p53 in vitro. However, only UbcH5B and C appear to contribute to the regulation of p53 protein levels in cells (177). It is an intriguing possibility that binding of mutant p53 to the RING domain Mdm2 promotes pairing with different E2, which may result in less efficient ubiquitination or promote other type of linkage of ubiquitin chain, and remains to be elucidated in future.

Binding of mutant p53 to the RING domain of Mdm2 might impair its ubiquitin ligase activity towards its other substrates. Mdm2 can autoubiquitinate itself (564) and Mdmx (588, 589). Therefore it is possible that mutant p53 may lead to accumulation of Mdm2 and Mdmx, which would inactivate wild type p53. In some tumor cell lines high levels of mutant p53 and high levels of Mdm2 are detected (518), supporting this idea.

The interesting question remains whether Mdm2 is able to interact with mixed tetramers of wild type and mutant p53 and which regions on Mdm2 would be involved in such interaction. It would be interesting to determine which protein, wild type or mutant, is dominant in determining whether Mdm2 interacts with the N-terminus or RING and whether Mdm2 is able to ubiquitinate and degrade the complex of wild type and mutant p53. This question can be investigated by using tagged versions of p53 proteins.

It would be also interesting to test whether the RING domain of Mdmx, which is highly similar to RING domain of Mdm2, plays a role in the interaction with mutant p53. Though Mdmx fails to ubiquitinate p53 on its own, Mdmx can restore the E3 activity of inactive Mdm2 mutants with point mutations in the extreme C-terminus and promote degradation of p53 (270). The interesting question whether Mdmx can ubiquitinate p53 mutants, which are not efficiently ubiquitinated by Mdm2, remains to be determined in future.

Results of this chapter show that though the ability of Mdm2 to ubiquitinate p53 mutants is compromised, it can still efficiently degrade p53 mutants, though through a different mechanism. The ability to degrade did not absolutely require N-terminal interaction between mutant p53 and Mdm2 and was also independent of the ability of Mdm2 to function as an E3. Previous reports have also suggested that the ubiquitination function of Mdm2 can be uncoupled from its ability to target to degradation (328-330). This study provides further evidence that Mdm2 can play a post-ubiquitination role in degrading p53. The details of such a function of Mdm2 are not yet clear, but have been suggested to involve cooperation with hHR23A – a protein thought to be an adaptor between ubiquitinated substrates and the proteasome (330, 331). Another study has also shown that Mdm2 can play a role in transport to the proteasomes by directly interacting with the C8 subunit of the 20S proteasomes (243). This mechanism of degradation is important for the degradation of another substrate of Mdm2 – Rb. It has been further shown here that Mdm2 can also interact with 19S proteasomal subunits. This data suggests that Mdm2 can form a complex with the proteasome and may play a role in the entry of the ubiquitinated p53 to the proteasome.

In summary, the data presented here suggest that there are differences in the mechanism of degradation of wild type p53 and mutant p53 by Mdm2. Mdm2 interacts

115

with wild type p53 via the corresponding N-termini, this activates E3 activity of Mdm2 and leads to efficient ubiquitination of p53. Ubiquitinated p53 is then delivered to the proteasomes by Mdm2. This step may require the binding of Mdm2 via the RING. It is possible that ubiquitination unfolds p53 to promote the secondary interaction of Mdm2 with p53. Mutant p53 is already unfolded and is ubiquitinated by another E3. Therefore the second interaction involving the RING of Mdm2 is promoted, which results in proteasomal degradation of mutant p53. This suggests Mdm2 plays a postubiquitination role in degrading mutant p53 and is able to mediate the delivery of mutant p53 to the proteasome.

The important observation of this chapter is the distinct the mechanism of Mdm2-mediated degradation of mutant p53 which can have an important implication for tumour therapy. It was found here that inhibition of the N-terminal interaction by treatment with nutlin can stabilize wild type p53 much more than mutant p53. It would be interesting to test the effect of the inhibitors of E3 activity of Mdm2 on the levels of wild type and utant p53 proteins. The prediction would be that wild type p53 will accumulate more than mutant p53, as its degradation is more dependent on the ubiquitin ligase activity of Mdm2. This remains for future investigations.

Chapter 4. Analysis of Mdm2-independent ubiquitination of p53 DBD mutants.

4   Analysis of Mdm2-independent ubiquitination of p53 DBD mutants.

The results described in chapter 3 showed that conformational p53 mutants were hyperubiquitinated by Mdm2-independent mechanism. It was therefore of interest to try and identify the E3 responsible for this ubiquitination. Wild type p53 is known to be ubiquitinated by many different E3s (83, 84, 178, 185, 354, 360, 361, 590) (Table 2), some of which may also ubiquitinate mutant p53. In this chapter the contribution of Mdm2-dependent and some Mdm2-independent mechanisms to ubiquitination of p53 DBD mutants was assessed.

4.1   Contribution of Mdm2-dependent and Mdm2-independent mechanisms to ubiquitination of mutant p53.

First, an siRNA-mediated approach to downregulate the expression of the ubiquitin ligases was carried out using pools of four siRNA oligonucleotides (Dharmacon). The effect of reduction of endogenous Mdm2, Cop1, ARF-BP1 (Fig.4.1, 4.2) and CHIP (Fig.4.3, 4.4) on the ubiquitination of wild type p53 and p53175H was examined in U2OS cells and H1299 cells. U2OS cells express endogenous wild type p53 and endogenous wild type Mdm2 and are efficiently transfected by siRNAs. The expression of the Mdm2, Cop1 and ARF-BP1 was down-regulated in both cell types by the siRNA oligonucleotides. To reduce the expression of Mdm2, previously published siRNA oligonucleotides were used. The down-regulation of these E3s was verified by the Western blotting analysis. Equal amount of total proteins was verified by actin or Hsp90 proteins levels. The expression of endogenous Cop1 could not be detected in H1299 cells. Wild type p53 was weakly ubiquitinated in U2OS cells, and down-regulation of Mdm2 reduced this ubiquitination of wild type p53 in U2OS cells, suggesting contribution of the endogenous Mdm2 (Fig.4.1). However, down-regulation of Cop1 and ARF-BP1 also reduced ubiquitination of wild type p53 in U2OS cells, suggesting that all these E3s target wild type p53 (Fig.4.1). The weak signal of ubiquitination of wild type p53 did not allow an assessment of the individual contribution of each of them. In H1299 in this experiment ubiquitination of wild type p53 was not detected (Fig.4.2), which is probably due to lower levels of expression of endogenous Mdm2 than in U2OS cells. Confirming the data shown above, p53175H was

FIGURE 4.1. Contribution of Mdm2, Cop1 and ARF-BP1 to ubiquitination of wild type p53 and p53175H in U2OS cells.
U2OS cells were transfected twice with non-targetting (ctrl) or Mdm2, Cop1, ARF-BP1 specific siRNA oligonucleotides with 24 h between transfections. Cells were transfected with wild type p53 or p53175H along with HA-ubiquitin. Cells were treated with MG132 24h later and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody. Direct Western blot analysis was carried out with Mdm2 Hsp90, Cop1, actin and ARF-BP1 specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

hyperubiquitinated in U2OS (Fig.4.1) and in H1299 cells (Fig.4.2) and the reduction of Mdm2 expression did not decrease its ubiquitination. Therefore, similarly to DKO and H1299, mutant p53 is ubiquitinated by Mdm2-independent mechanism in U2OS cells. Down-regulation of Cop1 weakly reduced ubiquitination of mutant p53 in U2OS cells (Fig.4.1), but not in H1299 cells (Fig.4.2). ARF-BP1 did not affect ubiquitination of mutant p53 in U2OS cells (Fig.4.1) and weakly increased the ubiquitination of mutant p53 in H1299 cells (Fig.4.2). This experiment was repeated two times and no striking effect of Cop1 or ARF-BP1 knock-down was detected.

FIGURE 4.2. Contribution of Mdm2, Cop1 and ARF-BP1 to ubiquitination of wild type p53 and p53175H in H1299 cells.
H1299 cells were transfected twice with non-targetting (ctrl) or Mdm2, Cop1, ARF-BP1 specific siRNA oligonucleotides with 24 h between transfections. Cells were transfected with wild type p53 or p53175H along with HA-ubiquitin. Cells were treated with MG132 24h later and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody. Direct Western blot analysis was carried out with Mdm2 Hsp90, Cop1, actin and ARF-BP1 specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

Next, the contribution of ubiquitin ligase CHIP was investigated. Conformational p53 mutants are known to interact with chaperones Hsp70 and Hsp90. CHIP ubiquitinates the substrates bound by these chaperones (355), and therefore is an attractive candidate for mutant-p53 specific ubiquitin ligase. The contribution of chaperone-associated ubiqutin ligase CHIP to ubiquitination of mutant p53 was examined (Fig.4.3, 4.4). Down-regulation of CHIP resulted in a pronounced reduction of ubiquitination of mutant p53 and also reduced the ubiquitination of wild type p53 to a lesser extent (Fig. 4.3). These results are in agreement with a previous report showing that CHIP can target to degradation both wild type and mutant p53 (355). To confirm this result the experiment was repeated with individual siRNA oligonucleotides. As alternative control GFP-specific siRNA oligonucleotides were used in this experiment. The CHIP protein levels were difficult to

detect (Fig.4.3), therefore in this experiment the expression of CHIP mRNA was analyzed by RT-PCR (Fig.4.4). Similarly to Fig.4.3 downregulation of CHIP expression significantly decreased ubiquitination of both p53 DBD mutants - p53175H and p53ΔV mutants (Fig. 4.4). This observation was reproduced in at least three independent experiments. The remaining ubiquitination of mutant p53 leaves the possibility that other unidentified ubiquitin ligases may contribute.

FIGURE. 4.3. Contribution of CHIP to ubiquitination of wild type p53 and p53175H.
U2OS cells were transfected twice with with non-targetting (ctrl) or with a a pool of CHIP specific siRNA oligonucleotides (Dharmacon) with 72 h between transfections. Cells were transfected with wild type p53 or p53175H along with HA-ubiquitin. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody. Direct Western blot analysis was carried out CHIP and actin specific antibodies.
IP: immunoprecipitation. IB: immunoblotting.

Conformational p53 mutants are specifically recognized by molecular chaperones cells (41, 453), which can present substrates to the ubiquitin ligase CHIP (C-terminal of Hsp-70-interacting protein) (591). To test whether interaction with Hsp70 is required for the specific ubiquitination of mutant p53, expression of Hsp70 was reduced by siRNA-mediated knockdown (Fig.4.5). Down-regulation of Hsp70 only partially inhibited ubiquitination of p53 mutants (Fig.4.5), suggesting that other molecular chaperones may be involved.
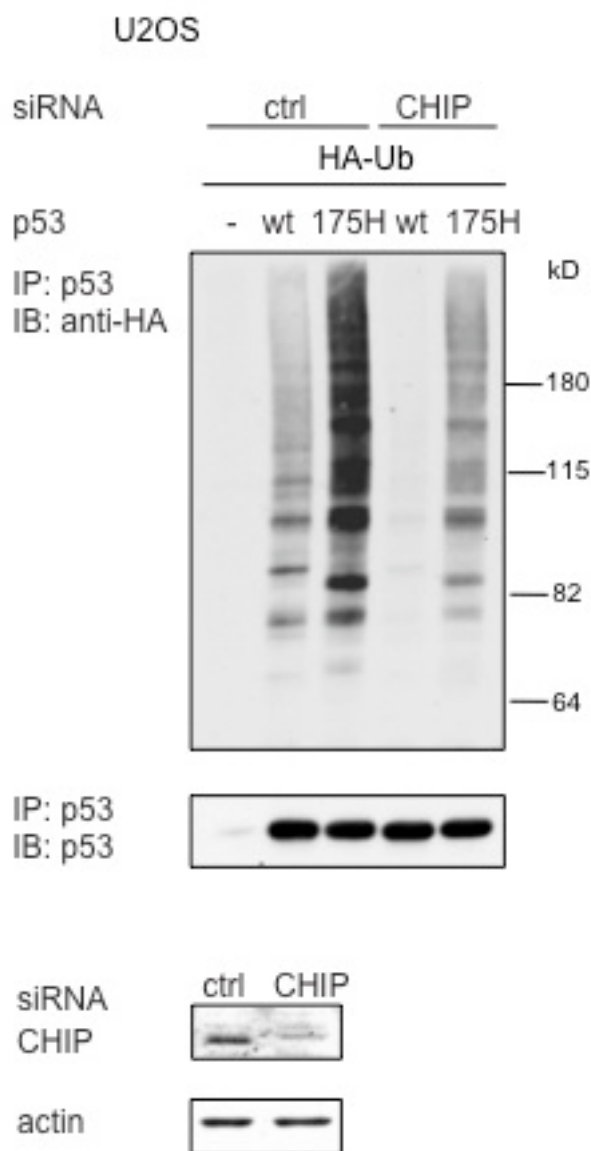
FIGURE. 4.4.
Contribution of CHIP to ubiquitination of wild type p53 and p53175H.
U2OS cells were transfected twice with with non-targetting (ctrl) or with a single pair of CHIP specific siRNA oligonucleotides with 72 h between transfections. Cells were transfected with wild type p53 or p53175H along with HA-ubiquitin. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody. RT-PCR analysis was carried out with CHIP and GAPDH specific primers.
IP: immunoprecipitation.
IB: immunoblotting.

The contribution of CHIP to ubiquitination of mutant p53 suggests that CHIP may play a role in regulation of stability of mutant p53. Tumour cells accumulate high levels of mutant p53 protein, suggesting that inactivation of CHIP would be an advantageous event in tumourigenesis. Consistent with this prediction, a locus 16p13 where the CHIP gene is located is frequently deleted in papillary carcinomas of the breast (592). The data of Oncomine database suggests that the expression of CHIP mRNA is reduced in several types of brain cancers (Oncomine www.oncomine.org) (Fig.4.6). Interestingly, data from GEO profiling database (NIH GEO profiling) suggest that downregulation of CHIP expression can be associated with advanced stage of colorectal cancer (Fig.4.6). Comparison of polysomal RNA from isogenic cell lines derived from primary colorectal cancer and its metastasis to the lymph node shows that CHIP is downregulated in metastasis (Fig.4.6). However, these studies have not analyzed status of p53 and this remains to be investigated in future.
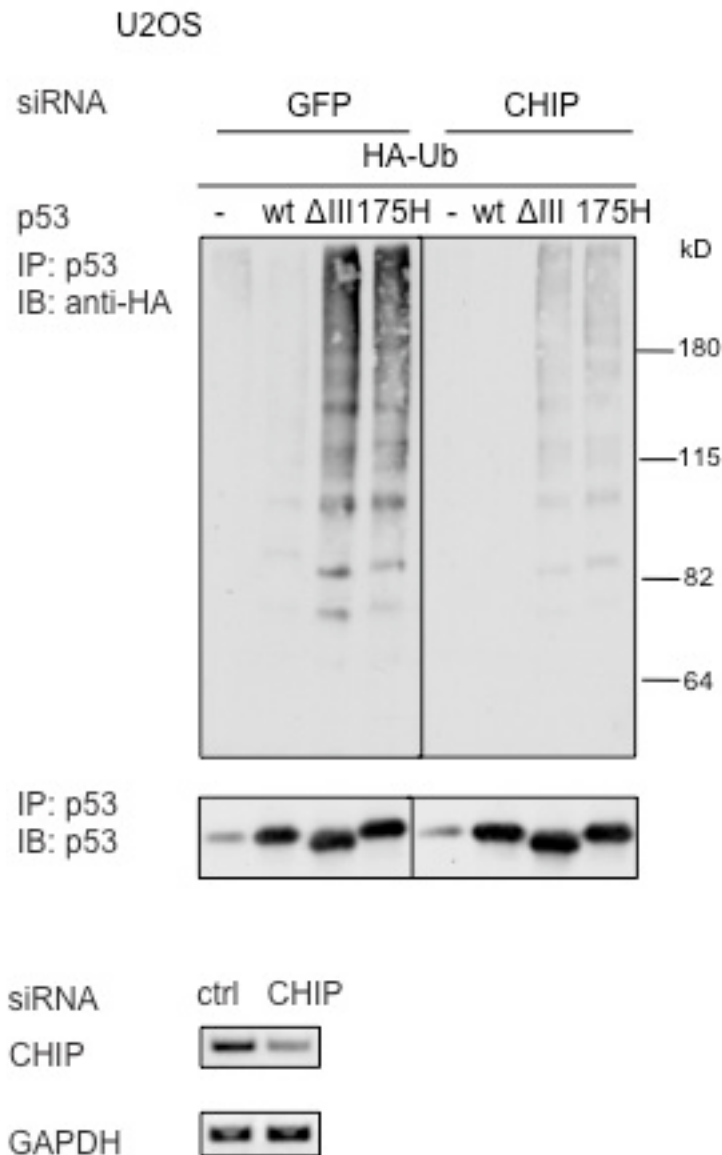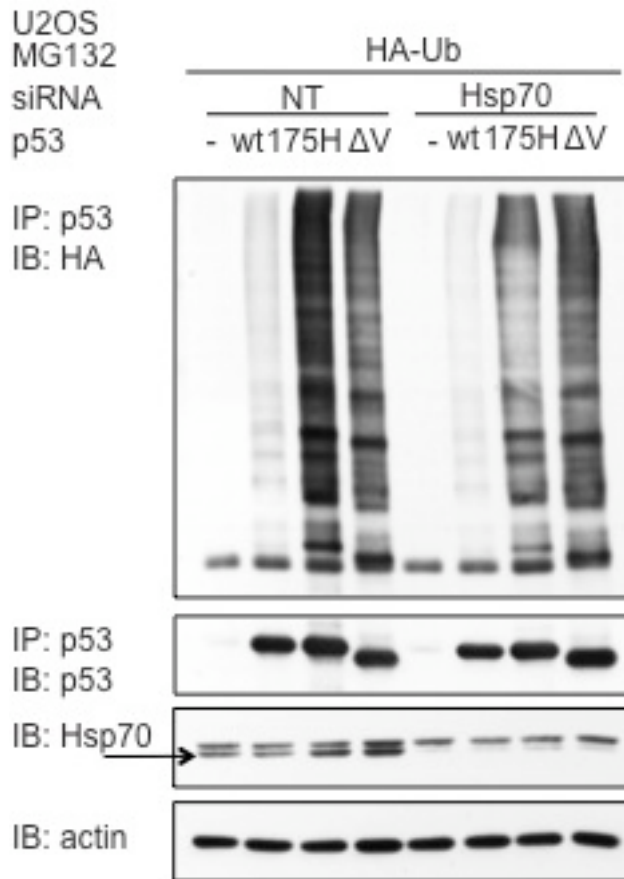
FIGURE. 4.5. Effect of siRNA-mediated reduction of Hsp70 expression on ubiquitination of wild type p53 and p53175H.

U2OS cells were transfected twice with with non-targetting (ctrl) or with Hsp70 specific siRNA oligonucleotides with 40 h between transfections. Cells were transfected with wild type p53 or p53175H along with HA-ubiquitin. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either an anti-HA antibody or the p53-specific CM1 antibody. Direct western blotting was carried out with actin or Hsp70 specific antibodies.

IP: immunoprecipitation. IB: immunoblotting.
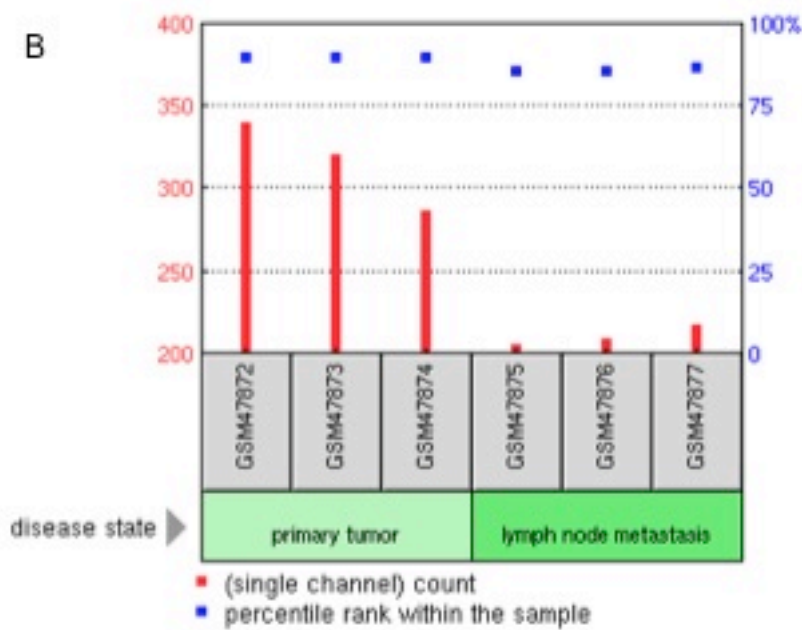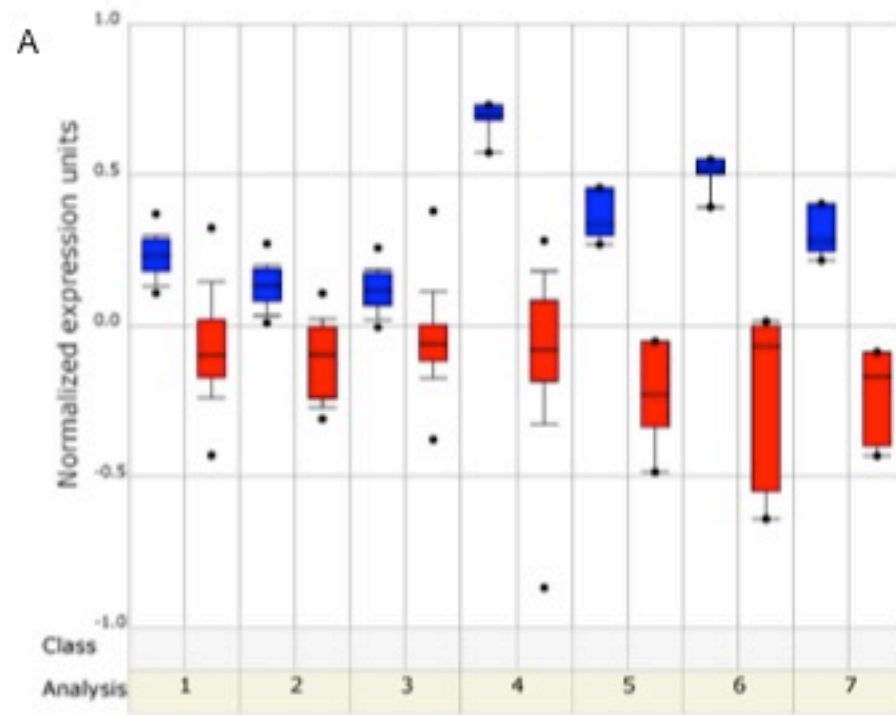
FIGURE 4.6. Anaysis of CHIP expression in cancers.
A. CHIP mRNA expression data in normal tissue (blue) and brain cancers (red) has
been obtained from Oncomine expression profiling database.
B. CHIP expression is downregulated in metastatic tumor. Graph shows comparison
of polysomal RNA from cell lines derived from human primary colorectal cancer and
lymph node metastasis was obtained from NIH GEO profiling database.

4.2 Increase in ubiquitination of p53 mutants is not reflected in increased degradation.

To investigate the consequences of hyperubiquitination of mutant p53 first the degradation rate of p53 mutants was tested. To this end half-life of wild type p53 and p53 mutants was measured in DKO cells. DKO cells were used in order to observe the effect of Mdm2-independent ubiquitination. Firstly, the half-life of wild type and mutant p53 was analyzed by blocking protein synthesis with cycloheximide treatment (Fig. 4.7). As a control, the levels of actin protein, which is known to have a long half-life in normal cells (48h) were monitored (593). The intensity of the protein bands was quantitated and the values of p53 were normalized to the values of actin. The results are presented on the graph. As expected, transfected wild type p53 had a prolonged half-life in Mdm2-null cells (Fig. 4.7), suggesting that p53 is not efficiently degraded by Mdm2-independent mechanisms. p53 DBD mutants with deletions of conserved boxes and point mutants p53175H and p53273H also had a long half-life, and with minor variation were slightly less stable than wild type p53 (Fig.4.7). This observation was reproduced in another independent experiment. To confirm this observation, the half-life of wild type p53, p53175H, p53ΔV and p53ΔIV proteins was also measured by pulse-chase with ^{35}S-methionine in DKO cells (Fig. 4.8). The intensity of p53 protein was quantified and presented as a graph. Similarly, wild type p53 had a prolonged half-life and all the p53 DBD mutants were stable similarly to wild type p53 in this experiment. The conclusion of this experiment was confirmed in another independent experiment. To confirm these observations in another cell type, the half-life of wild type p53 and p53 DBD mutants was measured by cycloheximide treatment in H1299 cells (Fig.4.9). Similarly to DKO, wild type p53 had a prolonged half-life in these cells, suggesting that endogenous Mdm2 in these cells does not degrade transfected wild type p53. Simirlarly to DKO, p53 DBD mutants were only slightly less stable than wild type p53 in these cells, but had quite long half-life and around 50% of the protein was present after 9 hours (Fig.4.9). In future it would be interesting to test the stability of wild type p53 and p53 DBD mutants in cells with higher levels of Mdm2 such as U2OS cells.

This suggests that though p53 mutants are ubiquitinated much more than wild type p53, they are not less stable, suggesting that ubiquitination does not target them efficiently to the proteasomes. Ubiquitination can promote nuclear export of p53 (371) and therefore localization of p53 mutants is analyzed in Chapter 5.

Analysis of half-life of p53
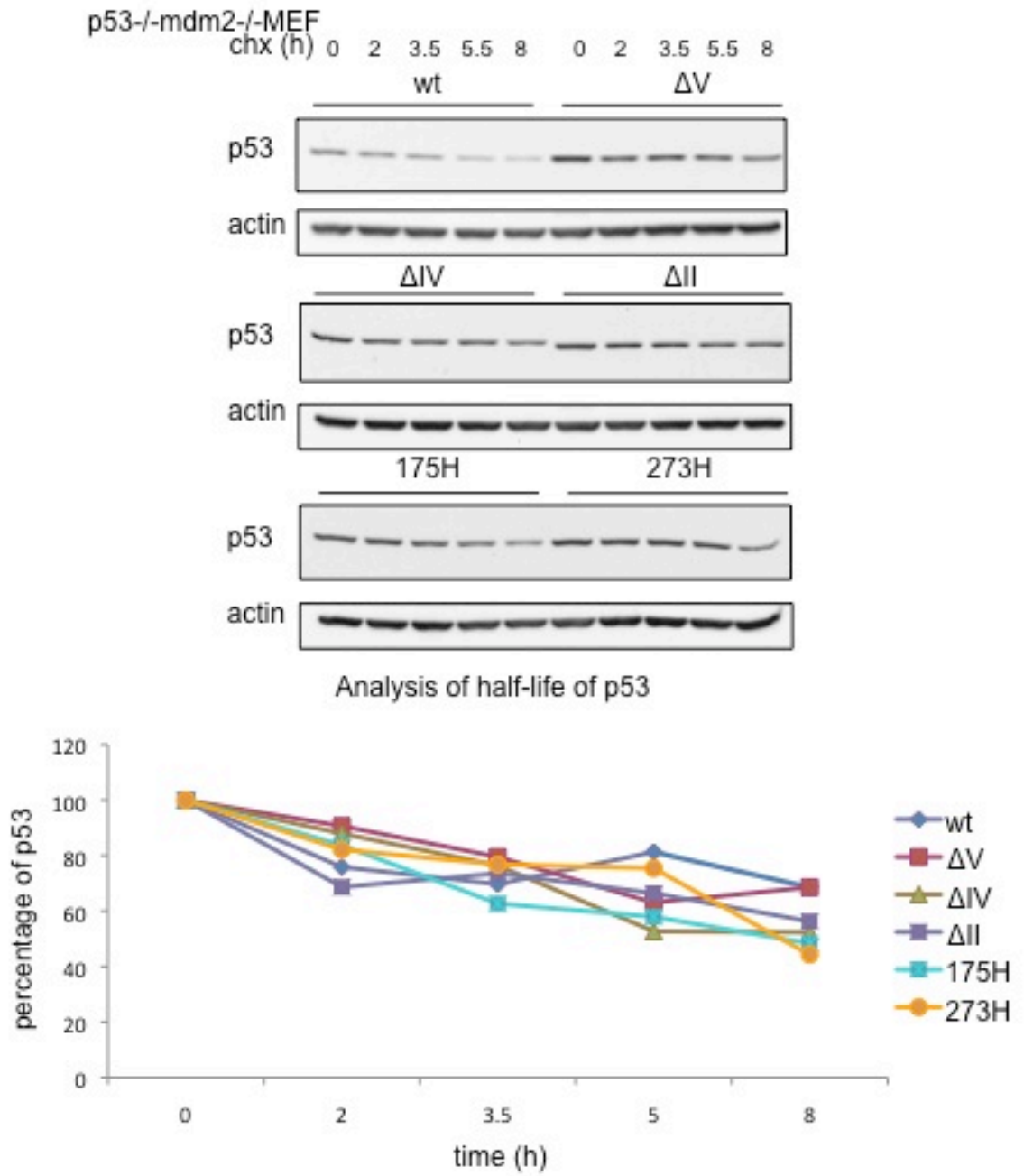
FIGURE 4.7. Analysis of half-life of p53 in p53-/-mdm2-/- cells by cycloheximide treatment.

p53-/-mdm2-/- cells were transfected with wild type p53 or indicated p53 DBD mutants. Cells were treated with cycloheximide 24h after transfection and collected at the indicated time points. Western blot analysis was carried out with the p53 specific (DO1) antibody and an anti-actin antibody. The results were quantitated using Scion image software (NIH) and the values of p53 were normalized to the values of actin.

126

p53-/-mdm2-/-MEF

Analysis of half-life of p53
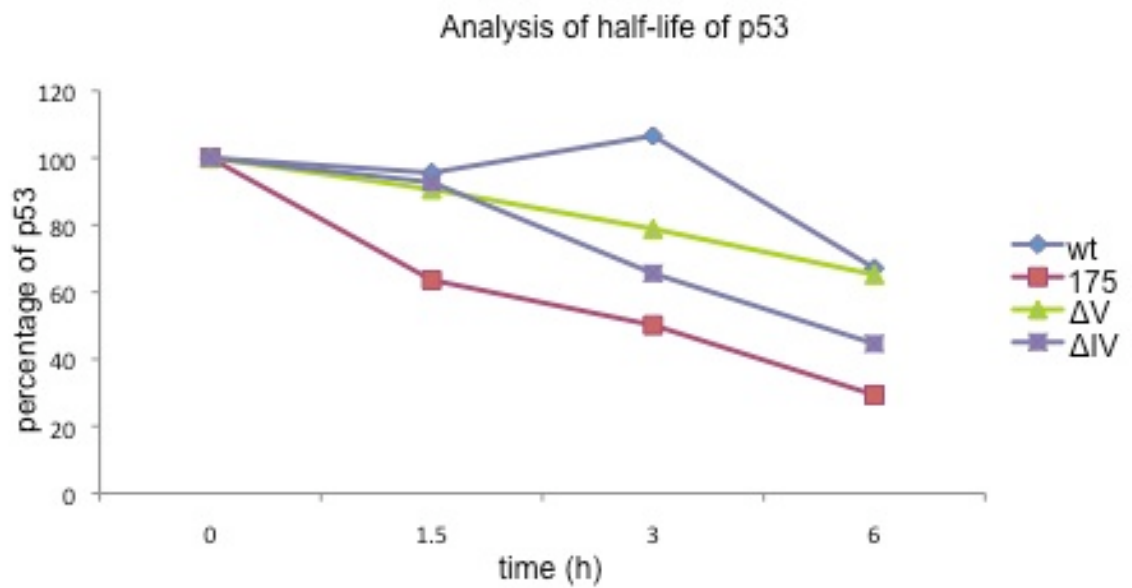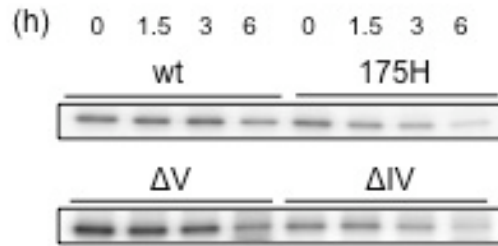
FIGURE 4.8. Analysis of half-life of p53 in DKO cells by pulse chase.
p53-/-mdm2-/- (DKO) cells were transfected with wild type p53 or indicated p53
DBD mutants. 24h after transfection DKO cells were pulsed [^{35}S] methionine/cysteine
and chased for the times indicated. Cell lysates were immunoprecipitated with p53
specific antibody DO1 and analysed by SDS-PAGE. The results were quantitated
using Scion image software (NIH).

127
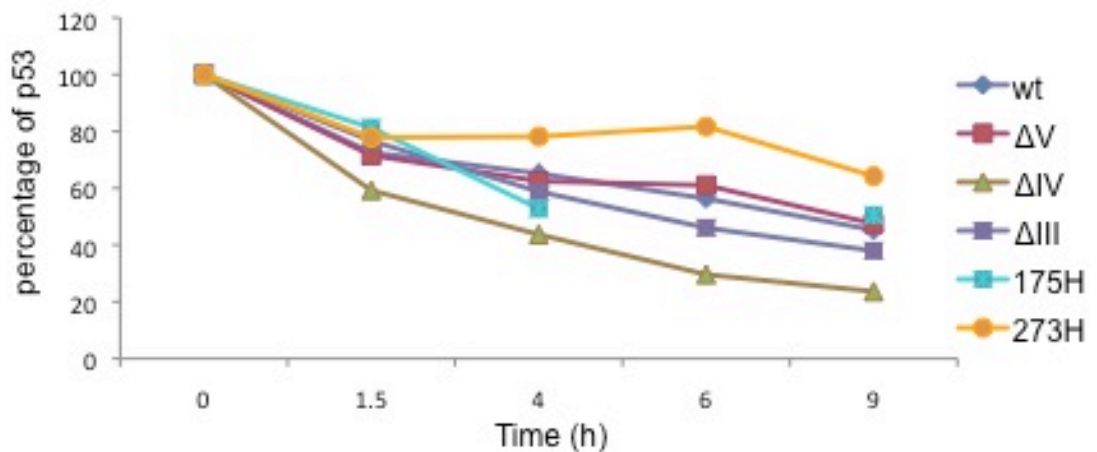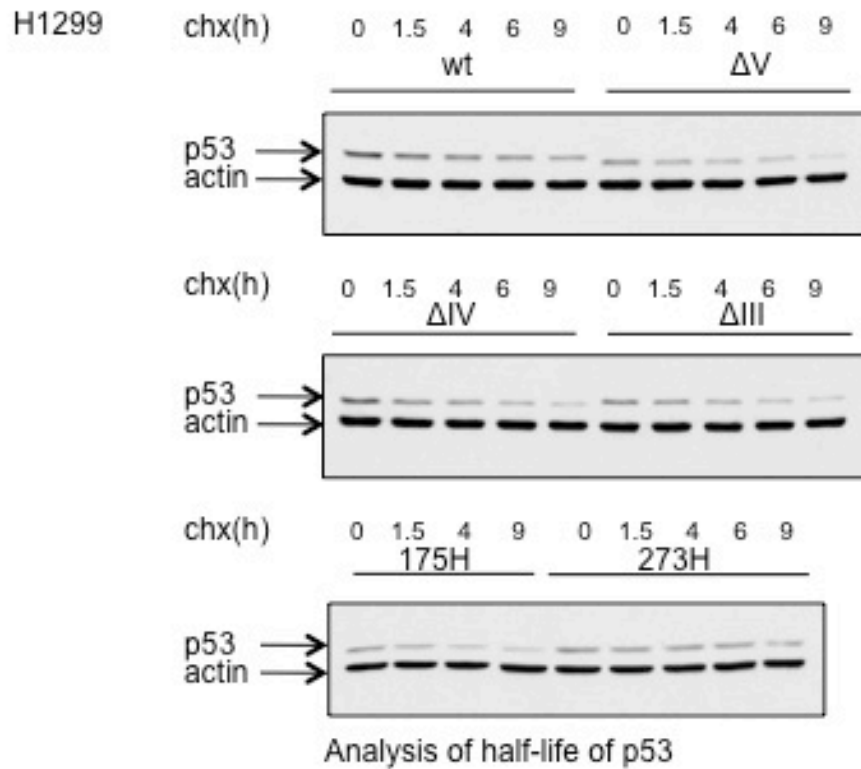
FIGURE 4.9. Analysis of half-life of p53 in H1299 cells by cycloheximide treatment.
H1299 cells were transfected with wild type p53 or indicated p53 DBD mutants. Cells were treated with cycloheximide 24h after transfection and collected at the indicated time points. Western blot analysis was carried out with the p53 specific (DO1) antibody and an anti-actin antibody. The results were quantitated using Scion image software (NIH) and the values of p53 were normalized to the values of actin.

4.3 Summary and discussion.

In this chapter it was found that despite the reduced sensitivity to Mdm2-mediated ubiquitination, conformational p53 mutants are recognized and ubiquitinated by another E3. The increased ubiquitination of some p53 mutants with altered conformation has been noted previously (594, 595) although here it has been shown that this effect is Mdm2-independent. A number of other E3s that can target wild type p53 have been described recently (83, 84, 178, 185, 354, 360, 361, 590) and it is possible that some of them are responsible for the ubiquitination of mutant p53. The contribution of some E3s to ubiquitination of wild type and mutant p53 has been evaluated here and these data suggest that E3 ligases might differ in their sensitivity to the conformation of the DBD. Consistent with previous studies, which have shown that both wild type and mutant p53s are targeted to the proteasomes by CHIP (355), here it is shown that CHIP ubiquitinates wild type p53 and mutant p53. These results suggest that CHIP may play a role in the ubiquitination and degradation of mutant p53, and reduced expression of CHIP in some cancers (Oncomine database) could contribute to the enhanced stability of mutant p53s. CHIP is implicated in the endoplasmic reticulum-associated degradation (ERAD), which represents an important means of quality control of its target proteins. Therefore ubiquitination of mutant p53 by CHIP can be part of the mechanism of p53 folding control. Given that the contribution of CHIP to ubiquitination of mutant p53 was only partial and the existence variety of E3s, there may be other E3s that target only mutant p53. It would be interesting to investigate if Mdmx contributes to ubiquitination of mutant p53. The other possibility is that wild type and mutant p53s differ in their sensitivity to deubiquitinases.

These data imply that Mdm2-independent ubiquitination does not result in efficient degradation of mutant p53 proteins, suggesting that it lacks a degradation signal. It is thought that polyubiquitination consisting of at least 4 ubiquitins linked via K48 target proteins to the proteasomes (202). In addition, there are 6 more lysines in the ubiquitin, which can be used in the formation of the ubiquitin chain and have different functional consequences on the substrate. It is possible that Mdm2-independent ubiquitination of mutant p53 is linked via alternative lysine and therefore does not target mutant p53 for degradation. This possibility remains to be checked in future. Ubiquitinated mutant p53 can be degraded by overexpression of Mdm2, suggesting it provides the necessary degradation signal.

Chapter 5. Localization of p53 DBD mutants.

5   Localization of p53 DBD mutants.

Ubiquitination of the C-terminus of p53 exposes the nuclear export signal (NES) and results in translocation of p53 to the cytoplasm (224, 371). Therefore it was of interest to check the localization of hyperubiquitinated mutant p53. The conformation of DBD of p53 can also be an important determinant of nuclear localization. Several studies have shown that temperature sensitive p53 mutant V135 localizes to the nucleus at 32°C when in wild type conformation but is in the cytoplasm at 37°C, when it is in "mutant" conformation, suggesting that conformation of p53 can be important determinant of localization of p53 (552, 596-598). Another study has correlated mutant conformation of mutant p53 with cytoplasmic localization and transformation potential of 3T3 cells (599).

In this chapter localization of p53 DBD mutants was analyzed.

5.1   Ubiquitinated p53 mutant proteins localize to the cytoplasm.

First, subcellular localization of p53 mutants was analyzed in DKO cells. As expected wild type p53 located to the nucleus in the majority of the cells. Though it was reproducibly seen that some small percentage of cells displayed wild type p53 in the cytoplasm. This could be due to overexpression of p53 in cells, which is not efficiently imported into the nucleus. p53273H mutant, which was not ubiquitinated in these cells (Figs. 3.11), was also located in the nucleus (Fig.5.1). However, the ubiquitinated p53 mutants 175H and ΔV were present in the cytoplasm in majority of cells with a variable degree of nuclear localization (Fig. 5.1). The results were quantitated by counting the cells with cytoplasmic p53 in three independent experiments. This suggests that conformational p53 DBD mutants locate to the cytoplasm. This result was reproduced in at least four independent experiments. To confirm the results in another cell type the experiments were repeated in U2OS cells. U2OS cells express endogenous wild type p53, which could complicate the analysis of localization of the transfected p53 with p53-specific antibodies. However no signal of endogenous p53 was detected by p53-specific antibodies (Fig.5.2). When wild type p53 was transfected, it was detected mostly in the nuclei in U2OS cells, as expected (Fig.5.2). Therefore it was possible to use U2OS cells to analyze localization of transfected wild type p53 and p53 DBD mutants. Cytoplasmic localization of p53175H and p53ΔV was also observed in U2OS cells (Fig. 5.3), though 175H displayed more nuclear localization in these cells compared to DKO (Fig. 5.3). As another method of looking at

localization of p53, the subcellular fractionation of U2OS cells transfected with wild type p53, p53175H or p53ΔV was carried out (Fig. 5.4). The fractionation of nuclear and cytoplasmic compartments was confirmed by the expression of nuclear protein lamin A/C and cytoplasmic protein Hsp70 (Fig. 5.4). The conclusion of the localization data was reflected by subcellular fractionation in U2OS cells, where most of the wild type p53 was in the nucleus, 175H protein was found in both nuclear and cytoplasmic fraction and p53 ΔV was present more in the cytoplasmic fraction than in the nucleus (Fig.5.4). It was consistently noticed that ΔV displayed more cytoplasmic localization than 175H (Fig. 5.1, 5.3). The U2OS cells appeared to be a better system to analyze the localization of p53 than DKO cells, as they were transfected with higher efficiency and cells retained a normal morphology after staining. Localization of contact mutant 273H to the nucleus suggests that unfolded conformation of mutant p53 is required for localization to the cytoplasm. Taken together with the results in previous chapters, it would appear that cytoplasmic localization of mutant p53 correlates with their ubiquitination.
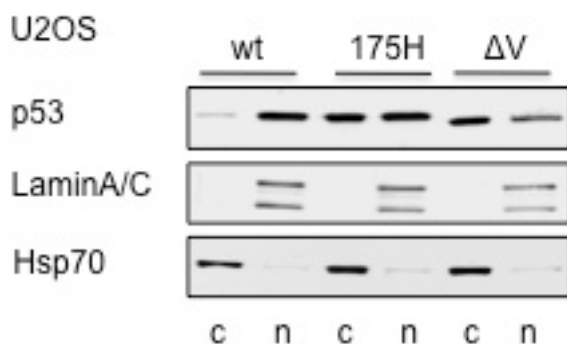
FIGURE 5.4. Mutant p53 is detected in the cytoplasmic fraction of U2OS cells. U2OS cells were transfected with wild type p53 or indicated p53 DBD mutants. Cells were fractionated and cytoplasmic (C) and nuclear (N) proteins were resolved by SDS-PAGE and analysed by Western blotting with p53-specific (DO1), Hsp70-specific and Lamin A/C specific antibodies.

5.2 Cytoplasmic localization is not due to enhanced export.

Cytoplasmic localization of an otherwise nuclear protein can be a result of enhanced nuclear export or less efficient nuclear import. To analyze the contribution of the nuclear localization (NLS) and nuclear export signals (NES) to localization of wild type p53 and p53 175H with mutated c-NES and NLS was analyzed (Fig. 5.5). Mutation of NLS, (produced by substituting Lys 305, 319, 320, 321 and Arg 306 to Ala) and has been shown to prevent nuclear import of p53 (224, 600). Mutation of c-NES (L348A and L350A) has been shown to prevent nuclear export of p53 (157). Subcellular localization (Fig.5.6) of these p53 mutants was analyzed in U2OS cells. To inhibit nuclear export cells were treated with leptomycin B, which blocks CRM-1-dependent nuclear export (601) (Fig.5.6). Percentage of cells with cytoplasmic p53 is presented on the graph (Fig.5.7).
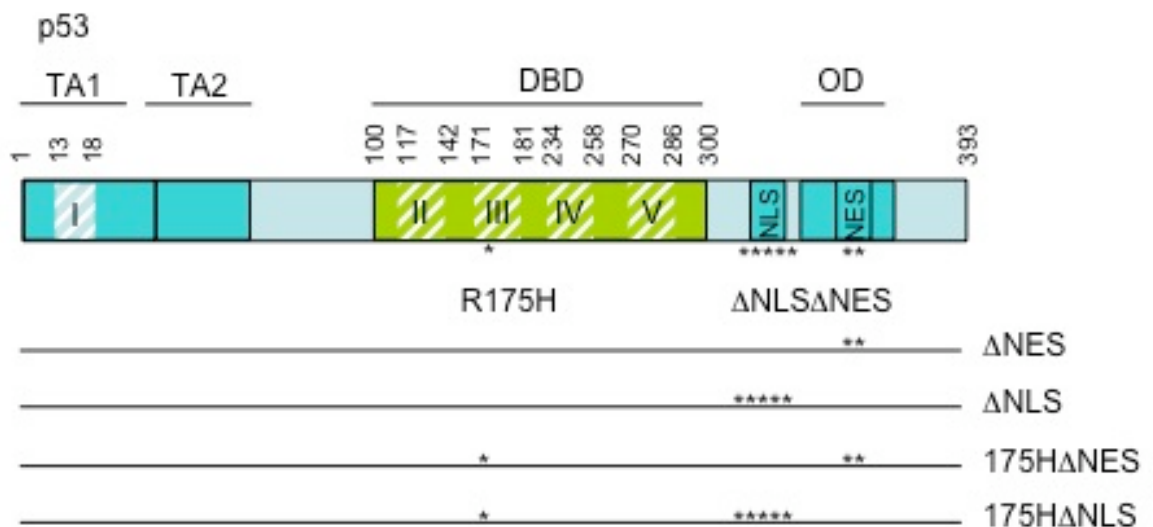
FIGURE 5.5. Schematic representation of the structure of p53 and p53 mutants.
The main domains of p53, nuclear export (NES), nuclear localization (NLS) signals and the location of the conserved boxes I, II, III, IV and V and the corresponding deletions, point mutations R175H and R273H.
TA - transactivation domain, DBD - DNA binding domain, PD - proline-rich domain, OD - oligomerization domain.

The average of four independent experiments for wild type and p53175H and of two independent experiments for p53 with mutation of NLS and NES is presented (Fig.5.7). Wild type p53 located to the nucleus with small percentage of the cells having cytoplasmic p53 (Fig.5.6). The cytoplasmic localization p53 was not prevented by treatment with leptomycin B or by mutating the c-NES (Fig.5.6, 5.7). This suggests that cytoplasmic fraction of wild type p53 is not imported into the nucleus, possibly due to overexpression. Leptomycin B treatment or mutation of c-NES did not decrease cytoplasmic localization of

175H in this experiment and 175H with mutation of c-NES located to the cytoplasm similarly to 175H p53 mutant. This suggests that cytoplasmic localization of 175H is not due to enhanced nuclear export (Fig.5.6, 5.7). p53ΔNLS and p53175H/ΔNLS were seen almost exclusively in the cytoplasm, with no cells displaying distinct nuclear staining of p53 (Fig.5.6). This suggests that both wild type and mutant p53 can be recognized by nuclear import machinery, which is mediated via c-terminal NLS and other NLSs do not appear to contribute. To confirm these results the experiments were repeated in the DKO cells (Fig.5.8, 5.9). Similarly to observations made in U2OS cells, blockage of nuclear export by leptomycin B treatment did not prevent cytoplasmic localization of p53ΔV or p53175H in these cells (Fig.5.8). 175H with mutation of NES displayed cytoplasmic staining similar to 175H, whereas 175HΔNLS located almost exclusively to the cytoplasm (Fig.5.9). These results confirm the finding in U2OS cells and suggest that mutant p53 localization to the cytoplasm is not due to enhanced nuclear export, but could be due to cytoplasmic sequestration.

In addition to C-terminal NES, p53 contains NES in its N-terminus (11-27aa). It is possible that due to binding of Mdm2 to the N-terminus of p53, n-NES is not easily accessible to nuclear export machinery. According to the binding data presented here Mdm2 can interact outside N-terminus on mutant p53, therefore the contribution of n-NES to nuclear export of mutant p53 was tested (Fig.5.10). To this end subcellular localization of p53 DBD mutants – 175H, ΔV, and the corresponding p53 mutants lacking box I - 175H/ΔI and ΔV/ΔI - was analyzed (Fig.5.10). It should be noted, that deletion of box I (13-18aa) does not cover the whole region of the described n-NES (11-27aa). The cells with cytoplasmic p53 were counted and presented on the graph (Fig.5.10). Deletion of box I did not affect localization of wild type p53, which was nuclear in most of the cells (Fig.5.10). The cytoplasmic localization of p53 DBD mutants was not decreased when n-NES was mutated (Fig.5.10).

These results suggest that mutant p53 localizes to the cytoplasm not due to enhanced nuclear export, but nuclear import may be less efficient. It should be also noted that the percentage of cells with wild type p53 in the cytoplasm did not change when nuclear export was inhibited by leptomycin B or N- or C-terminal NES were deleted, suggesting that some population of wild type p53 is also sequestered in the cytoplasm. This might suggest that a small fraction of wild type p53 adopts a mutant conformation in cells.

5.3 Mutant p53 is sequestered in the cytoplasm by interaction with Parc.

The results described above suggest that mutant p53 is sequestered in the cytoplasm, rather than showing enhanced nuclear export. Parc has been shown to play a role as a cytoplasmic anchor protein forming complexes with p53 (375). To test whether mutant p53 interacts with Parc, co-immunoprecipitation analysis following co-transfection of wild type p53 and p53 DBD mutants along with Parc or empty vector control in U2OS cells was carried out. In agreement with previous reports interaction between wild type p53 and Parc was very weak, which may be due to mostly nuclear localization of p53 (Fig.5.11). Although Parc was also less well expressed in this experiment. Deletion of the NLS on p53, which leads to localization of wild type p53 to the cytoplasm, promoted interaction with Parc (Fig.5.11). p53 DBD mutants, which locate to the cytoplasm, all interacted with Parc (Fig.5.11). Though p53175H/ΔNLS located more to the cytoplasm than p53175H, there was no increase in amount of Parc bound to p53175H/ΔNLS, probably due to saturation of Parc (Fig.5.11). The experiment was repeated two more times and similar observation was made. Consistent with previous reports (375), these data show that Parc interacts with cytoplasmic p53, independently of the mutation of DBD of p53. This result suggests that p53 mutants can be sequestered in the cytoplasm by interaction with Parc. This could be tested by downregulating Parc expression by siRNA-mediated approach. Parc is known to interact with the oligomerization domain of p53, and their interaction could be inhibited by a p53 C-terminal peptide (602).
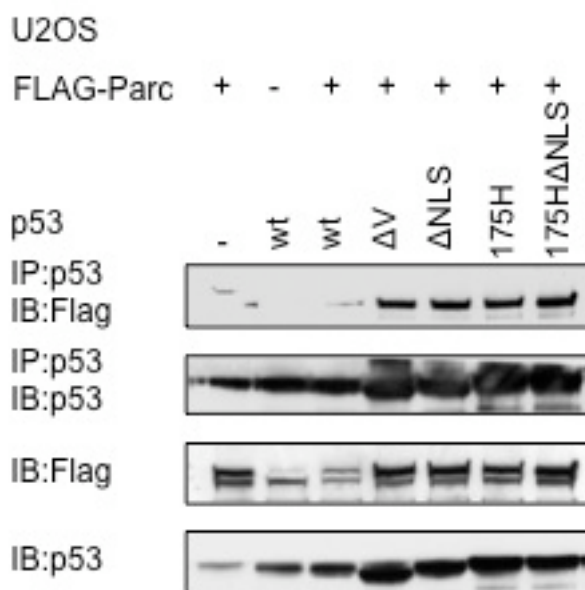
FIGURE 5.11. p53 DBD mutants interact with Parc.
U2OS cells were transfected with indicated p53 constructs and with FLAG-tagged Parc construct. Cell lysates were analyzed by immunoprecipitation with p53-specific antibodies and immunoblotting with p53-specific and FLAG-specific antibodies.

5.4 Localization of p53 mutants in H1299.

To extend the study to other cell types, the localization of p53 mutants was tested in H1299 cells (Fig.5.12). Intriguingly, in this cell type p53175H located to the nucleus in most cells (Fig.5.12). Wild type p53 and p53273H also localized to the nucleus (Fig.5.12). p53ΔV located to the cytoplasm in some cells but much less than in U2OS cells (Fig.5.12).
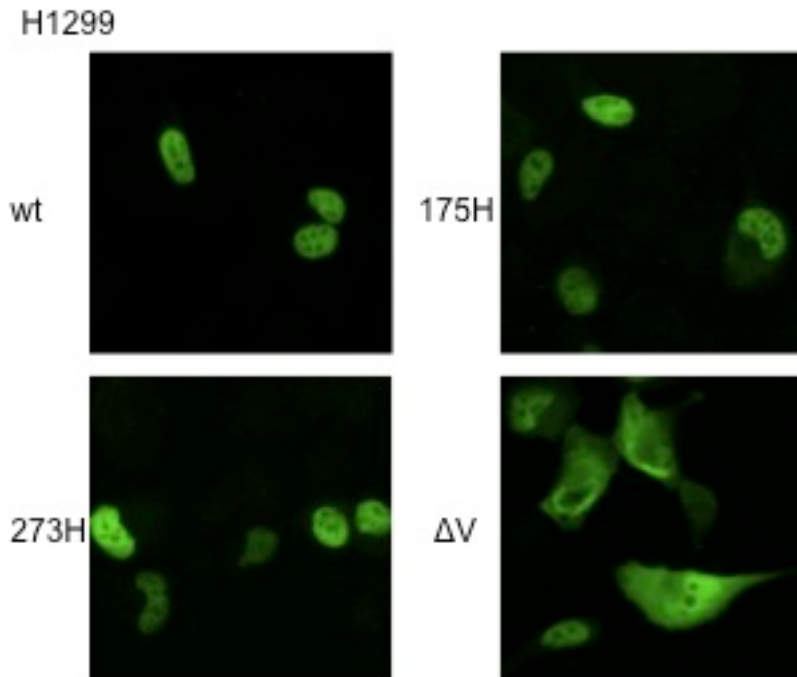
FIGURE 5.12. p53175H displays nuclear staining in H1299 cells.
H1299 cells were transfected with wild type p53 or indicated p53 DBD mutants. Cells were fixed and stained with anti-p53 DO1 and DAPI.

Therefore, cytoplasmic localization of p53 mutants seems to be dependent on the cell type. p53 DBD mutants were hyperubiquitinated in H1299 cells (Fig.3.12), implying that ubiquitination does not sequester p53 mutants in the cytoplasm. It is possible that ubiquitination is required but not sufficient for cytoplasmic localization. There are possibly other changes in H1299 cells which prevent cytoplasmic retention. It is possible that H1299 do not express enough Parc to sequester mutant p53 in the cytoplasm.

It is of interest to check where endogenous mutant p53 localizes in tumour-derived cell lines. The preliminary analysis of tumour cell lines expressing contact mutant p53273H mutant shows nuclear localization of this mutant, consistently with the experiments when 273H was transiently expressed (Fig.5.1, 5.3).

5.5 Summary and discussion.

Whereas wild type p53 localizes mostly to the nucleus, here it was found that ubiquitinated p53 DBD mutants display cytoplasmic localization in some cell types. The results shown in this chapter indicate that the unfolded conformation of mutant p53 is associated with cytoplasmic sequestration, as conformationally unfolded p53 mutants but not contact mutant 273H located to the cytoplasm. In agreement with the results presented here, another report has associated ubiquitination of p53 DBD mutants and their cytoplasmic localization (594). Interestingly, sequestration of wild type p53 in neuroblastomas has been recently shown to be due to hyperubiquitination of p53 (603). The results in this chapter suggest that mutant p53 is not subject to enhanced nuclear export and is sequestered in the cytoplasm. The mechanism for p53 sequestration in the cytoplasm has been suggested to involve interaction with Parc (375), which inhibits nuclear import. However, some fraction of p53 undergoes normal nuclear import, suggesting that there are two populations of mutant p53 in the cells, one is sequestered in the cytoplasm and the other fraction undergoes normal nucleo-cytoplasmic shuttling. This suggests that mutant p53 may exist in mutant conformation sequestered in the cytoplasm and wild type conformation, which locates to the nucleus. However, cytoplasmic sequestration of mutant p53 depends on the cell type and in other cells, like H1299, mutant p53 is efficiently imported to the nucleus. It is possible that the localization of mutant p53 to the cytoplasm is representative of protective mechanism against misfolded p53, and further in tumourigenesis, cells select for nuclear accumulation of mutant p53. This study suggests that ubiquitination of p53 mutants occurs in the cytoplasm. Mutant p53 may be ubiquitinated by the activity of cytoplasmic E3s. Interestingly, CHIP localizes to the cytoplasm and has been shown to cooperate with Parc in ubiquitination of its substrates. It would be interesting to determine what is the biological function of localization of mutant p53 to the cytoplasm and to extend the study to other tumour-derived cell lines expressing mutant p53.

Chapter 6. Interaction of p53 DBD mutants with p73.

6 Interaction of p53 DBD mutants with p73.

Several p53 DBD mutants can associate with p63 and p73 in cells, both endogenous and overexpressed, which leads to inactivation of p63/p73 (458-464). The interaction is mediated via the DNA binding domains of p53 and p63/p73 (461), though the exact binding site has not been mapped. In this chapter the contribution of the conserved boxes of DBD to the interaction with one of the family members p73 and the effect of the interaction on the stability of mutant p53 and p73 proteins were studied.

6.1 p53 with deletions of the conserved boxes of DBD interact with p73.

p53 with deletions of conserved boxes of DBD ΔV, ΔIV, ΔIII and ΔII adopt a "mutant" conformation (582) and therefore predicted to interact with p73. To detect the interaction and to test if any of the conserved boxes can be the binding site of p73, co-immunoprecipitation analysis of p53 DBD mutants with p73 following their co-expression in cells was carried out. The polymorphism status of p53 modulates the interaction and mutant p53 with 72R associates with p73 more efficiently than mutants with 72P polymorphism (459, 463). Therefore p53 mutants with 72R polymorphism were used in
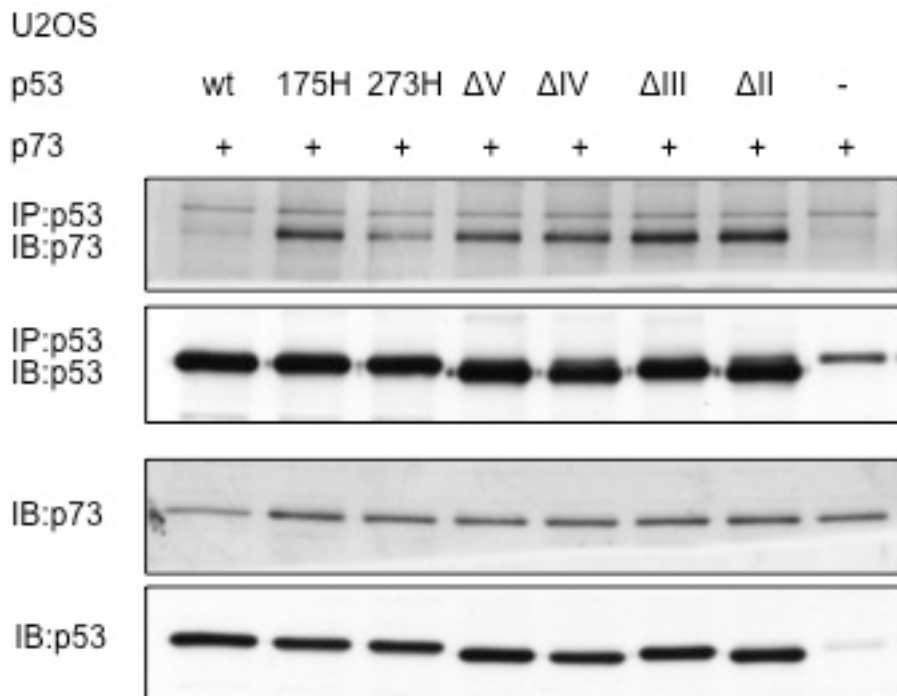
FIGURE 6.1. p53 DBD mutants interact with p73.
U2OS cells were transfected with wild type or indicated p53 DBD mutants and with HA-p73α. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and analyzed by immunoblotting with CM1 or HA-specific antibodies.

this experiment. U2OS cells were co-transfected with p53 ΔV, ΔIV, ΔIII and ΔII as well as p53175H and p53273H along with with p73α. Cell lysates were immunoprecipitated with p53-specific antibody and p73 was detected with a p73-specific antibody. In this experiment p73α was used, which is the full-length TAp73 transcriptionally active isoform. TAp73α has been previously reported to associate with several p53 DBD point mutants. Wild type p53 failed to interact with p73 whereas all p53 DBD deletion and point mutants interacted with p73α (Fig.6.1). This result was observed in three independent experiments. Contact mutant 273H interacted with p73 less well than conformational p53 mutants. In agreement with other reports, these data suggest that unfolded conformation of DBD of p53 unveils the cryptic p73 binding site, not accessible in wild type conformation of DBD.

6.2 Effect of interaction of p53 mutants with p73 on degradation of p53 and p73.

Mdm2 interacts with p73, however Mdm2 fails to degrade p73 or export p73 to the cytoplasm (416, 418). The effect of the interaction between mutant p53 and p73 on Mdm2-mediated degradation was analyzed. Specifically, the following questions were addressed. 1) Can interaction of p73 with mutant p53 lead to degradation of p73 by Mdm2? 2) Can p73 protect mutant p53 from being degraded by Mdm2? To address these questions, p73α was expressed along with Mdm2 and with wild type p53 or p53 DBD mutants ΔIII and ΔII (Fig.6.2A), which interacted with p73 better than other p53 deletion mutants. GFP was used to control for transfection efficiency. The apparent slower migration of GFP in the last lane is due to the fracture of the gel (Fig.6.2A). Consistent with the data presented above, both wild type and mutant p53 were efficiently degraded by Mdm2, regardless of expression of p73, suggesting that p73 does not protect mutant p53 from degradation (Fig.6.2A). Consistent with previous reports, p73 was resistant to degradation by Mdm2, whereas wild type p53 and p53 DBD mutants were efficiently degraded by Mdm2 (Fig.6.2A). Co-expression of p53 DBD mutants seemed to slightly reduce levels of p73, but this was difficult to assess due to strong signal of p73 on this gel. Therefore, the western blotting analysis was repeated with the smaller quantity of the cell lysates from this experiment using gel with broader lanes (Fig. 6.2B). This result more clearly shows that degradation of p53ΔIII was not affected by the presence of p73 (Fig.6.2B). Interestingly, though p73 was not degraded by Mdm2 when co-expressed with wild type p53, p73 protein levels decreased when p53ΔIII was present. This suggests that mutant p53

promotes degradation of p73 by Mdm2. Interestingly, it was noticed that p73 increases the expression of Mdm2 – both endogenous and transfected. The mechanism of increasing levels of transfected Mdm2 protein may involve protein stability. To analyze if p73 can stabilize Mdm2 protein, p73 mutant lacking Mdm2-interaction site or Mdm2 lacking N-terminus can be tested in future. These observations were reproduced in two independent experiments.
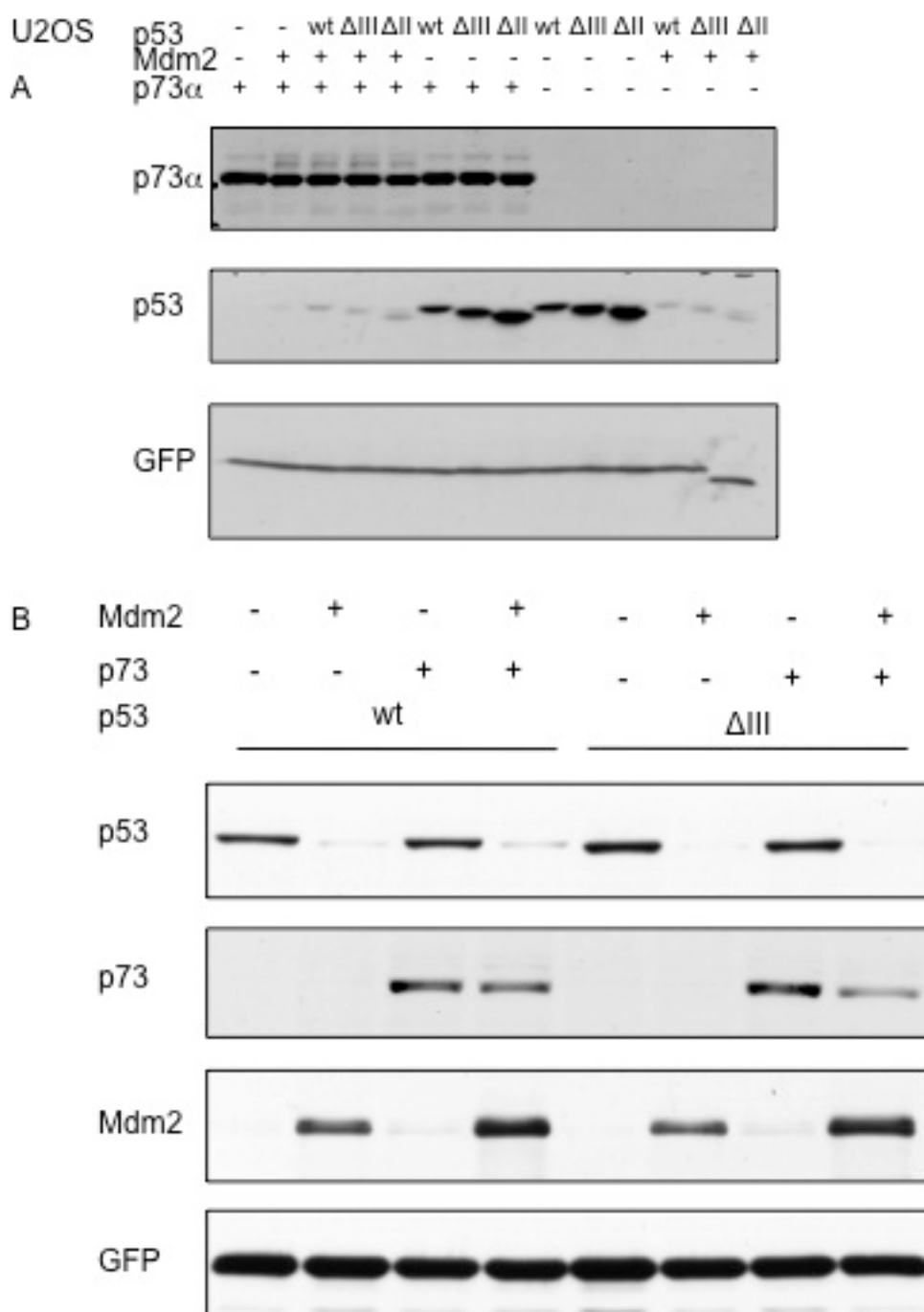
FIGURE 6.2. Effect of p53 and p73α on Mdm2-mediated degradation of p73α and p53. U2OS cells were transfected with wild type p53 or indicated p53 DBD mutants along with empty vector or wild type Mdm2 and with HA-p73α. GFP was co-transfected to control for transfection efficiency. A. Western blot analysis was carried out with p53-specific (DO1), HA-specific and GFP-specific antibodies. B. Three times less of the cell lysates of indicated samples were subject to repeat SDS-PAGE and Western blot analysis.

The effect of p53 DBD mutant on p73 degradation was quite weak, possibly due to rapid degradation of p53 by Mdm2. The aim of the next experiment was to confirm the result and to test if p53 175H could also promote degradation of p73 and increase the amount of p53 mutant bound to p73, p73 was co-expressed with Mdm2 and p53ΔII and p53175H were titrated in (Fig.6.3). Interestingly, when p53175H or p53ΔII mutants were expressed at higher levels the p73 protein levels decreased (Fig.6.3). In agreement with Figure 6.2B, this suggests that p53175H and p53ΔII promote degradation of p73 by Mdm2, however this needs to be confirmed by treatment with proteasome inhibitors or analysing half-life of p73. It will be interesting to test if ubiquitin ligase activity of Mdm2 is required for the ability to degrade p73 by testing Mdm2-464 mutant. These results suggest, that p53 mutant can promote degradation of p73 by Mdm2. In future, p53 and p73 mutants lacking the N-terminal binding to Mdm2 will be tested. The *in vivo* ubiquitination assay of p73 will also be set up to test if mutant p53 promotes ubiquitination of p73 by Mdm2.
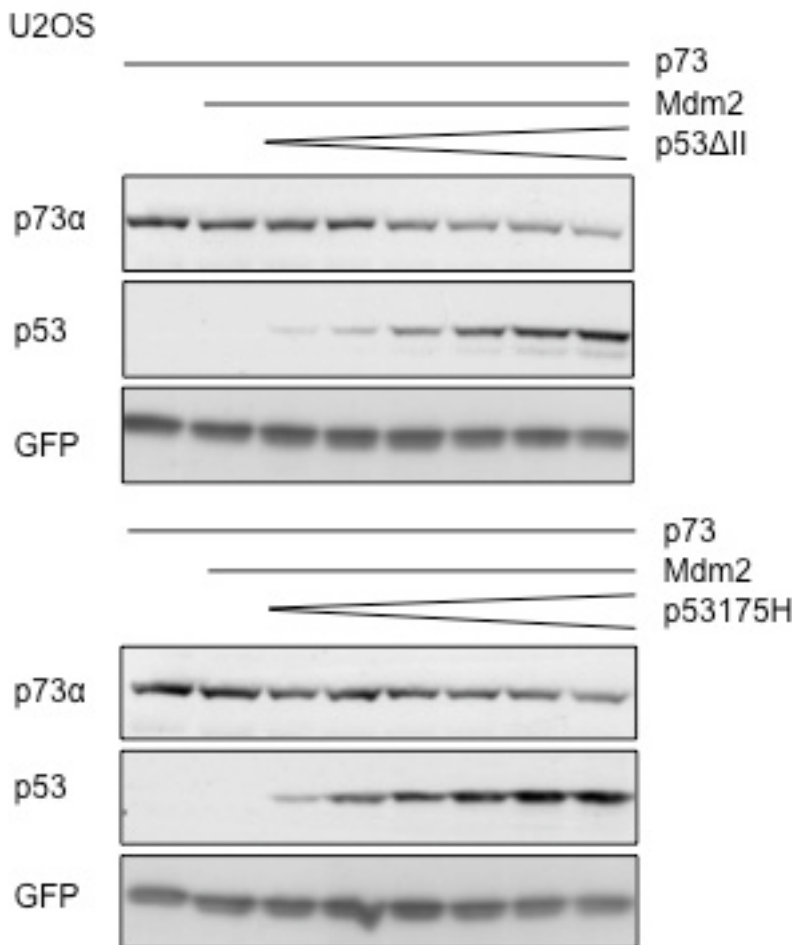
FIGURE 6.3. **Effect of p53 DBD mutants on degradation of p73α by Mdm2.**
U2OS cells were transfected with HA-p73α and empty vector or wild-type Mdm2 and with increasing amounts of p53ΔII (A) or p53175H (B) mutants. GFP was co-transfected to control for transfection efficiency. Western blot analysis was carried out with p53-specific (DO1), HA-specific and GFP-specific antibodies.

6.3 p73 inhibits ubiquitination of mutant p53.

As shown above, conformational p53 mutants are subject to Mdm2-independent ubiquitination and can interact with p73. Therefore it was tested if p73-specfic Itch ubiquitin ligase can target p53 mutants. To this end Itch expression was down-regulated by siRNA-mediated approach with previously published Itch-specific siRNA oligonucleotides and the ubiquitination of transfected p53175H was analyzed in U2OS cells. Ubiquitination of mutant p53 was only weakly reduced when Itch-specific siRNA was transfected. However, to confirm this the reduction of Itch expression needs to be verified (Fig. 6.4).
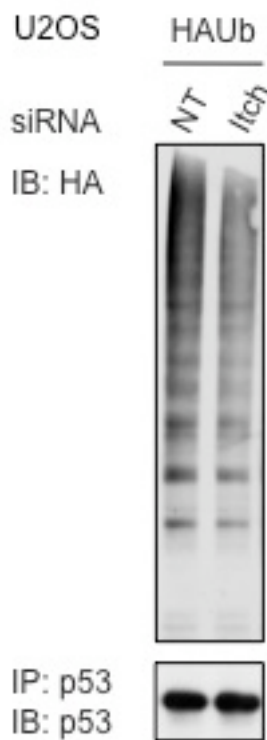
FIGURE 6.4. **siRNA-mediated knockdown of Itch expression does not affect the ubiquitination of p53175H.** U2OS cells were transfected with non-targetting (NT) or Itch-specific siRNA oligonucleotides. Cells were transfected with p53175H along with HA-ubiquitin 24h later. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either HA-specific or the p53-specific CM1 antibody.

Next, the effect of p73 on ubiquitination of mutant p53 was examined. To this end wild type p53 or p53175H were expressed in U2OS cells along with the p73 isoforms α or β and HA-ubiquitin and *in vivo* ubiquitination assay was carried out (Fig.6.5). p73β is a splice variant of TA p73 isoform, which is functionally very similar to p73α. p73β interacts with p53175H similarly to p73α. Interestingly both p73α and p73β decreased the ubiquitination of p53 175H, without any effect on the residual ubiquitination of wild type p53 (Fig.6.5). This observation was reproduced in two other independent experiments. However, p73 did not stabilize mutant p53 protein in cells (Fig.6.2). This could be explained by the previously described observation that ubiquitination does not efficiently degrade mutant p53. It would be interesting to confirm this observation in Mdm2-null cells and to test the effects of p73 on the Mdm2-mediated ubiquitination of mutant p53. A preliminary experiment suggests that p73α does not inhibit Mdm2-mediated ubiquitination of mutant p53.
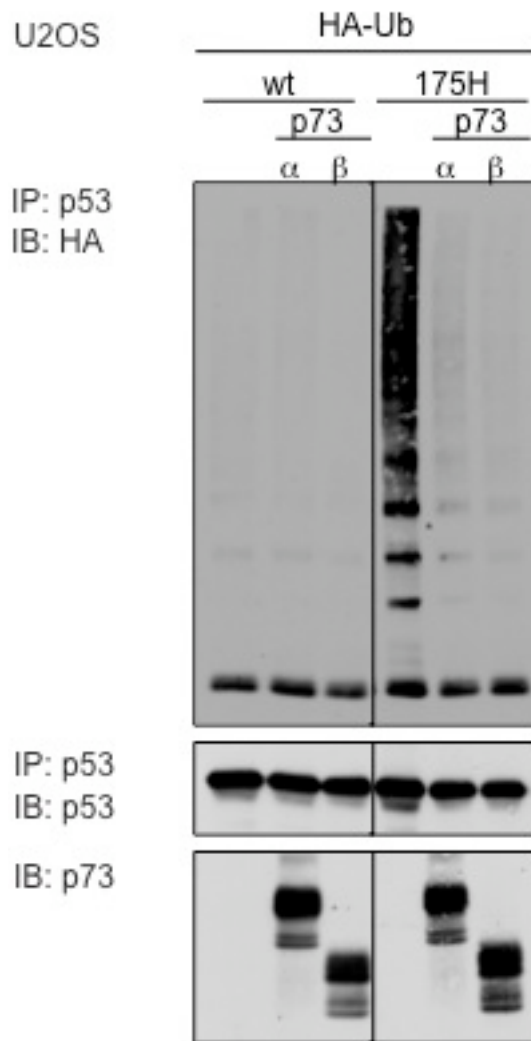
FIGURE 6.5. p73 inhibits ubiquitination of p53175H.
U2OS cells were transfected with wild type p53 or p53175H along with HA-tagged ubiquitin (HA-Ub) and with empty vector or FLAG-p73α or FLAG-p73β. Cells were treated with MG132 and lysed under denaturing conditions. Cell lysates were immunoprecipitated with p53-specific antibody (DO1) and Western blotted with either HA-specific or the p53-specific CM1 antibody. Direct western blotting analysis was carried out with p73-specific antibody (sc-20).

6.4 Localization of mutant p53 and p73.

To study if the interaction between mutant p53 and p73 affects the localization of each of the proteins, subcellular localization of wild type p53, p53175H or p53ΔV expressed along with p73α was analyzed U2OS cells (Fig.6.6). Similarly to results shown above, wild type p53 localized to the nucleus (Fig.6.6A) and p53175H and p53ΔV displayed some cytoplasmic localization (Fig.6.6B, C). Endogenous p73 was not detected in these cells with p73-specific antibody, however overexpressed p73 was clearly visible. In agreement with previous studies (373, 604, 605) transfected p73 resided in the nucleus in most cells with small percentage of cells where p73 was detected in the cytoplasm (Fig.6.6A). The colocalization of p73 with wild type p53 was visualized by confocal microscope by merging the channels of green (p53) and red (p73) fluorescence which displays colocalization as yellow color. The technical caveat of this experiment is that the intensity of the p53 staining was stronger than p73 in this experiment. Wild type p53 and p73 only partially colocalized in the nucleus (Fig.6.6A), suggesting that function of p73 may differ from p53. In addition, p73 was found in distinct structures in the nuclei of some cells (Fig.6.6A). The biological significance of these structures is not clear, however they seem to localize to heterochromatin regions, as seen by staining with DAPI (Fig6.6A). The pattern of localization of p53175H and p53ΔV mutants was similar to described above (Fig.5.2). The expression of p53 mutants did not relocalize p73, as it remained in the nucleus in most cells when p53175H and p53ΔV were co-expressed (Fig.6.6B,C). The p53 DBD mutants and p73 also partially colocalized in the nucleus (Fig.6.6B,C). In some cells mutant p53 colocalized with p73 in the distinct nuclear aggregates (Fig.6.6B,C). These findings are still preliminary. These data suggest that only a fraction of mutant p53 is involved in interaction with p73, which takes place mostly in the nucleus. In future experiments it will be important to determine the stoichiometry of the p53/p73 interaction. Though p73 inhibits ubiquitination of mutant p53, this might be indirect, as the data of this study suggests that ubiquitination of mutant p53 takes place in the cytoplasm.

6.5 Summary and discussion.

In this chapter the interaction of mutant p53 and p73 was analyzed. It was shown here that all p53 DBD mutants with deletions of the conserved boxes interacted with p73. This together with previous reports (461, 606) suggests that the altered conformation of DBD of p53 is the determinant for the interaction. As shown previously, all the p53 DBD deletion mutants have "mutant" conformation of DBD and similarly to tumour-derived conformational mutants display a cryptic epitope (213-217aa), recognized by the conformation-sensitive antibody. The possibility that this region is the binding site for p73 remains to be checked in future.

Whereas others have shown that Mdm2 inhibits transcriptional activity of p73, the data shown here suggest that mutant p53 can promote degradation of p73 by Mdm2, which is not degraded by Mdm2 on its own (416, 418). Studies of p53-p73 chimeras have shown that lack of the homology to the region of 92-112aa of p53 can be the reason for resistance of p73 to degradation by Mdm2 (419). The data presented here suggest that p73 can be degraded when in complex with mutant p53, which may provide such degradation signal. This could contribute to the inactivation of p73 by mutant p53. It would be interesting to test if mutant p53 can promote ubiquitination of p73 by Mdm2.

As shown here, p73 can inhibit the ubiquitination if mutant p53. It is not clear whether inhibition of ubiquitination by p73 can contribute to accumulation of mutant p53. p73 does not seem to protect p53 mutants from degradation by Mdm2, suggesting that p73 might not inhibit ubiquitination by Mdm2, which has been confirmed in the preliminary experiment.

Since the unfolded conformation of the DBD of p53 is the determinant of both binding to p73 and of ubiquitination of mutant p53, it is possible that the ubiquitin ligase and p73 compete for binding to the DBD of p53. However, this is unlikely, as localization data suggest that mutant p53 and p73 colocalize only partially. It is possible that p73 inhibits the ubiquitination of mutant p53 indirectly, by sequestering the ubiquitin ligase in the nucleus. Another interesting possibility could be that p73 promotes deubiquitination of mutant p53 in the nucleus. This also implies that ubiquitination does not sequester mutant p53 in the cytoplasm. This is also confirmed by the nuclear localization of p53 mutants in H1299 cells, in which ubiqitination of these mutants was clearly detected.

The interaction of mutant p53 and p73 has been suggested to contribute to oncogenic properties of mutant p53 and further characterization of the molecular mechanisms of the interaction and functional consequences can be important for clinical implications.

Chapter 7. Discussion.

7 Discussion.

Mutations in p53 are very frequently found in cancers, suggesting they are selected for during malignant progression. Many of the p53 mutations affect the DNA binding domain (DBD) of p53, which partially or completely distort its conformation (436, 579). Mutations of DBD result in loss of the tumour suppressive function of p53, by inactivating its transcriptional activity. Moreover, mutant p53 acquires novel functions and actively contributes to cancer progression, known as "gain-of-function" effect. Mutant p53 often accumulates to high levels in tumour cells (581), although the reason why mutant p53 is not degraded in cancer cells remains unclear. In mice engineered to express only mutant p53, stabilization of p53 is seen only in tumours but not in normal cells (484, 485). While these mutants do not activate expression of Mdm2, it is apparent that normal cells retain sufficient Mdm2 levels to keep the levels of mutant p53 protein low. Secondary events may occur which contribute to stabilization of mutant p53 in tumours. It is possible that the stabilization of mutant p53 in tumours is related to ARF expression – an inhibitor of Mdm2 – that is specifically activated in tumours (16). This idea is also supported by a recent observation that p16 is lost in a proportion of tumours in mice expressing mutant p53. By inhibiting E2F activity, the CDK inhibitor p16 leads to inhibition of ARF, a transcriptional target of E2F (520). However, siRNA mediated inhibition of ARF expression in a tumour cell line expressing high levels of mutant p53 did not decrease the stability of the p53 (Horn and Vousden, personal communication), suggesting that ARF is not the only determinant of mutant p53 stability. The ongoing DNA damage signalling in tumour cells can also contribute to accumulation of p53. It is therefore possible that although Mdm2 is able to control the basal levels of mutant p53, it fails to degrade high levels of mutant p53 after stress. However, clearly many tumours express high levels of Mdm2 and high levels of mutant p53, suggesting that other mechanisms account for accumulation of mutant p53. In addition, there are some tissue-specific components of regulation of stability of mutant p53, such as Mdm2-independent regulation of mutant p53 in liver (520). Therefore it appears that a number of different mechanisms can lead to accumulation of mutant p53.

Although p53 mutants are quite heterogeneous, they have common determinants of their structure and function, which divide them into conformational and contact mutants. The results described in this study suggest a distinct pathway that regulates the degradation of conformational p53 mutants. Here it has been demonstrated that mutant and wild type

p53 can be degraded through overlapping, but distinct pathways. Unlike wild type p53, mutant p53 is specifically ubiquitinated in Mdm2-independent manner. It is therefore possible that the selective stabilization of mutant p53 reflects a tumour-specific defect in the pathways that target mutant p53 for degradation. This study suggests there are differences in the degradation pathways that regulate the conformational and contact mutants. It is clear however that other factors contribute to the stability of contact mutant p53 forms in cancers, since p53s mutated at codon 273 – which are ubiquitinated like wild type p53 in this study – are frequently found to be stabilized in human cancers.

Although both wild type p53 and mutant p53 are degraded by Mdm2, there are clear differences in the mechanisms of degradation. Whereas wild type p53 degradation is entirely dependent on ubiquitination by Mdm2, the ubiquitin ligase activity of Mdm2 is less important for degradation of mutant p53. The results of this study propose the following model of degradation of wild type and mutant p53 (Fig.7.1). In the first step, the N-terminal domain of Mdm2 interacts with N-terminus of p53 which activates the ubiquitin ligase activity of Mdm2 leading to polyubiquitination of p53. In the next step polyubiquitination causes unfolding of p53, which promotes interaction of RING domain of Mdm2 with p53, possibly in its DBD. This leads to the next step in which Mdm2 delivers p53 to the proteasomes, which involves the interaction of Mdm2 with the proteasome. In case of mutant p53 the ubiquitination step by Mdm2 is less important. Mutant p53 is already unfolded and also highly ubiquitinated in cells by another E3. Therefore the second interaction involving RING domain of Mdm2 and mutant p53 is promoted. At this step Mdm2 delivers mutant p53 ubiquitinated by another E3 to the proteasomes.
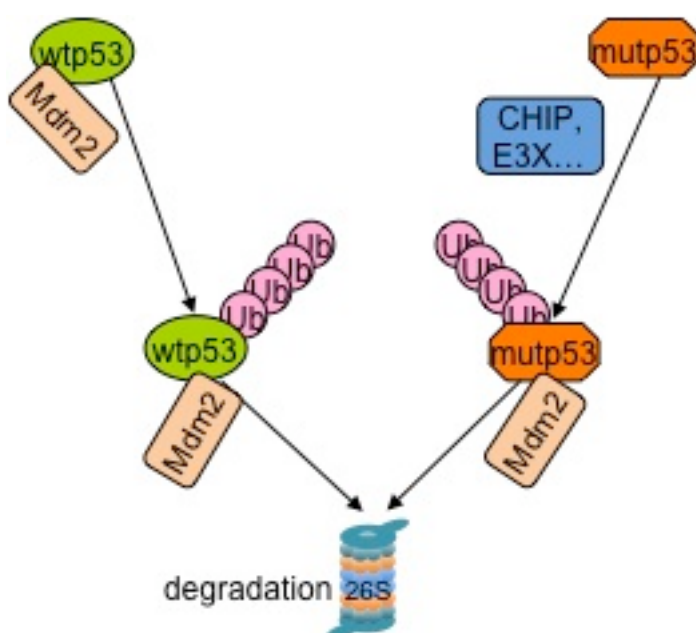
FIGURE 7.1. Model of degradation pathways of wild type and mutant p53.
This study suggests the following model of degradation of wild type p53 and mutant p53. Wild type p53 is ubiquitinated and targeted to the proteasomes for degradation mainly by Mdm2. Mutant p53 is ubiquitinated by other ubiquitin ligases, such as CHIP and others unknown (E3X). Mdm2 may play a role in delivery of the ubiquitinated mutant p53 to the proteasomes for degradation.

Hence, it is interesting to investigate whether this function of Mdm2 is compromised in tumours with mutant p53. The other possibility is that mutant p53 is not ubiquitinated in tumor cells, which could be due to loss of expression of mutant p53-specific E3.

This study shows that degradation of mutant p53 is dependent on Mdm2, however it does not require the N-terminal interaction of both proteins. This has an important potential implication for treatment of tumours with the inhibitor of N-terminal interaction - nutlin-3. Nutlin-3 may be a promising strategy for the treatment of tumours that retain wild type p53. However, given the oncogenic activity, further stabilization of mutant p53 would be unfavourable for treating cancer. Our study has shown that while nutlin-3 leads to efficient stabilization of wild type p53, it only weakly stabilizes mutant p53. This suggests that nutlin-3 can be used to treat tumours, which retain both wild type and mutant forms of p53.

Whereas degradation of wild type p53 is dependent on the E3 activity of Mdm2, the data shown in this study suggest that E3 activity is not absolutely required for the degradation of mutant p53. This suggests the possibility of using the inhibitors of E3 activity of Mdm2 to selectively stabilize wild type p53 but not mutant p53.

Conformationally altered p53 mutants are selectively ubiquitinated by other E3s, independent of Mdm2. Despite being ubiquitinated, mutant p53 is not efficiently degraded, which implies the existence of additional signals for proteasomal destruction. Mdm2 may provide such a signal, as it readily degrades mutant p53, ubiquitinated by other E3. A number of different ubiquitin ligases, which can target wild type p53 have been described recently (83, 84, 178, 185, 354, 360, 361, 590). In agreement with other reports (355, 455), CHIP contributes to ubiquitination of both wild type and mutant p53. CHIP is involved in protein folding control by ubiquitinating and degrading its substrates (607, 608). This suggests that ubiquitination of mutant p53 can be a part of the protective mechanism of the cell to eliminate misfolded p53. This also suggests an interesting possibility that mutant p53 may induce ER stress, which can be triggered by accumulation of misfolded proteins in cells. ER stress has been described to accompany different stages of tumour progression, however it has been unclear whether it inhibits or promotes tumour development (609). Recent report suggests that HRas induced senescence is mediated by ER-associated unfolded protein response, suggesting its anti-oncogenic role (610). It is possible that tumours overcome this barrier by deregulating the components of the ER associated degradation. This role of CHIP in ubiquitination of mutant p53 implies that CHIP can be inactivated in cancers, contributing to accumulation of mutant p53. In agreement with this

hypothesis, CHIP expression is reduced in some cancers, however it is not clear whether these cancers have mutant p53.

The contribution of CHIP seems to be partial, leaving the possibility that other E3s exist that specifically target mutant p53, or that wild type and mutant p53 differ in their sensitivity to deubiquitinases. Discovery of other ubiquitin ligases that specifically ubiquitinate mutant p53 can be addressed by analysis of specific binding partners of mutant p53 by proteomics approach. Alternatively, screening of all human ubiquitin ligases can be carried out using the siRNA library that targets all known human E3s for the effect of the down-regulation of their expression on mutant p53 ubiquitination.

Unlike wild type p53, which is mostly nuclear, it has been found here that the conformationally altered p53 mutants display enhanced cytoplasmic localization. The data presented here suggest that "mutant" conformation of the DBD of p53 is an important determinant for localization to the cytoplasm, as contact mutant locates to the nucleus similarly to wild type p53. Cytoplasmic localization of wild type p53 has been noted in neuroblastomas and has been attributed to enhanced nuclear export. However, according to the results presented in this study mutant p53 is sequestered in the cytoplasm and its nuclear import is reduced. The mechanism of such sequestration is not known, however it can involve interaction with Parc. It is likely, that cytoplasmic localization of mutant p53 requires the cryptic epitope in DBD, which can be involved in protein interactions. It should be noted that some population of mutant p53 undergoes functional nuclear import. The reason for heterogeneous behaviour of mutant p53 is not clear, and could be dependent on the stage of cell cycle. The findings of this study also suggest that mutant p53 residing in the cytoplasm is ubiquitinated. CHIP, which contributes to this ubiquitination of mutant p53, has been shown to locate to the cytoplasm. Interestingly CHIP has been shown to cooperate with Parc in ubiquitination of the substrates. There are many other cytoplasmic ubiquitin ligases, which may contribute to ubiquitination of mutant p53. Inhibition of ubiquitination of mutant p53 by p73 does not relocate mutant p53 to the nucleus, suggesting that ubiquitination does not sequester mutant p53 in the cytoplasm and is rather a consequence of cytoplasmic localization of mutant p53. Interaction of mutant p53 and p63/p73 occurs mostly in the nucleus, therefore the nuclear fraction of mutant p53 might be contributing to the gain-of-function effect. The function of mutant p53 in the cytoplasm is unknown. Although the cytoplasmic function of wild type p53 has been suggested to promote apoptosis, mutant p53 does not induce cell death. One speculation can be that keeping mutant p53 in the cytoplasm is the means to inactivate it, which could reflect the existence of the cellular mechanism to degrade misfolded p53. Inactivation of CHIP or Parc at later stages of tumourigenesis can lead to relocation of mutant p53 to the nucleus

where it can exert gain-of-function effect. This may indicate that the nuclear localization of mutant p53 would be favoured by tumour. Therefore, it would be interesting to check localization of mutant p53 in normal cells and in tumour cells in p53 knock-in mice.

The data presented here suggest that mutant p53 can target p73 for degradation by Mdm2, which can contribute to inactivation of p73 function. By inhibiting ubiquitination of mutant p53, p73 can play a role in regulation of stability of mutant p53 and contribute to its accumulation in tumours.

The data of this study suggest that the regulation of stability of mutant p53 differs from that of wild type p53. Identifying the E3 responsible for the hyper-ubiquitination of mutant p53 and investigation into the mechanism of Mdm2-mediated delivery to proteasomes may have important implication in understanding the mechanisms that specifically down-regulate mutant, but not wild type p53 in tumours. Further studies of the interaction of mutant p53 with its family members and its functional consequences can also provide the way of inactivation of the mutant p53 oncogenic function. This could potentially have therapeutic advantages, since mutant p53s are highly expressed in cancer cells and show a clear ability to promote various aspects of tumourigenesis, including metastatic spread (484, 485).

Whereas the strategy to develop cancer treatments for tumors with wild type p53 would be to stabilize p53, in case of tumors bearing mutant p53 the accumulation of mutant p53 will have adverse effects due to the gain-of-function activites of mutant p53. The differences in the mechanisms of degradation of wild type and mutant p53 suggest the possibility to develop cancer treatments that specifically stabilize wild type p53 but not mutant p53.

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