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Regulation of p53 by HRad23

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Abstract

Nucleotide excision repair (NER) is an important mechanism for removing a wide spectrum of different DNA lesions, particularly the ultraviolet (UV) induced lesions as cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts. Therefore, NER constitutes a crucial defense mechanism against DNA damage-induced cytotoxicity, mutagenesis and carcinogenesis. The proteins HRad23A and HRad23B seem to be important for nucleotide excision repair: they interact with the XPC-protein and allow DNA damage recognition. Interestingly mice lacking HRad23B show strong developmental defects and protein stabilizing effects that are not related to the nucleotide excision repair pathway. Thus, the nucleotide excision repair proteins HRad23 might have additional functions.

In this PhD thesis, other putative functions of HRad23 were investigated especially its role in the regulation of the p53 level after DNA damage induced by UVC irradiation.

The first major result concerns the regulation of HRad23 proteins itself: They are induced by UVC independently of transcription. Regarding the regulation of p53, it was found that overexpression of HRad23 leads to accumulation of p53 and as well, the reduction of HRad23 protein levels reduces the level of p53. This effect is specific for HRad23A and HRad23B, as other members of the UBL family have no effect on p53 stabilisation.

A last finding was that HRad23 inhibits the transcriptional machinery through a general mechanism, as p53-dependent and p53-independent promoters were inhibited. This, plus the fact that p53 is stabilized in presence of HRad23 and is transcriptionally inactive, might suggest that

upon UVC irradiation, HRad23 accumulates in the cell to allow DNA repair. Thus, p53 might be reactivated if the cells need to enter into apoptosis as a consequence of impossible repair.

In summary, this study shows that upon UVC irradiation, the HRad23 proteins are increased in their protein levels by non-transcriptional mechanism. This increase will lead to the accumulation of a transcriptionally inactive p53 that can be used to activate pro-apoptotic p53 target genes in case of too high damage.

Regulation von p53 durch Rad23

Zusammenfassung

Excisionsreparatur ist ein wichtiger Mechanismus zur Entfernung einer Vielzahl von DNA-Schäden, speziell von UV-induzierten Lesionen wie cyclobutan-Pyrimidin Dimeren und 6-4 Photoprodukten. Excisionsreparatur ist daher ein entscheidender Abwehrmechanismus gegen Cytotoxizität, Mutagenese und Carcinogenese, die durch DNA-Schäden ausgelöst werden. Die Proteine HRad23A und HRadB scheinen für die Excisionsreparatur wichtig zu sein: Sie interagieren mit dem XPC-Protein und ermöglichen die Erkennung des DNA-Schadens, der dann beseitigt werden kann. Interessanterweise zeigen aber HRad23-defiziente Mäuse Entwicklungsdefekte und proteinstabilisierende Effekte, die nichts mit Excisionsreparatur zu tun haben: HRad23 Proteine scheinen also noch andere Funktionen, zusätzlich zu ihrer Funktion in Excisionsreparatur, zu besitzen.

In dieser Doktorarbeit wurde speziell der Einfluss von HRad23 Proteinen auf die Regulation des p53 Proteins nach DNA-Schädigung durch UVC-Bestrahlung untersucht.

Ein erstes Resultat betrifft die Regulation der HRad23 Proteine selbst: Sie werden durch UVC unabhängig von Transkription induziert. Bezüglich der p53 Regulation zeigte sich, dass die Überexpression von HRad zur Akkummulation von p53 führt und umgekehrt verringerte Expression von HRad23 auch in einer Verringerung von p53-Protein resultiert. Dieser Effekt ist spezifisch für HRad23A und Hrad23B, weil andere Proteine der UBL-Familie den Effekt nicht zeigen.

p53 kann als Transkriptionsfaktor wirken. Diese Aktivität von p53 wird von HRad23-Proteinen inhibiert; generell scheinen HRad23-Proteine Transkription von vielen Promotoren zu unterbinden.

Zusammengenommen lassen sich meine Ergebnisse dahingehend interpretieren, dass die Akkumulation von HRad23A und HRad23B in Zellen nach UVC-Bestrahlung für DNA-Reparatur benötigt wird, und dass die Stabilisierung des p53 Proteins und der Block seiner Transkriptionsaktivität möglicherweise dazu führt, dass die Zellen in Apoptose gehen.

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Abbreviations

°C	degrees Celsius
Bax	BCL2 associated X protein
Bcl-2	B-cell lymphoma protein 2
CBP	CREB-binding protein
CDNA	complementary DNA
Cdks	cyclin-dependent kinases
cDNA	complementary DNA
CKI	Cdk-inhibitor protein
CPD	cyclobutane pyrimidine dimers
DBD	DNA-binding domain
DMEM	Dulgecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	protein-ubiquitin ligase
ECL	enhanced chemiluminescence
E. *coli*	*Escherichia coli*
EDTA	ethylenediamine-N,N-tetracetate
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
g	gram
GADD45	growth arrest and DNA damage inducible proteine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hour
HDAC	Histone Deacetylase classe 1

HPLIC	human Protein linking IAP with cytoskeleton
HRad23	human RADiation sensitive protein 23
HRP	horseradish peroxidase
HSB	high salt buffer
Ig	immunoglobulin
IP	immunoprecipitation
M	molar
mA	millamper
Mdm2	Murine double minute2
ml	milliliter
mRNA	messenger RNA
MW	molecular weight
NER	nuclear excision repair
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PBS	phosphate buffered saline
pRB	phosphorylated retinoblastoma
RNA	ribonucleic acid
RNAi	RNA-mediated interference
Rpm	revolution per minute
RT	room temperature
SDS	sodium-lauryl-sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
Tris	Tris-(hydroxymethyl)-aminomethane
μ	micro (10^6)
Ubc	ubiquitin conjugating enzyme

UVC	ultraviolet -C
V	volt
XPC	Xeroderma pigmentosum group C complementing protein

1 Introduction

One of the major causes of disease in modern society is cancer. Cancer develops in a multi-step process depending on genetic alterations that drive the progressive transformation of normal human cells into malignant derivatives (Renan 1993).Two classes of gene mutation have been identified in cancer development, including oncogene and tumor suppressor genes, like p53, with recessive loss of function. The activity of oncogenes and impaired function of tumour suppressor proteins leads to uncontrolled cell proliferation and escape from apoptotis signals. Such mutation can occur upon UV irradiation, which will introduce different types of damage into the DNA, notably cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs). If unrepaired, these photolesions can give rise to cell death, mutation induction, and onset of carcinogenic events, but the relative contribution of CPDs and 6-4PPs to these biological consequences of UV exposure is hardly known (Jans, Schul et al. 2005). Cells possess many properties to detect the damaged DNA and to try to repair it, or die. In order to survive with damages, they need to suppress apoptosis inducing signals (Sigal and Rotter 2000) and transform into cancer cell. One of the key players for keeping the genome integrity is the protein p53.

The protein p53 was first discovered in 1979, and has since been a subject of intense research. It has been demonstrated that mutation of p53 is associated with human cancers (Baker, Fearon et al. 1989; Nigro, Baker et al. 1989; Takahashi, Nau et al. 1989). It has become clear now that p53 is a major “guardian of the genome” (Lane 1992). The importance of p53 in safeguarding the genome has been demonstrated by genetic studies (Dumble, Donehower et al. 2003).

p53-deficient mice are indeed highly tumor susceptible, although it is not clear why these mice are mainly prone to lymphoma and skin cancer. Moreover, about 50% of human cancers carry mutated p53 (De Leo 2005), and human tumor cells retaining wild-type p53 often have defects in activating or responding to p53. It is likely that all cancer cells have a dysfunctional p53 system, which includes p53 and associated proteins as well as its upstream regulators and downstream effectors. In response to DNA damage and abnormal proliferation, p53 is accumulated and activated in cells, leading to cell cycle arrest. By stopping the DNA replication, the repair of damaged DNA is facilitated. If this repair is not possible, p53 will lead to the elimination of irreversible damaged DNA containing cells by apoptosis, and also of abnormally growing cells to prevent potential transformation. These activities are mediated through a variety of biochemical functions such as transcriptional activation, transrepression, DNA annealing which involves a large set of target genes and interacting proteins (Levine 1997; Amundson, Myers et al. 1998; Bates, Phillips et al. 1998; Bates, Ryan et al. 1998; Giaccia and Kastan 1998; Janus, Albrechtsen et al. 1999; Janus, Albrechtsen et al. 1999).

1.1 p53: a transcriptional activator

The level and activity of p53 is under normal conditions tightly regulated by mechanisms that will be described in another chapter. But under certain circumstances p53 gets activated, and this involves two different types of event. After different type of stresses that lead to DNA damage, increased level of p53 is a necessary step in order to allow cell cycle arrest, DNA repair or apoptosis. For example, after UV irradiation, p53 accumulates in the cells within 3 hours. This accumulated p53 will then

get activated and promote a transcriptional block allowing DNA repair (van Gijssel, Mullenders et al. 2003), or apoptosis. p53 is a transcriptional regulator. The main mechanism by which p53 exerts its function is by increasing or decreasing the transcription of several target genes, which will in turn be responsible for the effects of p53: cell cycle arrest (Kastan, Onyekwere et al. 1991; Di Leonardo, Linke et al. 1994), DNA repair (Fitch, Cross et al. 2003), or apoptosis (Lowe, Ruley et al. 1993). The main target genes of p53 and their action are described in table1. At first, p53's steady state level in cells increases due to enhanced translation event (Fu and Benchimol 1997) and enhanced stability of the protein (Maltzman and Czyzyk 1984). Second, p53 turns into an active transcription factor after post-translational modifications such as phosphorylation and acetylation (Appella and Anderson 2001). Coupled with the stabilisation of p53, phosphorylation events (Ser15) stimulate recruitment of transcription factor, such as p300, which possess co-activator function for p53 and histone acetyl-transferase function. This factor enhances transcription from p53-responsive promoters and also promotes acetylation of a cluster of C-terminal lysine residues in p53 that are normally targets for ubiquitylation (Gu and Roeder 1997).

Target gene	Regulation by p53	Effect	Reference
p21	+	Cell cycle arrest in G1 phase	Kim, 1997
Bax	+	Induction of apoptosis	Miyashita et al., 1995
GADD45	+	Cell cycle arrest in G2 phase	Chin et al., 1997
14-3-3sigma	+	Cell cycle arrest in G2 phase	Hermeking et al., 1997
Apaf1	+	Apoptosis after neuronal injury	Fortin et al., 2001
p53AIP1	+	Apoptosis	Oda et al., 2000
Bid	+	Apoptosis	Fischer et al, 2003
NOXA	+	Cytochrome c release	Seo et al., 2003
PUMA	+	Apoptosis	Nakano et al., 2001
Fas	+	Sensitize cell to pro-apoptotic signal	Munsch et al., 2000
DR5	+	Signal apoptotic death	Wu et al., 2000
PIDD	+	activation of caspase 2	Tinel et al., 2004
TRAF4	+	Apoptosis	Sax et al., 2003
Cyclin B1	-	Cell cycle arrest in G2	Innocente et al., 1999
Survivin	-	apoptosis	Hoffman et al., 2002

Table 1 Known p53 target genes and their physiological relevance.

1.2 p53-mediated apoptosis

The apoptotic mechanisms of p53 have been intensively dissected and multiple pathways have been identified. p53-promoted apoptosis is mediated by both sequence-specific transactivation dependent and independent pathways which cooperate to full apoptotic response (Amundson, Myers et al. 1998; Bates, Ryan et al. 1998; Sionov and Haupt 1999).

1.2.1 Sequence-specific transactivation independent apoptosis

The first indication for transactivation independent apoptosis was based on the observation that p53 can induce apoptosis in presence of RNA and

protein synthesis inhibitors (Caelles, Helmberg et al. 1994). Nevertheless the mechanisms underlying this apoptotic activity of p53 are poorly understood, although recent research may provide some clues. One of the possible mechanisms could be through direct interaction with apoptosis inducing proteins. The helicases XPB and XPD are required for p53-mediated apoptosis without impairing growth arrest by p53 (Wang, Vermeulen et al. 1996). It could be also proteins that interact with anti-apoptotic proteins, such as p53BP2 (Naumovski and Cleary 1996).

1.2.2 Sequence specific transactivation dependent apoptosis

Several p53-induced target genes can promote apoptosis, although the expression of each alone is insufficient to cause significant cell death (Gottlieb and Oren 1998). The apoptotic target genes may need to act in concert in order to mount a full apoptotic response. The apoptotic target genes can be divided into two major families. The first encodes proteins acting at the level of receptor signalling for apoptosis, while the second family includes proteins acting downstream by activating apoptotic effectors proteins.

1.3 Bax and apoptosis

Bax belongs to the Bcl-2 family of proteins, which consists of both anti- and pro-apoptotic molecules, and controls the mitochondrial function in apoptosis. Bax acting as a pro-apoptotic agent, leads to the release of apoptogenic molecules from mitochondria inducing mitochondrial outer membrane permeabilization. It is believed that Bax is a death effector and is absolutely required for mitochondrial apoptosis pathway (Wei, Zong et al. 2001; Zong, Lindsten et al. 2001). Under normal conditions, Bax exists as an inactive form mainly located in the cytosol, and can be

induced to undergo a conformational change and migrate to the mitochondria by apoptotic signals (Wolter, Hsu et al. 1997; Yamaguchi and Wang 2001). Upon activation, Bax will form an oligomere that will bind on the mitochondrial outer membrane and induces cytochrome c release (Waterhouse, Goldstein et al. 2001). This occurs after stabilization and activation of p53 upon stresses, which will in turn, if an apoptotic answer to the damage is needed, transactivate the Bax gene by a direct binding on an upstream regulatory region of the gene. The raised level of Bax will lead the cells to enter into apoptosis.

1.4 Cell cycle arrest

A cell reproduces by performing an orderly sequence of events in which it duplicates its content and then divides in two. This cycle of duplication and division is known as the cell cycle, and is the essential mechanism by which all living forms reproduce. The cell cycle is divided into four major events, a G1 phase, a S phase, a G2 phase, and a M phase. G1 and G2 are gaps in between the S and M phases. The S phase serves for DNA replication, and the M phase is for the nuclear division, followed by the cytoplasmic division. Eucaryotic cells have evolved a complex network of regulatory proteins, known as the cell-cycle control system. This cell cycle control system is a family of protein kinases known as cyclin-dependent kinases (Cdks). Activity of those kinases rises and falls during the cell cycle events. The Cdks activity is mainly controlled by other proteins known as cyclins, in association of the respective cyclin-Cdks will form the cyclin-Cdks complex, which will trigger the cell cycle events.

There are four cyclins:

G1/S cyclins bind Cdks at the end of G1and permit the DNA replication

S-cyclins bind Cdks during S phase and are required for DNA replication

M-cyclins promote the events of mitosis

G1-cyclins

The G1-cyclins, in most of the cells help to promote the passage through start or the restriction point in late G1. The different cyclin-Cdk complexes formed are G1-Cdk, G1/S-Cdk, S-Cdk, and M-Cdk.

The control of those checkpoints is made by Cdk-inhibitor proteins, (CKIs). The role of the CKIs is to inhibit the passage through the different checkpoints. Such CKIs are called p21, GADD45, or 14-3-3σ for example. The G1 checkpoint blocks progression from G1 to S phase by inhibiting the activation of G1/S-Cdks and S-Cdks complexes. The G2 checkpoint, that promotes the entry into the M phase by controlling the M-Cdks, and the M checkpoint that control correct cytokinesis.

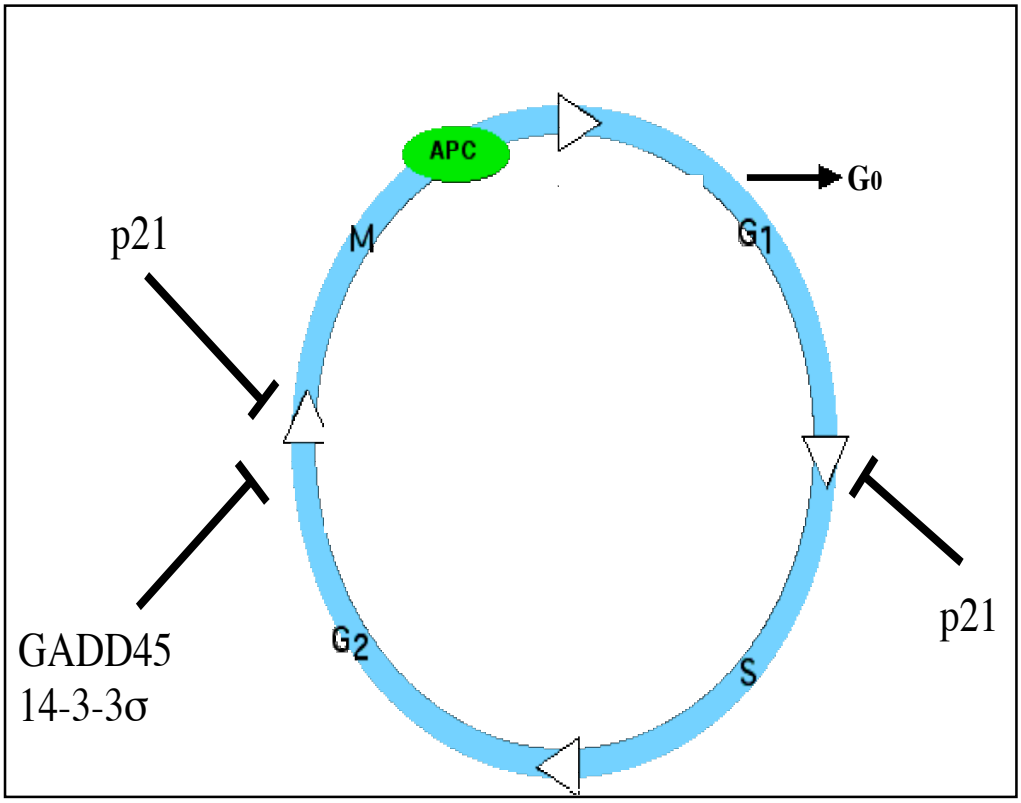


Figure 1.1 The different phases of the cell cycle. This picture represents the different phases of the cell cycle and their check points control. p21 is able to stop the cell cycle at two different step: at the G1 to s phase, and at the G2 to M phase.

In mammalian cells, DNA damage leads to the activation of the gene regulatory protein p53, which stimulates the transcription of several target genes. One of those genes encodes for a Cyclin-kinase inhibitor called p21, which binds to G1/S-Cdks and S-Cdks and inhibits their activities, blocking the entry into S phase.

P53 plays a role in regulating vital checkpoints during the G1 and G2 phases of G1 arrest (el-Deiry 1998), while the induction of GADD45 gene mediate the G2 arrest (Hermeking, Lengauer et al. 1997). These checkpoints prevent cells with damaged genomes from undergoing DNA replication or mitosis. P21 mediates p53-dependent G1 arrest by

inhibiting the activity of cyclin dependent kinases (CDKs), which phosphorylate the retinoblastoma (pRB) gene product. PRB sequesters, in its hypophosphorylated form, the E2F1 transcription factor, preventing transition from G1 to S phase (Hengstschlager, Braun et al. 1999). In addition, pRB recruits histone deacetylase (HDAC1), which blocks transcription by promoting nucleosome compaction (Brehm, Miska et al. 1998). P53 can also trigger growth arrest in a p21-independent manner. This occurs through the binding to Cyclin H, leading to the inhibition of the protein kinase complex CDK7/CyclinH1/Mat1 by p53, which activates the CDK2/CyclinA kinase required for the G1/S transition (Schneider, Montenarh et al. 1998).

In addition to G1 arrest, p53 has been shown to induce an efficient G2 arrest. P53 is able to inhibit the cyclinB/cdc2 complex through the induction of GADD45, which disrupts this complex, probably via a direct interaction with cdc2 (Wang, Zhan et al. 1999).

1.4.1 Involvement of p21 in cell cycle arrest

p21 was identified as a mediator of p53-induced growth arrest (el-Deiry, Tokino et al. 1993), a direct regulator of CDK activity (Harper, Adami et al. 1993), and a gene whose expression is induced in concomitance with cellular senescence (Noda, Ning et al. 1994).

p21 belongs to the family of Cip/Kip CkIs, which share significant sequence homology in their amino terminal region, and recognize a broad, but not identical range of cyclin/CDK targets (Sherr and Roberts 1999). The amino terminal domain of p21 is both necessary and sufficient to inhibit cyclin/CDK activity *in vitro* and *in vivo*. The carboxy-terminal

region of p21 associates with the proliferating nuclear antigen, subunit of DNA polymerase δ.

p21 can modulate different biological functions, and its role in acting as a negative regulator of cell growth is well established. It is shown to play a crucial role in mediating cell cycle arrest in the G1 phase in response to DNA damage (Macleod, Sherry et al. 1995). p21 is also responsible for blocking the re-entry of G2 cells in the S phase (Waldman, Lengauer et al. 1996), as well as for the G2 block induced by DNA damage prior to cytokinesis (Bunz, Dutriaux et al. 1998).

Besides the functions of p21 in cell cycle arrest and apoptosis, it plays an unexpected role in differentiation (Di Cunto, Topley et al. 1998), which may involve a number of mechanisms that depend on specific interaction of p21 with well known signalling proteins such as caspase3, calmodulin, and CDKs.

1.5 Regulation of p53

p53 has numerous target genes and numerous effects. Under normal condition, p53 remains at low level in the cells, and its activity when triggered by DNA damage, for example, needs only a subset of those genes. Therefore, p53 activity should be tightly.

Under normal conditions, p53 is not needed and is present at low level in the cell. In response to stresses, as DNA damage, p53 level and/or activity should increase rapidly, by blocking the degradation, as transcription/translation events need time. This occurs by submitting p53 to extensive post-translational modifications, including phosphorylation and acetylation, which modulate its stability and activities, whereby p53

is converted from a latent into an active protein (Meek 1998; Meek 1998; Sakaguchi, Herrera et al. 1998; Jayaraman and Prives 1999).

1.5.1 Regulation of p53 activity

The p53 protein can be converted from a latent form to an active form by a variety of mechanisms including interaction with damaged DNA, post-translational modifications, and protein-protein interaction.
DNA damage, such as double strand DNA break, mistmaches, and single-stranded DNA, can be recognized by the C-terminus domain of p53 (Janus, Albrechtsen et al. 1999; Janus, Albrechtsen et al. 1999). A single double strand break in DNA is sufficient to activate p53 (Huang, Clarkin et al. 1996). Further, the occupation of the C-terminus of p53 is believed to relieve it from negative regulation and thereby expose the DNA binding core of p53 (Hupp and Lane 1995; Jayaraman and Prives 1999). The different post-translational modifications, which regulate p53 activity are phophorylation, acetylation, and ubiquitylation events.

1.5.2 Phosphorylation

Phosphorylation of serines at the N-terminus of p53 increases its sequence-specific DNA binding, and consequently enhances the sequence-specific transaction of its target genes (Hu, Qiu et al. 1997; Shieh, Ikeda et al. 1997; Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Khanna, Keating et al. 1998; Sakaguchi, Herrera et al. 1998; Shieh, Taya et al. 1999; Tibbetts, Brumbaugh et al. 1999).

p53 activity is also modulated by phosphorylation at the C-terminus (Meek 1998; Meek 1998; Jayaraman and Prives 1999). The phosphorylation of each one of these serines sites is enough to increase the sequence-specific DNA-binding of p53 (Meek 1998; Meek 1998; Jayaraman and Prives 1999). Possibly these phosphorylations relieve p53 sequence-specific transactivation from negative regulation. Among the C-terminal serine residues, only one Serine 392 is phosphorylated following UV irradiation, but not infrared radiation (Kapoor and Lozano 1998; Lu, Taya et al. 1998), and this stabilizes the tetramer formation of p53 (Sakaguchi, Sakamoto et al. 1997).

1.5.3 Acetylation

Phosphorylation at the N-terminus enhances its binding to the transcriptional co-activator p300 (Lambert, Kashanchi et al. 1998) which acetylates p53 on Lysine 382(Sakaguchi, Herrera et al. 1998).
p53 can also be acetylated by another acectyl transferase within its nuclear localization sequence (Sakaguchi, Herrera et al. 1998). These acetylations increase the sequence-specific DNA-binding of p53 (Lambert, Kashanchi et al. 1998; Sakaguchi, Herrera et al. 1998).

1.5.4 Regulation of p53's level

The p53 protein is constitutionally expressed in the cell, but has a very short half-life (Blagosklonny 1997). Exposure of cells to DNA damage, such as ionising radiation, ultraviolet light or anti-cancer drugs, leads to a rapid accumulation of the p53 protein (Giaccia and Kastan 1998). But

under normal conditions, p53 remains at low level in the cell via degradation by the proteasome pathway.

1.5.5 p53: facing the ubiquitin/proteasome system

The ubiquitin/proteasome pathway was discovered some 20 years ago and was originally thought to eliminate abnormal and damaged proteins (Hershko, Ciechanover et al. 2000). Now we know that this pathway is responsible for the highly selective proteolysis of intracellular proteins in general cellular environments. The ubiquitin/proteasome pathway was also found to play proteolysis-independent roles, e.g. protein kinase activation and subnuclear trafficking (Pickart et al., 2001).
Degradation of a protein via the ubiquitin/proteasome pathway involves two successive steps:

1. Conjugation of multiple ubiquitin moieties to the protein substrate.
2. Targeted degradation of the ubiquitinated protein by the 26S proteasome complex.

Conjugation of ubiquitin to the substrate proceeds through three sequential steps using three groups of enzymes. Firstly, ubiquitin, a 76 aa, highly conserved and ubiquitously expressed protein, is activated to a thioester by ubiquitin-activating enzyme (E1), then transferred to one of several ubiquitin-conjugating enzyme (E2). Finally E2 collaborate with mainly protein-ubiquitin ligases (E3), which specifically bind to protein substrates, transferring ubiquitin to the α-amino group of a lysine residue in the substrate to generate an isopeptide bound. After creating the first

ubiquitin-conjugate, the polyubiquitinated protein is produced by progressive transfer of additional ubiquitins to lysine-48 of the previously conjugated ubiquitin by the same cascade. Most organisms have one E1, a significant but limited number of E2, and many E3 (Ciechanover, Orian et al. 2000).

The polyubiquitin chain serves as a recognition marker for the 26S proteasome. The proteasome is composed of two ATP containing 19S subunits for specifically recognizing polyubiquitin chains and one 20S core for degrading protein substrates (see figure 1.2).

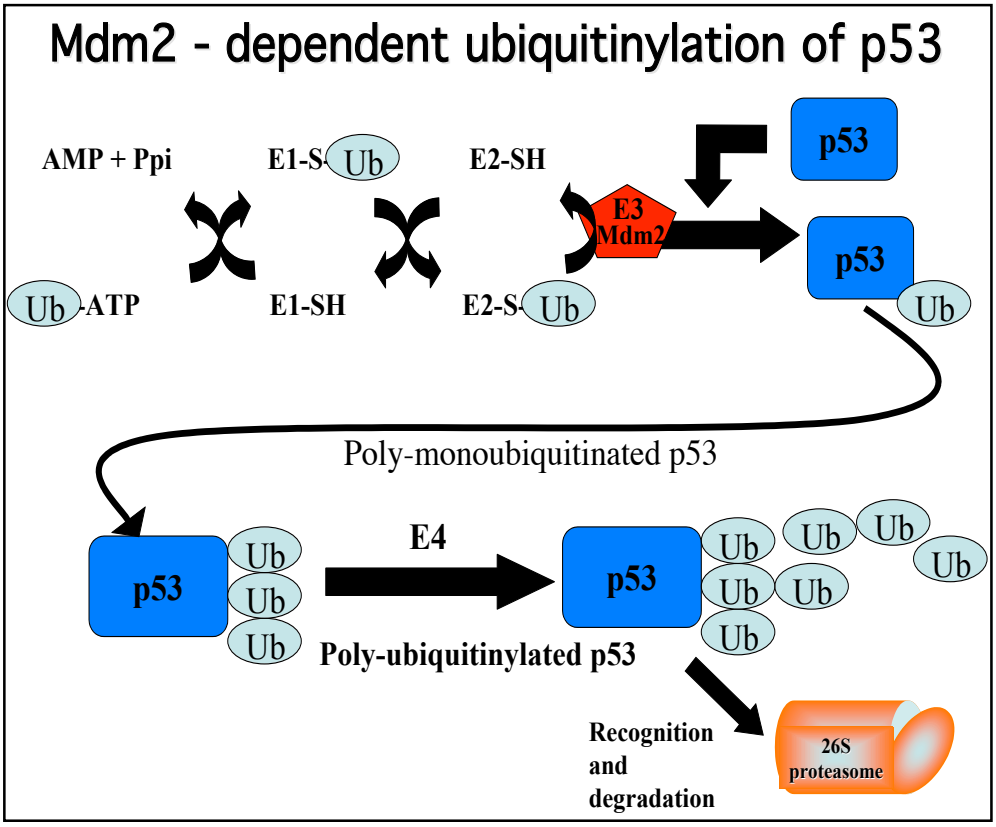


Figure 1.2 mdm2-dependent degradation of p53 by the 26S proteasome pathway.

1.5.6 E6-dependent degradation of p53

One of the players of p53 regulation is the Human Papilloma Virus E6 protein that can promote p53 degradation via the ubiquitin-proteasome system (Scheffner 1998).
E6 acts as an ubiquitin ligase for p53 (Scheffner 1998). However, since E6 is exprimed in *Human papilloma virus* infected cells, this mechanism of regulation of p53 levels is only limited.

1.5.7 Mdm2: main regulator of p53 degradation

The Murine double minute2 (Mdm2) gene was originally identified as one of three genes (mdm1, mdm2, and mdm3), which were overexpressed greater than 50 fold by amplification in a spontaneously transformed mouse Balb/c cell line (3T3-DM). The mdm2 genes are located on small, acentromeric extrachromosomal nuclear bodies, called double minute, which were retained in cells only when they provided a growth advantage. The product of the gene mdm2 was later shown to be the one responsible for transformation of cells when overexpressed (Cahilly-Snyder, Yang-Feng et al. 1987).
Soon after the identification of the mdm2 gene, the reason for its transformation potential was discovered. Mdm2 was shown to bind the tumor suppressor p53 and inhibit p53-mediated transactivation (Momand, Zambetti et al. 1992). These studies led to the hypothesis that overexpression of mdm2 was another mechanism by which the cell could inactivate p53 in the process of transformation.
The mdm2 gene consists of 12 exons with 2 different promoters that can generate 2 different proteins: the full length called p90 and a shorter form

called p76. The p76 is also initiated at the first internal ATG (19-21). From the protein sequence, the p53 interaction domain is encoded by the amino terminal 100 amino acids of Mdm2. This domain binds the amino terminal transactivation domain of p53. Thus, even if Mdm2 cannot degrade p53, it interferes with the ability of p53 to interact with the transcription machinery. Other motifs include a nuclear localization signal and a nuclear export signal. The signals shuttle Mdm2 back and forth between the cytoplasm and the nucleus and provide another way to control p53 (Freedman and Levine 1998; Roth, Dobbelstein et al. 1998).

Amino acids 464-471 can function as a nuclear localization signal (Lohrum, Ashcroft et al. 2000), although the biological significance of this regulation is unclear. The central acidic domain of Mdm2 is necessary for the interaction with the ribosomal protein L5 and with p300/CBP (CREB-Binding Protein). Recently, this domain was found to contribute to p53 degradation because Mdm2 mutant lacking part of this domain was unable to promote p53 degradation, but still could ubiquitylate it (Argentini, Barboule et al. 2001; Zhu, Yao et al. 2001) Downstream of the acidic domain is a Zinc finger domain of unknown function followed by the ring finger domain, responsible for its ubiquitin ligase activity.

Mdm2 is an ubiquitine ligase for p53 and its main function is the regulation of p53 done by its ubiquitylation and degradation. Thus, Mdm2 is the main protein that keeps p53 at low level in the cell. This system is constitutively active. Thus, when necessary, for example after DNA damage, p53 should be prevented from degradation by modifications or by interacting with other proteins that lead to its accumulation. A family of protein exist called the Ubiquitin-Like (UBL) proteins that are known to interact with ubiquitin as well with the proteasome, and may act as a linker in between the two events. Therefore,

these interacting partners might also sequester p53 from the proteasome after stresses that need a p53 response.

1.6 The UBL family of protein

The family of ubiquitin-like (UBL) domain proteins (UDPs) comprises a conserved group of proteins involved in a multitude of different cellular activities (Hartmann-Petersen and Gordon 2004). Some of them are involved in the nuclear excision repair pathway.

1.6.1 HPLIC

The human homolog of the yeast Dsk2 protein called hPLIC 1& 2 are ubiquitin-domain proteins, which are characterized by having a noncleavable ubiqutin-like domain as part of their structure (Jentsch and Pyrowolakis 2000; Li and Hochstrasser 2000) at the N-terminus. In addition, the PLIC proteins have a single ubiquitin associated domain (UBA) located at the extreme C-terminus.

1.6.2 HRad23

Another member of this UBL family, called human HRad23A & B is described as having a role in the nuclear excision repair pathway. The S. cerevisiae protein Rad23- for RADiation sensitive 23, is a multi-domain protein composed of 398 Amino acids (Watkins, Sung et al. 1993). It is highly conserved among eukaryotes and has two homologues

in human called HRad23A and HRad23B (Masutani, Sugasawa et al. 1994). Rad23 contains several domains called from N-terminal to C-terminal: a ubiquitin-like domain and two ubiquitin associated domain encompassing an XPC-binding domain (Rad4-binding domain in yeast). The ubiquitin-like domain shares 22% identical and 43% similar (identical plus conserved changes) residues with yeast ubiquitin (Watkins, Sung et al. 1993).

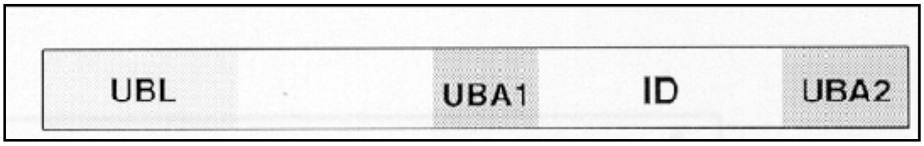


Figure 1.3. Domain organization of the HRad23 protein. The HRad23 protein is composed of four domains, a UBL (ubiquitin like) domain in its N-terminal part, two UBA (ubiquitin associated domains) encompassing a XPC binding domain, located at its C-terminal part.

Rad23 is a stable protein with a half-life of six hours in yeast (Watkins, Sung et al. 1993). The UBL domain was shown to interact with subunits of the proteasome (Schauber, Chen et al. 1998) and it seems to locate to the Rpn1 and Rpn2 subunits of the 19S base complex in yeast (Elsasser, Gali et al. 2002; Saeki, Sone et al. 2002) and with the subunit S5a (Hiyama, Yokoi et al. 1999) of the 19S lid complex in Human. This homology might be responsible for proteasome recognition and be involved in the Rad23 function as replacement of this UBL domain by ubiquitin functionally rescues Rad23-mediated DNA repair in a Rad23 deletion mutant (Watkins, Sung et al. 1993; Lambertson, Chen et al. 2003).

A possible role for Rad23 came from the observation that in yeast, deletion of Rad23 gene results in UVC sensitivity and inefficient DNA damage repair (Watkins, Sung et al. 1993). The UVC irradition was used,

as it creates damage that will be specifically repaired by the nuclear excision repair pathway, and Rad23 is one of those proteins involved in NER.

The same group also observed that a Rad23 lacking its UBL domain could not suppress these phenotypes, suggesting an important role for this domain in NER. It should be noted that Rad23 lacking both UBA domain supports DNA repair (Bertolaet, Clarke et al. 2001), meaning that the necessary domain for this function is the UBL domain.

The function of the UBA domain is related to recognition of the specific ubiquitinylated protein, and is not involved in DNA damage repair. It was shown that binding of Rad4 to the C-terminal part of Rad23 stimulates the assembly of the multi-subunit DNA repair complex on UV-damaged DNA (Guzder, Sung et al. 1998; Jansen, Verhage et al. 1998) which occurs, in Human, upon interaction of HRad23B and the XPC (xeroderma pigmentosum group C) protein (Sugasawa, Masutani et al. 1996; Sugasawa, Ng et al. 1997). Two groups reported that Rad4 and XPC are unstable proteins that are degraded by the proteasome in the absence of Rad23, in yeast, and as well in Human in the absence of Hrad23, resulting in DNA damage repair deficient phenotype (Lommel, Ortolan et al. 2002; Ng, Vermeulen et al. 2003).

In addition of its involvement in the nuclear excision repair pathway, a role for Hrad23 in the ubiquitin-proteasome system has been suggested. Keeping in mind that this role is so far under debate, Rad23 has been suggested to promote degradation. By simultaneous binding to the ubiquitinated substrate and the proteasome, Rad23 could act as a putative shuttle factor (Madura et al., 2002), a role already proposed for Dsk2 (Funakoshi, Sasaki et al. 2002) a homologue of Rad23 in yeast.

Rad23 can inhibit the disassembly of the ubiquitin chains, and therefore favour the degradation.

Second, Rad23 has also been shown to interfere with the elongation of the ubiquitin chains *in vitro,* and overexpression of Rad23 stabilizes certain proteolytic substrates in ubiquitin-conjugated forms *in vivo,* such as p53, suggesting a role in inhibition of degradation (Ortolan, Tongaonkar et al. 2000; Chen, Shinde et al. 2001).

The involvement of human and mouse Rad23 homologues in NER has been studied, but number of observations suggest other roles for these proteins. For example, mice lacking the HRad23 isoforme B, present strong developmental defect and surviving animal show impaired growth and male sterility (Ng, Vrieling et al. 2002).

Taking with the observation that the embryonic fibroblast from these mice are not UV sensitive, it was suggested that HRad23B has a separate role in the development, that can not be functionally compensated by the HRad23A isoforme, despite it might have a compensatory role in DNA damage repair.

1.7 Aim of my PhD project

The aim of my PhD project was to study putative other functions of HRad23 proteins.

I was more specifically interested in investigating the potential role of HRad23 in the regulation of p53 levels and function after DNA damage induced by UVC irradiation.

2. Material and methods

2.1 Materials

2.1.1 Chemicals

All general chemicals used, unless otherwise stated, supplied by Carl Roth GMBH & Co (Karlsruhe Germany), Merck (Darmstadt, Germany), Sigma Chemie Gmbh (Deisenhofen, Germany) and were of the highest purity grade. Aqueous solutions were prepared with water filtered with the Milli-Q plus water system (Millipore, Molsheim, Germany). Radioactivity was purchased from Amersham Pharmaciabiotech (Bukinghamshire, UK).

2.1.2 Primers

The primers were synthesized by MWG BiotechGmbH (Münche, Germany).

HRad23A FW
5´-gccggatccATGGCCGTCACCATCACGCTCAAA-3´
HRad23A RV
5´-GGGCGGCCGCTCATATGTCGTGGTCTTTGTAGGTCCTCGTCATCAAAGTTCTGACTCA-3´
HRad23B FW
5´-GCCGGATCCATGGAGGTCACCCTGAAGACCCTC-3´
HRad23B RV
5´-GGGCGGCCGCTCATATGTCGTGGTCTTTGTAGTCATCTTCATCAAAGTTCTGCTGTA-3´

2.1.3 Plasmids

Name	Insert/usage	Source
pcDNA3	empty vector	Dr.Blattner,Karlsruhe
pCMV-GFP	GFP cDNA	Dr. Göttlicher, Karlsruhe
pHis6-Ubi	His6-tagged Human Ubiquitin (8x)	Dr. Göttlicher, Karlsruhe
pcDNA3-p53	p53 cDNA	Dr. Blattner, Karlsruhe
pcDNA3-Mdm2	Mdm2 cDNA	Dr. Blattner, Karlsruhe
pcDNA3-hRAD23A	Human RAD23A	Dr. Blattner, Karlsruhe
pcDNA3-hRAD23B	Human RAD23B	Dr. Blattner, Karlsruhe,
pBAB18	Puromycin selection	Dr. Morrisson, Karlsruhe

2.1.4 DNA probes for Northern Blot analysis

All inserts were prepared by restriction digest from original plasmids and purified by EasyPure purification Kit (biozyme, Oldendorf, Gemany)

2.1.5 RNA oligonucleotides (small interfering RNA)

All RNA oligonucleotide duplexes were synthetised by Dharmacon Research Inc. (Lafayette, USA) and were >97% pure with no contaminating single-strand RNA. The specificity was proven by BLAST-search (NCBI program), and the sequence published in (Glockzin, 2004).

HRAd23A siRNA	5´-AAGAGCCCAUCAGAGGAAUCC-3´
HRad23B siRNA	5´-AAAGUCAGGCUGUGGUUGACC-3´
Control siRNA	(control duplex IX) was from Dharmacon.

2.1.6 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Invitrogen GmbH (Karlsruhe, Germany), Promega (Mannheim, Germany) or New England BioLabs (Beverly, USA), except notification.

2.1.7 Antibodies

Primary antibodies

The names and sources of primary antibodies used in this study are listed.

Name	**Source**
Actin, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-1616
Erk-1, rabbit polyclonal	Santa Cruz (Santa Cruz, USA), sc-93
c-Fos, rabbit polyclonal	UPSTATE (Biomol, Germany)
GR, rabbit polyclonal	Santa Cruz (Santa Cruz, USA), sc-1002
p53, DO-1, monoclonal	Dr. Blattner, karlsruhe
Mdm2, 4B2, monoclonal	Dr. Blattner, karlsruhe
PCNA, PC10, monoclonal	Dr. Blattner, karlsruhe

Secondary antibodies

Secondary antibodies used in this study are listed.

Name	Supplier
HRP conjugated rabbit anti-mouse IgG	DAKO (Glostrup, Denmark)
HRP conjugated goat anti-rabbit IgG	DAKO (Glostrup, Denmark)
HRP conjugated rabbit anti-goat IgG	DAKO (Glostrup, Denmark)

2.1.8 Cell lines

All media used for cell culture were purchased from Invitrogen (Karlsruhe, Germany). FCS was purchased from PAA laboratories GmbH (Linz, Austria).

The different cell line used in this study are listed below:

Name	Origin	Source	Medium
H1299	Lung carcinoma cell line	Dr.Blattner Karlsruhe, Germany	DMEM, 10% FCS
U2OS	osteosarcoma l cell line	Dr.Blattner Karlsruhe, Germany	DMEM, 10% FCS
NFB	normal fibroblast cell line	M.Litfin Karlsruhe, Germany	DMEM, 20% FCS

2.2 General methods

2.2.1 Restriction endonuclease digestion of DNA

Basically, one unit of restriction enzyme was used per μg of plasmid DNA. Reaction was carried out according to the manufacturer, and quality of the digest was controlled by gel electrophoresis.

2.2.2 Sub-cloning

The DNA insert of interest was released from the vector by proper digestion, purified by gel electrophoresis followed by the EasyPure kit, and ligated into the vector of interest having compatible sites.

2.2.3 DNA ligation

All DNA ligation were performed in 50μl as total volume and incubated one hour at 37 degree, using the ligase and buffer supplied by the manufacturer (Invitrogen, Karlsruhe, Germany).

2.2.4 Nucleic acid analysis by agarose gel electrophoresis

The required amount of agarose (SeaKem, Biozym Diagnostik, Hamln) was dissolved in electrophoresis buffer (TAE: 40mM TRIS, and 2mM EDTA,
ph 7.7). Ethidium bromide was added at a concentration of 0.3μg/ml.

The gel (when set) was run in electrophoresis buffer at 50 to 100V at room temperature. Samples were loaded onto the gel in loading buffer (10mM EDTA, 10% glycerol, 0.1% SDS, and 0.02% bromophenol blue). DNA was visualized by transillumination with 302nm radiation.

2.2.5 Isolation and purification of DNA from agarose gel

The DNA insert of interest was purified from the agarose gel using the EasyPure DNA purification kit (Biozymz, Oldendorf, Germany), following the instruction of the manufacturer.

2.2.6 Preparation of chemically competent E. coli

A colony of the type E.coli DH5α was incubated overnight at 37°C and with shaking 200 rpm in 5 ml of LB medium (10 g/L NaCl). The overnight culture was spread the next day in a 500 ml fresh LB and the bacteria were grown up to a density comprised in between 400 to 600nm at the OD_{600}.

2.2.7 Transformation of E. coli

Transformation of E. coli was used to propagate DNA plasmids or DNA ligation products. Basically, approximately one μg of plasmid or the total ligation mix were added to roughly 50 μl of chemically competent E. coli, after incubation of the two on ice for 20 min. after mixture and another

ice incubation for 30 min, the bacteria were heat-shocked for 45s at 42°C. Transformed bacteria were mixed with LB medium, (described above), in a proportion of 9 volume LB for 1 volume of bacteria, and incubated at 37°C for 30 min without shaking. Finally, the bacteria were platted on agar-LB plates containing the right antibiotics and allowed to grow 24 hours at 37°C.

2.2.8 Maxi-preparation of plasmid from bacteria

A 200 ml overnight culture of bacteria E. coli transformed with the plasmid of interest was used for the preparation of the plasmid and process with the Qiagen Plasmid Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction.

2.2.9 Mini-preparation of plasmid from bacteria

A 1,5 ml culture of bacteria transformed with the appropriate plasmid was pelleted in eppendorf for few seconds at 14000 rpm.

The obtained pelleted was resuspended in 150 µl of buffer P1 (10mM EDTA, 50mM Tris-HCL pH 8, and 0,4 mg/ml Rnase A). After incubation at room temperature for 5 min, 300 µl of buffer P2 (200mM NaOH, and 1% SDS) were added to lyse the bacteria for 3 min at room temperature, after shaking the eppendorf. Then, 225 µl of buffer P3 (3M potassium acetate pH 5,5) were added and the samples after being gently mixed were incubated on ice for 15 min, and centrifuged for 10 min at 14000 rpm. After centrifugation, the DNA-containing supernatant was removed

and precipitated by the addition of 2,5 volumes of pure ethanol, and centrifuged at 14000 rpm for 20 min. The DNA-pellet obtained was air-dried and resuspended in 50 µl of TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8).

2.2.10 Isolation of poly(A)+ RNA from mammalian cells

15 cm dish (Greinier, Frickenhausen, Germany) containing the cells at 80-90% confluency were washed twice in PBS, and lysed in 10 ml STE buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, and 0.8 % SDS). The lysate was homogenized with an ultraturax after the addition of proteinase K at a final concentration of 0.3 mg/ml, and incubated at 37°C for 45 min. After the incubation, the final concentration of NaCl was adjusted to 0.5M by adding 1 ml of 5M NaCl, and 1 ml (about 1 mg) of oligo-dT cellulose (type VII, Pharmacia, Uppsala, Sweden) swollen in HSB (10 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, and 0.1% SDS). The resulting mix was incubated overnight at room temperature to allow the binding of the poly(A)+ RNA to the cellulose. The oligo-dT cellulose was washed three times in 8 ml HSB buffer, and the poly(A)+ RNA were eluted with three times 1 ml of sterile water and then precipitated with an equal volume of isopropanol and 1/10 of 3 M Natrium acetat pH 4.8 at –20°C for 2 days. Samples were collected by centrifugation at 10000 rpm for 1 hour at 4°C and were washed twice with freshly prepared ethanol 70%. The obtained pellet were air dry at room temperature and resuspended in sterile water and stored at –80°C.

2.2.11 Determination of nucleic acid concentration

The concentration of DNA was determined by spectrotroscopic measurement of their optical density (OD) at 260nm and 280nm. The OD of 1 is equivalent to a concentration of 50 μg/ml of double strand DNA or to
a concentration of 40 μg/ml of RNA. Pure DNA and RNA in aqueous solution should have a ratio an $OD_{260/280nm}$ ration of 1.6/18.

2.2.12 Polymerase chain reaction (PCR)

PCR reactions were performed in a total volume of 50 μl according to the manufacturer's data sheet. The reactions were carried out in a PCR thermocycler (Perkin Elmer, Norwalk, USA), using the following cycling parameters: 95°C, 5 min, 1 cycle; 95°C, 1 min, 55°C, 30 sec, 72°C, 1 to 2 min for a total of 30 to 50 cycles; 72°C, 10 min as post-elongation step; 4°C, up to the analysis of the PCR products by gel electrophoresis.

2.2.13 Reverse transcription polymerase chain reaction (RT-PCR)

First strand cDNA synthesis

The first strand of cDNA was made using the Superscript II/Rnase H Reverse transcriptase kit (Life Technologies) according to the supplier's manual.

2.2.14 Amplification of first strand cDNA by PCR

In order to amplify a specific cDNA fragment, the standard PCR reaction was employed using the first strand cDNA reaction and a specific set of amplification primers. The reaction was also performed in 50 µl volume with specific reagents.

2.2.15 TOPO Blunt cloning

The plasmid vector pCRII BLUNT TOPO is supplied with blunt ends for direct cloning with the PCR products. The reactions for blunt cloning were performed according to protocols provided in the TOPO cloning Kit (Invitrogen, Groningen, Netherlands).

2.2.16 Cell culture

Mammalian cells were maintained at 37°C in an incubator (Forma scientific, Labortech GmbH, Göttingen, Germany) with 5% CO_2 and 95% humidity. All cells were grown in petri dishes, plates or flaskes (Greinier, Frickenhausen, Germany) depending on the application. The cells were allow to grow until they reached a confluence of 80-90%, and were

subsequently split by trypsinisation and re-seeded at a lower density. Trypsin treatment of the cells was performed by removal of the culture medium from the cells, followed by one wash Ca^{2+}/Mg^{2+} free PBS (137mM NaCl, 2.7mM KCl, 6.5mM Na_2 HPO_4, 1.5mM KH_2 PO_4). After removal of PBS, 0.25% trypsin was applied to the cells and the cells incubated at room temperature until they became detached. Fresh medium was added to the cells and they were centrifuge for 2 min at 1200 rpm. The cell pellet was therefore resuspended in new fresh medium in order to remove the trypsin and the cells were re-platted at the desired density in new flask. To prepare the cells for storage, logarithmically growing cells in a large flask were trypsinised, harvested by addition of medium and centrifuged at 1200 rpm for 2 min. After removing the medium, the pellet was resuspended in 90% FCS and 10% DMSO (Fluka ChemieAG. Switzerland) and placed in five 1ml cryovials tubes. After incubation on ice for 30 min, the cells were transferred to –80°C for short storage, and after few days, transferred to the liquid nitrogen for longer storage. To repropagate the cells, one aliquot was thawed, and the cells were washed once with fresh medium in order to remove the DMSO, and after centrifugation at 1200 rpm for 2 min, they were seeded in a new flask in fresh medium.

2.2.17 Transient transfection of cells

Adherent growing cells were seeded on petri dishes the day before transfection, that they reach semi-confluence on the day of transfection. Up to 6 µg total per 35 mm dish were mixed in 180 µl H_2O with 20 µl of 2.5M $CaCl_2$. 200 µl of 2xHSB buffer pH 7.05 (280mM NaCl, 1.5mM Na_2HPO4, 50mM Hepes) were added to the mix drop by drop while the resulting mixture was bubbled at the same time with a pateur pipette. The calcium-DNA precipitate was added to the cell, having 4 ml of new

medium, drop by drop, and was equally distributed in medium by gently shaking the dish. The next day the cells were washed once with 15% Glycerol-PBS, then by PBS only, and finally the cells were put in the incubator for additional hours with new medium within the period of 24-48 hours post-transfection.

2.2.18 Transient and stable transfection of cells: Fugene6™

Fugene6 (Roche, Mannheim, Germany) was used for DNA transfection of the cells according to the manufacturer's protocol. In case of stable transfection, the appropriate antibiotic was added in the medium depending on the selective marker on the plasmid.

2.2.19 Lipofection: oligofectamine™

Oligofectamine (Invitrogen, Karlsruhe, Germany) was used for the delivery of siRNA following the instruction of the manufacturer. In brief, the cells were seeded the day before to obtain a confluency of approximately 30% on the day of transfection. siRNA and oligofectamine were diluted and mixed in Optimem I (Invitrogen, Karlsruhe, Germany), incubated at room temperature to permit the complex to form, and added to the cells covered with medium free of FCS and antibiotics. 4 hours later the cells were washed and new FCS medium was added to the cells. The silencing was then observed after an incubation period of 24 hours to 48 hours.

2.2.20 Preparation of whole cell extracts

Cells were washed twice with ice cold Ca^{2+}/Mg^{2+} free PBS and harvested by scraping with a rubber policeman. The cells were centrifuged for few seconds at full speed to collect them, and the resulting pellet was resuspended in NP40-1% buffer (150mM NaCl, 50mM Tris pH8, 5mM EDTA pH8, 1% NP40) completed with fresh added proteases inhibitor cocktail (Roche, Mannheim, Germany), 1mM PMSF (Phenylmethylsulphonyl fluoride), and 1mM DTT. The suspension was left to stay on ice for 20 min, and the extract was cleared by centrifugation at 13200 rpm for 15 min in the cold. Finally, the protein concentration was measured. In some cases, the cells were directly harvested in 2X SDS-sample buffer (120mM Tris-HCl pH 6.8, 4% SDS, 20% v/v glycerol, 0.01% bromophenol blue, and 100mM β-Mercaptoethanol).

2.2.21 Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976) using BSA (Bovine Serum Albumin) as standard.

2.2.22 Separation of proteins in SDS-polyacrylamide gels

In this method developed by Laemmli (1970) denaturation of proteins by SDS allows their separation in polyacrylamide gel by their molecular weight. Gels were casted in the Bio-Rad apparatus (Bio-Rad, Mini

Protean II). The resolving gel contained 10 to 12% acrylamide depending on the experiment, and the stacking gel 5% according to Sambrock (Sambrock et al. 1989). Samples were run first at 20 mA per gel in the stacking gel, and 120 V in the separating gel.

2.2.23 Radioactive labeling of DNA probes

Radioactive labelling of DNA probes was performed with a Prime-it Kit (Stratagene, La Jolla, USA) and purified with Nick columns (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturers instructions.

2.2.24 Northern blot analysis

The method is based on the protocol of Lehrach et al.(1977). Poly(A)+RNA were separated in a 1% formaldehyde MOPS gel and then the gel was soaked in sterile H_2O for 5 min to remove the formaldehyde. After rinsing in 10X SSC, RNA was transferred to the membrane and hybridized with appropriate DNA probe as mentioned above. Signal was detected using MP Hyperfilm (Amersham Pharmacia, Buckinghamshire, UK).

2.2.25 Stripping DNA probes

To allow re-use of Northern blot, the radioactive labelled DNA was stripped by incubating the membrane in a strip buffer (0.1% SSC, 0.1% SDS) at 95°C for 20 min. the process can be repeated in case of a strong

signal. The membrane was then used for re-probing from the pre-hybridisation step as usual.

2.2.26 Western Blotting and detection of proteins with antibodies

At the end of the electrophoretic migration, the proteins were transferred onto a nitrocellulose Immobilon-P membrane (Millipore, Bedford, USA) from the gel by electroblotting in a transfer chamber from Bio-Rad, containing the transfer buffer (24mM Tris Base, 193M glycine, and 10% methanol). The blotting occurred at 30 V and overnight. The transfer completed, the membrane was incubated in PBS Tween20 0.05%, and china ink to visualize the protein bands. The membrane was thereafter pre-incubated in blocking solution (PBS Tween20 0.05%, supplemented with 5% non-fat milk) for 30 min to reduce unspecific binding. Primary antibody was diluted in the blocking solution at the concentration recommended by the supplier, usually 1/1000, for one hour at room temperature on an end-over-end wheel.

2.2.27 Stripping western blot membrane

In case of the detection of two different proteins the same size on the same membrane, then this one was stripped the following way. It was incubated for 30 min at 55°C in 50 ml of stripping solution (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.4 g DTT) with shaking. The membrane was thereafter washed in PBS-Tween20 and reprocessed as for normal antibodies detection.

2.2.28 Immunoprecipitation

Cells were harvested and lysed in lysis buffer (50mm ris pH 7.4, 100mm NaCl, 3mM $MgCl_2$, and 0.5% NP-40) containing 1X complete™ protease inhibitors cocktail (Roche, Mannheim, Germany). The cell lysate was incubated on ice for 30 min before clarification by centrifugation for 10 min at 10000g at 4°C. The antibody of interest was added to the supernatant for -1 hour at 4°C. 30µl of 50% protein G-agarose beads slurry was added and the samples was rotated at 4°C for further 2 hours. Immunocomplexes were then recovered by gentle centrifugation. After few washing steps with ice cold lysis buffer, 60µl of 2X Laemmli sample buffer was added and the samples were subjected to SDS-PAGE analysis.

2.2.29 *In vivo* ubiquitination assay

H1299 cells were split in 10 cm dishes the day before transfection to have the next day approximately 50% of confluence and were transfected with

the calcium phosphate method with the His6-Ubi. Cells were harvested 24 hours post-transfection. For some cases, 10 μM of MG132 (Calbiochem, LA Jolla, USA), a potent proteasome inhibitor, was added to the medium 4 hours prior to harvest the cells to enhance accumulation of the poly-ubiquitinated proteins. The cells were washed twice in ice cold PBS and scraped in 5 ml of PBS. 1 ml of the cells suspension was taken out to determine the total amount of the protein of interest by Western Blot. Remaining cells were resuspended in 7 ml of Guanidinium lysis buffer (100 mM $Na_2HPO_4/Na_2H_2PO_4$ pH8, 10 mM Tris-HCl pH 8, 6 M Guanidine-HCl, and 5 mM imidazole, 10 mM β-Mercaptoethanol freshly added) and 75 μl of Ni^{2+} -NTA-agarose beads (Qiagen, Hilden, Germany) were added to allow the binding of the His-ubiquitinated proteins. Tubes were sealed and rotated overnight at room temperature. The beads were precipitated at 2000 rpm for 5 min and washed and incubated for 15 min with the different buffers: Guanidinium-HCl buffer, Urea buffer (100 mM $Na_2HPO_4/Na_2H_2PO_4$ pH8, 10 mM Tris-HCl pH 8, 8 M urea, and 10 mM β-Mercaptoethanol freshly added), Buffer A with 0.2% Triton X-100 (100 mM $Na_2HPO_4/Na_2H_2PO_4$ pH6.3, 10 mM Tris-HCl pH 6.3, 8 M urea, 10 mM β-Mercaptoethanol freshly added), buffer A with 0.1% Triton X-100, buffer A without Triton X-100. Finally the His-ubiquitinated proteins were eluted from the beads by incubating in 100 μl of elution buffer (150 mM Tris-HCl pH 6.8, 200 mM imidazole, 5% SDS, 30% v/v glycerol, 0.72 M β-Mercaptoethanol) for 30 min at room temperature under light shaking. The supernatant was removed from the beads by centrifugation and subjected to Western Blot analysis.

2.2.30 Luciferase assay

This assay allows quick analysis of a promoter activity. The cells transfected with the reporter vector and plasmids expressing various transcription factors, were collected 24 hours post-transfection. After washing in PBS without Ca^{2+} /Mg^{2+}, the cell were lysed in 1 time passive lysis buffer (Promega). The lysate could be store at –80°C or analysed immediately for luciferase activity. 50 µl of the lysate were mixed with 350 µl coelenterazin buffer and 100 µl of coelenterazin buffer B (8 ml of coelenterazin buffer, 1 µl of 1mM coelenterazin diluted in 90% methanol and 0.1N HCL) for he normalisation of the transfection efficiency. Luciferase activity of the reporter gene was given by mixing 50 µl of the lysate in 350 µl of GLY-GLY buffer (25mM gly-gly, 15mM $MgSO_4$, 4mM EDTA pH 7.8) completed with 1mM DTT and 1mM ATP, and 100 µl of luciferin (0.28 mg/ml luciferin in gly-gly buffer as stock diluted 1/5 in gly-gly buffer). The value were given as fold induction and normalized on the renilla activity. The measurement was done with a luminometer (LB 9501, Berthold).

2.2.31 Ultraviolet radiation

Ultraviolet radiation (UV) (290-400 nm) is an electromagnetic radiation in between the visible light and x-rays. It is divided into three major wavelengths: UVA, UVB, and UVC. In the present work, I only used the UVC irradiation (200-280 nm), which is the shortest wavelength and present characteristics such as formation of 2-pyrimidine dimer, single or double DNA strand break, or DNA crosslink, that are prerequisite for activation of the DNA repair pathway.

For UV irradiation, the medium of culture was removed and kept, and the cells were washed twice with Ca^{2+}/Mg^{2+} free PBS. They were irradiated at 30 W/m^2 at a distance of 30 cm. After UV irradiation, the cells were covered back with the medium and let in the incubator for the appropriate time before they were harvested and processed as described before.

2.2.32 Flow cytometry (FACS)

Cells stained with annexin V FITC labelled and counterstained with propidium iodide were analysed by flow cytometry (FACS StarPlus, Becton Dickinson, Franklin Lakes, USA) using a specific UV filter. Data analysis was performed using the CellQuest pro software.

3 Results

3.1. Effect of UVC irradiation on p53 level

After DNA damage induced by UVC irradiation for example, the cells should either arrest their cell cycle and block transcription to allow repair to take place, or they should enter in apoptosis to protect their genome integrity. Under normal circumstances, p53 is kept at low level in the cell by mdm2. Therefore, after UVC irradiation, p53 should be prevented from degradation despite the presence of mdm2, to allow p53 to activate the transcription of its target genes, such as p21 and Bax, responsible for the cell cycle arrest and/or the entry in apoptosis. To address this question, I studied the effect of UVC irradiation on the mdm2-dependent degradation of p53 in p53 negative cells transfected with both p53 and mdm2 to avoid any possible interference of a regulation of endogenous mdm2. As expected, in the absence of UVC irradiation, mdm2 promotes a decrease in p53 level (figure 3.1, compare lane 2 and 1). After UVC irradiation, no increase in p53 level was observed (figure 3.1, lane3) as compared to the level of p53 in absence of UVC irradiation (figure 3.1, lane 1). This can be explained by the fact that in this experiment, p53 was already highly expressed in the cells, therefore no further increase could be detected. However after UVC irradiation, overexpression of mdm2 did not promote a decrease in p53 level (figure 3.1, lane 4).

This result shows that after UVC irradiation, p53 is protected from degradation by mdm2.

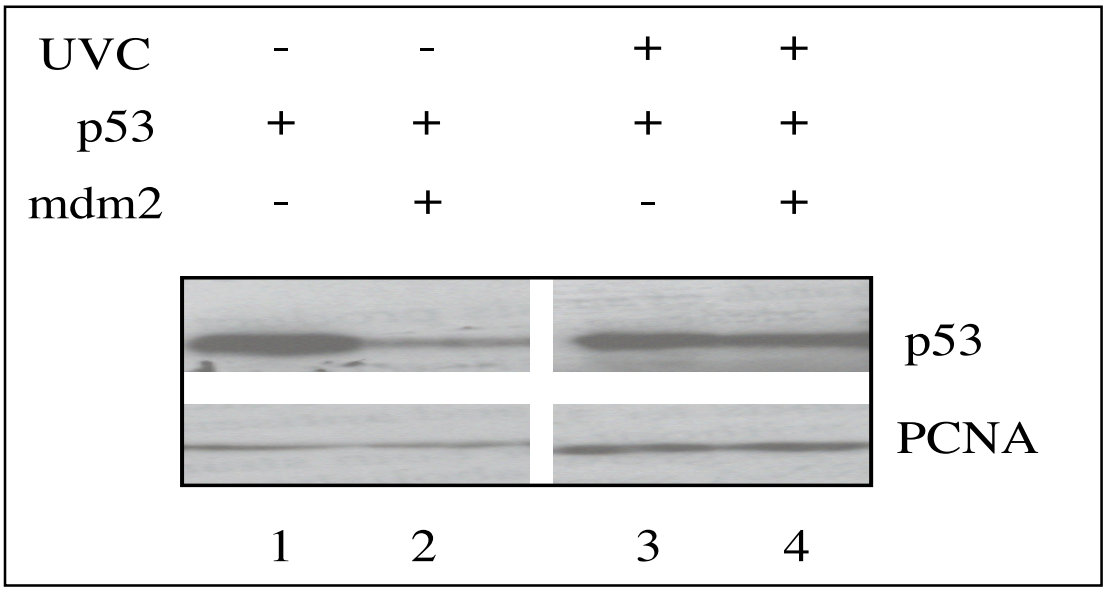


Figure 3.1. UVC irradiation protects p53 from mdm2-dependent degradation. p53 negative H1299 cells were transfected with a p53 expression vector, together with a mdm2 expression vector or empty vector. The cells were irradiated at 30 J/m2 12 hours post transfection, and, they were harvested. 10 hours post-irradiation. The level of p53 was determined by western blotting.

3.2. Effect of UVC irradiation on p53 transcriptional activity.

After DNA damage, p53 might play a role in DNA repair (Seo and Jung 2004), or might induce the transcription of some of its target genes. For instance, in cycling cells, p53 might induce p21 to stop the cell cycle and allow the DNA repair. Alternatively, p53 might induced proapoptotic genes, such as Bax, if the damage are too important. However, trancription has to be stopped to allow DNA repair. Thus, the transcriptional activity of p53 that has accumulated after DNA damage might be inhibited. The next point I wanted to address was to determine if p53 is transcriptionally active. To address this question I overexpressed p53 in p53 negative cell, to avoid variability of the endogenous level of p53, and measure the effect of UVC irradiation on its activity in a reporter gene assay. To ensure that any effect observed was due only to regulation of p53 activity, and not to variation of its level, the expression

of the reporter gene was normalized to the expression of p53. In the absence of UVC irradiation, overexpression of mdm2 reduced the transcriptional activity of p53 by about 50 percent (figure 3.2, lane 2). This is consistent with the observation that mdm2 interacts with the transcriptional domain of p53, and thus inhibits its activity (Haines, Landers et al. 1994; Chen, Lin et al. 1995; Haupt, Rowan et al. 1997; Freedman, Wu et al. 1999). In the absence of mdm2, UVC irradiation inhibited the transcriptional activity of p53 by about 20 percent (figure 3.2, compare lane 1 and 3). In presence of mdm2, the transcriptional activity was further decreased upon UVC irradiation (figure 3.2, compare lane 2 and 4).

Therefore, the p53 that is prevented from mdm2-dependent degradation presents a reduced transcriptional activity after UVC irradiation.

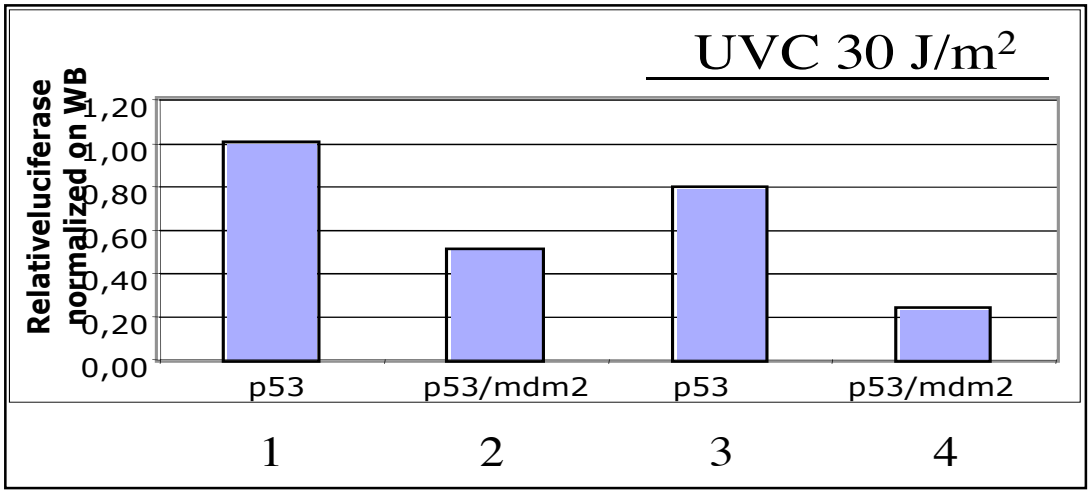


Figure. 3.2. p53 is transcriptionally inhibited after UVC irradiation. p53 negative cell line H1299 cells were transfected with a luciferase reporter gene controlled by the Bax promoter together with a p53 expression vector, with either an mdm2 expression vector or an empty vector. The cells were irradiated at 30 J/m2 12 hours post-transfection and the luciferase activity was determined 10 hours later. The luciferase activity was normalized to the level of p53 determined by western blot.

3.3. Relation between UVC irradiation and HRad23 proteins

3.3.1. Effect of UVC on HRad23A and B levels

Several arguments suggested that HRad23 might be responsible for stabilisation of p53. Indeed, yeast Rad23 competes with the ubiquitylation of several proteins leading to their accumulation (Raasi and Pickart 2003). It is also possible that HRad23 is interfering with mdm2, which would leads to p53 accumulation, since there is no other ubiquitin ligase known in the cell for p53. If so, one would expect that HRad23 might be upregulated after UVC irradiation. To address this question, I studied the level of endogenous HRad23 in fibroblasts after UVC irradiation. HRad23 was steadily detected in control non-irradiated cells. After UVC irradiation, the protein level of HRad23A presented an increase, which was related to the dose received (figure 3.3). The dose of 5 J/m^2 of UVC (figure 3.3, lane 2) was already sufficient to increase HRad23A level. The doses of 15 to 30 J/m^2 UVC (figure 3.3, lane 3 to 4) induced a further increase in the level of HRad23A. A similar effect was observed for HRad23B with a more pronounced response. When p53 levels were monitored, the same effect was observed, and the protein expression level of p53 followed the one of the HRad23 proteins.

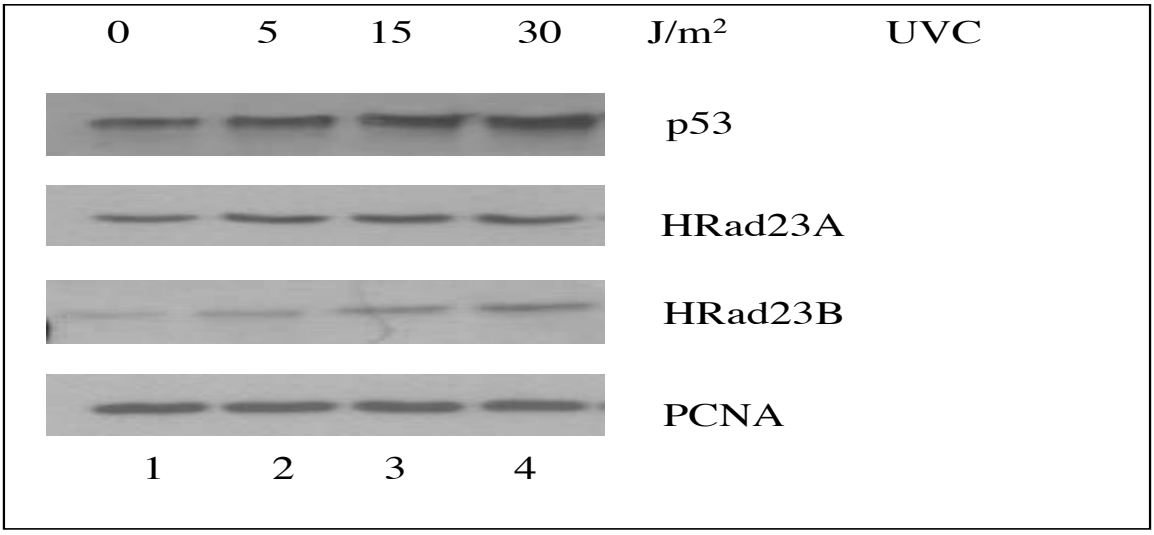


Figure 3.3. Endogenous level of p53 and HRad23A & B are increased after UVC irradiation. Fibroblast were irradiated with UVC at doses ranging from 0 to 30 J/M2 and harvested 4 hours post-irradiation. The levels of p53 and HRad23A and B were determined by western blotting.

I then studied the kinetics of the effect of UVC irradiation on HRad23 level (figure 3.4). HRad23 levels presented an increase that was deteced as early as one hour post-irradiation. This increase was sustained until 7 hours post irradiation and started to decrease slowly, although HRad23 levels were still elevated 9 hours post irradiation (figure 3.4).
Interestingly, the kinetics of the effect of UVC on the levels of p53 was retarded as compared to the levels of HRad23. The increase in p53 levels was detected only 3 to 4 hours post irradiation, and was maximal only at the latest time point of 9 hours (figure 3.4).

Altogether, these results show that UVC irradiation induces time and dose dependent increase in HRad23 protein level. Moreover, the increase in HRad23 levels precedes the increase of p53 levels, which is compatible with a putative role of HRad23 in the stabilisation of p53 after UVC irradiation.

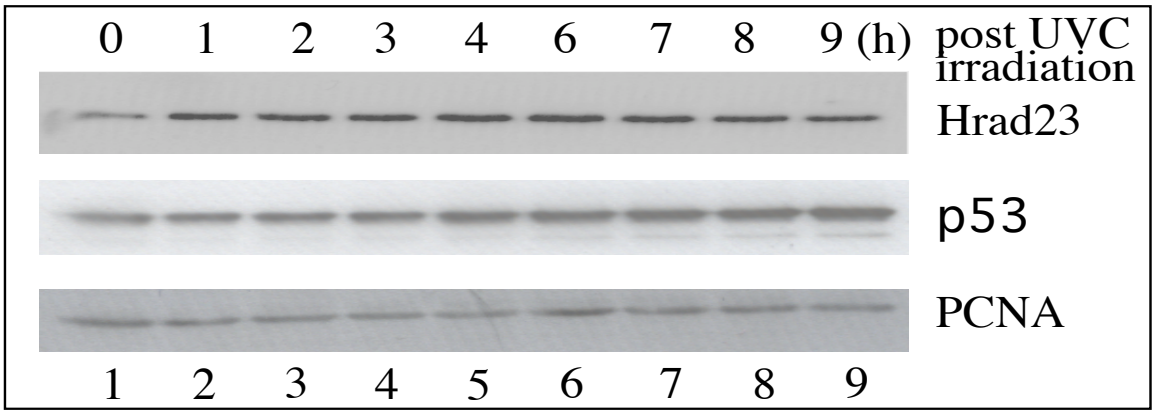


Figure 3.4. Endogenous level of HRad23 is increased prior to p53. Fibroblast were irradiated at 30 J/m^2 and harvested from 0 to 9 hours post-irradiation. The level of p53 and HRad23A were determined by western blotting.

3.3.2 Mechanism of HRad23 accumulation.

The next question I addressed was to study by which mechanism the level of HRad23 is increased. As a first step, I studied whether this increase occurs at the mRNA level. Fibroblasts were harvested following UVC irradiation and the level of HRad23 mRNA was quantified by Northern blot. UVC irradiation did not modify the level of HRad23 mRNA (figure 3.5A).

Thus, the increase at the protein level of HRad23 after UVC irradiation is neither due to an increase of the transcription of this gene nor to a stabilization of its mRNA.

Therefore, UVC irradiation increased HRad23 protein level by either a translational, or a post-translational event, such as a protection from degradation. To confirm this hypothesis, fibroblasts were treated with actinomycin D, an inhibitor of the transcription or with cycloheximide, an inhibitor of the translation and irradiated with UVC. In cells treated with actinomycin D, UVC irradiation still induces an increase in HRad23 protein levels, to the same extent as in the control cells (figure 3.5B). The

c-Fos immunoblot is a control for the actonomycin D treatment. This result confirms that the increase in HRad23 levels is not due to an increase in the transcription. In cells treated with cycloheximide, UVC irradiation did not promote any increase in HRad23 levels, confirming that the increase of HRad23 protein level is due to a translational or post-translational event.

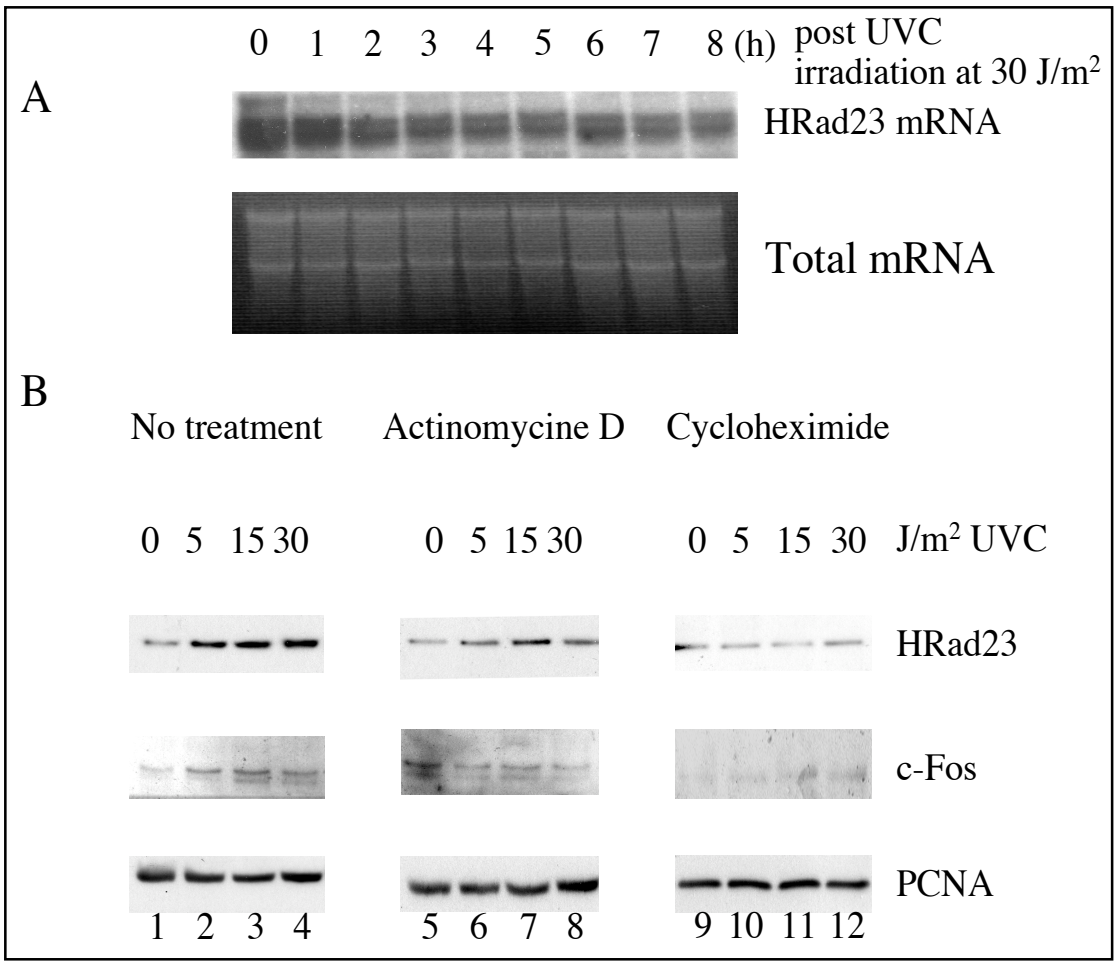


Figure 3.5. Endogenous level of HRad23 is increased by post-transcriptional events upon UVC irradiation. Fibroblast were UVC-irradiated at 30 J/m^2 and harvested from 0 to 8 hours post-irradiation. The level of endogenous HRad23 mRNA was detected by Northern blot (A). Fibroblast were UVC-irradiated from 0 to 30 J/m^2 directly after treatment and harvested 4 hours post-irradiation. The different protein levels were detected by western blot analysis (B).

HRad23 has been proposed to be degraded via the 26S proteasome pathway after ubiquitylation by the ubiquitin ligase E6AP (Kumar, Talis et al. 1999). One could therefore speculate that UVC irradiation promotes an inhibition of its ubiquitylation or an inhibition of its degradation by the proteasome. To test this, I studied the ubiquitylated pattern of HRad23 after UVC irradiation in cells transfected with HRad23, or with HRad23 and E6AP, and treated with the proteasome inhibitor to allow the accumulation and detection of ubiquitylated proteins. No ubiquitylated

form of HRad23 was observed in control non-irradiated cells (figure 3.6A, lane1). Thus, in resting conditions, the level of HRad23 is not regulated by ubiquitylation. After UVC irradiation, an increase in the level of HRad23 was detected (figure 3.6A, compare lane 1 and 2) but no ubiquitylation of HRad23 was observed. Since the protein E6AP was proposed to promote HRad23 ubiquitylation (Kumar, Talis et al. 1999), I used it as a control. Surprisingly, overexpression of E6AP did not induce HRad23 ubiquitylation, neither in control cells, nor following UVC irradiation. However, E6AP overexpression decreased the level of HRad23 (figure 3.6A, compare lane 3 and 1).

As a control for the ubiquitylation assay itself, I studied the effect of mdm2 on p53 ubiquitylation (figure 3.6B). In the conditions of the assay, overexpression of mdm2 induced multiubiquitylation of p53 (figure 3.6B)

Altogether, these results show that HRad23 protein levels are not regulated via ubiquitylation. Thus, the mechanism of UV-induced increase in HRad23 levels cannot be an inhibition of ubiquitylation.

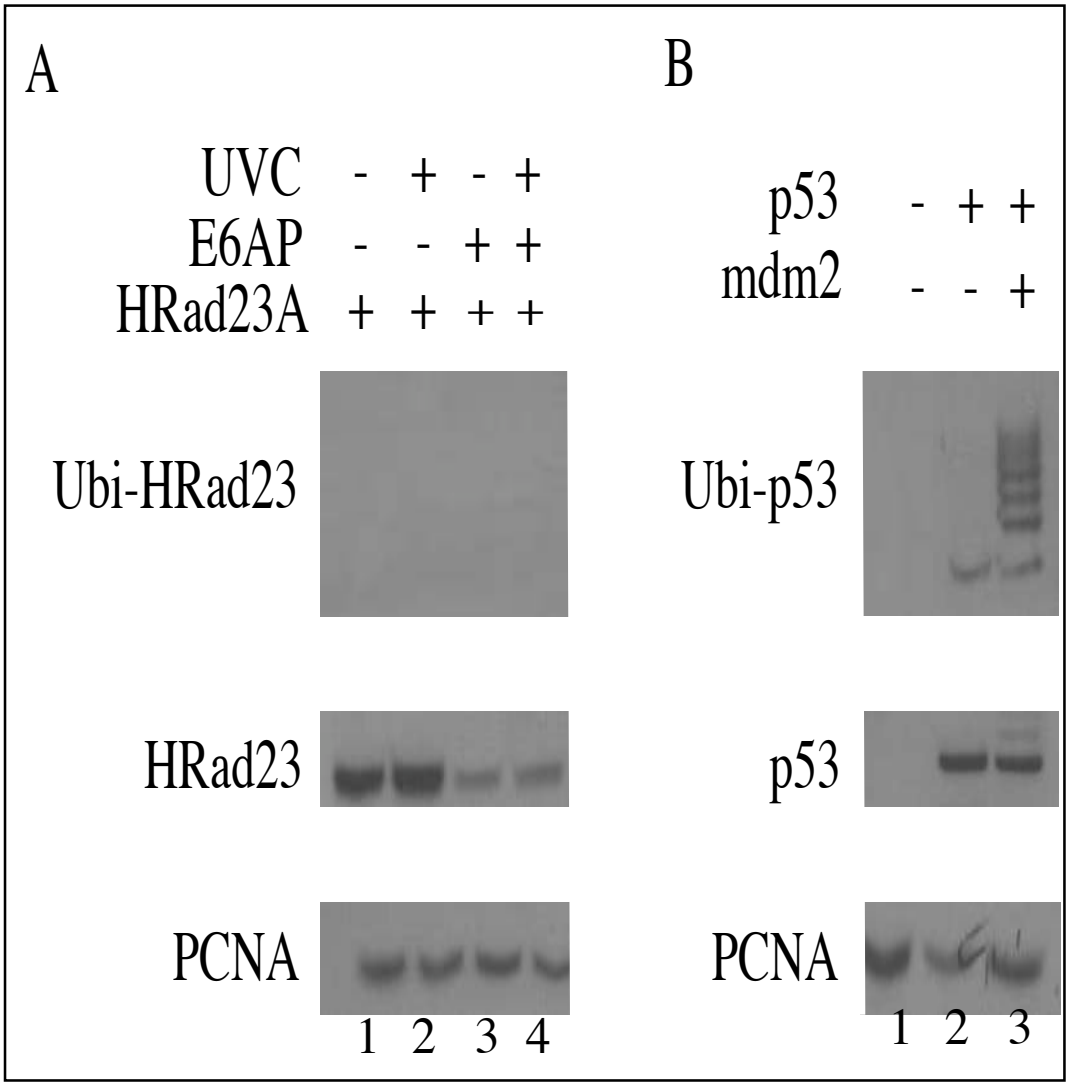


Figure 3.6. HRad23A is not ubiquitylated. p53 negative cell line H1299 was transfected with A) expression vectors for HRad23A and E6AP or empty vector, and B) expression vectors for p53 and mdm2 as the control. Cells were cotransfected with a His-ubiquitin vector allowing the purification of ubiquitin-conjugates. The cells were irradiated or not at 30 J/M2 and treated with proteasome inhibitor for 4 hours and harvested 24h post-transfection. Ubiquitin-conjugates were purified and analysed by western blot

3.4. Effect of HRad23 on p53 degradation

3.4.1. Effect of HRAd23 on mdm2-dependent degradation of p53

So far, I observed that after UVC irradiation both p53 and HRad23 levels were increased, and that HRad23 increase was preceding the one of p53. If the increase in HRad23 levels is involved in UVC-induced p53 stabilization, then overexpression of HRad23 might be sufficient to prevent
mdm2-dependent degradation of p53. To address this question, I used the p53 negative H1299 cell and I overexpressed p53 together with or without HRad23 and mdm2, and determine by Western blot the level of p53. In p53 negative cell line H1299 overexpression of mdm2 leads to a dramatic decrease of transfected p53 (figure 3.7A, lane 2) and overexpression of Hrad23 prevented the mdm2-dependent decrease in p53 levels (figure 3.7A, lane 3). HRad23 did not increase p53 levels in the absence of mdm2 (figure 3.7A, lane 4). Therefore, the overexpression of HRad23 is sufficient to protect p53 from mdm2-dependent degradation. Furthermore, several p53 species of higher molecular weight were observed in the presence of mdm2 together with HRad23 (figure 3.7A, lane 3). These might be ubiquitylated forms of p53, since they were not observed in the absence of mdm2 (figure 3.7A, lane 4). This might suggest that HRad23 protects p53 from mdm2-mediated degradation without inhibiting its ubiquitylation.

To investigate the specificity of this action of HRad23, I studied the effect of HPLIC1 and HPLIC2, two other members of the UBL family of proteins like HRad23, on mdm2-dependent degradation of p53 (figure 3.7B). HPLIC1 and 2 share the same structure as HRad23, except that

HPLIC1 and 2 possess only one UBA domain located in the C-terminal part of the protein.

When overexpressed, neither HPLIC1 nor HPLIC2 did prevent mdm2-induced p53 degradation (figure 3.7B). Moreover, in the absence of mdm2, HPLIC1 and 2 induced a slight increase in the level of transfected p53. Therefore, one cannot exclude a possible role for HPLIC1 and 2 in the regulation of p53 levels. However, since the protection effect on UVC on p53 degradation is mdm2 dependent (figure 3.1), one can exclude a role for HPLIC in the stabilisation of p53 upon UVC irradiation.

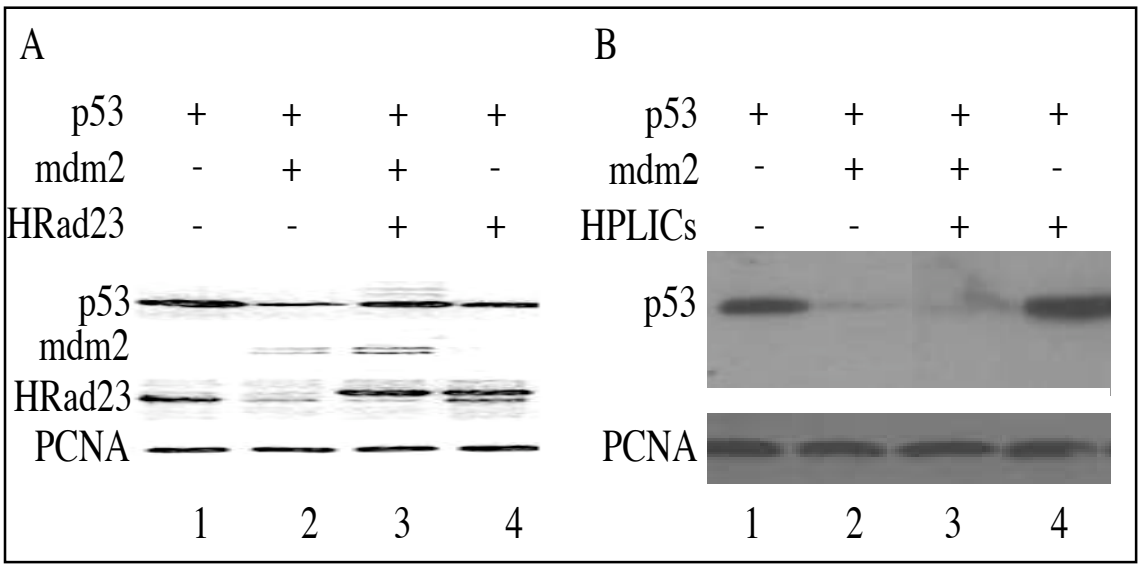


Figure 3.7. p53 is prevented from mdm2-dependent degradation by HRad23A and not by HPLIC. P53 negative cell line H1299 was transfected with expression vector for p53, mdm2,and HRad23 (A), or HPLIC (B). The cells were harvested 24 hours post-transfection. The different protein levels were determined by western blotting.

In the experiment above, I have shown that overexpressed HRad23 is able to promote p53 accumulation despite the presence of mdm2. The next question I wanted to address was to determine if endogenous of HRad23 was also able to protect p53 from degradation.

I therefore use a RNA interference approach, using small interfering oligonucleotides directed against HRad23A and B mRNAs (figure 3.8).

In p53 positive cells transfected with the siRNAs a decrease in the level of HRad23 proteins about 50 percent was observed (figure 3.8, compare lane 2 to lane 1) as compared to the cells transfected with a control siRNA targeting an irrelevant sequence (figure 3.8, lane 1). Furthermore, this specific silencing of HRad23 resulted in a decrease in the level of p53 (figure 3.8, lane 8). This result strongly suggests that endogenous HRad23 protects endogenous p53 from degradation.

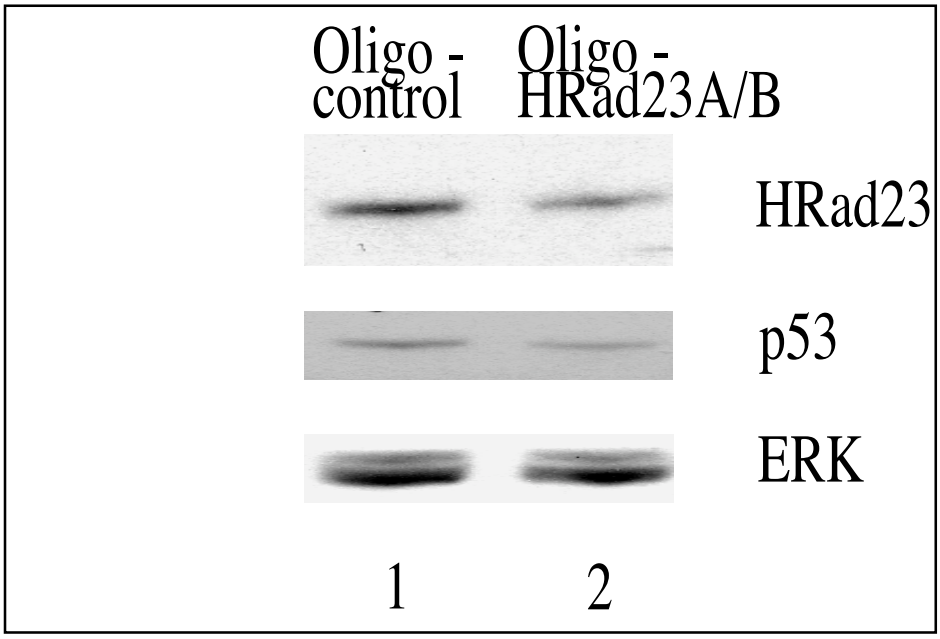


Figure 3.8. Depletion of HRad23 promotes a decrease in p53 level. U2OS cells were treated with indicated siRNAs for 24h, and were harvested. The different protein levels were determined by western blotting, and ERK was used as a loading control.

3.4.2. Effect of HRAd23 on E6-dependent degradation of p53.

Our results so far suggest that HRad23 specifically protects p53 from the degradation mediated by mdm2. To further investigate this specificity, I studied the effect of HRad23 on p53 degradation mediated by E6, the second ubiquitin ligase for p53. After infection by the *human papilloma virus type 16*, the viral protein E6 is able to act as an ubiquitin ligase for p53 and target it for proteasomal degradation (Scheffner, Huibregtse et al. 1993). As expected, the level of p53 is dramatically decreased in the presence of transfected E6 (figure 3.9, lane 2) as compared to the level of transfected p53 alone (figure 3.9, lane 1). However, in the presence of overexpressed HRad23, E6 was still able to promote a decrease in the level of p53 (figure 3.9, lane 3). It seems that Hrad23 has no effect on the degradation of p53 mediated by E6, meaning that HRad23 mechanism of action on p53 degradation is specific of mdm2.

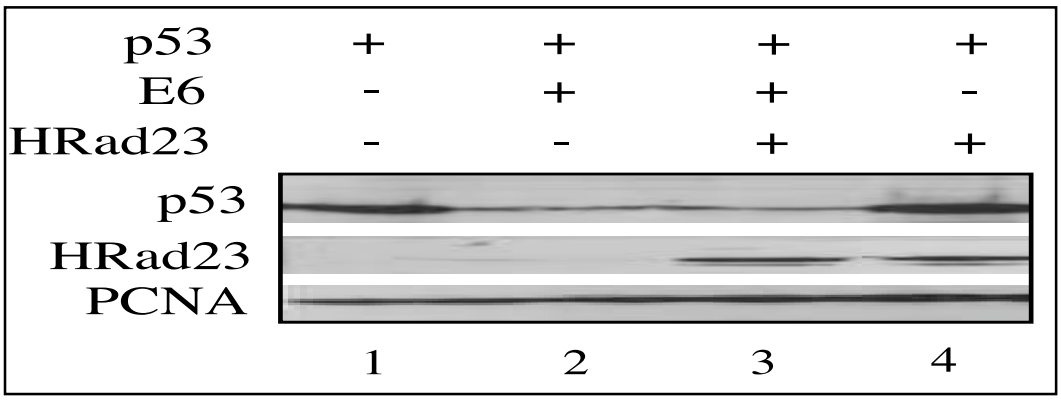


Figure 3.9. HRad23 does not prevent E6-mediated degradation of p53. p53 negative cells H1299 cells were transfected with p53, together with an expression vector for E6, and HRad23 or empty vector. The cells were harvested 24 hours post-transfection. The different protein levels were determined by western blot analysis.

3.4.3. Effect of HRad23 on p53/mdm2 interaction.

Since HRad23 inhibits p53 degradation mediated specifically by mdm2, one hypothesis is that HRad23 might disrupt the complex formed by p53 and mdm2. If HRad23 disrupt the complex, then mdm2 cannot act as the ubiquitin ligase for p53, which will be in turn stabilized. In order to answer this question, I studied the effect of HRad23 on the interaction between p53 and mdm2 by co-immonuprecipitation. p53 was immunoprecipitated at the first step, and the association with mdm2 was verified by Western blot.

In the absence of HRad23, an interaction was detected between p53 and mdm2 (figure 3.10A, lane 2). The mdm2 detected on the western blot is specific of the interaction with p53 and cannot come from unspecific binding of the endogenous mdm2 with the materials used for the co-immunoprecipitation since no mdm2 signal is observed when p53 is transfected alone (figure 3.10A, lane 1). The overexpression of increasing amounts of HRad23 did not disrupt the p53/mdm2 complex (figure 3.10A, lane 3 and 4). Thus HRad23-mediated inhibition of mdm2-induced p53 degradation is not resulting from a disruption of the p53/mdm2 interaction.

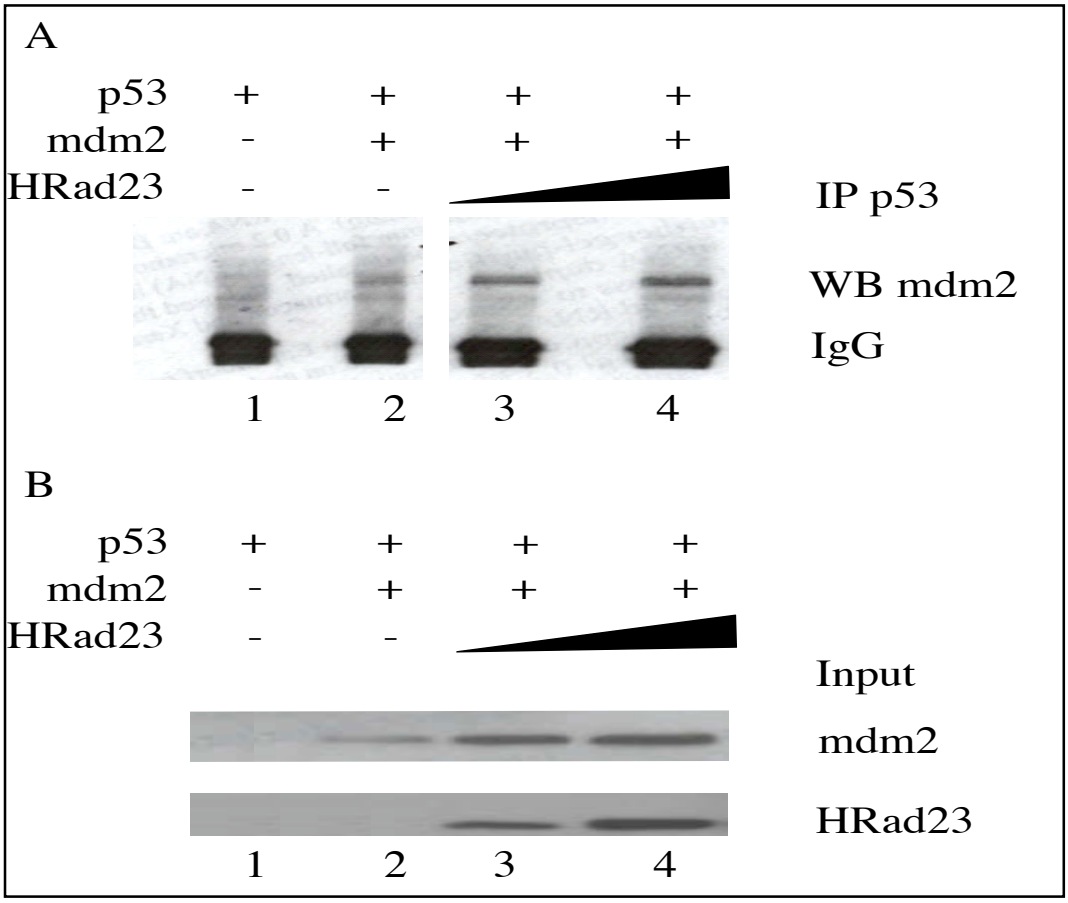


Figure 3.10. HRad23 does not disrupt the complex p53/mdm2. H1299 cells (p53 negative) were transfected with expression vector for p53, together with mdm2, and HRad23. The cells were harvested 24 hours post-transfection and the cell lysate was immunoblot for the different protein levels input (B), or immunoprecipitated with an anti-p53 antibody followed by mdm2 immunoblot (A).

3.4.4. HRad23 does not affect the polyubiquitylation of p53 by mdm2.

The next hypothesis was that HRad23 might prevent the polyubiquitylation of p53 mediated by mdm2. To address this question, I performed an ubiquitylation assay in which I looked for the ubiquitylation of p53 by mdm2 in the presence or absence of HRad23 (figure 3.11).
The cells were transfected with p53 in the presence of mdm2, then treated with proteasome inhibitor to prevent the degradation of ubiquitylated

form of p53 and allow their accumulation. In the presence of mdm2, ubiquitylated p53 appeared as multiple bands of different molecular weight, depending on the number of ubiquitin molecules bound (figure 3.11, lane 2). Overexpression of HRad23 did not inhibit the ubiquitylation of p53 mediated by mdm2 (figure 3.11, lane 3). On the contrary, much more multiubiquitylated form of p53 could be detected in the presence of HRad23. In cells only transfected with mdm2, ubiquitylated p53 was immediately degraded by the preoteasome, and could start to accumulate only when the proteasome inhibitor was added. In cells transfected with mdm2 and HRad23, ubiquitylated p53 accumulates much more. Since these cells were treated with the proteasome inhibitor for the same time, the increased accumulation of ubiquitylated p53 could have occurred only prior to the proteasome inhibitor treatment. One can therefore conclude that HRad23 prevents the degradation of ubiquitylated p53.

Altogether, these results show that HRad23 does not prevent mdm2-mediated ubiquitylation of p53, but rather inhibits the degradation of ubiquitylated p53.

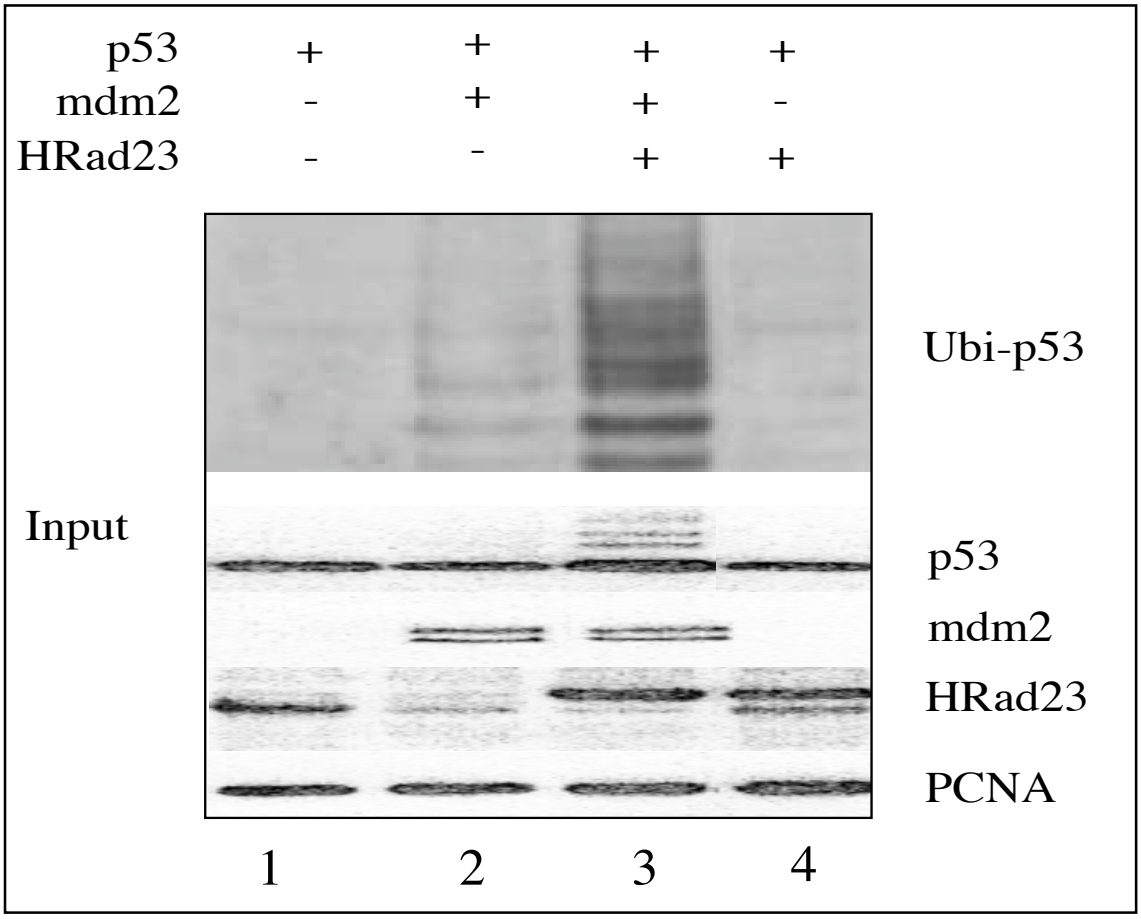


Figure 3.11. p53 is still ubiquitylated by mdm2 in presence of HRad23. P53 negative cell line H1299 was transfected with an expression vector for p53, together with mdm2 and HRad23 or empty vector and with a His-ubiquitin vector allowing. The purification of ubiquitin-conjugates.The cells were treated 4 hours with proteasome inhibitor and harvested 24 hours post-transfection. The different protein levels were determined by western blotting.

My results show that UVC irradiation induces an increase in HRad23 levels, which precedes the stabilisation of p53, and that HRad23 protects p53 from proteasome-mediated degradation. This strongly suggests that UVC-induced increase in HRAd23 levels might be responsible, at least in part, for the stability of p53 after UVC irradiation. This remains to be further demonstrated.

3.5. Effect of HRad23 on p53 transcriptional activity.

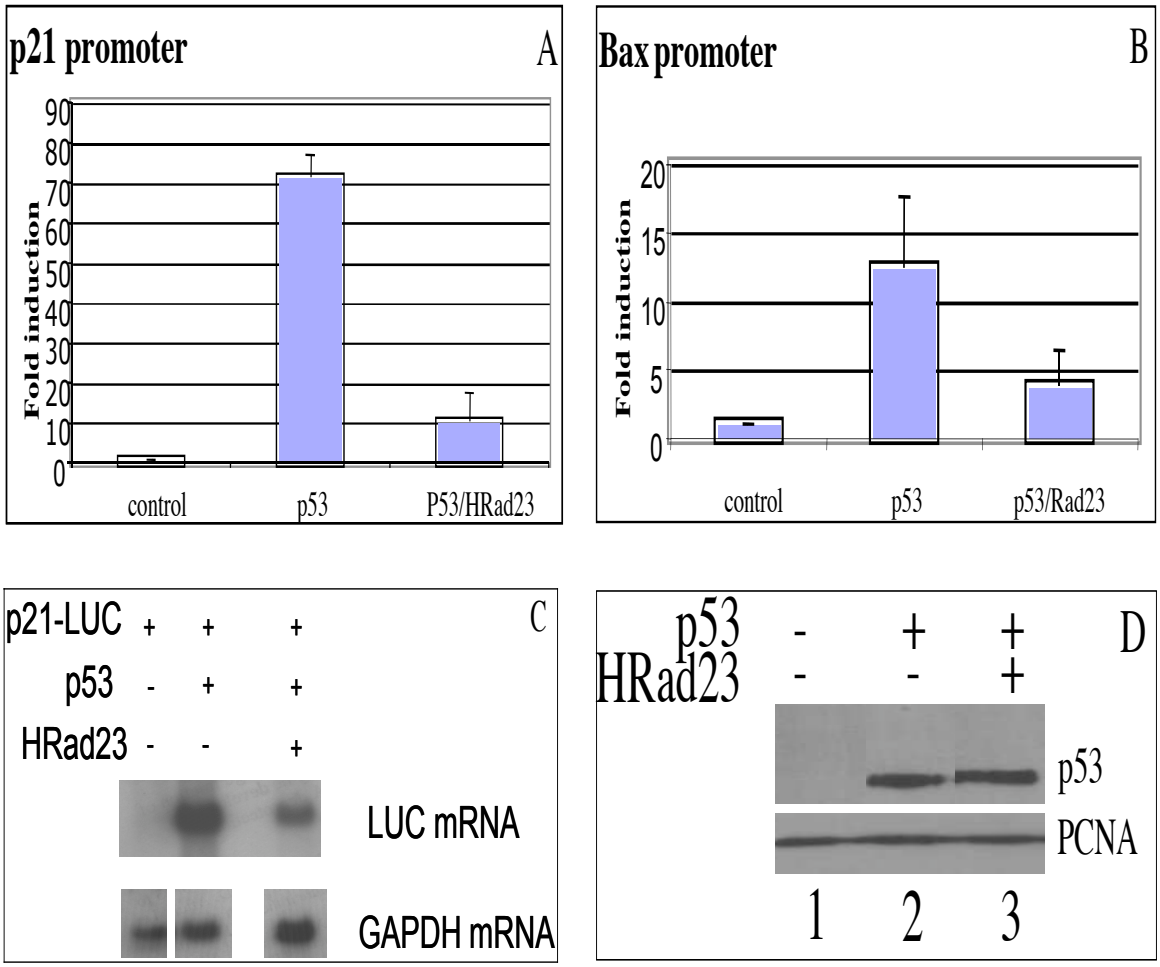
My results so far strongly suggest that the increase in the level of HRad23 upon UVC irradiation might participate in the stabilization of p53. Since p53 that accumulates upon UVC irradiation is transcripionally inative, the next question I wanted to address was to determine the effect of HRad23 on p53 transcriptional activity.

I therefore studied the effect of coexpressing HRad23 on the transcriptional activity of p53 in reporter gene assay. In p53 negative cells, overexpression of p53 strongly induced the expression of the reporter gene under the control of the p21 promoter (figure 3.12A). Overexpression of HRad23 did not modify the level of transfected p53 (figure 3.12D, compare lane 2 to lane 3) but strongly reduced p53-dependent transcriptional induction (figure 3.12A and B). The same inhibitory effect of HRad23 on p53-dependent transcription was observed using a reporter gene under the control of another p53-regulated promoter, the Bax promoter (figure 3.12B).

Since HRad23 interferes with the proteasome, and since the luciferase used as a reporter in this assay is regulated by the proteasome (data not shown), we were concerned that the inhibitory effect of HRad23 in the reporter gene assay might be due to an increased degradation of the reporter protein. I therefore studied the effect of HRad23 on the reporter gene directly at the mRNA level by northern blot. Overexpression of Had23 strongly inhibited the p53 mediated induction of luciferase mRNA (figure 3.12C, compare lane 3 to lane 2).

Since HRad23 promotes the accumulation of polyubiquitylated p53, one could argue that this observed inhibition of p53 transcriptional activity might be due to the polyubiquitylation itself, and not to a direct transcriptional inhibitory effect of HRad23. However, ubiquitylated-p53

accumulation is only observed in the presence of mdm2 (figure 3.11), and here, mdm2 is only present at the endogenous level, and is not able to polyubiquitylate the overexpressed p53 in a significant way (figure



3.12C). Thus, these results show that HRad23 inhibits the transcriptional activity of p53.

Figure 3.12. Effect of HRad23 on p53-dependent promoter. p53 negative cell line H1299 was transfected with the Luciferase gene driven by the p53 response element of the p21 promoter (A, D) or Bax promoter(B), together with expression vectors for p53, HRad23 or empty vector, or only with the expression vector for p53, together with Hrad23 or empty vector (D).The cells were harvested 24 hours post-transfection, and the transcriptional activity of p53 was measured by reporter gene assay (A and B), or the different protein levels were determined by Western blot (D), or the mRNA were determined by Northern blot (C).

3.5.1. HRad23 decreases transcriptional activity of p53-independent promoters.

The next question that needed to be answered was to know if HRad23-mediated transcriptional inhibition is specific for p53-dependent promoters, or if other promoters are also regulated.

To address this question, I performed reporter gene assay using the minimal collagenase I promoter that is activated by the transcription factor AP-1 after TPA induction, and the MMTV promoter, which is activated by the glucocorticoid receptor (figure 3.13).

In the absence of HRad23, TPA induced the AP-1 dependent reporter gene by about 4 times as compared to the basal activity (figure 3.13A, compare lane 2 to lane 1). Overexpression of HRad23, (figure 3.13A, lane 3) decreased the activity from approximately 50 percent. Similarly, the induction of the MMTV promoter by the glucocorticoid dexamethasone was strongly inhibited in the presence of HRad23 (figure 3.13B, compare lane 3 to lane 2).

Thus, HRad23 is able to inhibit not only p53-dependent transcription, but also p53-independent transcription.

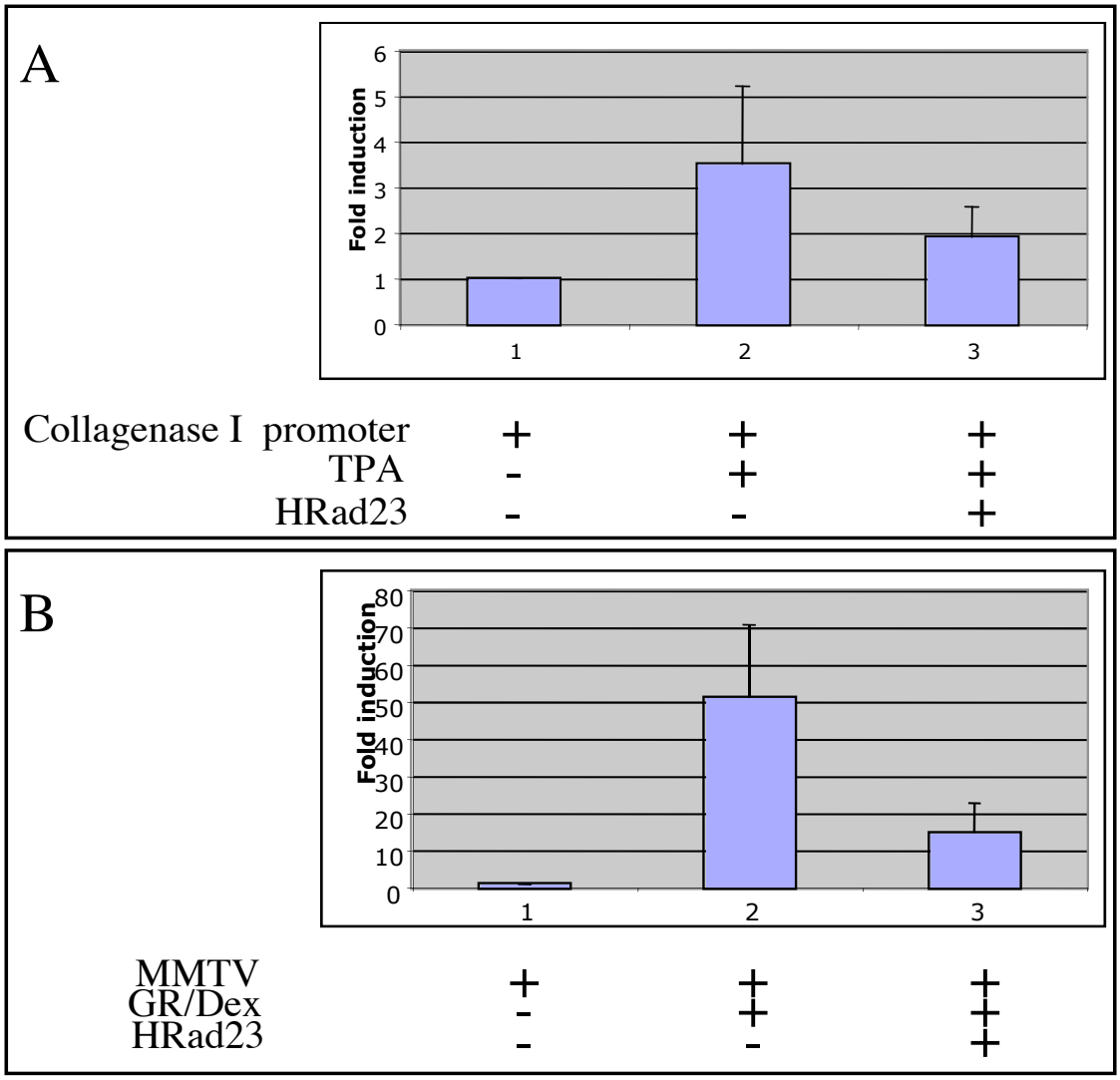


Figure 3.13. Effect of HRad23 on AP1 and MMTV promoter. p53 positive cell line U2OS was transfected with the Luciferase gene driven by the AP-1-dependent collagenase I promoter (A) or MMTV promoter (B), GR (in the case of the MMTV promoter), HRad23. The cells were induced for 8 hours by TPA (A), or for 12 hours by dexamethasone (B) and harvested 24 hours post-transfection.

The HRad23-mediated transcriptional inhibition is not dependent on the transcription factor used, but might target a general mechanism independently of the transcription factor, or directly the basal transcription machinery. To address this question, I studied the effect of HRad23 on the activity of a constitutive promoter, the RSV promoter, upstream of the luciferase gene by northern blot. The constitutive

expression of the reporter mRNA was strongly reduced by HRad23, to nearly undetectable levels (figure 3.14).

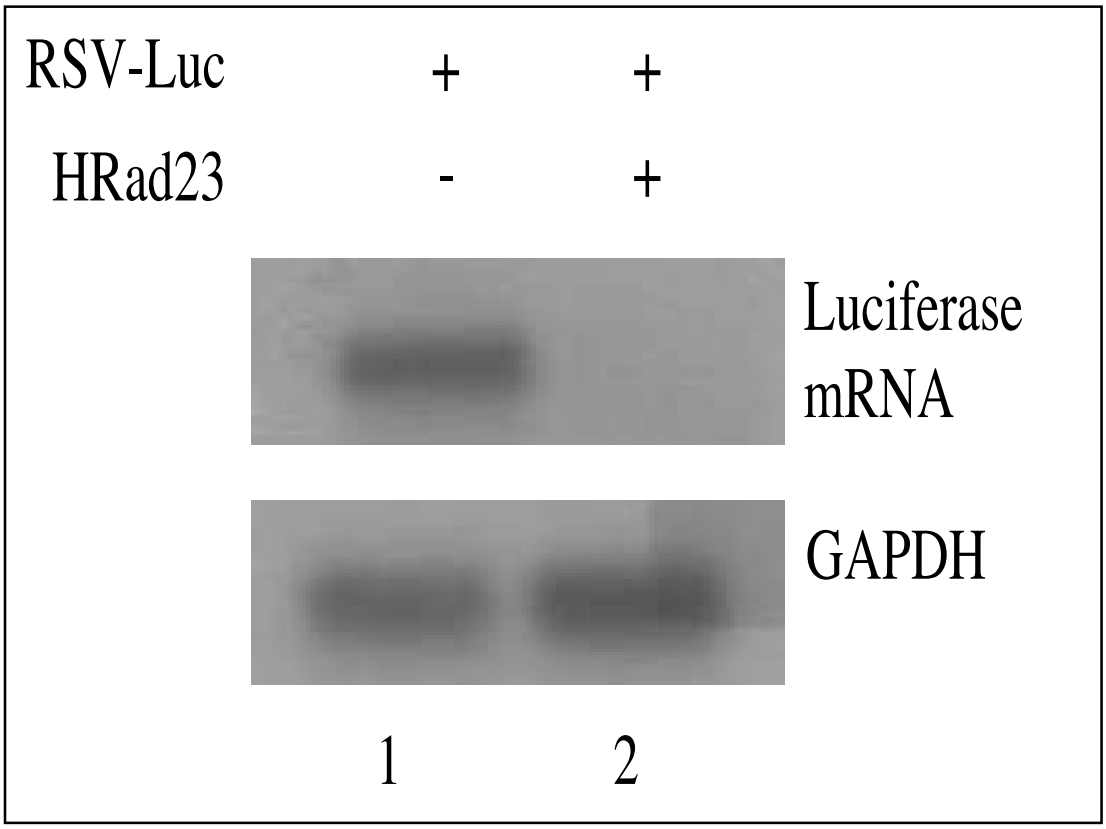


Figure 3.14. Inhibition of the transcriptional activity by HRad23. p53 positive cell line U2OS was transfected with the Luciferase gene driven by the constitutive RSV promoter, HRad23, and empty. The cells were harvested 24 hours post-transfection, the mRNA were prepared and subjected to Northern blot analysis.

3.5.2. HRad23 decreases endogenous gene transcription.

So far, the inhibitory effect of Hrad23 was studied in reporter gene assays. Therefore, the next question I addressed was to determine if HRad23 has a similar effect on endogenous gene. To answer this question I used two different cell line, a p53 positive and a p53 negative cell line to eliminate any effect of it, and stably transfect them with Hrad23. The activity of the endogenous gene c-Fos was compared between the stable HRad23 cell line versus the parental cell line.

The western blot (figure 3.15A) shows the overexpression of HRad23 in the p53 cell line stably transfected with HRad23. This cell line overexpressing HRad23 were growing more slowly as compared to the parental cell line, and the total mRNA content of these cells was always 30 to 40 percent lower than in parental cells. This might suggest that stable overexpression of HRad23 inhibits general transcription mechanism. I then specifically studied the expression of the c-Fos gene in those cells. In the case of the p53 negative cell line, after a period of starvation, in control non induced cells, the c-Fos mRNA was not detected both in the presence or absence of HRad23 (figure 3.15B, compare lane 1 and 2). TPA treatment strongly induced c-Fos mRNA in the control cell line (figure 3.15B, lane 3). This induction was strongly reduced in the cells stably transfected with HRad23 (figure 3.15B, lane 4). The same results were observed in the p53 positive cells (data not show). Therefore the inhibition of the transcription of p53-independent genes is not a secondary effect due to HRad23-mediated regulation of p53.

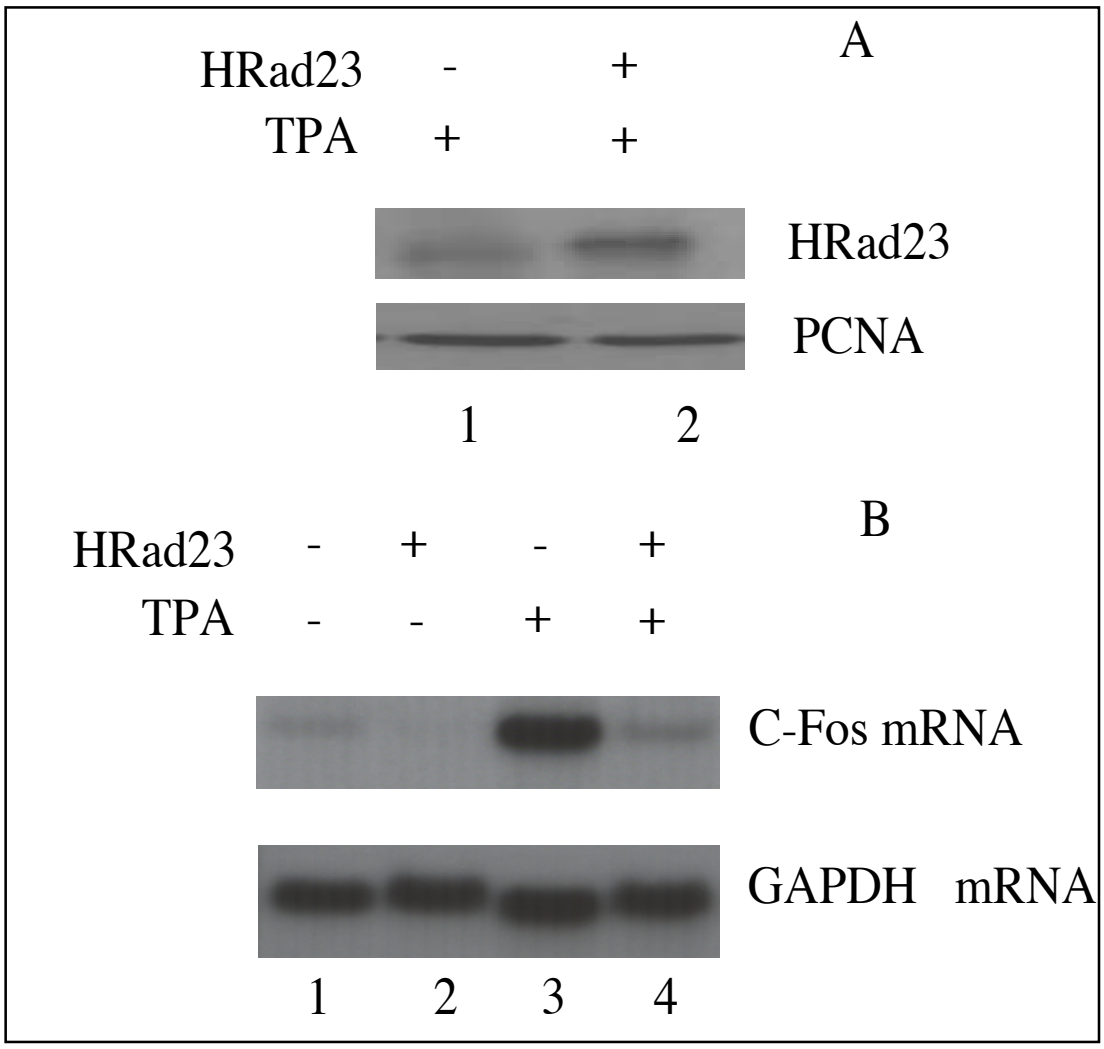


Figure 3.15. Inhibition of the transcriptional activity of endogenous gene by HRad23. p53 positive cell line U2OS, or p53 negative cell line H12299 were stably transfected with HRad23, or empty vector. After starvation, the cells were induced by TPA for 4 hours and were harvested. The mRNA were prepared and subjected to Northern blot analysis (B), or the protein level was analysed by western blot analysis (A).

3.5.3. A knock down of HRad23 increases gene transcription.

My results show that an increase in the level of HRad23 promotes an inhibition of transcription. The next question that needed to be answered was to determine if a decrease in endogenous HRad23 level could lead to an increase in transcription. Since downregulation of endogenous HRad23 level leads to a decrease in p53 level (figure 3.8), any effect on

p53 transcriptional activity would be very difficult to interpret. I therefore study the effect of downregulation of endogenous HRad23 on the transcriptional activity of the glucocorticoid receptor. Indeed, overexpression of HRad23 downregulates the transcriptional activity of the glucocorticoid (figure 3.13B), without regulating its level. As shown previously, transfection with siRNAs targeting HRad23A and B lead to a decreased in the level of HRad23, as compare to the control siRNA (figure 3.8). The level of GR was not affected by siRNA (data not shown). However, after silencing of HRad23, dexamethasone-induced transcription of the GR dependent regulated gene was significantly increased as compared to the control cells (figure 3.16).

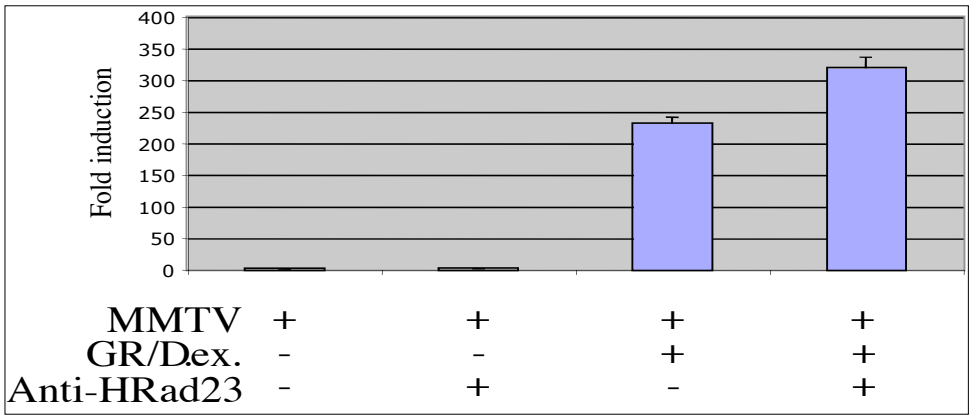
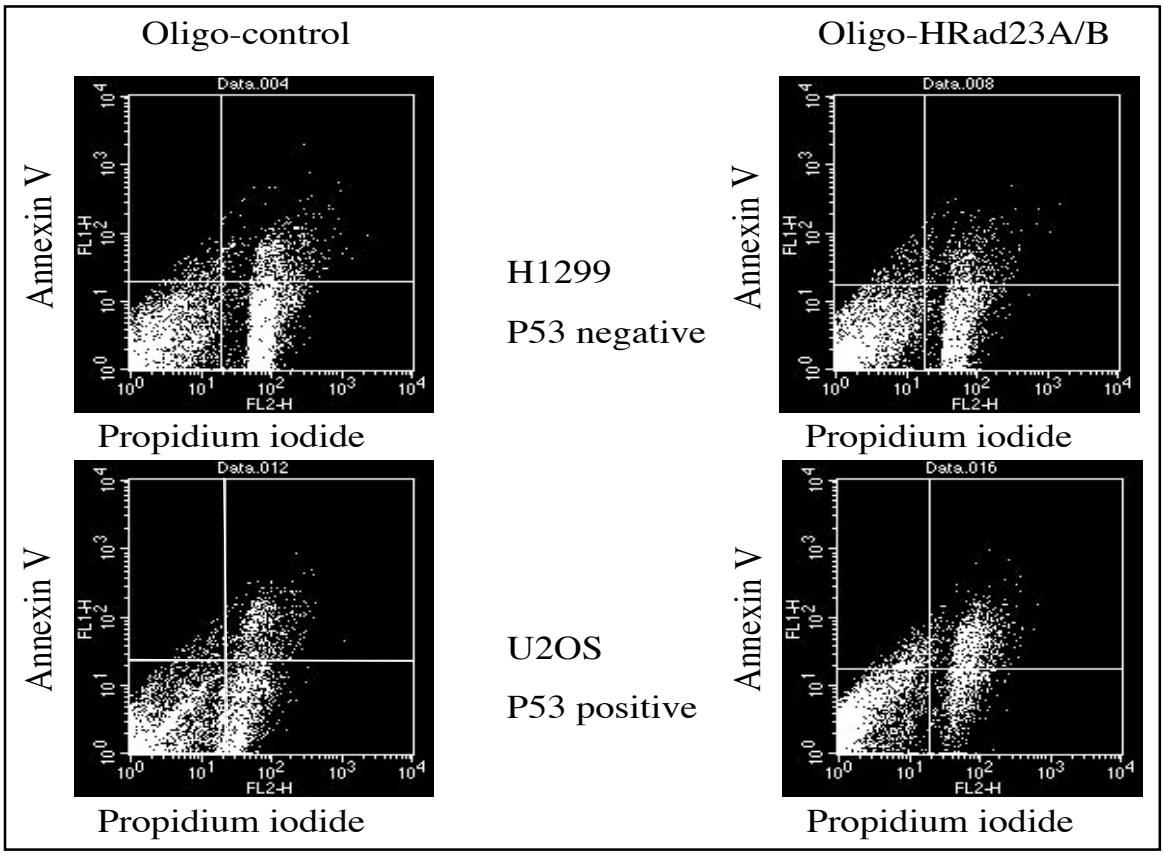


Figure 3.16. Knock down of HRad23 leads to an increase transcriptional activity. p53 positive cell line U2OS was transfected with the Luciferase gene driven by the MMTV promoter,GR, HRad23, or empty. The cells were harvested 24 hours post-transfection, and GR dependent transcriptional activity was measured by reporter gene assay.

3.6. Reduction of HRad23 leads to a p53-dependent apoptosis

Overexpression of HRad23 leads to the accumulation of a transcriptionally inactive p53. Since HRad23has a general inhibitory effect on the transcription, one can imagine that its depletion would lead to the reactivation of p53-dependent transcription. To answer this question, I studied the effect of the depletion of HRad23 on p53-mediated apoptosis. I then compared two different cell line, a p53 positive cell line: U2OS, and a p53 negative cell line: H1299. In the p53 negative cell line, no difference is visible in the apoptotic population of cells when HRad23 is reduced compared to the untreated cells. In the case of the p53 positive cell line, the DNA fragmentation-based assay confirm that an increase annexin V population was observed upon reduction of HRad23A and B levels compare to the parental cell line, suggesting an increase in the apoptosis(figure 3.17). Therefore, the depletion of HRad23 proteins by siRNA technique leads to a p53-dependent apoptosis.



	Apoptotic cells	
p53 negative cells H1299	13.8%	14.7%
p53 positive cells U2OS	9.7%	16.9%
	Oligo-control	Oligo-HRad23A/B

Figure 3.17. Reduction of Hrad23 leads to p53-dependent apoptosis. P53 positive and p53 negative cell line were transfected with oligo-controle or oligo-Hrad23 for 24 hours. The cells were incubated further 24. The apoptosis profiles were analysed with the flow cytometer and CellQuest pro software. One representative out of three similar experiments is shown.

My results show that overexpression of HRad23 inhibits transcriptional machinery in a general mechanism, as p53-dependent and p53-independent promoter were inhibited. On the contrary, the reduction of the HRad23 proteins levels increases the activity of the promoter tested, and also that this HRad23 depletion lead the cell to a p53-dependent apoptosis.

Altogether, theses results suggest that HRad23 might be responsible, or in part, in a general inhibition of the transcriptional machinery, but this remains to be further investigated.

4. Discussion

This thesis work presents major findings on the DNA repair proteins HRad23 and their role in the regulation of p53. Upon UVC irradiation, the level of the two HRad23 proteins is increased through a non-transcriptional mechanism. This work also describes a new function of HRad23, the protection of p53 from proteasome-mediated degradation. Furthermore, HRad23 inhibits p53-dependent and p53-independent transcription. These results strongly suggest a role of HRad23 in the regulation of p53 level and function upon DNA damage.

4.1. UVC increases HRad23 proteins level

A major finding in this work is that UVC irradiation increases HRad23 proteins level in a time dependent and dose dependent manner. HRad23 is involved in nuclear excision repair (Xie, Liu et al. 2004) and both HRad23 proteins are able to bind XPC, the human homologue of the yeast rad4 protein (Li, Lu et al. 1997). The complex HRad23/XPC recognizes DNA damage (Batty, Rapic'-Otrin et al. 2000; Batty and Wood 2000; Hey, Lipps et al. 2002; Janicijevic, Sugasawa et al. 2003), and initiates the recruitment of different other members of the nuclear excision repair machinery allowing the repair (Ng, Vermeulen et al. 2003; Tapias, Auriol et al. 2004). The role of the HRad23 proteins in nuclear excision repair is emphasised by the fact that the double knock out for those proteins leads to a repair deficiency (Ng, Vrieling et al. 2002). Thus, the increase of HRad23 after UVC might be a critical step to allow the repair of damaged DNA.

The mechanism by which HRad23 protein level is increased, is post-transcriptional. This was clearly demonstrated by the fact that UVC

irradiated cells did not show any increase levels of HRad23 mRNA, and also by the fact that an actinomycin D treatment had no effect on the increase of HRad23 protein level. This is coherent with an essential role of HRad23 after DNA damage. Indeed, it is possible that UVC irradiation can damage HRad23 gene itself. With such a mechanism, HRad23 protein level will still be able to increase and fulfil its role.

Therefore, one can ask by which mechanism HRad23 protein level is increased. It could be by protection from degradation, as HRad23 was described to be subjected to ubiquitination by E6AP (Kumar, Talis et al. 1999) and to proteasome-mediated degradation. However, I could observe no ubiquitination of HRad23 in proteasome inhibitor treated cells, in the presence or absence of UVC irradiation (figure 3.6). But overexpression of E6AP decreased HRad23 protein levels (figure 3.9). It might thus be possible that E6AP promotes degradation of HRad23 in an ubiquitin-independent manner. This is describes for other proteins such as p21 and c-Jun (Hoyt and Coffino 2004). Thus, we cannot exclude at the moment a role for E6AP in the regulation of HRad23 levels after UVC irradiation. It would therefore be interesting to study the level of E6AP after UVC, or to see whether overexpression of E6AP prevents the UVC-induced increase in HRad23 levels.

Alternatively, the increase of HRad23 levels after UVC irradiation might result from a stimulation of HRad23 translation. A RNA binding protein was found to stabilize specific transcript after genotoxic stress involved in cell survival and, or to enhance their translation (Yang and Carrier 2001). Indeed, a protein synthesis inhibitor cycloheximide blocks HRad23 increase after UVC irradiation (figure 3.5), suggesting a *de novo* translation. However, a steady state level of a protein corresponds to an *equilibrium* between its synthesis and its degradation. If UVC irradiation promotes the inhibition of HRad23 degradation, increase in its level is

due to a break of this equilibrium, only the result of *de novo* synthesis observed, and protein synthesis inhibitor like cycloheximide would abolish the observed increase. Thus, further investigations are required to conclude on the mechanism of UVC-induce increase in HRad23 levels.

4.2. HRad23 protects p53 from degradation

A second major finding of my work is related to a new function of HRad23 in the regulation of p53 levels. I could show that overexpression of HRad23 leads to p53 accumulation (figure 3.7A), and that its depletion by RNA interference reduced its levels (figure 3.8). Moreover, this effect is specific of HRad23, as the related protein HPLIC had no effect on p53 stabilization (figure 3.7B). This effect of HRad23 is also specific for mdm2-dependent degradation (figure 3.6), as the second ubiquitin ligase for p53, called E6, was still able to promote p53 degradation in presence of HRad23 (figure 3.9).

A mechanism for p53 protection from degradation might be that HRad23 blocks the recognition of ubiquitylated p53 by the proteasome. Indeed, others in our group have shown that *in vitro,* HRad23 interacts only with ubiquitylated p53 (Glockzin, Ogi et al. 2003). Since the first step in proteasome mediated degradation is the recognition of the multiubiquitylated substrate (Crews 2003), one can easily imagine that binding of HRad23 to the ubiquitylated p53 might prevent its recognition by the proteasome.

If true, the protective effect of HRad23 should be independent of the mechanism by which p53 has been ubiquitylated. However, my results show that HRad23 protects p53 from degradation only when it is ubiquitylated by mdm2, and not by E6. This might suggest that HRad23 is not able to interact with p53 when it is ubiquitylated by E6. This is not

the case. Indeed, p53 ubiquitylated by E6 can interact in vitro with HRad23 (Glockzin, Ogi et al. 2003). Alternatively, p53 that was ubiquitylated by mdm2 might be recognized by the proteasome in a different manner that p53 ubiquitylated by E6. This might be the case, since mdm2 and E6 ubiquitylate p53 in a different manner, on different residues. (Camus, Higgins et al. 2003). Thus, interaction with HRad23 might have a different effect on the recognition of polyubiquitylated p53 by the proteasome.

A possible physiological relevance for p53 stabilization by HRad23 might come from the fact that upon UVC irradiation, HRad23 protein level is increase. Therefore, one might speculate that this increase participates in the augmentation of p53 levels after DNA damage. However, other mechanisms have been described as UVC induced increase in p53 levels. For instance, it has been reported that after DNA damage, phosphorylation events on N-terminal part of p53 leads to its stabilisation (Siliciano, Canman et al. 1997).

Thus the exact contribution of the UVC-induced increase in HRad23 levels in the augmentation of p53 levels still has to be documented.

4.3. HRad23 inhibits transcription

The third major finding of this work is that HRad23 inhibits transcription. My results strongly suggest that HRad23 level increases upon UVC irradiation and participates in the stabilization of polyubiquitylated p53. Since p53 that accumulates upon UVC irradiation is transcripionally inactive, one might argue that polyubiquitylated p53 is transcriptionally

inactive. However, I have shown that Hrad23 in absence of mdm2 decreases p53-dependent transcriptional activity without interfering with its level. Moreover, p53 is not polyubiquitylated in absence of mdm2. Thus, the p53-dependent transcriptional inhibition is not due to its polyubiquitylation.

Another possibility is that the interaction between HRad23 and the polyubiquitylated p53 might prevent DNA binding or the transactivation function of p53. However, HRad23 interacts with p53 through its polyubiquitylated tail (Glockzin, Ogi et al. 2003)(Glockzin et al., 2003), and neither with the DNA binding domain, nor with the transactivation domain of p53. It seems therefore unlikely that HRad23 inhibits p53 by blocking its DNA binding domain or its transactivation domain.

Moreover, HRad23 inhibits not only p53-dependent transcription, but also p53-independent transcription such as AP-1, GR (figure 3.13), suggesting a more general mechanism by which HRad23 inhibits transcription. Therefore, something common to these transcription factors has to be targeted by HRad23. The CBP/p300 protein for instance is a co-activator for p53 (Livengood, Scoggin et al. 2002; Dornan, Shimizu et al. 2003; Dornan, Shimizu et al. 2003), for AP-1 (Zanger, Radovick et al. 2001), and for GR (Kino, Nordeen et al. 1999). Moreover, Hrad23 has been shown to interact with p300/CBP in vitro (Zhu, Wani et al. 2001).

Thus, HRad23-mediated inhibition of the transcription might participate in its functions in DNA repair. Upon DNA damage, the cells need to repair or to enter into apoptosis. After UVC irradiation the level of p53 is increased, which will lead to the activation of p53 pro-apoptotic target. Therefore those genes should not be activated directly upon p53 increase to avoid apoptosis before the cells have the time to repair the DNA.

Indeed, I observed that p53 accumulated after UVC irradiation is transcriptionally inactive (figure 3). Since HRad23 inhibits p53-

dependent transcription, UVC mediated increase in HRad23 levels might contribute to the transcriptional inhibition of p53. Thus, a high level of HRad23 after UVC irradiation might protect the cells from apoptosis. Indeed downregulation of basal levels of HRad23 is sufficient to induce a p53-dependent apoptosis (figure 3.17).

Since HRad23 mediates inhibition of transcription is not restricted to p53-dependent transcription, but is more general, another relevance of UVC-mediated increase in HRad23 levels might be to have a broader inhibitory effect on transcription. This transcription inhibition will allow the nucleotide excision repair to occur upon DNA damage. In case of too high damage, the cells could still enter into apoptosis since p53 is present at high levels.

Summary of the project

In conclusion this work shows that upon UVC irradiation, proteins of the nucleotide excision repair pathway called HRad23A and B, are increased in their protein level by a mechanism that is not transcriptional. This increase will in turn leads to the augmentation of the p53 protein by protection of its degradation. Moreover, the accumulated HRad23 has a general inhibitory effect on p53-dependent and p53-independent transcription.

Therefore, I would suggest that upon UVC irradiation leading to the activation of HRad23 proteins involved in the nucleotide excision repair pathway, the effect exerted by HRad23 might allow the cells to repair their DNA damage, keeping as a safety a high level of p53 that can be used to activate pro-apoptotic p53 target genes if the DNA damages are too high.

5 References

Amundson, S. A., T. G. Myers, et al. (1998). "Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress." Oncogene **17**(25): 3287-99.

Appella, E. and C. W. Anderson (2001). "Post-translational modifications and activation of p53 by genotoxic stresses." Eur J Biochem **268**(10): 2764-72.

Argentini, M., N. Barboule, et al. (2001). "The contribution of the acidic domain of MDM2 to p53 and MDM2 stability." Oncogene **20**(11): 1267-75.

Baker, S. J., E. R. Fearon, et al. (1989). "Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas." Science **244**(4901): 217-21.

Banin, S., L. Moyal, et al. (1998). "Enhanced phosphorylation of p53 by ATM in response to DNA damage." Science **281**(5383): 1674-7.

Bates, S., A. C. Phillips, et al. (1998). "p14ARF links the tumour suppressors RB and p53." Nature **395**(6698): 124-5.

Bates, S., K. M. Ryan, et al. (1998). "Cell cycle arrest and DNA endoreduplication following p21Waf1/Cip1 expression." Oncogene **17**(13): 1691-703.

Batty, D. P. and R. D. Wood (2000). "Damage recognition in nucleotide excision repair of DNA." Gene **241**(2): 193-204.

Batty, D., V. Rapic'-Otrin, et al. (2000). "Stable binding of human XPC complex to irradiated DNA confers strong discrimination for damaged sites." J Mol Biol **300**(2): 275-90

Bertolaet, B. L., D. J. Clarke, et al. (2001). "UBA domains of DNA damage-inducible proteins interact with ubiquitin." Nat Struct Biol **8**(5): 417-22.

Blagosklonny, M. V. (1997). "Loss of function and p53 protein stabilization." Oncogene **15**(16): 1889-93.

Brehm, A., E. A. Miska, et al. (1998). "Retinoblastoma protein recruits histone deacetylase to repress transcription." Nature **391**(6667): 597-601.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science **282**(5393): 1497-501.

Caelles, C., A. Helmberg, et al. (1994). "p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes." Nature **370**(6486): 220-3.

Cahilly-Snyder, L., T. Yang-Feng, et al. (1987). "Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line." Somat Cell Mol Genet **13**(3): 235-44.

Camus, S., M. Higgins, et al. (2003). "Differences in the ubiquitination of p53 by Mdm2 and the HPV protein E6." FEBS Lett **536**(1-3): 220-4.

Canman, C. E., D. S. Lim, et al. (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science **281**(5383): 1677-9.

Chen, J., J. Lin, et al. (1995). "Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene." Mol Med **1**(2): 142-52.

Chen, L., U. Shinde, et al. (2001). "Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly." EMBO Rep **2**(10): 933-8.

Ciechanover, A., A. Orian, et al. (2000). "The ubiquitin-mediated proteolytic pathway: Mode of action and clinical implications." J Cell Biochem **77**(S34): 40-51.

Crews, C. M. (2003). "Feeding the machine: mechanisms of proteasome-catalyzed degradation of ubiquitinated proteins." Curr Opin Chem Biol **7**(5): 534-9.

De Leo, A. B. (2005). "p53-based immunotherapy of cancer. Approaches ro reversing unresponsiveness to T lymphocytes and preventing tumor escape." Adv Otorhinolaryngol **62**: 134-50.

Di Cunto, F., G. Topley, et al. (1998). "Inhibitory function of p21Cip1/WAF1 in differentiation of primary mouse keratinocytes independent of cell cycle control." Science **280**(5366): 1069-72.

Di Leonardo, A., S. P. Linke, et al. (1994). "DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts." Genes Dev **8**(21): 2540-51.

Dornan, D., H. Shimizu, et al. (2003). "The proline repeat domain of p53 binds directly to the transcriptional coactivator p300 and allosterically controls DNA-dependent acetylation of p53." Mol Cell Biol **23**(23): 8846-61.

Dornan, D., H. Shimizu, et al. (2003). "DNA-dependent acetylation of p53 by the transcription coactivator p300." J Biol Chem **278**(15): 13431-41.

Dumble, M. L., L. A. Donehower, et al. (2003). "Generation and characterization of p53 mutant mice." Methods Mol Biol **234**: 29-49.

el-Deiry, W. S. (1998). "p21/p53, cellular growth control and genomic integrity." Curr Top Microbiol Immunol **227**: 121-37.

el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-25.

Elsasser, S., R. R. Gali, et al. (2002). "Proteasome subunit Rpn1 binds ubiquitin-like protein domains." Nat Cell Biol **4**(9): 725-30.

Fitch, M. E., I. V. Cross, et al. (2003). "The DDB2 nucleotide excision repair gene product p48 enhances global genomic repair in p53 deficient human fibroblasts." DNA Repair (Amst) **2**(7): 819-26.

Freedman, D. A. and A. J. Levine (1998). "Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6." Mol Cell Biol **18**(12): 7288-93.

Freedman, D. A., L. Wu, et al. (1999). "Functions of the MDM2 oncoprotein." Cell Mol Life Sci **55**(1): 96-107.

Fu, L. and S. Benchimol (1997). "Participation of the human p53 3'UTR in translational repression and activation following gamma-irradiation." Embo J **16**(13): 4117-25.

Funakoshi, M., T. Sasaki, et al. (2002). "Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome." Proc Natl Acad Sci U S A **99**(2): 745-50.

Giaccia, A. J. and M. B. Kastan (1998). "The complexity of p53 modulation: emerging patterns from divergent signals." Genes Dev **12**(19): 2973-83.

Glockzin, S., F. X. Ogi, et al. (2003). "Involvement of the DNA repair protein hHR23 in p53 degradation." Mol Cell Biol **23**(24): 8960-9.

Gottlieb, T. M. and M. Oren (1998). "p53 and apoptosis." Semin Cancer Biol **8**(5): 359-68.

Gu, W. and R. G. Roeder (1997). "Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain." Cell **90**(4): 595-606.

Guzder, S. N., P. Sung, et al. (1998). "Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA." J Biol Chem **273**(47): 31541-6.

Haines, D. S., J. E. Landers, et al. (1994). "Physical and functional interaction between wild-type p53 and mdm2 proteins." Mol Cell Biol **14**(2): 1171-8.

Harper, J. W., G. R. Adami, et al. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-16.

Hartmann-Petersen, R. and C. Gordon (2004). "Integral UBL domain proteins: a family of proteasome interacting proteins." Semin Cell Dev Biol **15**(2): 247-59.

Haupt, Y., S. Rowan, et al. (1997). "p53 mediated apoptosis in HeLa cells: transcription dependent and independent mechanisms." Leukemia **11 Suppl 3**: 337-9.

Hengstschlager, M., K. Braun, et al. (1999). "Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle." Mutat Res **436**(1): 1-9.

Hermeking, H., C. Lengauer, et al. (1997). "14-3-3 sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell **1**(1): 3-11.

Hershko, A., A. Ciechanover, et al. (2000). "Basic Medical Research Award. The ubiquitin system." Nat Med **6**(10): 1073-81.

Hey, T., G. Lipps, et al. (2002). "The XPC-HR23B complex displays high affinity and specificity for damaged DNA in a true-equilibrium fluorescence assay." Biochemistry **41**(21): 6583-7.

Hiyama, H., M. Yokoi, et al. (1999). "Interaction of hHR23 with S5a. The ubiquitin-like domain of hHR23 mediates interaction with S5a subunit of 26 S proteasome." J Biol Chem **274**(39): 28019-25.

Hoyt, M. A. and P. Coffino (2004). "Ubiquitin-free routes into the proteasome." Cell Mol Life Sci **61**(13): 1596-600.

Hu, M. C., W. R. Qiu, et al. (1997). "JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases." Oncogene **15**(19): 2277-87.

Huang, L. C., K. C. Clarkin, et al. (1996). "Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest." Proc Natl Acad Sci U S A **93**(10): 4827-32.

Hupp, T. R. and D. P. Lane (1995). "Two distinct signaling pathways activate the latent DNA binding function of p53 in a casein kinase II-independent manner." J Biol Chem **270**(30): 18165-74.

Janicijevic, A., K. Sugasawa, et al. (2003). "DNA bending by the human damage recognition complex XPC-HR23B." DNA Repair (Amst) **2**(3): 325-36.

Jans, J., W. Schul, et al. (2005). "Powerful Skin Cancer Protection by a CPD-Photolyase Transgene." Curr Biol **15**(2): 105-15.

Jansen, L. E., R. A. Verhage, et al. (1998). "Preferential binding of yeast Rad4.Rad23 complex to damaged DNA." J Biol Chem **273**(50): 33111-4.

Janus, F., N. Albrechtsen, et al. (1999). "The dual role model for p53 in maintaining genomic integrity." Cell Mol Life Sci **55**(1): 12-27.

Janus, F., N. Albrechtsen, et al. (1999). "Different regulation of the p53 core domain activities 3'-to-5' exonuclease and sequence-specific DNA binding." Mol Cell Biol **19**(3): 2155-68.

Jayaraman, L. and C. Prives (1999). "Covalent and noncovalent modifiers of the p53 protein." Cell Mol Life Sci **55**(1): 76-87.

Jentsch, S. and G. Pyrowolakis (2000). "Ubiquitin and its kin: how close are the family ties?" Trends Cell Biol **10**(8): 335-42.

Kapoor, M. and G. Lozano (1998). "Functional activation of p53 via phosphorylation following DNA damage by UV but not gamma radiation." Proc Natl Acad Sci U S A **95**(6): 2834-7.

Kastan, M. B., O. Onyekwere, et al. (1991). "Participation of p53 protein in the cellular response to DNA damage." Cancer Res **51**(23 Pt 1): 6304-11.

Khanna, K. K., K. E. Keating, et al. (1998). "ATM associates with and phosphorylates p53: mapping the region of interaction." Nat Genet **20**(4): 398-400.

Kino, T., S. K. Nordeen, et al. (1999). "Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300." J Steroid Biochem Mol Biol **70**(1-3): 15-25.

Kumar, S., A. L. Talis, et al. (1999). "Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination." J Biol Chem **274**(26): 18785-92.

Lambert, P. F., F. Kashanchi, et al. (1998). "Phosphorylation of p53 serine 15 increases interaction with CBP." J Biol Chem **273**(49): 33048-53.

Lambertson, D., L. Chen, et al. (2003). "Investigating the importance of proteasome-interaction for Rad23 function." Curr Genet **42**(4): 199-208.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-6.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-31.

Li, L., X. Lu, et al. (1997). "XPC interacts with both HHR23B and HHR23A in vivo." Mutat Res **383**(3): 197-203.

Li, S. J. and M. Hochstrasser (2000). "The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein." Mol Cell Biol **20**(7): 2367-77.

Livengood, J. A., K. E. Scoggin, et al. (2002). "p53 Transcriptional activity is mediated through the SRC1-interacting domain of CBP/p300." J Biol Chem **277**(11): 9054-61.

Lohrum, M. A., M. Ashcroft, et al. (2000). "Identification of a cryptic nucleolar-localization signal in MDM2." Nat Cell Biol **2**(3): 179-81.

Lommel, L., T. Ortolan, et al. (2002). "Proteolysis of a nucleotide excision repair protein by the 26 S proteasome." Curr Genet **42**(1): 9-20.

Lowe, S. W., H. E. Ruley, et al. (1993). "p53-dependent apoptosis modulates the cytotoxicity of anticancer agents." Cell **74**(6): 957-67.

Lu, H., Y. Taya, et al. (1998). "Ultraviolet radiation, but not gamma radiation or etoposide-induced DNA damage, results in the phosphorylation of the murine p53 protein at serine-389." Proc Natl Acad Sci U S A **95**(11): 6399-402.

Macleod, K. F., N. Sherry, et al. (1995). "p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage." Genes Dev **9**(8): 935-44.

Maltzman, W. and L. Czyzyk (1984). "UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells." Mol Cell Biol **4**(9): 1689-94.

Masutani, C., K. Sugasawa, et al. (1994). "Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23." Embo J **13**(8): 1831-43.

Meek, D. W. (1998). "Multisite phosphorylation and the integration of stress signals at p53." Cell Signal **10**(3): 159-66.

Meek, D. W. (1998). "New developments in the multi-site phosphorylation and integration of stress signalling at p53." Int J Radiat Biol **74**(6): 729-37.

Momand, J., G. P. Zambetti, et al. (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell **69**(7): 1237-45.

Naumovski, L. and M. L. Cleary (1996). "The p53-binding protein 53BP2 also interacts with Bc12 and impedes cell cycle progression at G2/M." Mol Cell Biol **16**(7): 3884-92.

Ng, J. M., W. Vermeulen, et al. (2003). "A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein." Genes Dev **17**(13): 1630-45.

Ng, J. M., H. Vrieling, et al. (2002). "Developmental defects and male sterility in mice lacking the ubiquitin-like DNA repair gene mHR23B." Mol Cell Biol **22**(4): 1233-45.

Nigro, J. M., S. J. Baker, et al. (1989). "Mutations in the p53 gene occur in diverse human tumour types." Nature **342**(6250): 705-8.

Noda, A., Y. Ning, et al. (1994). "Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen." Exp Cell Res **211**(1): 90-8.

Ortolan, T. G., P. Tongaonkar, et al. (2000). "The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly." Nat Cell Biol **2**(9): 601-8.

Pickart, C. M. and M. J. Eddins (2004). "Ubiquitin: structures, functions, mechanisms." Biochim Biophys Acta **1695**(1-3): 55-72.

Raasi, S. and C. M. Pickart (2003). "Rad23 ubiquitin-associated domains (UBA) inhibit 26 S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains." J Biol Chem **278**(11): 8951-9.

Renan, M. J. (1993). "How many mutations are required for tumorigenesis? Implications from human cancer data." Mol Carcinog **7**(3): 139-46.

Roth, J., M. Dobbelstein, et al. (1998). "Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein." Embo J **17**(2): 554-64.

Saeki, Y., T. Sone, et al. (2002). "Identification of ubiquitin-like protein-binding subunits of the 26S proteasome." Biochem Biophys Res Commun **296**(4): 813-9.

Sakaguchi, K., J. E. Herrera, et al. (1998). "DNA damage activates p53 through a phosphorylation-acetylation cascade." Genes Dev **12**(18): 2831-41.

Sakaguchi, K., H. Sakamoto, et al. (1997). "Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53." Biochemistry **36**(33): 10117-24.

Schauber, C., L. Chen, et al. (1998). "Rad23 links DNA repair to the ubiquitin/proteasome pathway." Nature **391**(6668): 715-8.

Scheffner, M., J. M. Huibregtse, et al. (1993). "The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53." Cell **75**(3): 495-505.

Scheffner, M. (1998). "Ubiquitin, E6-AP, and their role in p53 inactivation." Pharmacol Ther **78**(3): 129-39.

Schneider, E., M. Montenarh, et al. (1998). "Regulation of CAK kinase activity by p53." Oncogene **17**(21): 2733-41.

Seo, Y. R. and H. J. Jung (2004). "The potential roles of p53 tumor suppressor in nucleotide excision repair (NER) and base excision repair (BER)." Exp Mol Med **36**(6): 505-9.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-12.

Shieh, S. Y., M. Ikeda, et al. (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**(3): 325-34.

Shieh, S. Y., Y. Taya, et al. (1999). "DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization." Embo J **18**(7): 1815-23.

Sigal, A. and V. Rotter (2000). "Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome." Cancer Res **60**(24): 6788-93.

Siliciano, J. D., C. E. Canman, et al. (1997). "DNA damage induces phosphorylation of the amino terminus of p53." Genes Dev **11**(24): 3471-81.

Sionov, R. V. and Y. Haupt (1999). "The cellular response to p53: the decision between life and death." Oncogene **18**(45): 6145-57.

Sugasawa, K., C. Masutani, et al. (1996). "HHR23B, a human Rad23 homolog, stimulates XPC protein in nucleotide excision repair in vitro." Mol Cell Biol **16**(9): 4852-61.

Sugasawa, K., J. M. Ng, et al. (1997). "Two human homologs of Rad23 are functionally interchangeable in complex formation and stimulation of XPC repair activity." Mol Cell Biol **17**(12): 6924-31.

Takahashi, T., M. M. Nau, et al. (1989). "p53: a frequent target for genetic abnormalities in lung cancer." Science **246**(4929): 491-4.

Tapias, A., J. Auriol, et al. (2004). "Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors." J Biol Chem **279**(18): 19074-83.

Tibbetts, R. S., K. M. Brumbaugh, et al. (1999). "A role for ATR in the DNA damage-induced phosphorylation of p53." Genes Dev **13**(2): 152-7.

van Gijssel, H. E., L. H. Mullenders, et al. (2003). "Blockage of transcription as a trigger for p53 accumulation by 2-acetylaminofluorene DNA-adducts." Life Sci **73**(14): 1759-71.

Waldman, T., C. Lengauer, et al. (1996). "Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21." Nature **381**(6584): 713-6.

Wang, X. W., W. Vermeulen, et al. (1996). "The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway." Genes Dev **10**(10): 1219-32.

Wang, X. W., Q. Zhan, et al. (1999). "GADD45 induction of a G2/M cell cycle checkpoint." Proc Natl Acad Sci U S A **96**(7): 3706-11.

Waterhouse, N. J., J. C. Goldstein, et al. (2001). "Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process." J Cell Biol **153**(2): 319-28.

Watkins, J. F., P. Sung, et al. (1993). "The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function." Mol Cell Biol **13**(12): 7757-65.

Wei, M. C., W. X. Zong, et al. (2001). "Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death." Science **292**(5517): 727-30.

Wolter, K. G., Y. T. Hsu, et al. (1997). "Movement of Bax from the cytosol to mitochondria during apoptosis." J Cell Biol **139**(5): 1281-92.

Yamaguchi, H. and H. G. Wang (2001). "The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change." Oncogene **20**(53): 7779-86.

Zhu, Q., J. Yao, et al. (2001). "Mdm2 mutant defective in binding p300 promotes ubiquitination but not degradation of p53: evidence for the role of p300 in integrating ubiquitination and proteolysis." J Biol Chem **276**(32): 29695-701.

Zong, W. X., T. Lindsten, et al. (2001). "BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak." Genes Dev **15**(12): 1481-6.