Journal of Cellular Biochemistry 100:1276–1287 (2007)

The Kinetics of p53-Binding and Histone Acetylation at Target Promoters do not Strictly Correlate With Gene Expression After UV Damage

Roberta Magrini, Debora Russo, Gilberto Fronza, Alberto Inga, and Paola Menichini*

Molecular Mutagenesis Unit, Department of Translational Oncology,
National Cancer Research Institute (IST), Largo R. Benzi, 10, 16132 Genova, Italy

Abstract We have addressed the correlation between sequence-specific DNA binding by the tumor suppressor p53 and transactivation of various target genes, in the context of UV irradiation responses. In A549 cells (p53WT), p53 occupancy at the *p21*, *mdm2*, and *puma* promoters increased significantly after UV irradiation. In contrast, *p21* mRNA levels did not change, *mdm2* mRNA decreased and both p21 and mdm2 proteins were downregulated shortly after UV. At later times, higher p53 occupancy correlated with enhanced expression of these two genes both at mRNA and protein levels. In the p53 mutant cell lines LX1 (R273H) and SKMes1 (R280K), no significant p53-binding was detected at the gene targets analyzed. Accordingly, p21 and mdm2 proteins were not upregulated after UV irradiation. The kinetics of histone acetylation did not strictly correlate with gene expression. In fact, high levels of acetylated H3 (AcH3) and, particularly, acetylated H4 (AcH4) histones were found shortly after UV irradiation on *p21* and *mdm2* promoters. At the later time point, when transactivation was detected, acetylation levels decreased significantly although remaining higher than basal levels. Our results indicate that p53 transcription-dependent and -independent responses are activated with different kinetics after UV, possibly relating to the repair of UV-induced DNA damage. Based on the histone acetylation pattern we hypothesize that the DNA repair function of p53, associated to global genome repair and foci of DNA damage, may be relevant for all p53-binding sites, including those where occupancy by p53 is also associated to transcriptional modulation. J. Cell. Biochem. 100: 1276–1287, 2007.

Key words: p53 transactivation; ChIP; histone acetylation; UV irradiation; DNA repair

The tumor suppressor p53 plays a key role in the control of genome integrity. In response to stress signals, p53 is post-translationally modified resulting in higher protein stability and in changes in the potential for protein–protein (and p53::DNA) interactions. These modifications, concomitantly with stress-dependent changes in activity/availability of many cofactors, results in the activation of defined gene expression programs. As a sequence-specific transcription factor p53 is recruited to and can directly modulate the transcription of several genes associated with cell cycle arrest (e.g., *p21*, *Gadd45*, *cyclin G*, *14-3-3-σ*), senescence, apoptosis (e.g., *bax*, *aip1*, *puma*, *noxa*), modification of its stability/activity (*mdm2*), and DNA repair (e.g., *p53R2*, *p48*) [Vogelstein et al., 2000; Harris and Levine, 2005; Wei et al., 2006]. In addition, p53 can modulate gene transcription through interaction with co-activators and co-repressors possessing histone modifying activities [Espinosa and Emerson, 2001; Espinosa et al., 2003; Liu et al., 2003] and, as recently showed, through transcription independent activities [Yee and Vousden, 2005]. Interestingly, a number of studies suggest that p53 can function as a chromatin accessibility factor not

Roberta Magrini and Debora Russo contributed equally to the work.

Grant sponsor: Italian Association for Cancer Research (AIRC); Grant sponsor: Ministero della Salute; Grant sponsor: Fondo per gli Investimenti della Ricerca di Base (MIUR-FIRB); Grant number: RBNE014975; Grant sponsor: Alleanza Contro il Cancro (ACC).

Roberta Magrini's present address is Cancer Biology Unit, Sienabiotech S.p.A., Via Fiorentina, 1, 53100 Siena, Italy.

*Correspondence to: Paola Menichini, Molecular Mutagenesis Unit, Department of Translational Oncology, National Cancer Research Institute (IST), Largo R. Benzi, 10, 16132 Genova, Italy. E-mail: paola.menichini@istge.it

Received 2 June 2006; Accepted 1 August 2006

DOI 10.1002/jcb.21122

only to promote gene transcription but also to modulate the chromatin structure and facilitate the removal of DNA damage from global genome [Rubbi and Milner, 2003; Allison and Milner, 2004].

Mutations of the *p53* gene are one of the most common genetic alterations found in human cancer, and in many cases are considered crucial for tumor development and progression. A cancer cell carrying mutated p53 is generally unable to arrest its cell cycle and/or enter the apoptotic pathway after exposure to endogenous or environmental stresses. Furthermore, resistance to chemotherapy is frequently observed in tumors with mutant p53 compared to those carrying wild-type p53 [Soussi and Beroud, 2001; Szymanska and Hainaut, 2003; Soussi and Lozano, 2005], although results can differ according to the nature of the treatment [Blandino et al., 1999; Menendez et al., 2006]. Accumulating evidence indicates that different p53 mutations may have different impact on the activation of cellular functions normally regulated by wild-type p53. A specific p53 mutant protein may retain wild-type functions towards a subset of promoters, or may even acquire new transactivation activities [Sigal and Rotter, 2000; Weisz et al., 2004; Bossi et al., 2006].

By using a sensitive yeast assay [Flaman et al., 1995] it has been shown that tumor-derived p53 mutants possess heterogeneous transcriptional activities towards different promoter sequences containing p53 response elements (RE) [Campomenosi et al., 2001; Monti et al., 2002, 2003]. Indeed, p53 can be considered a master gene of diversity since different mutations of the *p53* gene may lead to simultaneous changes in target selectivity and in the transcription of *p53* regulated genes [Resnick and Inga, 2003]. In the ~50% of cancers where the *p53* gene maintains a wild-type sequence and transcription patterns, other events can result in p53 inactivation, such as the overexpression of negative modulators, the loss of important p53 cofactors, or the alteration of survival/proliferation pathways that are dominant over p53.

Studies aiming at understanding the mechanisms dictating transactivation specificities in the p53-regulated network have shown that p53 promoter occupancy correlated with but cannot fully explain kinetics or extent of target gene expression changes [Pan and Haines, 2000; Szak et al., 2001; Kaeser and Iggo, 2002; Espinosa et al., 2003]. The correlation was even less stringent when p53-induced biological responses were considered. For example, the binding of wild-type p53 to consensus sequences in some pro-apoptotic genes (i.e., *bax*, *pig3*, *aip1*, and *puma*) did not necessarily correlate with induction of apoptosis, suggesting that interactions with co-activators or changes in other cellular survival pathways may regulate the apoptotic program [McCormick, 1999; Kaeser and Iggo, 2002]. Although limited by being unable to capture the dynamicity of protein–DNA interactions, chromatin immunoprecipitation (ChIP) experiments have provided means of addressing in vivo binding of p53 to specific target sites [Pan and Haines, 2000; Szak et al., 2001; Kaeser and Iggo, 2002] or to the entire genome [Wei et al., 2006]. These experiments have shown that the intrinsic DNA-binding affinity of p53 RE can be related to p53-dependent changes in promoter architecture. While basal level of p53 can be sufficient for recruitment of p53 at high affinity sites, resulting in assembly of pre-initiation complexes, low affinity binding sites seem to require higher levels of p53 protein, achieved in stressed conditions. At these target sites the assembly of a pre-initiation complex and its transformation into an elongation complex may require additional events which are independent or only loosely correlated with p53 occupancy rates [Szak et al., 2001; Espinosa et al., 2003].

In this study, we have addressed the correlation between the binding of endogenous wild-type and mutant p53s to specific promoter regions of endogenous checkpoint and apoptotic target genes, their chromatin state, and the consequences of UV damage on their expression, using three lung cancer-derived human cell lines. One cell line retains wild-type p53 [A549 (p53WT)], while the other two carry only the mutated alleles [LX1 (p53R273H) and SKMes1 (p53R280K)] as previously determined by denaturing gradient gel electrophoresis [Loprevite et al., 1997] and checked by the yeast functional assay (data not shown). Our results indicate that the extent of binding to chromatin is only one determinant for p53 transactivation in vivo and interactions between UV damage-specific responses and p53 activity may regulate the level of p21 and mdm2 proteins. In particular, p53 transcription-dependent and -independent responses can be activated with

different kinetics after UV exposure, likely to allow an efficient repair of UV-induced DNA damage.

MATERIALS AND METHODS

Cell Culture and Treatment

A549 (human epithelial-like lung adenocarcinoma; p53wt) and SKMes1 (human epithelial-like lung squamous carcinoma; p53R280K) cell lines were grown in D-MEM (GIBCO Invitrogen, Milano, Italy) containing 10% fetal bovine serum (Sigma-Aldrich, Milano, Italy). LX1 (human epithelial-like lung squamous carcinoma; p53R273H) cells were cultured in RPMI 1640 (GIBCO Invitrogen) supplemented with 10% bovine serum (Sigma-Aldrich). For all cell lines, the medium contained 100 UI/ml of penicillin and streptomycin (MP Biomedicals, Irvine). Cells were maintained at 37°C in 5% CO_2 at 100% humidity. All cell lines were periodically checked for mycoplasma infections. Cells were UV-C irradiated at 50–80% confluency in the absence of medium with a germicidal lamp emitting 254-nm light at a fluence rate of 0.13 $J/m^2/s$, as monitored by a UVX digital radiometer (Ultraviolet Products, San Gabriel, CA).

Western Blots

To prepare total extracts, cells were washed twice with cold PBS and lysed in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 10 mM EDTA, 1 mM DDT, and protease inhibitors (0.5 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A). Cell lysates were incubated 20 min at 4°C in rocking and centrifugated at 14,000 rpm at 4°C for 5 min. Supernatants were collected and protein concentration was determined using the Bradford assay (Bio-Rad, Milano, Italy). Usually 20–30 μg of total proteins were resolved on 7.5–12% SDS–PAGE and transferred to PVDF Hybond-P membrane (Amersham, Milano, Italy). Membranes were blocked with 5% non-fat dry milk in 0.1% Tween 20 in PBS for 1 h, then incubated 1 h (or overnight) at 4°C with the appropriate first antibody. The following antibodies were employed: anti-p53 (CM1, Novocastra, Newcastle upon Tyne, UK); anti-β-actin (AC-74, Sigma-Aldrich); anti-bax (N-20), anti-mdm2 (SMP14), and anti-p21 (C-19) (Santa-Cruz Biotechnology, Milano, Italy). Subsequently, membranes were incubated with peroxidase-conjugate anti-mouse or anti-rabbit secondary antibodies (Sigma-Aldrich). Detection was carried out with Supersignal West Pico chemiluminescent substrate (Pierce, Rockford).

Chromatin Immunoprecipitation (ChIP) Assay

Subconfluent cells were UV- or mock-irradiated and processed at different times after UV exposure. Genomic DNA and proteins were cross-linked at room temperature by the addition of formaldehyde (1% final concentration) directly into culture medium. After 10 min, the cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M for 5 min at room temperature. Cells were washed 2× with cold PBS, lysed with 1 ml of cold buffer A (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP40, 1 mM DDT, 0.5 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A) and collected by scraping. Cell lysates were kept 20 min at 4°C in rotation and centrifuged 5 min at 2,000 rpm to pellet the nuclei. Pellets were resuspended in 50 μl/10^6 cells of cold buffer B (as buffer A but with 500 mM NaCl instead of 10 mM) and incubated 30 min on ice. Lysates were sonicated by using a microtip on a Branson sonicator. Insoluble materials were removed by 5 min centrifugation at 4°C at 14,000 rpm and the supernatant transferred to new tubes. To visualize the shearing efficiency, an aliquot of fixed and sonicated chromatin was phenol extracted and DNA samples were run in agarose gel. An optimal result yields chromatin fragments that are distributed around 600 bp. Usually, 100 μl aliquots of chromatin were diluted 10-fold with DBP buffer (350 mM NaCl, 50 mM Tris HCl pH 8, 5 mM EDTA, 1% NP40, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μg/ml sonicated single-stranded salmon sperm DNA, 1 μg/μl BSA), and incubated overnight in rotation at 4°C with 10 μl of antibody-coated paramagnetic protein G beads (Dynal Biotech, Oslo, Norway). To preabsorbe antibodies to the beads, 1 μg of FL-393 antibody (Santa-Cruz Biotecnology, CA) for p53, anti-acetyl-histone H3 and anti-acetyl-histone H4 (Upstate, Dundee, UK) for acetylated H3 (AcH3) and acetylated H4 (AcH4) histones or no antibody and 10 μl of beads were added to 1 ml of DBP buffer, incubated 4 h at 4°C in rotation, washed 2× with DBP buffer and resuspended in 10 μl of DBP buffer. Following immunoprecipitation, beads were washed four times with 0.5 ml of

DBP buffer, resuspended in 125 μl of LB buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS, 16 ng/μl ssDNA, 1 μg/μl BSA) and incubated 10 min at 85°C. After a brief centrifugation, supernatants were transferred to new tubes and incubated 6 h at 65°C to reverse cross-links. A 10% aliquot of fixed-sonicated chromatin before immunoprecipitation (10 μl) was processed as the IP fractions and used to estimate the input target DNA. Samples were diluted with one volume of water and incubated 1 h at 50°C with 2 μl Proteinase K 20 mg/ml (GIBCO Invitrogen). DNA was purified from the input and the immunoprecipitated chromatin by extraction with phenol/chloroform and isopropanol precipitated with glycogen (GIBCO Invitrogen) as a carrier. Pellets were resuspended in 100 μl of H_2O and aliquots of 5 μl were analyzed by real-time PCR.

Quantitative ChIP-PCR

DNA obtained from input chromatin and chromatin immunoprecipitated with anti-p53, anti-acetyl-histone H3, and anti-acetyl-histone H4 antibodies was amplified by real-time PCR (RotorGene 3000, Corbett Research, Sidney, Australia) using probes and primers specific for sequences that flank the promoter of *p21* (~2.2-kb upstream from transcriptional start site, TSS), *mdm2* (~0.7-kb downstream TSS in intron 1), *puma bax* (~0.5-kb upstream of TSS for both), and *aip1* genes, according to Kaeser and Iggo [2002]. The positions of the probes were determined using Ensemble v37 (http://www.ensembl.org/Homo_sapiens/index.html). The amplification of three dilutions of the input chromatin was used to make a standard curve of target DNA present in the input chromatin. For p53-binding determination, the amplification of *gapdh* sequences was used as negative control and to evaluate the specificity of the assay. Reactions were performed in 25 μl triplicates using the qPCR Mastermix (Eurogentec, Seraing, Belgium) according to manufacturer's instructions with primers and probes used at 600 and 200 nM, respectively. For all targets, the PCR cycles were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. For *gapdh*, an annealing step at 55°C for 30 s was used followed by an extension at 60°C for 30 s. A sample not containing the antibody was always amplified and the values occasionally detected (ranging from 0.08 to 0.2%) were subtracted. When determining p53-binding, the amount of target DNA immunoprecipitated, indicating the extent of p53-binding to its target, was expressed as percentage of input DNA. For the determination of histone acetylation, the level of acetylation in the *gapdh* gene was used to normalize for the overall AcH3 and AcH4 levels. Thus, the amount of H3 and H4 acetylation at each promoter was expressed as percentage of input target DNA/percentage of input *gapdh* DNA.

RNA Preparation and RT-PCR Analysis

Total RNA was extracted by using the RneasyMini kit (Qiagen, Milano, Italy) and 1 μg of RNA was reverse transcribed with the *Reverse*-iT™ 1st Strand Synthesis kit (ABgene, Surrey, UK) and random decamers. Quantitative RT-PCRs were run in 25 μl triplicates in a RotorGene 3000 (Corbett Research) using the RealMasterMix Probe (Eppendorf, Milano, Italy) according to manufacturer's instructions and primers and probes as in Kaeser and Iggo (2002), except for *gapdh*: gapdh fw 5′-GAAGGTGAAGGTCGGAGTC-3′; gapdh rv 5′-GAAGATGGTGATGGGATTTC-3′; gapdh probe 5′-6FAM-CAAGCTTCCCGTTCTCAGCC-3′. Five percent of the cDNA reaction was used as template for real-time PCR. For *p21*, *mdm2*, and *bax* the conditions were 95°C for 15 min, then 45 cycles of: 95°C for 20 s, 58°C for 20 s, and 68°C for 20 s. For *gapdh,* an annealing temperature of 52°C was used. The variation of gene expression in UV- compared to mock-irradiated cells was calculated by using the Comparative Quantitation Analysis software of the Rotor-Gene 3000. The software calculates the relative concentration of each sample compared to a calibrator sample chosen by the user. The mock-irradiated A549 sample was used as calibrator for all cell lines.

RESULTS

p53-Binding to *p21, mdm2, puma, bax,* and *aip1* Promoters After UV Irradiation

In order to evaluate the binding of mutant and wild-type p53 proteins to effector genes in vivo, we first determined the p53 protein level in the cell lines before and after a UV treatment. As expected, in response to UV irradiation (30 J/m^2), p53 was induced only in A549 (with a maximum level 16 h after treatment), while both in LX1 and SKMes1 cells, the level of mutated p53 proteins was already high before

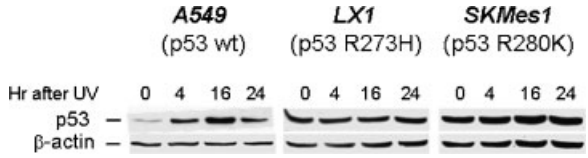

Fig. 1. Time course of p53 expression in lung cancer cells. Western blots showing the level of wild-type and mutant p53 protein in A549, LX1, and SKMes1 lung cancer cells after 30 J/m^2 UV-C irradiation. p53 induction was observed only in A549 cells. β-actin was used as loading control. Results are representative of at least three independent experiments.

UV and did not change after UV exposure (Fig. 1).

The extent of p53-binding to different target promoters, was evaluated by ChIP. For each cell line, the extent of binding measured at the *gapdh* locus (which does not contain p53-binding sites) was taken as background. This binding was very low (below 0.05% for A549 and below 0.2% for LX1 and SKMes1 cells) and did not show any significant modulation following p53 induction. In A549 cells, the binding of wild-type p53 significantly increased after UV irradiation at the *p21*, *mdm2*, and *puma* promoters (Fig. 2). At 16 h after treatment, when the p53 protein reached its maximum level, a 4.1-, 6.9-, and 4.2-fold increase was observed for *p21*, *mdm2*, and *puma,* respectively. For the *bax* and *aip1* promoters an increase of binding was measured at 16 h after UV, but it remained below the percentage of binding measured at *p21*, *mdm2*, and *puma* promoters before irradiation (0.75, 0.2, and 0.39%, respectively; please note the different scale of Y-axis). Thus, the ChIP assay performed in human cells expressing endogenous wild-type p53, allowed us to distinguish between p53 target genes that give strong binding (*p21*, *mdm2*, *puma*) and target genes giving weak/background binding (*bax*, *aip1*). Our results are consistent with those reported by Kaeser and Iggo [2002] using different cell lines, including the lung cancer derived H1299 expressing wild-type p53 from an inducible cassette. Clearly, occupancy increases as p53 protein accumulates after treatment. A strong binding even before the UV stress was observed at *p21* promoter sequence, suggesting that p53 is recruited before UV treatment. Indeed, it has been shown that a pre-assembled initiation complex containing a paused RNA pol II is present at the *p21* promoter before induction of p53 by DNA damage and early *p21* gene activation does not require high level of p53 [Espinosa et al., 2003].

In LX1 and SKMes1 cells, expressing mutant p53, the amount of immunoprecipitated target DNAs was comparable to the non-specific binding observed at *gapdh* (Fig. 2). Thus, p53R273H

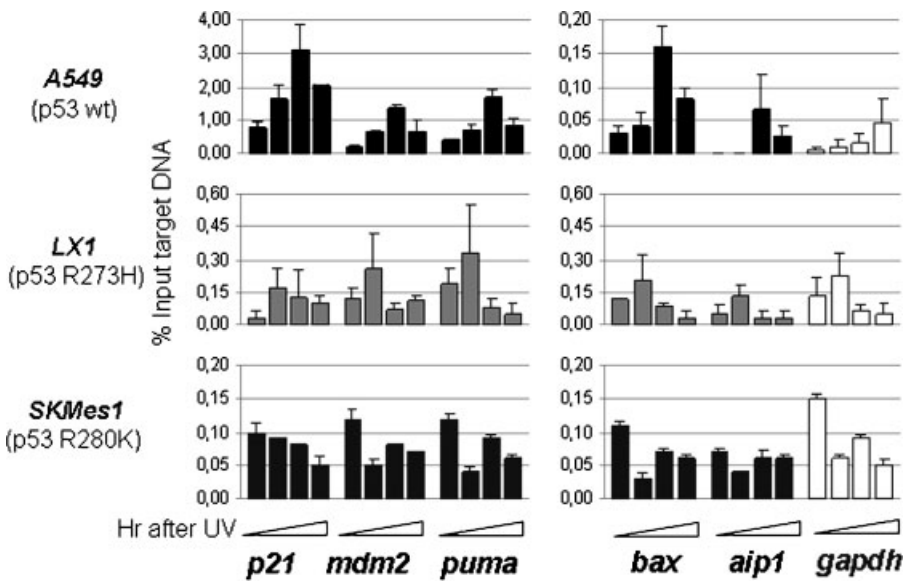

Fig. 2. Extent of binding of wild-type and mutant p53 to target genes in vivo. Quantitative ChIP results on A549, LX1, and SKMes1 at 0, 4, 16, 24 h after 30 J/m^2 UVC are shown. Primers specific for the region flanking the p53 REs in the promoter of *p21, mdm2, puma, bax,* and *aip1* genes were used. Amplification of *gapdh* was used to evaluate the amount of background binding and the ChIP specificity. For each target, the p53-binding was expressed as the percentage of the input chromatin. These data represent the average of two independent experiments. The error bars show the range of values for two independent experiments each assessed with three technical replicates.

and p53R280K proteins, although expressed at high level in these tumor cells, are not able to bind significantly or to be indirectly recruited at the gene targets analyzed.

Expression of *p21, mdm2,* and *bax* Genes: Activation of p53-Dependent and p53-Independent Responses After UV Irradiation

To determine whether the binding of p53 to the promoter of effector genes correlated with their expression, total RNA was isolated before the cross-linking treatment and used in quantitative RT-PCR. The fold change of mRNA expression for *p21*, *mdm2*, and *bax* genes after UV treatment is reported in Figure 3A. Values obtained with LX1 and SKMes1 were corrected to the expression levels in mock-irradiated A549 cells, as described in Materials and Methods.

In A549, a 2.8-fold increase of *p21* mRNA was detected at 16 h after UV, in agreement with the increase in p53-binding at the promoter. At 4 h time point, a twofold increase in p53 occupancy did not result in significant mRNA increase. In LX1 much lower amount of *p21* mRNA was detected, although an increase could be measured at later time. In SKMes1, *p21* transcription was also low and did not change after treatment. The low *p21* transcription detected in these mutated cell lines correlated with the lack of p53-binding at the promoter. For *mdm2*, a down modulation was observed at 4 h post-UV in A549 (Fig. 3A), followed by an increase at 16 and 24 h. Instead, p53 occupancy increased soon after UV irradiation (Fig. 2). In LX1 and SKMes1, no increase of expression after treatment was detected (Fig. 3A), in agreement with the lack of p53 occupancy at the *mdm2* promoter (Fig. 2). However, contrary to *p21* mRNA levels, *mdm2* expression is maintained at similar levels in the two p53 mutant cell lines, suggesting that

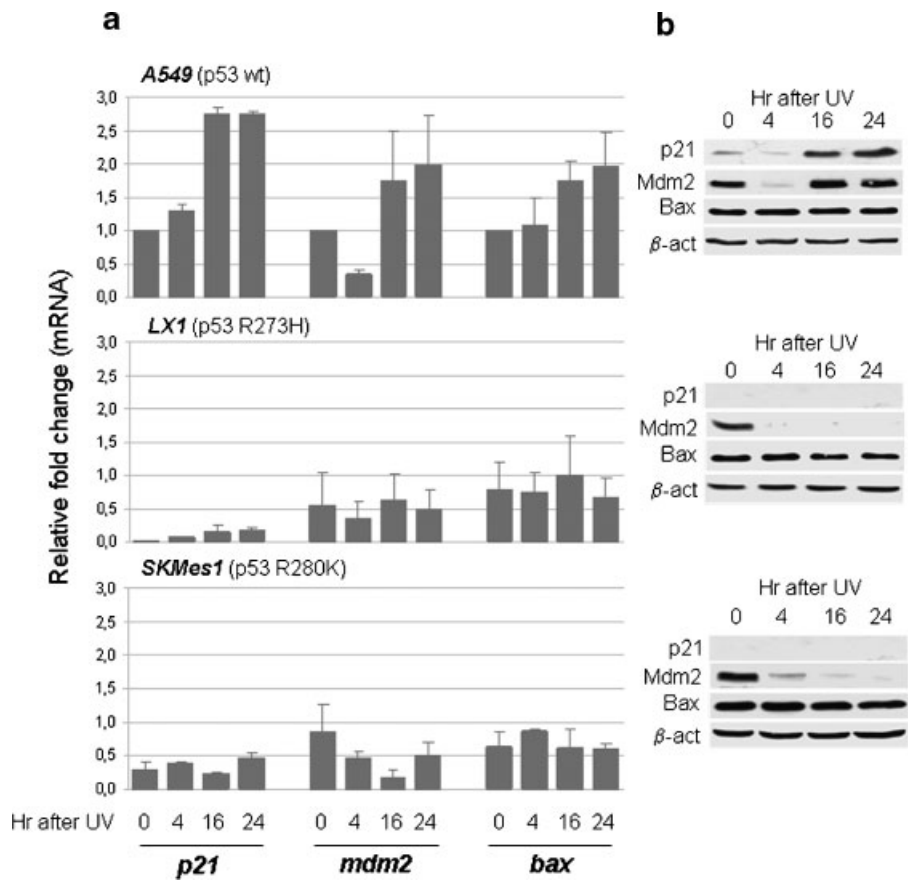

Fig. 3. Expression of p53 target genes. **A**: Real-time RT-PCR showing the level of *p21, mdm2,* and *bax* mRNA after exposure to 30 J/m^2 UVC. For each target, the level of mRNA was normalized to that of *gapdh*. The fold change was calculated using the Comparative Quantitation software of the RotorGene 3000 as described in Materials and Methods. Data represent the average of two independent experiments. The error bars show the range of values for two experiments. **B**: Western blots showing the level of p21, mdm2, and bax proteins at different times after 30 J/m^2 UVC. β-actin was used as loading control. Results are representative of at least three independent experiments; up to four experiments were made to confirm slight changes.

the *p21* promoter is highly dependent on active p53 or that there has been selection for sustained *mdm2* expression in the tumorigenesis processes. For the pro-apoptotic gene *bax*, the transcription increase two fold at 24 h post-UV in A549, while in cells carrying mutant p53, the mRNA level did not change up to 24 h.

The protein amounts for the various p53 target genes did not completely follow gene transcription (Fig. 3B). In the A549 cells, both p21 and mdm2 proteins decreased 4 h after UV irradiation compared to untreated cells but then increased at the 16 and 24 h time points. In LX1 and SKMes1 cell lines, the p21 protein was not detectable, while mdm2 was present before UV irradiation, decreased significantly 4 h post-treatment but did not recover after 16 or 24 h. Bax protein was detected in all cell lines and appeared to be independent from UV treatment and p53 status.

To better highlight the correlation between p53 occupancy levels to the patterns of mRNA and protein expression during the UV response, the results obtained in A549 cells for *p21* and *mdm2* genes were summarized in Figure 4. The main discrepancy was observed 4 h after UV when a p53-binding-independent response induced a downregulation of both p21 and mdm2 proteins. This negative regulation seemed to occur at different levels for the two genes. For the *p21* gene product, both p53-binding and mRNA expression were upregulated, suggesting that UV stress acted at a translational/post-translational levels. On the other hand, *mdm2* regulation occurred also at the transcriptional level, as the drop of the protein correlated with a drop of mRNA, although p53 occupancy increased already in the early time point. At later times, p53-binding contributed to the expression of these two genes and both mRNA and protein levels raised. The lack of both proteins in p53 mutant cell lines, where we could not measured a significant p53-binding, were consistent with the p53-binding-dependent upregulation of *p21* and *mdm2* genes at the two later time points analyzed.

p53-Dependent Histone Acetylation After UV Irradiation

Transcriptional regulation by p53 may be strongly influenced by chromatin structure, DNA topology, and recruitment of specific co-factors to binding sites that may direct the final outcome of p53-binding [Espinosa and Emerson, 2001; Espinosa et al., 2003]. The role of histone modifying enzymes and histone acetylation in transcriptional regulation has been widely demonstrated [Kadam and Emerson, 2002 and ref. therein]. Recently, it has been shown that when p53 binds to the RE in the *p21* promoter, the level of *p21* induction may reflects the ability of p53 to interact with histone acetyltransferase-containing proteins like p300/CBP and the extent of acetylated histones on the promoter [Liu et al., 2003]. Thus, we investigated whether the decrease of *mdm2* transcription observed in A549 shortly after UV irradiation could be linked to a low level of AcH3 and AcH4 histones on the promoter.

To measure the kinetics of histone acetylation at *p21* and *mdm2* promoters, quantitative

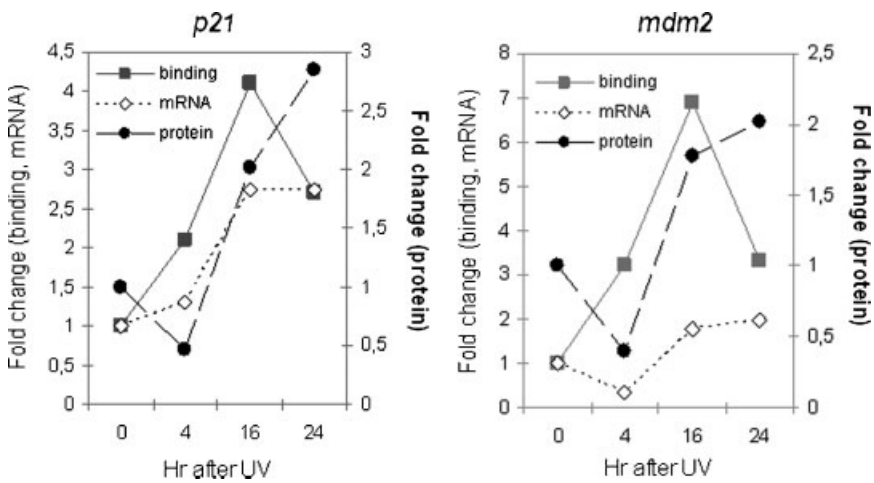

Fig. 4. Correlation between p53 occupancy, mRNA and protein expression during the UV response. The average fold change of p53-binding to the promoters, the mRNA and the protein levels for *p21* and *mdm2* genes in A549 cells are reported.

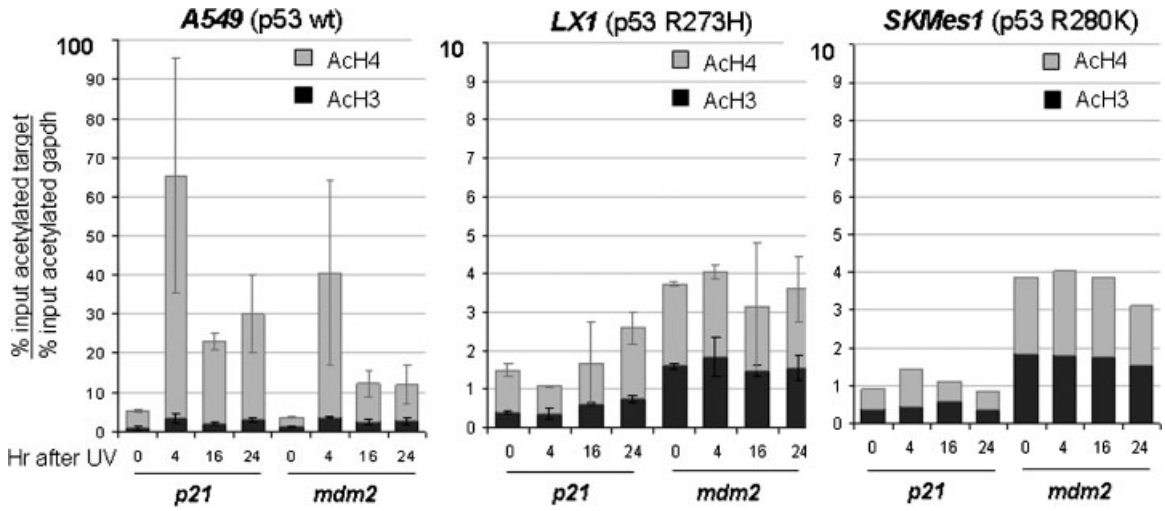

Fig. 5. Histone acetylation at the *p21* and *mdm2* promoters. A549 cells carrying wild-type p53 were treated with 30 J/m^2 UV-C and processed for ChIP assays using anti-acetyl-histone H3 and anti-acetyl-histone H4 antibodies. Primers that amplify the *p21* and *mdm2* promoter regions containing the p53 REs were used to determine the extent of H3 and H4 acetylation at these target sites before and after UV irradiation. The level of AcH3 and AcH4 in the *gapdh* gene was used to normalize for the overall acetylation levels.

ChIP-PCRs on the same fixed and sonicated extracts used to measure p53-binding, were performed (Fig. 5). In A549 cells, a sharp increase of AcH4 histones (about 14-fold) was measured at both promoters 4 h after UV irradiation, followed by a decrease at 16 and 24 h. Although the overall level of histone acetylation was lower for *mdm2* than for *p21* promoter, the amount of AcH4 histones on *mdm2* promoter 4 h after UV was the highest in the 24 h experimental window, suggesting that the decrease of *mdm2* transcription, observed shortly after treatment, could not depend on a low level of acetylated histones on the promoter.

Since it has been observed that the ability to induce histone acetylation was impaired in p53 mutants [Liu et al., 2003], we measured the kinetics of histone acetylation also in LX1 and SKMes1 cells. Interestingly, a complete different pattern was observed. First, the overall level of acetylated histones was much lower (~1/10th) than in cells carrying wild-type p53 and this was mainly due to a decrease in AcH4. Second, we did not observe a marked increase of acetylation after treatment, suggesting that p53 is responsible for much of the recruitment of histone acetylases on those promoters upon acute stress. Consistent with the expression levels, the *p21* promoter was less acetylated than the *mdm2* promoter. Moreover, while a slight increase of AcH4 was determined at 24 h in LX1, no changes were found in SKMes1 (Fig. 5). The acetylation levels of the *mdm2* were comparable in untreated cells independent of p53 status, but only in p53 wt A549, an increase in acetylation followed by gene upregulation was observed.

DISCUSSION

The goal of this work was to better investigate the contribution of p53-binding to the activation of some known p53 effector genes involved in crucial cellular pathways, like cell cycle control and apoptosis, in human tumor cells after a genotoxic treatment. We studied the ability of endogenously expressed wild-type and mutant p53s to bind in vivo the promoter sequences of genes normally activated by wild-type p53, that is, *p21*, *mdm2*, *bax*, *puma*, and *aip1*, the acetylation state of their promoters, and their transcription.

Following UV irradiation, p53 protein level increased and a strong binding of wild-type p53 to the promoters of *p21*, *mdm2*, and *puma*, was observed. Our DNA-binding data confirm the notion that the promoters of *p21*, *mdm2*, and *puma* genes contain strong binding sites for wild-type p53, while the p53 consensus sequences in the promoters of *bax* and *aip1* give weak/background binding [Kaeser and Iggo, 2002]. On the other hand, the two mutated proteins were unable to bind all the

targets analyzed. Both p53-R273H and p53-R280K lack transactivation functions as determined in model systems (http://www-p53.iarc.fr/index.html; http://p53.free.fr/). However, reports differ as R273H appeared to be competent for DNA binding in vitro as well as for transactivation of reporter constructs in some human carcinoma cell lines as SW620, HT-29, MDA-MB-468, K562, but not in H1299 [Park et al., 1994; Friedlander et al., 1996]. The conflicting results on DNA binding and transactivation activity for the R273H mutant could be due to the fact that the conformation of the p53-R273H protein is nearly identical to the conformation of the wild-type [Ory et al., 1994]. The differences between our results and some of the previously published studies could be related to the experimental set up, as in vitro DNA binding and ectopic reporter assays may not accurately recapitulate results with endogenous gene targets. However, an influence of the cellular environment and cell type on the activity of this mutant could not be excluded.

The mRNA and protein expression analysis showed that p53-binding to the promoter is necessary but insufficient for the activation of p53 effector genes and revealed an interplay between p53-dependent and p53-independent cellular response to UV irradiation. Already 4 h post-UV irradiation p53 occupancy at the *p21* promoter increased, consistent with the increase in p53 protein levels. In contrast, *p21* protein decreased. This decrease, as well as the uncoupling of *p21* mRNA and protein expression, is consistent with previous reports [Butz et al., 1998; Allan and Fried, 1999; Wang et al., 1999; Rieber and Strasberg Rieber, 2000; Izumaru et al., 2004], and indicates a strong *p21* modulation at the post-transcriptional level following DNA damage. Recently, it has been shown that only UV doses below 40 J/m^2 led to p21 protein degradation in human, mouse, and rat cells independent from *p21* transcription and p53 status (wild-type or mutant) [Bendjennat et al., 2003]. According to a model, p21 degradation would allow PCNA to participate in DNA repair. However, early recruitment of p21 protein to DNA damage sites, dependent on the interaction with PCNA, has been found in HeLa cells and in normal fibroblasts proficient in nucleotide excision repair (NER), but not in NER-deficient XP-A fibroblasts, suggesting a direct involvement of p21 in DNA repair [Perucca et al., 2006].

Also for *mdm2* some discrepancies among p53-binding, mRNA expression and protein level were found. At short times after UV, although the p53-binding at the promoter increased, both the mRNA and protein expression decreased and were upregulated only at later time points. This may indicate that at an initial level of induction, p53 is bound to the promoter but this binding is not sufficient to trigger transcription or there are factors that block transcription initiation. Alternatively (or in addition), messenger RNA instability could determine the initial mRNA and, consequently, protein drop. A disparity between p53-binding and gene expression after UV irradiation, has been also reported in WI-38 fibroblasts and in NT2 teratocarcinoma cells exposed to 10–20 J/m^2 UV-C, but not after γ-rays exposure [Kaeser and Iggo, 2002], suggesting that a specific "early" UV damage response leads to the repression of *mdm2* transcription. It has been reported that the timing of *mdm2* induction after UV treatment is UV dose-dependent. At UV doses higher than 20 J/m^2 the induction of *mdm2* could be delayed to allow p53 to arrest the cell cycle and perform DNA repair before the resumption of normal rates of DNA synthesis [Wu and Levine, 1997]. However, the regulation of *mdm2* after UV is still unclear and many factors, including the cell type and the properties of the promoters, may influence the timing of *mdm2* induction [Kim et al., 1999; Perry, 2004]. Interestingly, for all cell lines under study, mdm2 protein was found highly expressed in mock-irradiated cells. This suggest that the early regulation of mdm2 protein, as well as the basal level of mdm2 expression, have been conserved in the three cell lines, regardless of their p53 status and their cellular transformation pathway.

When the pro-apoptotic *bax* gene was studied, we found that in A549 the mRNA expression increased about twofold after stress, while in LX1 and SKMes1 cells no p53-binding and no variation in mRNA expression were found. Constant high levels of bax protein were detected before and after UV irradiation in all cell lines. This indicates that an accumulation of the bax protein occurred in these tumor cells, independently from p53 transactivation and from UV exposure. There are several explanations for this lack of correlation, including for example the net balance between pro- and anti-apoptotic BH3 proteins. In addition, there is

evidence showing that p53 may have transcription-independent activities [Yee and Vousden, 2005] and one such reported activity is the direct activation of bax by cytosolic p53 [Chipuk et al., 2004], which could be impacted by the specific nature of the p53 protein.

In our study with human cancer cell lines the main discrepancy between p53 promoter occupancy, mRNA and protein expression of target genes was observed at short time after UV treatment. Possible players in the modulation of transcription are histone modifying enzymes and the levels of acetylated histones [Kadam and Emerson, 2002]. It has been shown that the acetylation state of the promoter and the ability of p53 to interact with histone acetyltransferase-containing proteins may influence *p21* induction [Liu et al., 2003]. Thus, we investigated whether the decrease of *mdm2* transcription observed in A549 shortly after UV irradiation could be linked to a low H3 and H4 acetylation levels. We found that this was probably not the case, since high level of AcH3 and AcH4 histones were measured at 4 h post-UV on *p21* as well as on *mdm2* promoters. However, we cannot rule out that the level of histone acetylation in other, not explored, regulatory regions of *mdm2* may influence its early transcription rate. The difference in the extent of histone acetylation measured at the *mdm2* and *p21* promoters could represent a feature of the promoter itself. Indeed, heterogeneous patterns of histone acetylation for different p53 target gene promoters following 5-FU treatment has been demonstrated [Kaeser and Iggo, 2004].

We think that the peak of H4 histone acetylation detected soon after irradiation can reflect two parallel, p53-dependent UV-induced responses, one of which is not linked to transcriptional regulation but can be instead related to DNA repair. It has been proposed that p53 can function as a chromatin accessibility factor, by mediating histone acetylation and recruitment of p300 to sites of NER [Rubbi and Milner, 2003; Allison and Milner, 2004]. According to this model, an inhibition of transcription elongation induced by UV lesions may trigger p53-dependent global chromatin relaxation and facilitate DNA repair. Indeed, it has been shown that homozygous p53 mutant cell were deficient in global genomic repair (GGR) of cyclobutane pyrimidine dimers while the heterozygous primary LFS cells exhibited normal GGR [Ford, 2005]. Interestingly, a recent study showed that a new tumor suppressor gene, p33ING2, significantly enhances NER in a p53-dependent manner by rapidly inducing histone H4 acetylation, chromatin relaxation, and the recruitment of the damage recognition factor xeroderma pigmentosum group A protein to photolesion sites [Wang et al., 2006].

While the DNA repair ancillary function of p53 has been associated to GGR and DNA damage foci, we hypothesize it has implications for all p53-binding sites, including those proximal to TSS, where occupancy by p53 is also associated to transcriptional modulation. Following the repair of UV-induced lesions, the original state of chromatin condensation has to be restored but a higher level of AcH3 and AcH4 could be maintained at sites that needs to be engaged in p53-dependent transcription as part of the genotoxic response. Notably, in cells expressing a mutant p53 unable to bind its target promoters, the recruitment of acetylated histone on the promoters was not observed. The extent and the kinetics of histone acetylation associated with transcriptional activation are influenced by the location of the enhancer site responsible for the recruitment of chromatin modifiers relative to the proximal promoter, as revealed by high-resolution ChIP, also in the case of p53-mediated transcription [Barlev et al., 2001; Espinosa and Emerson, 2001; Espinosa et al., 2003]. However, the same pattern of H4 acetylation changes was apparent independent of the position relative to the TSS of the DNA region containing the p53 REs that we probed (~2.2-kb upstream from TSS for *p21*; ~0.7-kb downstream TSS in intron 1, for *mdm2*). This observation was confirmed with *puma* and *bax* genes, where a proximal promoter region (~0.5-kb upstream of TSS) was analyzed in the ChIP experiments (data not shown). These findings further suggest that a transcription-independent UV-response is acting at short times after irradiation at the promoter of p53 effector genes containing p53 REs.

Our results on *p21* and *mdm2* modulation also indicate that an interaction between the p53-independent UV responses and p53 activity may occur. Shortly after UV, degradation of p21 proteins could be related to DNA repair while degradation of mdm2 and delayed *mdm2* transcription could affect *mdm2*-related ubiquitination of p53 and possibly of histone H2B [Minsky and Oren, 2004] and may promote DNA repair processes. At later times and depending on the

extent of residual DNA damage [Christophorou et al., 2005], p53-dependent transcription may develop to elicit specific cellular responses.

ACKNOWLEDGMENTS

R.M. was recipient of a fellowship from the National Institute for Cancer Research. D.R. was recipient of a PhD programme fellowship of the University of Genova. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Ministero della Salute, Fondo per gli Investimenti della Ricerca di Base (FIRB), and Alleanza Contro il Cancro (ACC).

REFERENCES

Allan LA, Fried M. 1999. p53-dependent apoptosis or growth arrest induced by different forms of radiation in U2OS cells: p21WAF1/CIP1 repression in UV induced apoptosis. Oncogene 18:5403–5412.

Allison SJ, Milner J. 2004. Remodelling chromatin on a global scale: A novel protective function of p53. Carcinogenesis 25:1551–1557.

Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL. 2001. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell 8:1243–1254.

Bendjennat M, Boulaire J, Jascur T, Brickner H, Barbier V, Sarasin A, Fotedar A, Fotedar R. 2003. UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. Cell 114:599–610.

Blandino G, Levine AJ, Oren M. 1999. Mutant p53 gain of function: Differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene 18:477–485.

Bossi G, Lapi E, Strano S, Rinaldo C, Blandino G, Sacchi A. 2006. Mutant p53 gain of function: Reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene 25:304–309.

Butz K, Geisen C, Ullmann A, Zentgraf H, Hoppe-Seyler F. 1998. Uncoupling of p21WAF1/CIP1/SDI1 mRNA and protein expression upon genotoxic stress. Oncogene 17: 781–787.

Campomenosi P, Monti P, Aprile A, Abbondandolo A, Frebourg T, Gold B, Crook T, Inga A, Resnick MA, Iggo R, Fronza G. 2001. p53 mutants can often transactivate promoters containing a p21 but not Bax or PIG3 responsive elements. Oncogene 20:3573–3579.

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303: 1010–1014.

Christophorou MA, Martin-Zanca D, Soucek L, Lawlor ER, Brown-Swigart L, Verschuren EW, Evan GI. 2005. Temporal dissection of p53 function in vitro and in vivo. Nat Genet 37:718–726.

Espinosa JM, Emerson BM. 2001. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell 8:57–69.

Espinosa JM, Verdun RE, Emerson BM. 2003. p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. Mol Cell 12:1015–1027.

Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, Sappino AP, Limacher IM, Bron L, Benhattar J, et al. 1995. A simple p53 functional assay for screening cell lines, blood, and tumors. Proc Natl Acad Sci USA 92:3963–3967.

Ford JM. 2005. Regulation of DNA damage recognition and nucleotide excision repair: Another role for p53. Mutat Res 577:195–202.

Friedlander P, Haupt Y, Prives C, Oren M. 1996. A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. Mol Cell Biol 16:4961–4971.

Harris SL, Levine AJ. 2005. The p53 pathway: Positive and negative feedback loops. Oncogene 24:2899–2908.

Izumaru S, Arima N, Toyozumi Y, Kato S, Morimatsu M, Nakashima T. 2004. Down-regulation of p21Waf-1 protein facilitates IR- and UV-induced apoptosis in human squamous carcinoma cells. Int J Oncol 24:1245–1255.

Kadam S, Emerson BM. 2002. Mechanisms of chromatin assembly and transcription. Curr Opin Cell Biol 14:262–268.

Kaeser MD, Iggo RD. 2002. Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci USA 99:95–100.

Kaeser MD, Iggo RD. 2004. Promoter-specific p53-dependent histone acetylation following DNA damage. Oncogene 23:4007–4013.

Kim E, Rohaly G, Heinrichs S, Gimnopoulos D, Meissner H, Deppert W. 1999. Influence of promoter DNA topology on sequence-specific DNA binding and transactivation by tumor suppressor p53. Oncogene 18:7310–7318.

Liu G, Xia T, Chen X. 2003. The activation domains, the proline-rich domain, and the C-terminal basic domain in p53 are necessary for acetylation of histones on the proximal p21 promoter and interaction with p300/CREB-binding protein. J Biol Chem 278:17557–17565.

Loprevite M, Varesco L, Favoni R, Ferrara GBS, Moro F, Ottaggio L, Fronza G, Campomenosi P, Abbondandolo A, Cutrona G, Roncella S, Albini A, Aluigi MG, Pozzi S, Pera C, Biticchi R, Gismondi V, Grossi F, Pennucci MC, Ardizzoni A. 1997. Analysis of K-ras, p53, bcl-2 and Rb expression in non-small cell lung cancer cell lines. Int J Oncol 11:1203–1208.

McCormick F. 1999. Cancer therapy based on p53. Cancer J Sci Am 5:139–144.

Menendez D, Inga A, Resnick MA. 2006. The biological impact of the human master regulator p53 can be altered by mutations that change the spectrum and expression of its target genes. Mol Cell Biol 26:2297–2308.

Minsky N, Oren M. 2004. The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression. Mol Cell 16:631–639.

Monti P, Campomenosi P, Ciribilli Y, Iannone R, Inga A, Abbondandolo A, Resnick MA, Fronza G. 2002. Tumour p53 mutations exhibit promoter selective dominance over wild type p53. Oncogene 21:1641–1648.

Monti P, Campomenosi P, Ciribilli Y, Iannone R, Aprile A, Inga A, Tada M, Menichini P, Abbondandolo A, Fronza G.

2003. Characterization of the p53 mutants ability to inhibit p73 beta transactivation using a yeast-based functional assay. Oncogene 22:5252–5260.

Ory K, Legros Y, Auguin C, Soussi T. 1994. Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation. Embo J 13:3496–3504.

Pan Y, Haines DS. 2000. Identification of a tumor-derived p53 mutant with novel transactivating selectivity [In Process Citation]. Oncogene 19:3095–3100.

Park DJ, Nakamura H, Chumakov AM, Said JW, Miller CW, Chen DL, Koeffler HP. 1994. Transactivational and DNA binding abilities of endogenous p53 in p53 mutant cell lines. Oncogene 9:1899–1906.

Perry ME. 2004. Mdm2 in the response to radiation. Mol Cancer Res 2:9–19.

Perucca P, Cazzalini O, Mortusewicz O, Necchi D, Savio M, Nardo T, Stivala LA, Leonhardt H, Cardoso MC, Prosperi E. 2006. Spatiotemporal dynamics of p21CDKN1A protein recruitment to DNA-damage sites and interaction with proliferating cell nuclear antigen. J Cell Sci 119:1517–1527.

Resnick MA, Inga A. 2003. Functional mutants of the sequence-specific transcription factor p53 and implications for master genes of diversity. Proc Natl Acad Sci USA 100:9934–9939. Epub 2003 Aug 8.

Rieber M, Strasberg Rieber M. 2000. Apoptosis-inducing levels of UV radiation and proteasome inhibitors produce opposite effects on p21(WAF1) in human melanoma cells. Int J Cancer 86:462–467.

Rubbi CP, Milner J. 2003. p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage. Embo J 22:975–986.

Sigal A, Rotter V. 2000. Oncogenic mutations of the p53 tumor suppressor: The demons of the guardian of the genome. Cancer Res 60:6788–6793.

Soussi T, Beroud C. 2001. Assessing TP53 status in human tumours to evaluate clinical outcome. Nature Rev Cancer 1:233–240.

Soussi T, Lozano G. 2005. p53 mutation heterogeneity in cancer. Biochem Biophys Res Commun 331:834–842.

Szak ST, Mays D, Pietenpol JA. 2001. Kinetics of p53 binding to promoter sites in vivo. Mol Cell Biol 21:3375–3386.

Szymanska K, Hainaut P. 2003. TP53 and mutations in human cancer. Acta Biochim Pol 50:231–238.

Vogelstein B, Lane D, Levine AJ. 2000. Surfing the p53 network. Nature 408:307–310.

Wang JA, Fan S, Yuan RQ, Ma YX, Meng Q, Goldberg ID, Rosen EM. 1999. Ultraviolet radiation down-regulates expression of the cell-cycle inhibitor p21WAF1/CIP1 in human cancer cells independently of p53. Int J Radiat Biol 75:301–316.

Wang J, Chin MY, Li G. 2006. The novel tumor suppressor p33ING2 enhances nucleotide excision repair via inducement of histone H4 acetylation and chromatin relaxation. Cancer Res 66:1906–1911.

Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH, Ruan Y. 2006. A global map of p53 transcription-factor binding sites in the human genome. Cell 124:207–219.

Weisz L, Zalcenstein A, Stambolsky P, Cohen Y, Goldfinger N, Oren M, Rotter V. 2004. Transactivation of the EGR1 gene contributes to mutant p53 gain of function. Cancer Res 64:8318–8327.

Wu L, Levine AJ. 1997. Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. Mol Med 3:441–451.

Yee KS, Vousden KH. 2005. Complicating the complexity of p53. Carcinogenesis 26:1317–1322.