The role of p53.S389 phosphorylation in DNA damage response pathways and tumorigenesis

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Chapter 4

Lack of p53.S389 phosphorylation predisposes mice to develop 2-acetylaminoflurene-induced bladder tumors but not ionizing radiation-induced lymphomas

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Abstract

Cellular activity of the tumor suppressor protein p53 is primarily regulated by post-translational modifications. Phosphorylation of the COOH-terminus, including Ser389, is thought to result in a conformational change of the p53 protein, enhancing DNA binding and transcriptional activity. *In vitro* studies presented here show that, in addition to UV radiation, Ser389 is phosphorylated upon exposure to 2-acetylaminofluorene (2-AAF). Both agents induce bulky DNA adducts repaired by nucleotide excision repair (NER). In contrast, ionizing radiation, known to induce DNA damage not repaired by NER, does not result in Ser389 phosphorylation. Previously, we have shown that p53.S389A mutant mice, lacking the Ser389 phosphorylation site, are sensitive to developing UV-induced skin tumors. Here, we show that p53.S389A mice are also prone to developing 2-AAF-induced urinary bladder tumors, whereas no increased tumor response was found upon ionizing irradiation. These results provide evidence for our hypothesis that phosphorylation of Ser389 is important for activation of p53 to exert its function as a tumor suppressor not exclusively upon the presence of UV-induced DNA damage, but also upon exposure to other bulky adduct-inducing agents. Analysis of 2-AAF- and UV-induced tumors from p53.S389A mice revealed the presence of additional *p53* mutations, indicating that lack of Ser389 phosphorylation by itself is not sufficient to abrogate p53 function in tumor suppression.

In addition, analyses of skin tumors of p53.S389A mice revealed an interesting hotspot mutation previously found exclusively in NER-deficient mice and patients.

Introduction

Cancer is caused by loss of controlled cell growth, due to mutational inactivation or activation of genes involved in cell cycle regulation. Several mechanisms are known to be critical in the prevention of cells from becoming cancerous. Of these, cell cycle control and DNA repair result in the removal of DNA damage before mutations are formed, whereas the process of apoptosis eliminates cells with sustained DNA damage. These cellular responses are mediated, at least in part, by the p53 tumor suppressor protein[1-3].

The levels of p53 protein in normal unstressed cells are very low due to rapid protein degradation mediated by MDM2 [4]. However, in response to DNA damage or other sources of cellular stress, p53 protein levels increase and its transcriptional activity is greatly increased [5]. The key mechanism of p53 activation is post-translational modification, including (de)phosphorylation, acetylation, and sumoylation [1-3]. Several sites have been shown to be potential targets for modifications, and affect p53 functions in a variety of ways. For example, phosphorylation at its NH2-terminus disrupts the interaction of p53 with the MDM2 protein, thereby preventing degradation and thus stabilizing the p53 protein [6-8]. Modifications of the p53 COOH-terminus are thought to result in a conformational change, thereby enhancing DNA-specific binding and transcriptional activity [9;10]. The specific patterns of p53 post-translational modifications seem to be dependent on the specific type of agent to which the cell is exposed to. At the COOH-terminal regulatory domain, Ser315 is phosphorylated after exposure to both UV-light and ionizing radiation [9;11]. This is in contrast with Ser392 (equivalent to mouse Ser389), which is specifically phosphorylated upon UV radiation [12;13]. Kinases responsible for Ser389 phosphorylation include CKII, p38 Map kinase, and PKR [13-16].

To enable the *in vivo* functional analysis of a specific p53 phosphorylation site, we recently generated mice with a single point mutation in the *p53* gene, resulting in a serine to alanine

substitution at amino acid residue 389 (p53.S389A mutation) [17]. Absence of the Ser389 site resulted in reduced DNA binding capacity and reduced expression levels of p53 target genes, indicating that the transcription-regulating activity of p53 is adversely affected. In addition, the apoptotic response was impaired in cells lacking the Ser389 phosphorylation site. In line with these *in vitro* findings, the p53.S389A mutation resulted in an impaired p53 function *in vivo*, as mice carrying the mutation were more susceptible to develop skin tumors upon exposure to UV-light [17]. Both survival and latency time of spontaneous tumor development in these mutant mice were, however, not altered, indicating that adverse effects of lack of Ser389 phosphorylation are linked to exposure to genotoxic stress, i.e., UV-induced bulky DNA adducts.
Apart from UV-light, a variety of genotoxic agents are known to induce bulky DNA adducts, all of which are substrates for nucleotide excision repair (NER) [18-20]. Exposure to this class of compounds might result in the activation of p53 through a uniform pattern of specific post-translational modification events, including phosphorylation of Ser389. If so, absence of the Ser389 phosphorylation site could result in an adversely affected p53 tumor suppressor function after exposure to NER-related agents. To test this hypothesis, we did a carcinogenicity study with p53.S389A mice that were exposed to an additional NER-specific compound, 2-acetylaminofluorene (2-AAF). As a control, we analyzed tumor development of p53.S389A mice upon exposure to ionizing radiation, inducing non-NER-related DNA damage. Our *in vitro* and *in vivo* studies suggested that Ser389 phosphorylation is not exclusively involved in p53 activation after UV-light, but rather seems related to a broader class of compounds inducing similar types of DNA damage.

Materials and Methods

Western blot analysis

Wild-type and $p53^{-/-}$ mouse embryonic fibroblasts (MEF) were expanded in culture flasks (Greiner) and plated at a density of 1.2 $*10^6$ cells/10 cm dish (plate). Twenty-four hours later, cells were washed with PBS and exposed to 5 Gy ionizing radiation, 20 J/m^2 UV-C light or 12 µmol/L NA-AAF. Six hours later, Western blot analyses were done as described previously by Bruins *et al.*[17]. Briefly, p53 immunoprecipitation was done with an anti-p53 mouse monoclonal antibody Ab-4 (Oncogene Research Products, San Diego, CA). The concentrated samples were separated on polyacrylamide electrophoresis gels (NuPAGE, Invitrogen, Carlsbad, CA) and transferred to Hybond polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The original lysates were also separated and transferred as described above. Subsequently, membranes were incubated with either anti-p53 mouse antibody Ab-1 (Oncogene Research Products) or anti-phospho-p53 rabbit polyclonal antibody Ser392 (Cell Signaling, Beverly, MA) which is known to cross-react with mouse Ser389. Primary antibodies were detected by incubating the membrane with horseradish peroxidase-linked sheep antimouse IgG or horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Pharmacia Biotech). The membranes with remnants were incubated with an anti-actin affinity-purified goat polyclonal antibody I-19-HRP (Santa Cruz, Santa Cruz, CA). Staining was done by using enhanced chemiluminescence plus reagent (Amersham Pharmacia Biotech). The membranes were scanned using a PhophorImager (Storm 860, Molecular Dynamics, Sunnyvale, CA).

Mice

P53.S389A mice [17] were crossed with $p53^{+/-}$ mice [21] to obtain $p53^{S389A/-}$ mice. For the

survival experiments, the 2-AAF and ionizing radiation exposure experiments, mice were all in a mixed 129Sv/C57BL/6 background. For the UV-B light experiment, mice were crossed into a hairless background (SKH:HRA).

In vivo 2-acetylaminofluorene treatment

For the 2-AAF study, wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice (ages 6-9 weeks) were treated for 39 weeks with 300 ppm 2-AAF in the diet, and were subsequently fed a normal diet for 2 weeks. All treatment groups consisted of 30 mice per genotype (15 males and 15 females). The control groups consisted of 10 mice per genotype (5 males and 5 females) and were fed a normal diet for 41 weeks. Mice that became moribund or lost >20% of their bodyweight were sacrificed intercurrently.

In vivo exposure to ionizing radiation

The ionizing radiation study was done with wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice (ages 6-9 weeks). All groups, consisting of 15 mice (approximately equal numbers of males and females), were exposed to a single dose of 5 Gy of X-rays with a dose rate of 0.31 Gy/minute at 200 kV. Control mice of all genotypes were followed as they aged (cf. 17). Mice that became moribund or lost >20% of their bodyweight were sacrificed intercurrently. All surviving mice were scheduled for autopsy 54 weeks after the radiation.

In vivo UV irradiation

For the chronic UV-B irradiation experiment, hairless wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice (ages 6-9 weeks) were exposed daily to a UV-B light dose of 600 J/m^2 using Philips TL12 lamps as previously described [17]. The UV-irradiated wildtype, $p53^{S389A/+}$, $p53^{S389A/-}$, and $p53^{+/-}$ groups consisted of 14 mice per genotype (7 males and 7 females), the UV-irradiated p53.S389A group consisted of 20 mice (approximately equal numbers of males and females). Mice that had developed a tumor >3 mm, became moribund, or lost >20% of their bodyweight were sacrificed. Necropsy and (histo)pathology. Upon necropsy, tissues and macroscopically found tumors were fixed in 3.8% neutral buffered formaldehyde, and a slice of the tumors was snap-frozen in liquid N_2. For histopathologic analysis, the formaldehyde-fixed samples were embedded in paraffin wax, cut into 5 µm sections and stained with H&E. Sequence analysis of the *p53* gene. Frozen skin tumors of UV-treated mice were analyzed for acquired *p53* mutations by direct sequencing. For this, DNA was isolated using the DNA Isolation Kit for blood/ bone marrow/ tissue (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Part of the *p53* gene, spanning exons 2 to 8, were amplified using the HotStarTaq Master Mix Kit (Qiagen, Valencia, CA). First, exons 2 to 4 or exons 5 to 8 were amplified using the following primers:

Exons 2 to 4: 5'-CAAGTTATGCATCCATACAG-3' and
5'-TCAGGGCAAAACTAAACTCT-3'

Exons 5 to 8: 5'-GAGGGCGTCCAATGGTGCTT-3' and
5'-GGTGGGCAGCGCTGTGGAAGG-3'

Subsequently, regions spanning exon 2, exons 3 to 4, exons 5 to 6, or exons 7 to 8 were re-amplified using the following primers:

Exon 2: 5'-CAAGTTATGCATCCATACAG-3' and
5'-TTTGTTTCTCTCAGGCAAGG-3'

Exons 3 to 4: 5'-GCCTGGGATAAGTGAGATTC-3' and
5'-TCAGGGCAAAACTAAACTCT-3'
Exons 5 to 6: 5'-AGTTCCCCACCTTGACACCT-3' and
5'-GTCAACTGTCTCTAAGACGCA-3'
Exons 7 to 8: 5'-TTCCCGGCTGCTGCAGGTCA-3' and
5'-AGGAGAGAGCAAGAGGTGACT-3'

Purified PCR products (QIAquick PCR Purification Kit, Qiagen) were directly sequenced using the Big Dye Terminator Sequence kit version 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3700 DNA sequencer. The primers were the same as used for re-amplification PCR (see above).

Statistical analyses

The statistical significance of tumor responses were calculated with the Kaplan-Meyer test for UV-light and ionizing radiation studies, and with one-tailed Fisher's exact test for the 2-AAF carcinogenicity study. The level of statistical significance was taken as $p \leq 0.05$.

Results

Phosphorylation of p53.S389 upon *in vitro* exposure to genotoxic agents

We have previously shown that p53.S389 is phosphorylated *in vitro* upon exposure to UV light [17]. In the present study, we analyzed p53 protein after exposure to NA-AAF (a reactive metabolite of 2-AAF) to analyze whether p53.S389 phosphorylation is also associated with exposure to another NER-specific agent. For this purpose, wild-type and $p53^{-/-}$ MEFs were exposed to 12 μmol/L NA-AAF, 20 J/m² UV light or 5 Gy ionizing radiation and levels of total p53 protein and p53 protein phosphorylated at Ser389 were determined (Figure 1). In response to NA-AAF treatment, UV irradiation and ionizing radiation, the total p53 protein levels increased significantly in wild-type MEFs as compared with the expected low p53 levels in untreated wild-type MEFs. In contrast, Ser389 phosphorylation was detectable upon NA-AAF treatment and UV irradiation, whereas no phosphorylation at this site was found upon ionizing irradiation. Similar observations were made at additional time points as well as after exposure to different doses (data not shown and [17]).

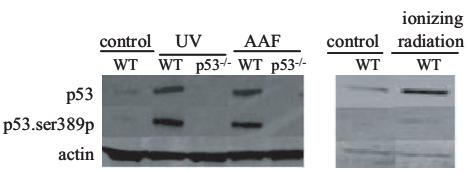

Figure 1 - p53 protein levels in MEFs upon exposure to ionizing radiation, UV-light or NA-AAF.
MEFs were exposed to 20 J/m² UV-light, 12 μM NA-AAF or 5 Gy ionizing radiation. Six hours later, total p53 protein levels and Ser389 phosphorylated p53 protein levels were visualized by Western blotting. The residuals of the samples were incubated with an anti-actin antibody to quantify the total protein levels present in the samples.

2-Acetylaminofluorene–induced tumor responses in p53.S389A mice

In line with our previously described chronic UV-light study with p53.S389A mutant mice [17], we analyzed the tumor responses of p53.S389A mice after exposure to the NER-specific compound 2-AAF. This study revealed that p53.S389A mice are more susceptible than wild-type mice to develop 2-AAF–induced urinary bladder lesions (see Table I). An increased number of both atypical foci as well as transitional cell tumors was observed in p53.S389A mice as compared with wild-type mice ($p = 0.05$, see supplementary Figure 3A-C for typical examples).

Table I - 2-AAF-induced tumor development in p53.S389A mice

Genotype	WT		$p53^{S389A/+}$		p53.S389A		$p53^{S389A/-}$		$p53^{+/-}$	
dose 2-AAF (in ppm)	0	300	0	300	0	300	0	300	0	300
Urinary bladder										
number examined	**10**	**30**	**9**	**25**	**10**	**28**	**10**	**26**	**10**	**26**
Atypia		1		3		5		12		11
Transitional cell papilloma						3		2		2
Carcinoma in situ						1		2		
Transitional cell carcinoma								2		8
Tumor incidence (%)	**0**	**0**	**0**	**0**	**0**	**14†**	**0**	**23†**	**0**	**38*†**
Liver										
number examined	**10**	**30**	**10**	**25**	**10**	**27**	**10**	**26**	**10**	**29**
Hypertrophy/pleomorphisms										
Hepatocellular adenoma		2		1				1	1	3
Hepatocellular carcinoma								1		3
Tumor incidence (%)	**0**	**7**	**0**	**4**	**0**	**0**	**0**	**8**	**10**	**21**

Tumor incidences were calculated by dividing the total number of tumors (either transitional cell papilloma, carcinoma in situ and transitional cell carcinomas in urinary bladder; or hepatocellular adenomas and carcinomas) by the number of mice examined.
* $p \leq 0.05$ as compared to untreated control mice, † $p \leq 0.05$ as compared to 2-AAF treated WT mice
WT = wild-type

$P53^{S389A/+}$ mice developed some atypical lesions, but no urinary bladder tumors. Both $p53^{S389A/-}$ and $p53^{+/-}$ mice showed a significantly increased urinary bladder tumor response as compared with wild-type mice upon exposure to 2-AAF ($p = 0.007$ and $p = 0.0001$, respectively). The 2-AAF-induced urinary bladder tumor incidence of $p53^{+/-}$ mice (38%) is in line with our previous results (29%) [22]. No significant difference was found between the tumor incidences of $p53^{+/-}$ as compared with $p53^{S389A/-}$ mice.

In liver, 2-AAF treatment resulted in a low incidence of hepatocellular tumors in all genotypes tested (see Table I; supplementary Figure 3D and E for typical examples). According to these results, the absence of the Ser389 phosphorylation site clearly did not affect 2-AAF-induced liver tumor development.

Previously, immunohistochemical studies indicated that ~50% of 2-AAF-induced urinary bladder tumors obtained from $p53^{+/-}$ mice carried additional *p53* mutations in the remaining wild-type allele [22]. This high incidence of *p53* mutations was also observed in this study, as two out of five urinary bladder tumors obtained from $p53^{+/-}$ mice were positive for both the CM5 antibody (recognizing up-regulated wild-type and mutated p53 protein) and Pab240 antibody (specific for mutated p53 protein only; see supplementary Table III; and supplementary Figure 4A and B for typical examples and supplementary Materials and Methods for protocol). In addition, we detected CM5- and Pab240-positive fields in 2-AAF–induced urinary bladder tumors obtained from both $p53^{S389A/-}$ (two out of five tumors analyzed) and p53.S389A (two out of four tumors analyzed) mice, indicating that additional *p53* mutations were acquired in the process of tumor development in these mice. No clear difference was found in the number or size of p53-positive fields in mice carrying the Ser389 mutation (p53.S389A as well as $p53^{S389A/-}$ mice) as compared with $p53^{+/-}$ mice (supplementary Table III).

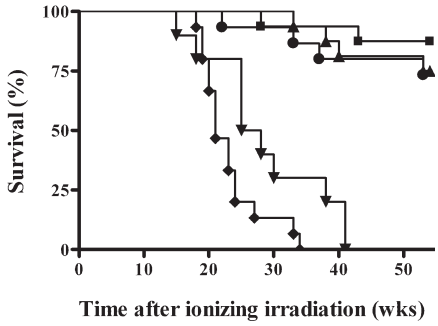

Figure 2 - Survival of ionizing irradiated mice
Wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice were exposed to a single dose of ionizing radiation (5 Gy), and monitored for the development of tumors. Mice were scheduled for autopsy at 54 weeks after the gamma treatment.
wild-type (■), $p53^{S389A/+}$ (▲), p53.S389A(●), $p53^{S389A/-}$(◆), $p53^{+/-}$ (▼)

Survival of p53.S389A mice upon exposure to ionizing radiation

Clearly, as shown in Figure 1 and described before [17], exposure to ionizing radiation does not result in phosphorylation of Ser389. To analyze whether absence of Ser389 phosphorylation effects ionizing radiation–induced tumor development *in vivo*, we exposed wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice to a single dose of 5 Gy radiation. No difference was found between the survival of wild-type, $p53^{S389A/+}$ or p53.S389A mice as 87%, 73%, and 73%, respectively, survived at least 54 weeks after the ionizing irradiation (Figure 2). However, $p53^{+/-}$ mice showed a severely reduced survival after irradiation (50% survival at 24 weeks, $p < 0.0001$ as compared with wild-type mice), which is in line with observations made by others [23;24]. Survival of $p53^{S389A/-}$ mice did not significantly differ from that of $p53^{+/-}$ mice (50% survival at 21 weeks), again indicating that absence of the Ser389 phosphorylation site has no effect on ionizing radiation–induced tumor development in mice. The irradiation protocol used resulted in the development of predominantly lymphoblastic lymphomas (either generalized or residing in the thymus) as all intercurrently sacrificed mice had developed this tumor type, irrespective of the p53 genotype.

Ionizing radiation–induced lymphomas have previously been shown to display loss of heterozygosity at a high frequency in $p53^{+/-}$ mice [23]. In line with these results, we found loss of heterozygosity in four out of five (80%) of the lymphomas isolated from $p53^{+/-}$ (see supplementary Figure 5 for typical examples). Also, seven out of eight (88%) of the lymphomas obtained from $p53^{S389A/-}$ mice displayed loss of heterozygosity. Thus, lack of Ser389 phosphorylation in mice does not affect incidence, latency time, and underlying molecular events of ionizing radiation-induced tumor development.

UV-induced skin tumor development in p53.S389A mice

We previously showed that p53.S389A mutant mice developed skin tumors significantly earlier than their wild-type littermates upon exposure to UV light, indicating that the absence of Ser389 phosphorylation influences the onset of skin tumor development [17](supplementary Figure 6). In the present study, we compared UV-induced tumor responses of $p53^{S389A/-}$ mice and $p53^{+/-}$ mice. The tumor latency time of $p53^{S389A/-}$ mice was slightly, but not significantly, reduced compared with $p53^{+/-}$ mice (supplementary Figure 6). Comparing the tumor response of p53.S389A mice with that of $p53^{S389A/-}$ animals revealed that absence of Ser389 phosphorylation resulted in a less severe cancer phenotype compared with complete absence of one p53 allele ($p53^{S389A/-}$ mice).

Acquired *p53* gene mutations in UV-induced skin tumors of p53.S389A mice

Alteration of p53 function by acquired gene mutations in UV-induced skin lesions is a common molecular event in both mice and humans [25-30]. Here, we investigated whether UV-induced skin tumors of p53.S389A mice are, like bladder tumors, accompanied by the formation of additional p53 mutations. For this purpose, we analyzed skin tumors obtained from wild-type, $p53^{+/-}$, p53.S389A, and $p53^{S389A/-}$ mice by direct sequencing of the *p53* gene (Table II). Consistent with previous studies [25-30], *p53* mutations were predominantly found at dipyrimidine sites and originated from C to T transitions, irrespective of the genotype of the mice. In addition, the majority of mutations were found on the nontranscribed strand (NTS) of the *p53* gene, demonstrating the reduced efficiency of NER in non-transcribed DNA in mice [31]. Again, p53 genotype of the mice did not seem to affect this strand bias (see Table II).

Table II - p53 mutation spectrum in UV-light induced skin tumors of p53S389A mice

p53 mutation					Genotype			
exon	codon	base change[a]	aa change	strand[b]	WT	p53.S389A	$p53^{+/-}$	$p53^{S389A/-}$
2	16	CCT CTT	pro leu	NTS	1 (13%)			
2	25	TTA TAA	leu stop	NTS	1 (13%)	1 (5%)		
2	26	TGG TGA	trp stop	TS			1 (6%)	
4	51	GAG TAG	glu stop	TS				1 (5%)
4	62	CGA TGA	arg stop	NTS			1 (6%)	2 (10%)
4	97	CAA TAA	gln stop	NTS		1 (5%)		
4	110	TTC TTG	phe leu	NTS				1 (5%)
4	119	GTT GGT	val gly	NTS			2 (12%)	3 (15%)
4	122	ACG ATG	thr met	NTS			1 (6%)	1 (5%)
5	124	TCT TTT	ser phe	NTS		1 (5%)	1 (6%)	1 (5%)
5	127	CTC TTC	leu phe	NTS		1 (5%)		
5	132	TGC CGC	cys arg	NTS			1 (6%)	1 (5%)
5	148	CCT CTT	pro leu	NTS		1 (5%)	1 (6%)	
5	149	CCA TCA	pro ser	NTS	1 (13%)	1 (5%)	2 (12%)	
5	156	GCC GTC	ala val	NTS		1 (5%)		
5	173	TGC CGC	cys arg	NTS		1 (5%)		
5	174	CCC CTC	pro leu	NTS		1 (5%)		
5	176	CAT TAT	his tyr	NTS	1 (13%)	1 (5%)		
6	187	CCT TCT	pro ser	NTS		1 (5%)		
6	189	CAG TAG	gln stop	NTS	1 (13%)	1 (5%)		
6	200	CCC CTC	pro leu	NTS				1 (5%)
6	210	CGC TGC	arg cys	NTS	1 (13%)	6 (30%)	2 (12%)	1 (5%)
7	238	TCC TTC	ser phe	NTS	1 (13%)	2 (10%)		2 (10%)
7	239	TGC TTC	cys phe	NA				1 (5%)
7	247	CCT CTT	pro leu	NTS		1 (5%)		
7	255	GAA TAA	glu stop	TS		1 (5%)		
8	267	TTT TCT	phe ser	NTS	1 (13%)	1 (5%)		1 (5%)
8	270	CGT TGT	arg cys	NTS	3 (38%)	7 (35%)	3 (18%)	1 (5%)
8	271	GTT GAT	val asp	NTS	1 (13%)			
8	275	CCT ACT	pro thr	NTS	1 (13%)	1 (5%)	1 (6%)	1 (5%)
# tumors analyzed					8	20	17	20
# mutations found					13	31	16	18
%tumors with mutation					88%	95%	82%	85%
# mutations / tumors					1.6	1.6	0.9	0.9

[a] Mutated nucleotides are underlined

[b] Strand is indicated as transcribed (TS) or nontranscribed (NTS) for mutations that occur at possible dipyrimidine dimers. NA, absence of dipyrimidine sequences at the mutation site

WT = wild-type

In line with previously described studies [25-30], 88% of the skin tumors obtained from UV-irradiated wild-type mice and 82% of the skin tumors obtained from UV-irradiated $p53^{+/-}$ mice harbored *p53* mutations. The observed *p53* mutation spectra in skin tumors from wild-type mice was comparable to the spectrum described before, and included among others, the well-known mutation hotspot in mouse skin cancer at codon 270 [25-30]. Interestingly, sequence analysis of tumors obtained from p53.S389A mice revealed that, despite the presence of the mutation at codon 389, additional *p53* gene mutations were acquired upon UV irradiation. The percentage of tumors from p53.S389A mutant mice harboring an additional *p53* mutation was not significantly changed compared with the percentage of wild-type tumors. However, the spectrum of *p53* mutations seemed altered compared with the spectrum observed in skin tumors from wild-type mice. First, in UV-induced skin tumors from p53.S389A mutant mice, *p53* gene mutations were found more dispersed over the *p53* gene. Second, an Arg-to-Cys mutation at codon 210 was found in 6 out of 20 (30%) p53.S389A tumors analyzed, whereas this mutation was found at a very low frequency in wild-type, $p53^{+/-}$ and $p53^{S389A/-}$ mice (13%, 12%, and 5%, respectively). With respect to the $p53^{+/-}$ and $p53^{S389A/-}$ mice, *p53* mutations seemed to be present at the 5' part of the *p53* gene in contrast with wild-type mice. In addition, the number of mutations per tumor was much lower in $p53^{+/-}$ and $p53^{S389A/-}$ mice as compared with wild-type, which might be attributed to the presence of only one p53 allele in these mice.

Discussion

In the present study, we show that Ser389 is phosphorylated *in vitro* upon the introduction of NER-specific DNA damages by UV light and 2-AAF. *In vivo* studies revealed that phosphorylation of this site is necessary, at least in part, for appropriate functioning of the p53 protein, since absence of the Ser389 phosphorylation site in mice resulted in increased tumor susceptibility in skin and urinary bladder upon exposure to both UV light or 2-AAF, respectively. In contrast, exposure to ionizing radiation did not result in phosphorylation of Ser389 *in vitro* and, in turn, not in an increased tumor response in mice lacking the Ser389 site. These results clearly provide evidence for our hypothesis that phosphorylation of Ser389 is important for activation of p53 to exert its function as a tumor suppressor not exclusively upon the presence of UV-induced DNA damage, but also after exposure to other NER-specific agents known to induce bulky adducts. P53 activation upon the presence of these bulky DNA adducts may be triggered by a common signal leading to phosphorylation of p53.S389. It is possible that the blockage of mRNA synthesis by bulky DNA adducts is involved in the induction of this p53 response since it was previously shown that this blockage is involved in p53 activation upon exposure to UV light [32]. It remains to be elucidated whether there is also a correlation between the presence of other bulky DNA adducts, like those induced by 2-AAF, inhibition of mRNA synthesis, and subsequent activation of p53.

The absence of the Ser389 phosphorylation event in mice resulted in a less severe cancer phenotype upon UV light radiation compared with the absence of an entire p53 allele [17]. In accordance, the 2-AAF carcinogenicity study revealed that homozygous absence of the Ser389 site resulted in a urinary bladder tumor incidence intermediate to that observed in wild-type and $p53^{+/-}$ mice. Apparently, also after exposure to 2-AAF, p53 is still—although to a lesser extent—activated in response to DNA damage in the p53.S389A mutant mice, presumably through other post-translational modifications. In line with this, other p53 knock-in mouse models with mutations in phosphorylation sites also displayed such intermediate cellular responses, underlining the idea

that redundancy of post-translational modifications occur [33-35]. The fact that no increased 2-AAF–induced liver tumor response was found in p53.S389A mice could be explained with our previous study in which we showed that hepatocytes have to be completely devoid of any p53 protein activity to become sensitive to a genotoxic compound in a p53-dependent manner [22]. Again, this points towards a redundancy of post-translational modifications.

In both the UV light and 2-AAF carcinogenicity studies, tumor responses of $p53^{+/-}$ and $p53^{S389A/-}$ mice were not significantly different. Possibly, the absence of a p53 allele already results in such an overt tumor phenotype, that a potential additional effect of lack of Ser389 phosphorylation cannot be detected in these assays. However, the chronic 2-AAF exposure study did not enable the analysis of tumor latency, which might have revealed differences in the onset of 2-AAF-induced urinary bladder tumors in $p53^{+/-}$ and $p53^{S389A/-}$ mice. $P53^{S389A/-}$ mice did show a higher tumor incidence than p53.S389A mice, again indicating that p53 lacking Ser389 phosphorylation is still able to exert some of its tumor-suppressive function.

We and others have previously shown that p53-dependent apoptosis in both bladder and skin tissue is a crucial step in preventing tumor development [22;36]. In addition, p53.S389A mutant cells have a reduced apoptotic response *in vitro* upon exposure to UV light irradiation [17]. Possibly, p53.S389A mice also show a reduced apoptotic response after exposure to either UV light or 2-AAF *in vivo*, which might result in increased levels of cells with sustained DNA damage, ultimately resulting in an increased mutational load. This might explain the extensive amount of additional p53 mutations observed in both UV-induced skin tumors as well as 2-AAF–induced urinary bladder tumors of p53.S389A mice. Presumably, these mutations are necessary to completely alter p53 function.

The mutations found in UV-induced skin tumors obtained from p53.S389A mice included mutations frequently found in wild-type tumors, like the hotspot mutation at codon 270 (this study and [25-30]). Intriguingly, another *p53* mutational hotspot, present at high frequency in tumors obtained from UV irradiated p53.S389A mice, was found at codon 210. This mutation was recently described as a signature for defective NER, since high numbers of this mutation were found in UV-induced skin tumors of $Xpc^{-/-}$ and $Xpa^{-/-}$ mice [26;37]. We and others [38] also detected the Arg-to-Cys mutation at codon 210 in UV-induced skin tumors of wild-type and $p53^{+/-}$ mice, although at a relatively low frequency. Apparently, this mutation is preferentially formed in UV-exposed skins of NER-deficient mice. Since lack of Ser389 phosphorylation results in a *p53* mutational phenotype in skin tumors more or less comparable to that observed in NER-deficient mouse models, it is tempting to speculate that phosphorylation of p53 and NER are interrelated. Upon the introduction of NER-specific DNA damage, Ser389 is specifically phosphorylated, resulting in the activation of p53 to induce specific cellular responses. One of these responses might be the activation of NER, either direct or indirectly, resulting in efficient repair of DNA damage. As a result, lack of Ser389 phosphorylation can result in molecular and/or cellular defects also observed in NER-deficient cells and mice. In line, *in vitro* data also showed evidence that p53 plays a role in the regulation of DNA repair [39]. In fact, it has been shown that p53 is required for efficient global genome repair following UV irradiation [40-42]. The exact mechanism by which p53 is involved in the NER pathway is not fully understood. P53 has been shown to regulate, at least in human cells, the expression of NER genes such as *XPC* and *p48* (*XPE* [43;44]), suggesting that p53 regulates NER through transcriptional activation of genes involved in DNA damage recognition. P53 has also been shown to directly bind certain repair factors, including XPB and XPD, as well as damaged DNA itself, indicating that it may

act as a direct repair factor [45-47]. This is, however, still inconclusive since no localization of p53 to sites of DNA damage following UV irradiation was found [48]. Previously, we have shown that the transactivating activity of the p53 protein upon UV irradiation is impaired in p53.S389A cells [17]. Therefore, one can envisage that the p53 protein, activated through phosphorylation of Ser389, is involved in transcriptional activation of NER genes. Although p53 does not regulate *p48* in mouse cells [49], it might regulate other genes involved in DNA repair, like *Gadd45* or *Xpc* [50].

The studies described here regarding compound-specific phosphorylation of Ser389, reveal new thoughts on p53 activation in general. Possibly, a specific type of DNA damage induced by a class of compounds with a specific mode of action, results in a unique cascade of post-translational modification events of p53. What these modifications have in common is that p53 is activated, however, the specific p53-induced cellular response will be dependent on the modification pattern of the protein. Of these, cell cycle arrest, apoptosis, and the DNA repair responses most favorable for removal of the specific type of DNA damage present in the cell will be most prominent. Future studies exposing p53.S389A mutant mice and cells to other NER-related and non-NER-related DNA damaging agents will be aimed at understanding the relationship between the type of DNA damage, p53 modification, transcriptional activation of target genes, cellular responses, and eventually tumor development.

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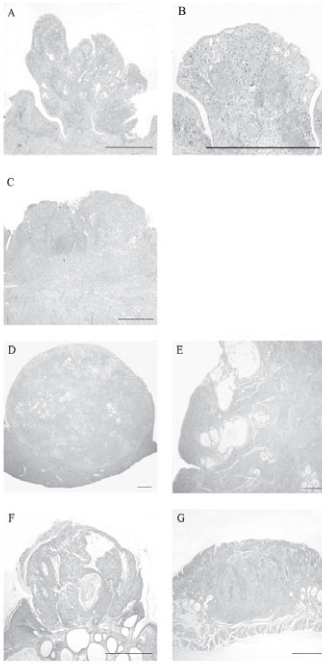
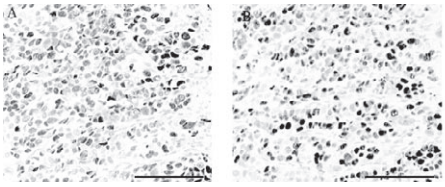
Supplementary tables and figures

Sup. Figure 3 - Histopathological features of carcinogen-induced tumors of p53.S389A mice



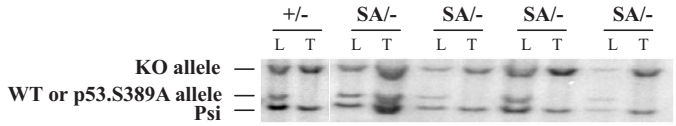
A) 2-AAF-induced transitional cell papilloma showing exophytic pattern of growth, well differentiated epithelium and no invasion.
B) 2-AAF-induced carcinoma in situ showing pleomorphic and atypical epithelial cells from basal layer up to surface. Invasion of lamina propria not yet obvious.
C) 2-AAF-induced transitional cell papilloma with pleomorphic picture, local atypia and local invasion of muscular wall.
D) Nodular hepatocellular adenoma compressing adjacent liver tissue.
E) Pleomorphic and invasive hepatocellular carcinoma involving major part of the liver.
F) Squamous cell papilloma consisting of well differentiated squamous epithelium without invasion of the dermis.
G) Squamous cell carcinoma showing moderately differentiated squamous sheets invading the dermis.
Bar represents 500 μm.
For color figure, see page 182.

Sup. Figure 4 - Immunohistochemical detection of p53 protein in a 2-AAF induced urinary bladder transitional cell carcinoma by CM5 and Pab240 antibody



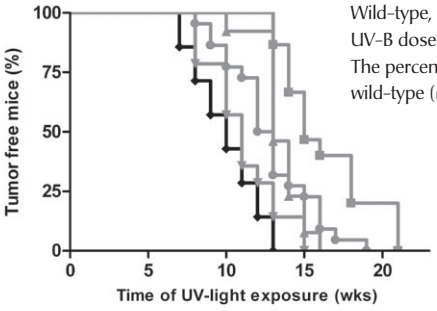
Slides were prepared as described in material and methods. Immunohistochemical staining was performed with the polyclonal CM5 (A) and Pab240 (B) antibody.
Bar represents 100 μm.
For color figure, see page 182.

Sup. Figure 5 - Loss of heterozygosity analysis (LOH) in gamma-induced lymphoblastic lymphomas of $p53^{+/-}$ and $p53^{S389A/-}$ mice



Representative examples of LOH analyses in control lung (L) tissue and lymphoblastic lymphoma (T) tissue. 'Psi' represents p53 pseudogene, +/- = $p53^{+/-}$, SA/- = $p53^{S389A/-}$

Sup. Figure 6 - UV-induced skin tumor development in mice



Wild-type, $p53^{S389A/+}$, p53.S389A, $p53^{S389A/-}$, and $p53^{+/-}$ mice were exposed to a daily UV-B dose of 600 J/m². Mice were scheduled for autopsy after 25 weeks of treatment. The percentage of skin tumor bearing animals is depicted.
wild-type (■), $p53^{S389A/+}$ (▲), p53.S389A(●), $p53^{S389A/-}$(◆), $p53^{+/-}$ (▼)

Sup. Table III - Detection of (mutant) p53 protein in urinary bladder tumors with CM5 and Pab240 antibody

		CM5 (and Pab240)				
	tumor	-	+	++	+++	total
p53.S389A	TCP	1	1 (1)	1 (1)		3
	CiS	0	1			1
	TCC	nd				0
$p53^{S389A/-}$	TCP	1				1
	CiS	1			1 (1)	2
	TCC	1		1 (1)		2
$p53^{+/-}$	TCP	1	1			2
	CiS	nd				0
	TCC	0	2 (1)	1 (1)		3

Mice were treated with 2-AAF for 9 months. Urinary bladder tumors were analyzed for p53 reactivity with the CM5 antibody as described in material and methods. The CM5 staining was scored either negative (-, <5%) or positive (+; 5-24%, ++; 25-75%, +++; >75%). The fields which were positive for the CM5 antibody were scored for Pab240 antibody. Number of Pab240 positive tumors is indicated between parentheses.

nd = not determined