# Constitutive and DNA Damage Inducible Activation of *pig3* and *MDM2* Genes by Tumor-Derived p53 Mutant C277Y

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## Abstract

**The *p53* gene is compromised in most human cancers by point mutation. Evidence is accumulating that these alterations frequently do not result in a complete loss of the sequence-specific transcriptional regulatory function of p53. Here, we describe the transcriptional activity of the p53 mutant C277Y isolated from a Ewing's sarcoma with high constitutive *pig3* expression. Transient transfection of this mutant into a p53 null cell line resulted in activation not only of the *pig3* but also of the *MDM2* gene compatible with the presence of constitutively expressed MDM2 transcripts initiated from the P2 promoter in the p53-C277Y hemizygous Ewing's sarcoma cell line. Expression of endogenous *pig3* and *MDM2* genes was further enhanced on irradiation of this cell line. Here, suppression of p53-C277Y by RNAi reduced *pig3* promoter activity, RNA, and protein expression. Reporter gene assays revealed that the potential of p53-C277Y to up-regulate *MDM2* expression was similar to wild-type p53, whereas activation of the *pig3* promoter was at least 5-fold increased over wild-type p53. The pentanucleotide microsatellite sequence present in exon 1 of the *pig3* gene was found to be responsible for p53-C277Y-mediated activation. In concordance with a role of PIG3 protein for cell death, we showed residual apoptotic activity of p53-C277Y to which the described Ewing's sarcoma cell line was found to be resistant. p53-C277Y has previously been reported to bind to DNA with altered sequence specificity and to be unable to activate generic p53 target genes in yeast-based functional assays. Our results, therefore, show that a p53 mutant may behave differently when tested in its authentic cellular context. (Mol Cancer Res 2004;2(5):296–304)**

## Introduction

The tumor suppressor protein p53 is a powerful transcription factor that plays an important role in cell cycle regulation, DNA repair, differentiation, senescence, and angiogenesis. It functions as a tetramer that can bind to about 300 different promoter elements in the human genome (1). p53 acts as both a sequence-specific activator as well as an unspecific repressor for many TATA box-containing genes (2). p53 is activated in response to cellular stress signals evoking either growth arrest or apoptosis. Disruption of p53 function by either mutation or protein inactivation through interaction with viral or cellular oncogenes is the most frequent alteration observed in human cancer (3). Most alterations are missense mutations that affect amino acid residues either directly contacting DNA or providing structural integrity to the core domain for sequence-specific DNA binding resulting in nuclear p53 accumulation and loss of transcriptional activity (4). Heterozygous germ-line p53 mutations predispose to a wide range of tumor types with early onset characterizing Li-Fraumeni syndrome (5). In addition to the loss of tumor suppressor function, some mutations may also result in a gain of oncogenic activity of p53 interfering with apoptosis or growth suppression (6) and increasing metastatic potential (7). The basis for the oncogenic function of mutant p53 is still not clear but, probably, both a dominant-negative effect toward wild-type (wt) p53 in the multimeric complex and possibly other p53 family members, as well as altered transcriptional activity on a specific subset of genes, may be involved (8). Individual mutations may differentially affect sequence-specific binding of p53 to distinct target genes, as has been shown for *p21* and *bax* gene promoters (4, 9). For several p53 repressed genes, including *PCNA* (10), *HSP70* (11), and *MDR-1* (12), specific p53 mutations have been reported to result in their activation. Some p53 mutations result in DNA binding with altered structural preference as exemplified by the mutant p53-G245S which binds to non-B-DNA conformations (13). Phenotypically, introduction of different p53 mutants into p53-null H1299 lung adenocarcinoma cells resulted in increased chemoresistance (14). The p53-D281G mutant isolated from human prostatic cancer has been reported to switch the apoptotic function of the tumor suppressor to a Bcl2-insensitive cell death program (15). As reported recently, mutant p53 proteins promote gene amplification independently of their capacity to inactivate the wt p53 protein (16). This effect was found to be dependent on conservation of the ability of mutant p53 to interact with topoisomerase I, and, in the absence of transcriptional activity, was interpreted as a gain of function in destabilizing genomic integrity (17).

In a yeast-based mutagenesis screen, several p53 variants have been selected that bind to and transcriptionally activate from an altered DNA binding motif (TTT CATG AAA) but not from a wild-type site (18). Among them, C277Y has been known as a naturally occurring mutation in Ewing's sarcoma (19) and several other human tumors, including osteosarcoma,

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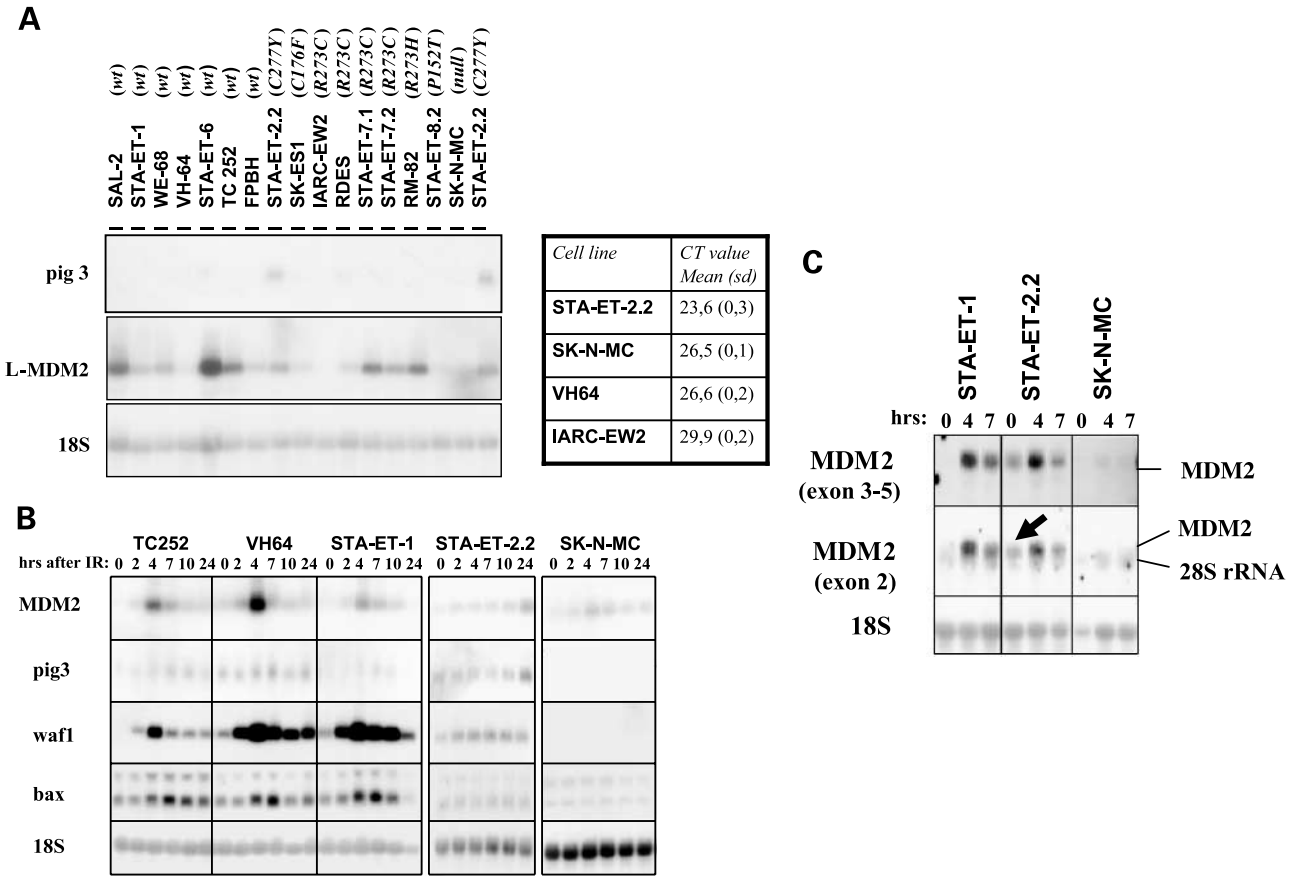
leiomyosarcoma, and carcinomas of the bladder, stomach, colon, breast, and liver, generally representing 0.3% of all mutations described in human cancers (20). In the yeast system, this mutant was capable of activating the *waf1*, but neither the human *mdm2* gene (*MDM2*), *cyclin G*, *gadd45*, nor *bax* promoters (21). Here, we report that when tested in the authentic cellular background (Ewing's sarcoma), the C277Y mutant strongly activates *MDM2* and the proapoptotic gene *pig3*.

## Results

### *Constitutive Activation of pig3 and MDM2 Genes in a Ewing's Sarcoma Cell Line Carrying a Hemizygous p53-C277Y Mutation*

During our studies on the function of the p53 pathway in Ewing's sarcoma family tumors (EFT; refs. 22, 23), we tested for the constitutive and activated expression of p53 downstream target genes on Northern blots. Among 16 EFT cell lines (7, 8, and 1 cell lines wild-type, mutant, and null for p53, respectively), STA-ET-2.2 showed a remarkably elevated constitutive *pig3* expression. By real-time quantitative PCR, an 8- to 80-fold difference in pig3 RNA levels was found between STA-ET-2.2 cells and a p53 null (SK-N-MC) or a wt p53–expressing (VH64) EFT cell line, respectively an EFT cell line carrying a p53 R273C mutation (IARC-EW2; Fig. 1A). Using a probe (spanning *MDM2* exons 3 to 5) that recognizes both basal (L-) and p53 inducible (S-) MDM2 transcripts, constitutive MDM2 RNA levels were found to be highly variable between the cell lines. On irradiation, all wt p53 cell lines showed pronounced inducibility of *MDM2* gene activity peaking at 4 hours postirradiation (Fig. 1B). The subsequent decrease in MDM2 RNA levels cannot be attributed to the induction of apoptosis because VH64 cells are completely radio-resistant (23). In p53 null SK-N-MC cells, only a weak p53-independent increase over background levels was observed for MDM2 RNA with a maximum at around 7 hours postirradiation (Fig 1B). In contrast, in STA-ET-2.2 cells, MDM2 RNA gradually increased up to 24 hours postirradiation. Interestingly, reactivity




| Cell line | CT value Mean (sd) |
|---|---|
| STA-ET-2.2 | 23,6 (0,3) |
| SK-N-MC | 26,5 (0,1) |
| VH64 | 26,6 (0,2) |
| IARC-EW2 | 29,9 (0,2) |

**FIGURE 1.** Constitutive and irradiation-induced MDM2 and pig3 RNA levels in EFT cell lines. **A.** *Left*, Northern blot of untreated EFT cell lines consecutively probed with a *pig3* and an *MDM2* exon 3 to 5 probe. The status of the endogenous *p53* gene is indicated in *parentheses*. For the cell line STA-ET-2.2, which is the only one that displays high basal *pig3* expression levels, two independent samples (*lanes STA-ET-2.2*) have been included to demonstrate reproducibility of the result. *Right*, quantification of *pig3* expression by real-time PCR. Mean cycle threshold (CT) values and SDs (*sd*) for three independent determinations are shown. For SK-N-MC, VH64, and IARC-EW2 cells, cycle threshold (CT) values were between 2.9 and 6.3 cycles lower than for STA-ET-2.2 corresponding to an 8- to 80-fold expression difference. **B.** Induction of p53 downstream genes *MDM2*, *pig3*, *waf1*, and *bax* in EFT cells by X-irradiation (6 Gy). For MDM2 RNA, an exon 3 to 5 probe detecting both constitutive (L-MDM2) and p53 inducible (S-MDM2) transcripts has been used. **C.** Demonstration of MDM2 induction by irradiation in wt p53 (*STA-ET-1*) and p53-C277Y (*STA-ET-2.2*) but not in p53 null (*SK-N-MC*) EFT cell lines using the *MDM2* exon 3 to 5 probe and identification of S-MDM2 transcripts initiated from the p53 responsive P2 promoter by hybridization to an *MDM2* exon 2 probe. Note that in STA-ET-2.2 cells, an S-MDM2 transcript is already detectable in unirradiated cells (*arrow*). In addition, the *MDM2* exon 2 probe shows some cross-hybridization to 28S rRNA. Hybridization of the Northern blots in **A**, **B**, and **C** to an 18S rRNA specific probe is shown as a loading control.

with an exon 2 probe that is specific for MDM2 transcripts initiated from the p53 inducible P2 promoter (24) indicated constitutive P2 promoter activity in untreated STA-ET-2.2 cells that was further increased on X-irradiation (Fig. 1C). Expression of the *pig3* gene was variably induced by X-ray treatment in the three wt p53 cell lines and absent from the p53 null cell line SK-N-MC. Remarkably, significant *pig3* induction was observed in STA-ET-2.2 cells at 24 hours (Fig. 1B). In addition, basal waf1 RNA levels comparable to wt p53 cell lines and, on irradiation, a slight increase in *waf1* expression was observed in this cell line, while *bax* inducibility was restricted to wt p53 cell lines only.

STA-ET-2.2 cells carry a hemizygous p53-C277Y mutation leading to nuclear accumulation of p53 (19) that has previously been shown to adopt an altered DNA binding specificity when tested on artificial p53 binding elements (18) and to retain some activity on the *waf1* promoter in a yeast-based assay (21). Thus, we speculated that elevated constitutive and irradiation inducible activity of *pig3* and *MDM2* genes in STA-ET-2.2 cells may be assigned to the expression of p53-C277Y in this cell line.

To directly test this hypothesis, we transiently introduced p53-C277Y and, for comparison, wt p53, mutant p53-R273H, and empty expression vectors into the p53-null EFT cell line SK-N-MC and tested for *pig3* and *MDM2* expression 24 hours posttransfection. Because wt p53 overexpression results in apoptosis of SK-N-MC cells (23) that can be blocked by the general caspase inhibitory peptide z-VAD.fmk (22), the experiment was done both in the absence and the presence of the caspase inhibitor. Thus, we were able to monitor p53-induced gene expression that is not affected by apoptotic loss of cell viability. As illustrated in Fig. 2, transfection of p53-C277Y was similarly effective in *pig3* gene induction as wt p53, while activation of *MDM2* transcription was clearly detectable but

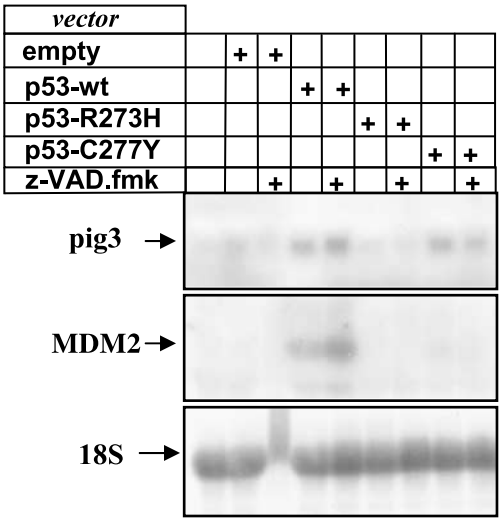



**FIGURE 2.** Induction of endogenous *MDM2* and *pig3* genes in p53 null SK-N-MC cells by transfection of wild-type and p53-C277Y but not by p53-R273C and empty expression vectors. A Northern blot is shown that has been hybridized consecutively to the probes indicated to the *right* of the figure. Because SK-N-MC cells are highly sensitive to p53-induced apoptosis, cells were incubated in the absence and the presence of the general caspase inhibitory peptide z-VAD.fmk (40 μmol/L) to avoid loss of cell viability. RNA was extracted 24 hours posttransfection.

| *construct* | *CT value Mean (sd)* |
|---|---|
| – | 22,0 (0,3) |
| **pSR** | 21,2 (0,1) |
| **pSR.p53** | 24,7 (0,2) |

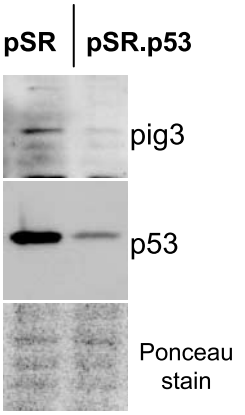



**FIGURE 3.** Modulation of PIG3 RNA (*left*) and protein expression (*right*) in STA-ET-2.2 cells by small inhibitory RNA against p53. Cells were infected with a retroviral small hairpin RNA vector (*pSR.p53*) or empty vector control (*pSR*) and subjected to puromycin selection 1 day after infection. At 6 days, total RNA and protein was extracted and subjected to Western blot analysis. For protein loading control, the Western blot was stained with Ponceau S. On suppression of p53 expression, cycle threshold (CT) values for *pig3* expression increased by 3.5 cycles corresponding to an about 10-fold decrease in expression. Concomitantly, a significant decrease in PIG3 protein was seen.

weaker than in wt p53–transfected SK-N-MC. No effect on *pig3* and *MDM2* expression was observed after transfection of p53-R273H or empty vector. z-VAD.fmk treatment was capable of further increasing induced pig3 and MDM2 RNA levels in wt p53 but not in p53-C277Y–expressing cells. These results corroborate the potential of p53-C277Y to activate *pig3* and *MDM2* genes.

To confirm the link between constitutive *pig3* gene activity and expression of p53-C277Y in STA-ET-2.2 cells, p53 siRNA was expressed from a retroviral small hairpin RNA expression vector (Fig. 3). Six days after infection, endogenous p53-C277Y protein levels were clearly reduced. Concomitantly, a significant decline in PIG3 RNA (about 10-fold) and protein expression was observed, providing further evidence for p53-C277Y-activated expression of *pig3* in STA-ET-2.2 cells.

### *p53-C277Y Activates pig3 and MDM2 Promoters*

To quantitatively address the transcriptional potential of p53-C277Y, we did reporter gene assays in comparison to wt p53 and to the R273H mutant using the wt p53 responsive promoters from *MDM2*, *pig3*, and *rgc* genes in SK-N-MC cells lacking endogenous p53 expression. Co-transfection of reporter and p53 expression constructs with a green fluorescent protein (GFP) expression plasmid and subsequent flow cytometric assessment of GFP expression indicated similar transfection efficiencies and viability of transfected cells when measured 24 hours posttransfection. At 44 hours, the proportion of dead cells significantly increased in wt p53–transfected cells due to extensive apoptosis (data not shown and Fig. 7). Figure 4A shows equal expression levels of p53 proteins in the presence of co-transfected reporter vectors. As illustrated in Fig. 4B, reporter gene assays done at 24 hours posttransfection showed similar activation of *pig3* and *rgc* reporter constructs by wt p53, whereas *MDM2* promoter activity was approximately 10 times higher. The activities of mutant p53 proteins toward these

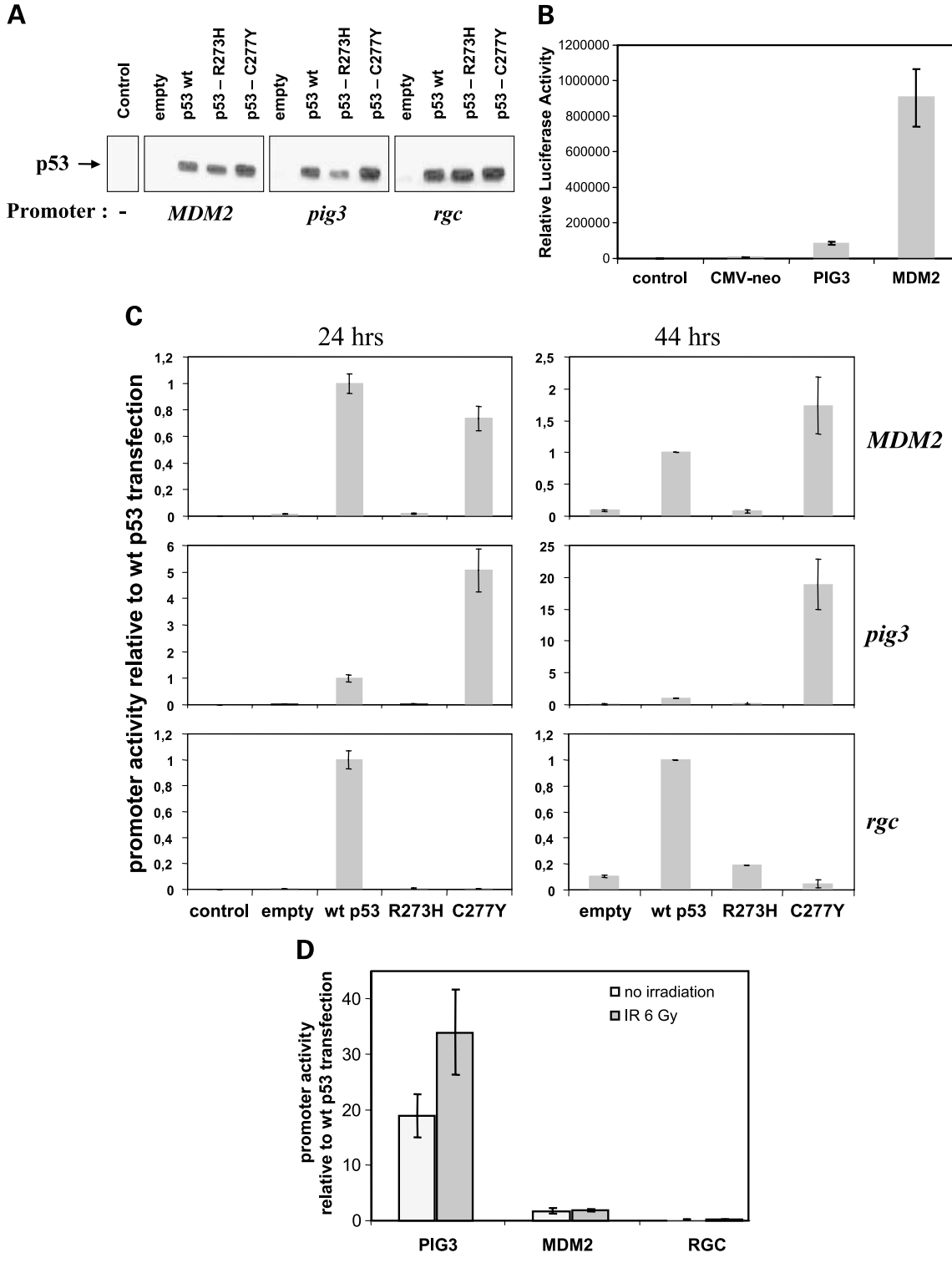



**FIGURE 4.** Transcriptional activity of wt p53, p53-R273H, and p53-C277Y on *pig3*, *MDM2*, and *rgc* promoters. **A.** Immunoblot of p53 protein in transfected SK-N-MC cells demonstrating similar expression levels for wild-type and mutant p53 in reporter gene assays. Cells were harvested 24 hours after transfection. *Lanes 1 to 4*, transfected *MDM2* promoter; *lanes 5 to 8*, transfected *pig3* (6 kb) promoter (pBR322pI-LUC-pig3); *lanes 9 to 12*, transfected *rgc* promoter. **B.** Direct comparison of *pig3*, *MDM2*, and *rgc* promoter activity for transfected wt p53 measured by luciferase reporter gene assay. *Columns*, mean values of triplicate experiments; *bars*, SDs. **C.** Transcriptional activity on *pig3* (6 kb), *MDM2*, and *rgc* promoters of mutant p53-R273H and C277Y proteins relative to wt p53 as measured 24 and 44 hours after transfection. *Control*, no reporter vector; *empty vector*, promoterless pGL3. **D.** Influence of ionizing irradiation (6 Gy) on the transcriptional activity of p53-C277Y on different promoter response elements. Cells were irradiated with 6 Gy 20 hours posttransfection and luciferase activity was determined 24 hours later.

promoters were evaluated relative to wt p53 24 and 44 hours after transfection (Fig. 4C). In concordance with the results obtained for the endogenous genes (Fig. 2), a strong induction of *MDM2* and *pig3* promoter-driven luciferase activity by the C277Y but not by the R273H mutant was observed. Significantly, induction of *pig3* promoter activity at 24 hours after transfection by p53-C277Y was even 5 times higher than by the wild-type protein. *MDM2* promoter activity in response to p53-C277Y closely approached wt p53–mediated activation (about 75% of wt). In contrast, the *rgc* response element was activated by neither of the two mutants. A further increase in relative *MDM2* and *pig3* promoter-driven reporter gene activities in C277Y versus wt p53–transfected cells at 44 hours has to be assigned at least in part to increased cellular mortality of wt p53–transfected cells. Cellular stress evoked by X-irradiation further contributed to increased *pig3* promoter induction by p53-C277Y but did not affect the activities of either *MDM2* or *rgc* promoters in transient transfection assays (Fig. 4D).

In addition, we tested the ability of synthetic small inhibitory RNA to p53 (siRNA-p53) to modulate *pig3* promoter activity in STA-ET-2.2 cells (Fig. 5). A *pig3* promoter-driven luciferase reporter vector was co-transfected with either synthetic siRNA-p53 or, for control, an irrelevant mismatched siRNA into STA-ET-2.2 cells. In concordance with previously published results (25) 30 hours post transfection, a clear reduction in p53 protein levels was observed with the specific siRNA but not with the irrelevant siRNA (not shown). Concomitantly, a 3-fold reduction in luciferase activity was observed in siRNA-p53–transfected cells. Taken together, these results are in concordance with a direct activation of *MDM2* by p53-C277Y and imply a gain of transcriptional function of this mutant on the *pig3* promoter.

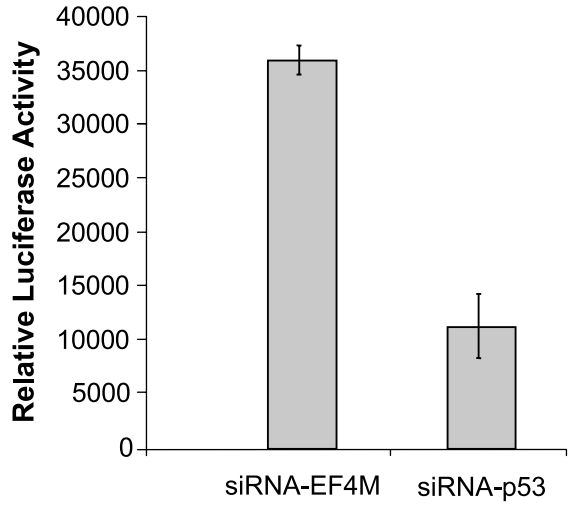



**FIGURE 5.** Reduction of *pig3* (6 kb) promoter activity in STA-ET-2.2 cells by RNAi-mediated suppression of endogenous p53 expression. *Columns*, mean values of triplicate co-transfection experiments with a *pig3*-promoter-driven luciferase reporter vector (pBR322pI-LUC-pig3) and either siRNA to p53 (*siRNA-p53*) or an irrelevant siRNA (*siRNA-EF4M*); *bars*, SD.

### *p53-C277Y Activates pig3 via the Pentanucleotide Microsatellite Sequence*

The *pig3* promoter region contains a putative p53 binding site between residues −328 and −312 that has previously been considered to be responsible for *pig3* responsiveness to wt p53 (26). More recently, a microsatellite sequence between positions +442 and +516 has been suggested to mediate regulation of the gene by p53 (27). To test which portion of the *pig3* upstream region is responsible for p53-C277Y-dependent activation, we compared four different reporter gene constructs in co-transfection experiments with expression plasmids for p53-C277Y, wt p53, and p53-R273H (Fig. 6). While the activity of p53-R273H in the reporter assay was about 100- to 170-fold lower than that of wt p53 with any of the constructs, p53-C277Y induced luciferase activity 4- to 12-fold higher than wt p53. The highest reporter activities were obtained using a 1.4-kb promoter fragment containing both the canonical p53 binding motif and the pentanucleotide microsatellite sequences. Mutation of the canonical p53 binding motif in the construct Pig3-delA did not significantly affect responsiveness to either wt p53 or p53-C277Y, indicating that this sequence is not involved in the regulation of the *pig3* gene by either wild-type or mutant p53. Confirming previously published results (27), the microsatellite sequence comprised by 15 repeats of the TGYCC pentanucleotide motif was sufficient for reporter gene activation by wt p53. Intriguingly, p53-C277Y induced luciferase activity 12 times better than wt p53 from this construct. These results establish that p53-C277Y mediates *pig3* gene activation via the microsatellite contained in exon 1 of the gene.

### *p53-C277Y Retains Residual Apoptotic Activity*

Because *pig3* gene activation has been reported to be associated with the apoptotic function of p53 (28), we tested the ability of ectopically overexpressed p53-C277Y to induce cell death in four different EFT cell lines that differ in their endogenous *p53* gene status. The apoptotic activity was tested 44 hours posttransfection in comparison to wt p53, to the DNA binding mutant p53-R273H, to the transactivation domain double mutant p53-22Q23S, and to the empty vector control (Fig. 7). Although some variability in the cell death-inducing activity of p53 expression constructs was observed in cell lines sensitive to wt p53, p53-C277Y showed the highest apoptotic activity among the three p53 mutants tested, which was significantly increased over the empty vector control. In contrast, in concordance with our previously published results (23), STA-ET-2.2 cells expressing endogenous p53-C277Y were completely resistant to all constructs, including wt p53. Insensitivity of this cell line to the deleterious effects of p53 accumulation is likely due to high Bcl2 and low APAF1 levels in this cell line (23) and may be envisaged as a protection mechanism against the residual apoptotic activity of p53-C277Y considering constitutively high p53 protein levels in this cell line (29).

## Discussion

In contrast to other tumor suppressors, which are usually completely abrogated by large deletions or frameshift mutations in cancer cells, point mutations resulting in single amino-acid substitution prevail for p53. Some of these variants retain the ability to activate a subset of p53 target genes. Most of these

transcriptional "gain of function" mutations are considered to confer growth advantage to tumor cells and to contribute to cancer progression. Examples are p53-C174Y, which was shown to induce the *fos* promoter restricting growth suppression of transfected Saos-2 cells but failing to transactivate the *MDM2*, *waf1*, *bax*, and *cyclin G* promoters (30), and p53-D281G active on the human epidermal growth factor (*EGFR*) and human multiple drug resistance genes (*MDR-1*) and inducing increased expression of endogenous NF-κB and PCNA proteins (31). Using a yeast-based screening assay, two classes of p53 mutants retaining partial transactivation activity have been defined (21). The first group showed sequence-specific DNA binding activity to *p21*, and occasionally to *SCS* and *cyclin G* promoters even at physiologic temperature (37°C), whereas the second group of mutants with amino acid substitution mostly positioned in the β-scaffold of the p53 protein retained transcriptional activity only at significantly sub-physiologic temperatures. p53-C277Y belongs to the first group affecting an amino acid residue that directly contacts DNA and that regulates DNA binding affinity in a redox state-dependent manner (32). Replacement of cystein 277 by either histidine or tyrosine results in altered DNA binding specificity as shown previously using artificial promoters (18). Interestingly, however, the amino acid residue at position 402 in *Caenorhabditis elegans* p53 corresponding to mammalian C277 is a tyrosine. It is intriguing to speculate that the replacement of an amino acid at a specific DNA contact site during evolution did not completely change target selectivity of p53. We show here that p53-C277Y is capable of transactivating *MDM2* and *pig3* in the authentic tumor cell milieu, albeit with delayed kinetics, although in the yeast-based assay, this mutant did not activate these promoters when tested

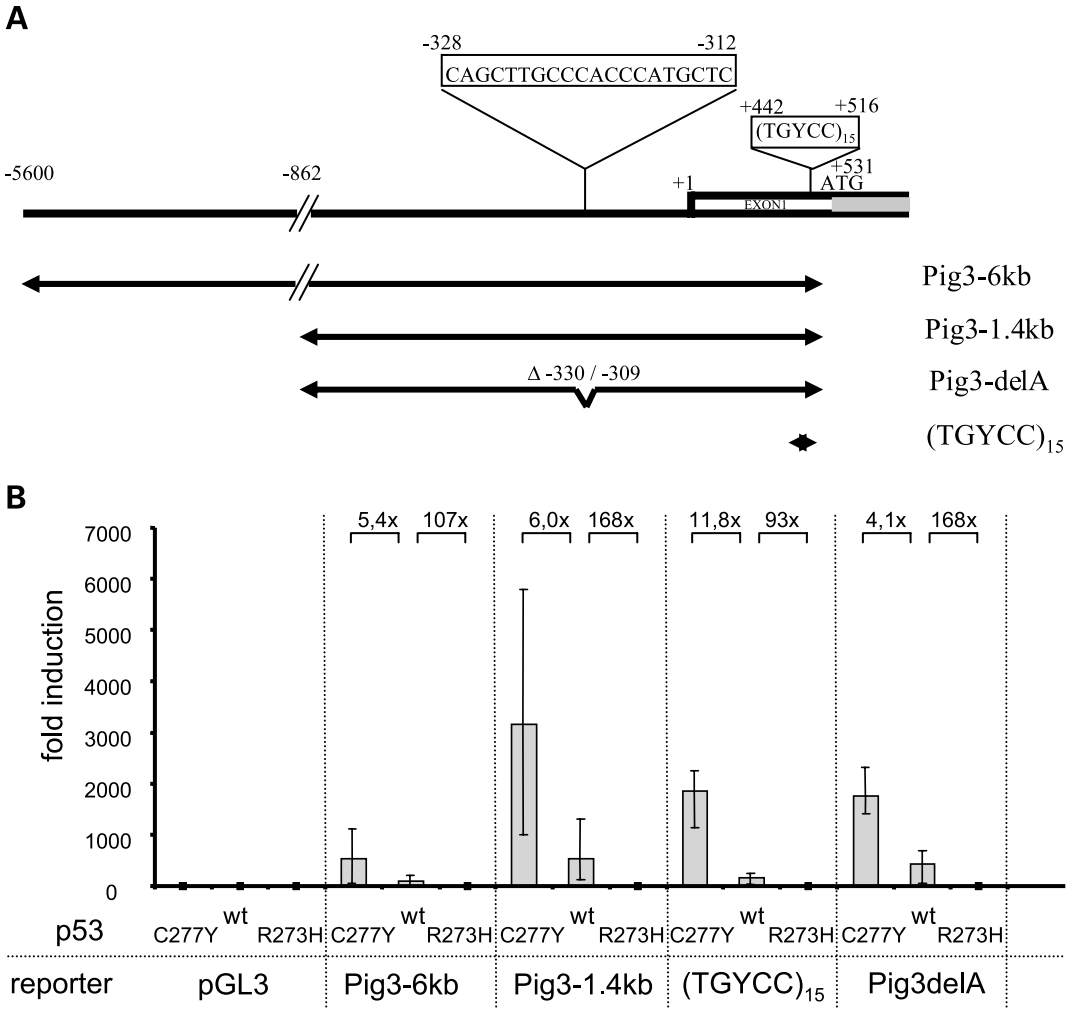



**FIGURE 6.** p53-C277Y activates the *pig3* promoter via a pentanucleotide microsatellite sequence. **A.** Schematic representation of the four different *pig3* reporter-driven luciferase expression constructs used relative to the structure of the *pig3* gene upstream region: Pig3-6kb (pBR322pI-LUC-pig3) and Pig3-1.4kb contain both the canonical p53 binding site between −328 and −312 (32) and a microsatellite sequence (+442 to +516) comprised by 15 copies of the TGYCC motif (34). Pig3-delA is identical to Pig3-1.4kb except for a 9-bp deletion within the p53 consensus binding site (34). $(TGYCC)_{15}$ contains only the microsatellite sequence. **B.** Luciferase activity in SK-N-MC cells co-transfected with either p53-C277Y, wt p53 (*wt*), or p53-R273H and the different reporter constructs as indicated on *bottom* of the figure. pGL3 was used as an empty luciferase reporter control. Luciferase activity is expressed as fold activation over empty effector expression vector control (pCMVNeo). Mean values and range of three independent experiments, each of which done in triplicate, are shown. The fold differences in luciferase activity between p53-C277Y and wt p53 and wt p53 and p53-R273H are indicated on *top* of the respective columns.

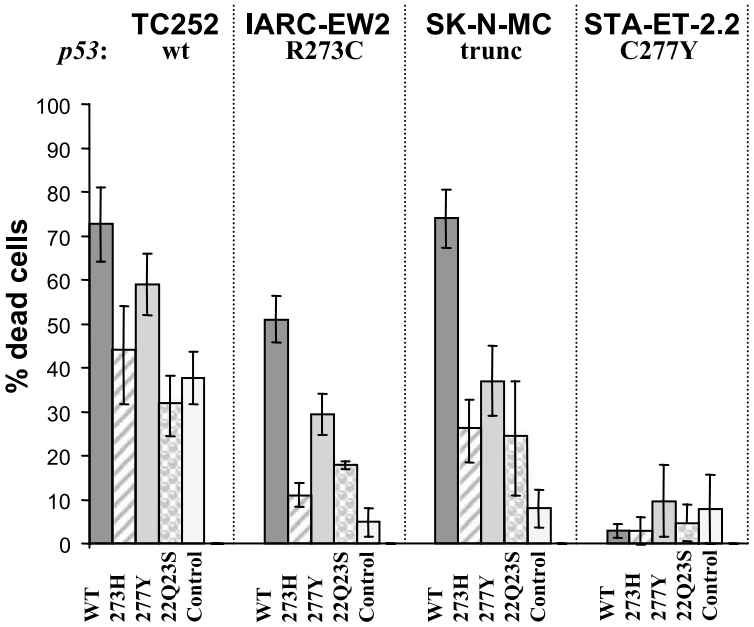



**FIGURE 7.** Apoptotic activity of EFT cell lines with different endogenous *p53* gene status (as indicated on *top* of the figure) to forced expression of ectopic wt or mutant p53 protein (as indicated on the *bottom* of the figure). The percentage of dead cells in the cultures was assessed 44 hours posttransfection. A mean of six independent transfections was done per construct. *Columns*, proportion (mean values) of cells with a sub-$G_1$ DNA content (apoptotic cells) 44 hours posttransfection relative to the total number of cells positively staining for p53 or LacZ above the background of pCMVNeo transfected cells; *bars*, SDs.

at 20°C, 30°C, and 37°C.[2] In another study using a yeast transactivation assay, 16 of 77 tumor-derived p53 mutants displayed preferential binding to *p21*, but not to *bax* or *pig3* promoters (33). p53-C277Y clearly differs from this group of mutants in that it strongly activated the *pig3* promoter and that the transactivating ability was further increased on a DNA damaging stimulus. Szak et al. (26) defined the *pig3* promoter as a low-affinity p53 binding site that may require interaction with additional factors possibly explaining delayed kinetics of *pig3* induction by wt p53 (34). Here, the p53 binding element was located by chromatin immunoprecipitation-PCR to a region between nucleotides −441 and −166 containing a putative p53 binding site (−328 to −309; ref. 28). Recently, however, a pentanucleotide polymorphic microsatellite sequence further downstream (+442 to +516) has been shown to bind to p53 and to be necessary and sufficient to mediate *pig3* gene activation (27). Our reporter gene assays not only confirm this observation for wt p53 but extend it to p53-C277Y which activated 12 times better than wt p53 from the isolated microsatellite.

It has been proposed that the inability of tumor-derived p53 mutants to induce apoptosis is correlated with a deficiency in *pig3* gene activation, suggesting a direct involvement of PIG3 in p53-mediated cell death (27). In fact, we show residual apoptotic activity of p53-C277Y in transient transfection assays. These results suggest that tumors harboring this mutant have to be protected from p53-induced cell death by additional genetic alterations conferring resistance. In fact, the two cell lines, STA-ET-2.1 (not shown) and STA-ET-2.2, established from the tumor, from which p53-C277Y was isolated, were completely resistant to wt p53–induced cell death. Interestingly, these two cell lines were the only ones of a series of seven EFT cell lines showing a significantly increased Bcl2/Bax expression ratio and a low level of APAF1 expression (23).

[2] R. Brachmann, personal communication.

In summary, p53-C277Y has been known for its altered DNA binding specificity on artificial promoters in in vitro tests. We provide the first evidence for constitutive and inducible transcriptional activity of p53-C277Y on *MDM2* and a gain of function of this mutant on *pig3*, in the authentic tumor cell context that so far has escaped detection in yeast-based assays.

## Materials and Methods

### *Cell Lines*

The cell line SK-N-MC was kindly provided by J. Biedler (Memorial Sloan Kettering Cancer Center, New York). The cell lines VH64, RM82, and WE68 were kindly supplied by F. Van Valen (Department of Pediatrics, University of Muenster, Germany), FPBH by M. Vetterlein (Institute of Tumour Biology and Cancer Research, University of Vienna, Austria), and SAL2 by G. Hamilton (Department of Surgery, University of Vienna, Austria). TC252 and IARC-EW2 were generously provided by T. Triche (Department of Pathology, Children's Hospital, Los Angeles, CA) and G.M. Lenoir (International Agency for Research on Cancer, Lyon, France), respectively. SK-ES1 and RDES were obtained from the American Type Culture Collection (Rockville, MD). The STA-ET series of cell lines was established at the Children's Cancer Research Institute (Vienna, Austria). The *p53* gene status of these cell lines as indicated in Fig. 1A has previously been described (19). All EFT cell lines were grown on fibronectin-coated plastics in RPMI 1640 supplemented with 10% fetal calf serum at 37°C.

### *Plasmid DNAs and siRNAs*

The luciferase reporter plasmid pBR322pI-LUC-pig3 carrying an approximately 6-kb–long *pig3* gene promoter fragment (~−5600 to ~+550) was kindly provided by C. Polyak (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; ref. 28). Reporter plasmids carrying either a

1.4-kb promoter fragment from −861 to +546 (pGL3PIG3), or a derivative of this fragment from which the canonical p53 binding site between −330 and −309 has been deleted by oligonucleotide-directed mutagenesis (delA), or a microsatellite sequence comprised by 15 repeats of the TGYCC motif between +442 and +516 of the *pig3* gene, have been generously provided by Matthias Dobbelstein (Institute of Virology, Philipps University, Marburg, Germany; ref. 27). The plasmid carrying the *MDM2* gene promoter pmdm2-luc was kindly supplied by J.C. Bourdon (Department of Surgery and Molecular Oncology, Ninewells Hospital and Medical School, University of Dundee, Dundee, United Kingdom). The luciferase reporter plasmid carrying the *rgc* promoter was prepared by insertion of a double-stranded 22-oligomer TTTGCCTTGCCTGGACTTGCCTTT to the *Kpn*I/*Mlu*I site within the plasmid pGL3-Basic. A GFP expression plasmid was constructed by insertion of the cytomegalovirus promoter into the multiple cloning site of pEGFP-1 (Clontech Laboratories, Palo Alto, CA). All mammalian expression vectors were based on pCMVNeo (35). The wt p53 and p53-R273H expression plasmids pC53-SN3 and pC53-4.2N3 were kindly supplied by B. Vogelstein (John Hopkins Oncology Center, Baltimore, MD). The p53-22Q23S encoding vector pRc/CMV-hp53 has been obtained from A. Levine (Princeton University, Princeton, NJ). The C277Y mutant that was cloned from the STA-ET-2.2 cell line (18) was inserted for expression into the *Bam*H1 site of pCMV-Neo.

The sequence of the synthetic siRNA against p53 has previously been described (25). The sequence of the control irrelevant siRNA EF4M was CACCCACGGUCCUUCACAC with dTdT 3′ overhangs on both strands. The p53 specific retroviral small hairpin RNA expression vector pSR.p53 was generated by cloning the siRNA expression cassette from pSUPER.p53, purchased from OligoEngine (Seattle, WA), into pSUPER.retro (OligoEngine).

### *Transfections and Reporter Gene Assay*

DNA (and DNA + siRNA) transfer into logarithmically growing EFT cells (0.5 to 1.0 × $10^6$ cells) was accomplished in six-well plates by lipofection of Qiagen purified plasmid DNA (Hilden, Germany) using LipofectAMIN PLUS reagent (Invitrogen, Paisley, United Kingdom) followed by 3 hours incubation in serum-free Optimem medium according to the manufacturer's instructions. Cells were washed with PBS and lysed 24 or 44 hours after transfection and luciferase activity was measured using Bright-Glo Luciferase Assay System (Promega, Madison, WI).

### *Irradiation and Cytotoxicity Measurement*

X-ray treatment of EFT cell lines (6 Gy) was done using a Phillips RT100 irradiation device (1.7 mm aluminum filter, 100 kV) at 12.5 Gy/minute. Irradiation of the transfected cells was done 12 hours after transfection. After treatment, cells were incubated at 37°C for the indicated times. Cytotoxicity was assessed by a vital dye exclusion test. Because dead and dying cells tend to detach from the culture plate, the floating and attached cells were separately collected, stained with trypan blue, and counted.

### *Northern Blot Analysis and Real-Time Quantitative PCR*

RNA extraction and Northern blotting were done according to standard procedures. The probes for human *MDM2* exons 3 to 5 were generated by PCR amplification using primers ex3F (TGTCAATACCAACATGTCTG) with ex5R (CTGTG-CTCTTTCACAGAGAAG). The probe for human *MDM2* exon 2 was generated by PCR amplification using primers ex2F (CTGTGTTCAGTGGCGATTGGA) with ex2R (GGATCAG-CAGAGAAAAAGTGG). The *pig3* gene probe was kindly supplied by Dale Haines (The Fels Institute for Cancer Research and Molecular Biology, Philadelphia, PA). Where indicated, cells were incubated in the presence of 0.2 mol/L Z-Val-Ala-DL-Asp-fluoromethyl ketone (z-VAD.fmk; Bachem, Bubendorf, Switzerland) to monitor the expression of p53 downstream genes in the absence of p53-induced apoptosis.

For quantification of *pig3* gene expression, real-time quantitative PCR was done using pig3-forward (ACCGCCTT-CCAGCTGTTACA) and pig3-reverse (CACTCAGTCCTG-CATGGATTAGC) primers. PCR reactions were set up in a total volume of 25 μL, including 5 μL template cDNA (corresponding to 100 ng), 12.5 μL TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 900 nmol/L of each of the primers, and 200 nmol/L FAM-labeled probe (CTTGTGGGAAATGTTCAGGCTGGAGACTATG). Amplification was carried out using the ABI Prism 7700 or 7900 Sequence Detecting System (Applied Biosystems) for a total of 50 cycles. After an initial denaturation step for 10 min at 95°C, each cycle consisted of denaturation for 15 seconds at 95°C, and annealing and primer extension for 60 seconds at 60°C.

### *Immunoblot Analysis*

For protein expression analysis, cells were harvested 24 hours posttransfection and lysed in a buffer containing 7 mol/L urea, 100 mmol/L DTT, 50 mmol/L HEPES (pH 7.6), 100 mmol/L NaCl, 0.05% Triton X-100, 50 mmol/L NaF, and protease inhibitor mixture (Roche, Vienna, Austria). One microgram of total protein was separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and probed with the DO-1 monoclonal antibody kindly provided by B. Vojtesek (Masaryk Memorial Cancer Institute, Brno, Czech Republic). The antibody to PIG3 (Ab-1) was purchased from Oncogene Research Products (Boston, MA).

### *Flow Cytometric Evaluation of Transfection Efficiencies*

Fluorescence-activated cell sorter analyses were done with a FACSCalibur (BD, Sunnyvale, CA). Twenty-four or 44 hours after co-transfection with pCMV-EGFP, p53, and reporter luciferase plasmid, both adherent and floating cells were harvested and the percentage of GFP-positive viable cells was evaluated.

### *Flow Cytometric Evaluation of p53-Induced Cell Death*

DNA content in transiently p53 or control-transfected cells was determined as previously described (23). Briefly, 44 hours after transfection adherent cells were combined with cells that had detached from the plastic and were fixed with 70% ice-cold

ethanol for at least 1 hour. After rehydration and washing in PBS, cells were stained with antibodies CM1 or DO1 (a kind gift of B. Vojtesek, Masaryk Memorial Cancer Institute, Brno) for detection of p53 or with an antibody to β-galactosidase (Z3781, Promega), followed by propidium iodide (50 μg/mL). FITC-conjugated secondary anti-rabbit (F205) or anti-mouse (F313) antibodies were from DAKO (Vienna, Austria). DNA content in FITC-positive and -negative cells was assessed by two-color fluorescence-activated cell sorter analysis.

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## References

1. Zhao R, Gish K, Murphy M, et al. Analysis of *p53*-regulated gene expression patterns using oligonucleotide arrays. Genes Dev 2000;14:981-3.

2. Mack DH, Vartikar J, Pipas JM, Laimins LA. Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. Nature 1993; 363:281-3.

3. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997; 88:323-31.

4. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations [see comments]. Science 1994;265:346-55.

5. Malkin D. p53 and the Li-Fraumeni syndrome. Biochim Biophys Acta 1994; 1198:197-213.

6. Lassus P, Bertrand C, Zugasti O, et al. Anti-apoptotic activity of p53 maps to the COOH-terminal domain and is retained in a highly oncogenic natural mutant. Oncogene 1999;18:4699-709.

7. Hsiao M, Low J, Dorn E, et al. Gain-of-function mutations of the *p53* gene induce lymphohematopoietic metastatic potential and tissue invasiveness. Am J Pathol 1994;145:702-14.

8. Sigal A, Rotter V. Oncogenic mutations of the *p53* tumor suppressor: the demons of the guardian of the genome. Cancer Res 2000;60:6788-93.

9. Flaman JM, Robert V, Lenglet S, Moreau V, Iggo R, Frebourg T. Identification of human *p53* mutations with differential effects on the bax and p21 promoters using functional assays in yeast. Oncogene 1998;16:1369-72.

10. Deb S, Jackson CT, Subler MA, Martin DW. Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J Virol 1992; 66:6164-70.

11. Tsutsumi-Ishii Y, Tadokoro K, Hanaoka F, Tsuchida N. Response of heat shock element within the human HSP70 promoter to mutated *p53* genes. Cell Growth & Differ 1995;6:1-8.

12. Dittmer D, Pati S, Zambetti G, et al. Gain of function mutations in *p53.* Nat Genet 1993;4:42-6.

13. Koga H, Deppert W. Identification of genomic DNA sequences bound by mutant p53 protein (Gly245→Ser) *in vivo.* Oncogene 2000;19:4178-83.

14. Blandino G, Levine AJ, Oren M. Mutant *p53* gain of function: differential effects of different *p53* mutants on resistance of cultured cells to chemotherapy. Oncogene 1999;18:477-85.

15. He M, Rennie PS, Dragowska V, Nelson CC, Jia W. A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53. FEBS Lett 2002;517:151-4.

16. El Hizawi S, Lagowski JP, Kulesz-Martin M, Albor A. Induction of gene amplification as a gain-of-function phenotype of mutant p53 proteins. Cancer Res 2002;62:3264-70.

17. Albor A, Kaku S, Kulesz-Martin M. Wild-type and mutant forms of p53 activate human topoisomerase I: a possible mechanism for gain of function in mutants. Cancer Res 1998;58:2091-4.

18. Gagnebin J, Kovar H, Kajava AV, et al. Use of transcription reporters with novel p53 binding sites to target tumour cells expressing endogenous or virally transduced p53 mutants with altered sequence-specificity. Oncogene 1998; 16:685-90.

19. Kovar H, Auinger A, Jug G, et al. Narrow spectrum of infrequent p53 mutations and absence of MDM2 amplification in Ewing tumours. Oncogene 1993;8:2683-90.

20. Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: new online mutation analysis and recommendations to users. Hum Mutat 2002;19:607-14.

21. Di Como CJ, Prives C. Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in a yeast-based assay. Oncogene 1998;16:2527-39.

22. Kovar H, Jug G, Printz D, Bartl S, Schmid G, Wesierska-Gadek J. Characterization of distinct consecutive phases in non-genotoxic p53-induced apoptosis of Ewing tumor cells and the rate-limiting role of caspase 8. Oncogene 2000;19:4096-107.

23. Kovar H, Pospisilova S, Jug G, Printz D, Gadner H. Response of Ewing tumor cells to forced and activated p53 expression. Oncogene 2003;22:3193-204.

24. Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. A functional p53-responsive intronic promoter is contained within the human *mdm2* gene. Nucleic Acids Res 1995;23:2584-92.

25. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550-3.

26. Szak ST, Mays D, Pietenpol JA. Kinetics of p53 binding to promoter sites *in vivo.* Mol Cell Biol 2001;21:3375-86.

27. Contente A, Dittmer A, Koch MC, Roth J, Dobbelstein M. A polymorphic microsatellite that mediates induction of PIG3 by p53. Nat Genet 2002;30: 315-20.

28. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. Nature 1997;389:300-5.

29. Kovar H, Auinger A, Strehl S, Gadner H. p53 Alterations in Ewing tumours: evidence for mutation preceding metastatic spread. Clin Chem Enzymol Commun 1993;5:259-64.

30. Preuss U, Kreutzfeld R, Scheidtmann KH. Tumor-derived p53 mutant C174Y is a gain-of-function mutant which activates the fos promoter and enhances colony formation. Int J Cancer 2000;88:162-71.

31. Deb D, Scian M, Roth KE, et al. Hetero-oligomerization does not compromise 'gain of function' of tumor-derived p53 mutants. Oncogene 2002; 21:176-89.

32. Buzek J, Latonen L, Kurki S, Peltonen K, Laiho M. Redox state of tumor suppressor *p53* regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. Nucleic Acids Res 2002;30:2340-8.

33. Campomenosi P, Monti P, Aprile A, et al. *p53* mutants can often transactivate promoters containing a *p21* but not Bax or PIG3 responsive elements. Oncogene 2001;20:3573-9.

34. Flatt PM, Polyak K, Tang LJ, et al. p53-dependent expression of PIG3 during proliferation, genotoxic stress, and reversible growth arrest. Cancer Lett 2000 Aug. 1;156:63-72.

35. Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990; 249:912-5.