IFI16 and NM23 Bind to a Common DNA Fragment Both in the *P53* and the *cMYC* Gene Promoters

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ABSTRACT

In the melanoma M14 cell line, we found that the antimetastatic protein NM23/nucleoside diphosphate kinase binds to the promoters of the oncogene cMYC and of P53, a gene often mutated in human cancer (Cervoni et al. [2006] J. Cell. Biochem. 98:421–428). In a further study, we find now that IFI16, a transcriptional repressor, in both promoters binds to the G-rich fragment that also binds NM23/NDPK. These fragments possess non-B DNA structures. Moreover, by sequential chromatin immunoprecipitation (re-ChIP) we show that the two proteins (IFI16 and NM23/NDPK) are simultaneously bound in vivo to the same DNA fragments. Since P53 stimulates apoptosis and inhibits cellular growth, and cMYC promotes cell growth and, in several instances, also apoptosis, the presence of NM23 and IFI16 on the same DNA fragments suggests their common involvement in the reduced development of some tumors. J. Cell. Biochem. 106: 666–672, 2009.   © 2009 Wiley-Liss, Inc.

NM23/nucleoside diphosphate kinase (NM23/NDPK or NM23) is a ubiquitous enzyme, produced by expression of the *NM23* genes in mammals. The most abundant human isoforms are NDPK A and NDPK B, encoded by genes *NM23-H1* and *NM23-H2*, respectively.

These hexameric enzymes transform nucleoside diphosphates into triphosphates by an ATP-dependent reaction [Lacombe et al., 2000].

The NM23/NDPK proteins have many other functions that are not necessarily connected with their catalytic activity, the best known being the property to suppress the metastatic potential of tumor cells (hence the gene name: no metastasis). These antimetastatic properties have been diffusely reported [Steeg, 2004], but the biochemical mechanism by which the protein works is still unknown and the object of great speculation.

In a previous study [Cervoni et al., 2006] performed in vivo on M14 cells, a melanoma cell line with intermediate metastatic potential [van Muijen et al., 1991; Meije et al., 2002], we found that

the promoters of several genes containing a G-rich sequence related to a non-B DNA conformation [Postel, 2003; Siddiqui-Jain et al., 2002] are natural targets of NM23.

The promoters of two of these genes, a tumor/metastasis suppressor gene (*P53*) and an oncogene (*cMYC*), have been the subject of further investigation, with the aim of finding other proteins that bind to NM23, or directly interact with it, in complexes involved in the transcription of the target gene and in the antimetastatic role of NM23. Both genes were chosen for their known participation in crucial cellular functions (cell cycle, cell differentiation, and apoptosis).

P53 is very often mutated in human cancer [Vogelstein et al., 2000], while *cMYC* [Marcu et al., 1992] promotes cell growth, proliferation and in some cases induces apoptosis [Conzen et al., 2000].

The first result of this study was the finding that IFI16 (Interferon-inducible myeloid differentiation transcriptional activator), a transcriptional repressor, binds in vitro to regions of either *P53*

or *cMYC* promoters that also bind NM23. Moreover, by sequential chromatin immunoprecipitation (re-ChIP) we found that the two proteins cross-linked in vivo to the same DNA fragment in the *P53* and in the *cMYC* promoters, suggesting a common intervention of NM23 and IFI16 in cell growth and apoptosis regulation.

MATERIALS AND METHODS

M-280 sheep anti-rabbit IgG, Protein G, and M-280 Streptavidin Dynabeads were obtained from Invitrogen, rabbit IgG was from Sigma–Aldrich. Proteinase K and *cis*-diammine dichloro platinum II (*cis*-DDP) were from Sigma–Aldrich, Benzonase, and DNase-free RNase A were from Roche Applied Science. The oligonucleotides used were synthesized by PRIMM.

Recombinant NDP kinase A and B were purified as described [Lascu et al., 1997; Gonin et al., 1999] on Q-Sepharose columns and stored as precipitates in saturated ammonium sulfate solutions.

The anti-IFI16 antibody was obtained from Santa Cruz Biotechnology while polyclonal antibody against NDP kinase was raised in rabbits immunized with NDP kinase B by Davids (Regensburg, Germany) and was purified by affinity chromatography using a HiTrap NHS activated column (GE Healthcare) [Phang-Ba et al., 1998]. These antibodies cross-react with the highly homologous NDPkinase A, thus allowing simultaneous detection of both isoforms, which migrate differently on SDS–polyacrylamide gel electrophoresis.

siRNA for IFI16 was purchased from QIAGEN and Lipofectamine™ RNAiMAX from Invitrogen.

M14 CELLS
The cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM Na pyruvate, in humidified atmosphere containing 5% CO_2.

NUCLEAR EXTRACTS
The cells were scraped, harvested by centrifugation and washed twice with PBS. The cells were then lysed in hypotonic lysis buffer (10 mM HEPES at pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM DTT) containing a protease inhibitor cocktail (Roche). After incubation on ice for 15 min, nuclei were collected by centrifugation at 1,500g at 4°C for 10 min. The nuclei were further purified by incubation with lysis buffer containing 0.2% (v/v) Triton X-100 on ice for 10 min. The nuclei were pelleted as described above and washed twice in hypotonic lysis buffer. The nuclear extracts were obtained by treating the nuclear fraction in ice for 30 min with 10 mM HEPES, pH 7.9, 10 mM KCl, 0.42 M NaCl, 20% (v/v) glycerol, containing the inhibitor cocktail, followed by centrifugation at 10,000g for 10 min.

AFFINITY PURIFICATION BY DNA IMMOBILIZED ON DYNABEADS
M-280 Streptavidin Dynabeads were washed three times in buffer A (5 mM Tris–HCl, pH 8, 0.5 mM EDTA, 1 M NaCl).

The biotinylated DNA fragments of the *P53* promoter (−345/−235) and of the *cMYC* promoter (−150/−363), containing the binding site for NM23 (G-rich sequence), were prepared by PCR using the following primers: Bio-*P53*-fw: 5′-aggatccagctgagagcaaa-3′; *P53*-rv: 5′-AGAGCTGTGAGGGCAGAATT-3′; Bio-*cMYC*-fw: 5′-AGGCGCGCGTAGTTAATTCA-3′; *cMYC*-rv: 5′-TCGCATTA-TAAAGGGCCGGT-3′. The amplification generated a single band displaying the predicted size. The PCR product was purified by NucleoSpin Extract II (Macherey–Nagel).

The 50 bp biotinylated double-stranded oligonucleotide of the *P53* promoter (−325/−275), containing the G-rich sequence was obtained from PRIMM.

These biotinylated DNA fragments were incubated with the beads (200 pmol/mg beads) for 30 min at 4°C in buffer A. The beads were then washed twice with buffer A, incubated with 50 µM biotin for 30 min at 4°C, washed three times with buffer A, and three times with buffer B (20 mM Tris–HCl, pH 8, 1 mM EDTA, 10% (v/v) glycerol, 0.5 mM DTT, 50 mM NaCl, 0.1% (v/v) Nonidet P-40) containing a protease inhibitor cocktail and resuspended in buffer B at 10 mg/ml. The nuclear extracts in buffer B were preincubated for 5 min with *E. coli* DNA, which was in a 500-fold excess in respect to the immobilized DNA. These nuclear extracts were mixed with the DNA-coated beads and incubated for 60 min at 4°C. The beads were then isolated using a magnetic separator (Invitrogen) and washed four times in buffer B and once in 10 mM Tris–HCl, pH 8, 1 mM EDTA. The specifically bound proteins were eluted with 400 mM NaCl or with Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10 mM glycerol, 5 mM dithiothreitol) at 65°C for 10 min, separated by SDS–gel electrophoresis in 10% or 15% polyacrylamide, and subjected to Western blot analysis with specific antibodies or to protein identification by mass spectrometry. Control experiments were carried out binding the beads to an irrelevant double-stranded DNA fragment or after saturating them with biotin.

CHROMATIN IMMUNOPRECIPITATION (ChIP) AND RE-ChIP ASSAYS
In ChIP experiments DNA-protein cross-linked complexes were formed in M14 cells by the use of *cis*–DDP and the immunoprecipitation procedure was carried out as described previously [Cervoni et al., 2003]. Antibodies used for ChIP and re-ChIP were anti-NDPK, anti-IFI16, and pre-immune immunoglobulin G (IgG) as negative control.

The immunocomplexes were eluted from the beads in 50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% SDS at 65°C for 10 min.

In re-ChIP experiments, after the elution of the primary immunocomplex obtained with anti-NDPK, the eluate was diluted two times with RIPA buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 0.1% Na-deoxycolate and 0.1% Triton X-100) and immunoprecipitated with anti-IFI16 antibody.

The DNA, from ChIP or re-Chip assays, was recovered from the purified immunocomplexes, after the incubation with 500 µg/ml Proteinase K for 2 h at 37°C, by a treatment with 1.5 M thiourea at 50°C for 30 min to reverse the cross-linkage, and purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The amount of immunoprecipitated DNA was determined by ethidium bromide fluorescence quantification according to Sambrook et al. [1989].

The proteins were recovered from the purified immunocomplexes, after the incubation with 0.25 U/ml Benzonase over night at 37°C, by a treatment with 0.1 M thiourea at 90°C for 30 min to

reverse the cross-linking with *cis*-DDP. The proteins were separated by SDS–gel electrophoresis in 10% or 15% polyacrylamide and subjected to Western blot analysis with specific antibodies.

PCR AMPLIFICATION OF IMMUNOPRECIPITATED DNA

We confirmed the presence of specific DNA sequences by subjecting the immunoprecipitated DNA to PCR. PCR was carried out in a 50 μl volume in the presence of 5 ng of immunoprecipitated DNA, using a Perkin-Elmer GeneAmp PCR System 2400. ChIP and re-ChIP DNAs were analyzed by PCR using *P53* promoter primers (fw: 5′-AGG ATC CAG CTG AGA GCA AA-3′; rv: 5′-AGA GCT GTG AGG GCA GAA TT-3′), *MYC* promoter primers (fw: 5′-AGG CGC GCG TAG TTA ATT CA-3′; rv: 5′-TCG CAT TAT AAA GGG CCG GT-3′), and as a negative control *P53* exon 2 primers (fw: 5′-TGG AAG TGT CTC ATG CTG GA; rv: 5′-CTT CCC ACA GGT CAC TGC TA-3′) and *ING* exon 1a (fw: 5′-GTT AGG TCC TGG TCG GGT TT-3′; rv: 5′-GCC TTG CCT GTC ACT TTT TC-3′). The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and quantified using a Kodak Image Station. The linear range of PCR product amplification was determined, and the amount of ChIP-DNA template was optimized.

PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

Selected bands were manually excised from SDS–PAGE and submitted to trypsin proteolysis. Briefly, after four destaining steps using 5% (30 min), 50% (two times, 30 min each), and 100% (10 min) acetonitrile in 25 mM ammonium bicarbonate, about 165 ng of trypsin (modified porcine variant, Promega, Madison, WI), solubilized in 15 μl of a 25 mM ammonium bicarbonate digestion buffer, were added to vacuum-dried gel band. Digestion was performed at 37°C overnight. An aliquot (1 μl) of peptide mixture was mixed with the same volume of α-cyano-4-hydroxy-*trans*-cinnamic acid matrix solution (10 mg/ml) in 70% acetonitrile containing 0.1% TFA (v/v) and spotted onto a MALDI target plate. MALDI-ToF analyses were performed in a Voyager-DE-STR instrument (Applied Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and operating in reflector mode. Mass data were obtained by accumulating several spectra from laser shots with an accelerating voltage of 20 kV. Two tryptic autolytic peptides were used for the internal calibration (m/z 842.5100 and 2807.3145). Selected ion signal was submitted to fragmentation by post source decay (PSD), after isolation of selected precursor ions using timed ion selector (TIS), performing 10 steps of the reflectron voltage; for each individual step the voltage was decreased by 25% of the previous step. The individual segments were automatically stitched together. The PSD fragment ions were measured as isotopically averaged masses. Calibration was performed with PSD spectra of angiotensin.

Identification by peptide mass fingerprint and by PSD analyses were performed using the Mascot search engine (v. 2.2) against the entire SwissProt database (v. 56.5, 402482 sequences), considering up to one missed cleavage, 50 ppm measurements tolerance, oxidation at methionine (variable modification), a mass tolerance of ±0.25 Da for the precursor ions and ±0.8 Da for the fragmentations.

RNA INTERFERENCE (siRNA)

The day before transfection, 2.5×10^4 M14 cells were seeded per well of a 96-well plate in 100 μl of a complete culture medium (containing serum and antibiotics). The cell cultures were grown under normal growth conditions (typically 37°C and 5% CO_2). The transfection was carried out according to the manufacturer's instructions and the siRNA was used at 160 nM final concentration. The M14 cells were incubated with the transfection complexes under their normal growth conditions and gene silencing was monitored at the mRNA level after 24, 48, and 72 h. The level of mRNA from an untreated sample was used a control reference.

TOTAL RNA PREPARATION AND REAL–TIME PCR

The untreated (as control) and treated cells (after 24, 48, and 72 h) were harvested and total RNA was isolated with TRIzol (Invitrogen) following the manufacturer's instructions. The reverse transcription of total RNA was conducted with SideStep™ II QPCR cDNA Synthesis Kit (Stratagene), according to the manufacturer's instructions. *P53*, *cMYC* and *IFI16* expression was evaluated with specific primers (QuantiTect® QIAGEN) by the Real-time PCR as described below. Real-time PCR was performed using a MJ MiniOpticon Detection System (BioRad Laboratories, Ltd.) with SYBR green fluorophore by means of Brilliant® SYBR® Green QPCR Master Mix (Stratagene). The protocol used was: denaturation (95°C for 5 min), amplification repeated 40 times (95°C for 30 s, 55°C for 30 s, 72°C for 30 s).

A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. PCR fluorophore acquisition temperatures were set from 40°C to 95°C, reading every 0.5°C. All reactions were carried out in at least duplicate. *GAPDH* (RT²-PCR primers from SuperArray) and *RPS27A* (QuantiTect® QIAGEN) genes were used as reference for normalization and the relative quantification was analyzed using Gene Expression Analysis for iCycler iQ Real-time PCR Detection System Software, Version 1.10 (BioRad Laboratories, Ltd.).

RESULTS

PROTEINS BINDING IN VITRO TO THE P53 AND cMYC PROMOTERS

Biotinylated probes obtained by PCR from melanoma M14 DNA, as previously described in Materials and Methods Section, were immobilized on streptavidin-coupled Dynabeads. These probes, containing sequences of *P53* (110 bp) and *cMYC* (213 bp) promoters (Fig. 1), are characterised by G-rich sequences that were found, in a previous study [Cervoni et al., 2006], bound to NM23 after cross-linking.

The beads were subsequently incubated with total nuclear extracts, collected, washed, and finally treated with Laemmli buffer at 65°C for 10 min. The proteins bound on the DNA fragments were in this way detached and recovered.

By Western blotting NM23/NDPK A and B were found present, as expected, in the proteins affinity-bound on the *P53* and *cMYC* promoter fragments (Fig. 2).

A

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  1 gggagaaaac gttagggtgt ggatattacg gaaagccttc ctaaaaaatg acatttaact
 61 gatgagaaga aaggatccag ctgagagcaa gcgcaaaagc tttcttcctt ccacccttca
121 tatttgacac aatgcaggat tcctccaaaa tgattccac  caattctgcc ctcacagctc
181 tggcttgcag aatttttccac cccaaaatgt tagtatctac ggcaccaggt cggcgagaat
241 cctgactctg caccctcctc cccaactcca tttcctttgc ttcctccggc aggcggatta
301 cttgcccctta cttgtcatgg cgactgtcca gctttgtgcc aggagcctcg caggggttga
361 tgggattggg gtttttcccct cccatgtgct caagactggc gctaaaagtt ttgagcttct
421 caaaagtcta gagccaccgt ccagggagca ggtagctgct gggctccggg gacactttgc
481 gttcgggctg ggagcgtgct ttccacgacg gtgacacgct tccctggatt gg
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B

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1921 tggtacgcgc gtggcgtggc ggtgggcgcg cagtgcgttc tcggtgtgga gggcagctgt
1981 tccgcctgcg atgatttata ctcacaggac aaggatgcgg tttgtcaaac aatactgcta
2041 cggaggagca gcagagaaag ggagagggtt tgagagggag caaaagaaaa tggtaggcgc
2101 gcgtagttaa ttcatgcggc tctcttactc tgtttacatc ctagagctag agtgctcggc
2161 tgcccggctg agtgtcctcc ccaccttccc caccctcccc accctcccca taagcgcccc
2221 tcccgggttc ccaaagcaga gggcgtgggg gaaaagaaaa aagatcctct ctcgctaatc
2281 tccgcccacc ggccctttat aatgcgaggg tctggacggc tgaggacccc cgagctgtgc
2341 tgctcgcggc cgccaccgcc gggccccggc cgtccctggc tcccctcctg cctcgagaag
2401 ggcagggctt ctcagaggct tggcgggaaa aagaacggag ggagggatcg cgctgagtat
2461 aaaagccggt tttcgggggct ttatctaact cgctgtagta attccagtga gaggcagagg
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Fig. 1. The maps show the promoters of *P53* (A) and *cMYC* (B). Long arrows (⟶) show the primers that amplified a 110 bp region of the *P53* promoter and a 213 bp region of the *cMYC* promoter. Broken arrows show the 50 bp region of the *P53* promoter used in one of the experiments. Bold capital letters show the GC-rich sequences in the *P53* e *cMYC* promoters.

In order to identify other components of the protein mixtures detached from the target DNA fragments, an aliquot of sample was subjected to SDS–PAGE and stained with Coomassie Brilliant Blue (Fig. 2).

Some of the more concentrated protein bands were excised from the gels and trypsin-digested as described in Materials and Methods Section. The tryptic peptide mixtures obtained were analyzed by MALDI-TOF mass spectrometry. Peptide mass fingerprint analysis allowed identification of several proteins, among which the 100 kDa protein IFI16 (code: Q16666) a putative transcriptional repressor [Johnstone et al., 1998].

The identity of this protein on the *P53* promoter and its presence on the *cMYC* promoter was confirmed by Western blotting with the relative antibody (Fig. 2).

With the purpose to gather more knowledge on the binding of IFI16 to the NM23-binding DNA fragment, a shorter (50 bp) biotinylated DNA fragment of the *P53* promoter containing the G-rich sequence was linked to Dynabeads and incubated with nuclear extracts. By Western blotting IFI16 was found bound also to this shorter oligonucleotide (Fig. 3).

We were unable to demonstrate the existence of a direct association of the two proteins by coimmunoprecipitation. Either the two proteins actually do not interact with each other, or the interaction is so weak that it does not withstand the usual coimmunoprecipitation procedure.
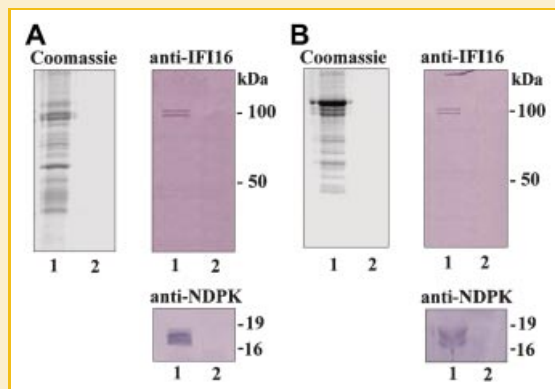

Fig. 2. SDS–PAGE and Western blotting with anti–IFI16 or anti–NDPK of nuclear proteins interacting with the *P53* (A) and *cMYC* (B) promoters. Lane 1: nuclear proteins interacting with the Dynabeads bound oligonucleotide; lane 2: control with Dynabeads not linked to oligonucleotides. Migration of molecular mass markers is also indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
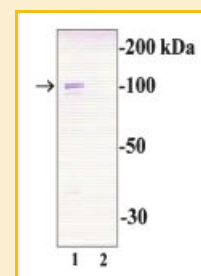

Fig. 3. Western blotting with anti–IFI16 of the nuclear proteins interacting with the 50 bp *P53* promoter (−325/−275). Lane 1: nuclear proteins interacting with the Dynabeads bound oligonucleotide; lane 2: control with Dynabeads not linked to oligonucleotides. Arrow indicates the presumptive position of the antigen. Migration of molecular mass markers is also indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
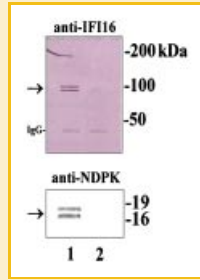
Fig. 4. Western blotting with anti–IFI16 and anti–NDPK of the proteins immunoprecipitated after cross-linking with *cis*-DDP. Lane 1: proteins immunoprecipitated with anti–NDPK; lane 2: control (proteins immunoprecipitated with rabbit IgG heavy chain). Arrows indicate the presumptive position of the respective antigens. Migration of molecular mass markers is also indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IN VIVO CROSS–LINKING AND IMMUNOPRECIPITATION

In order to verify the existence in vivo of the affinities established in vitro, chromatin immunoprecipitation (ChIP) experiments were performed on viable M14 melanoma cells cross-linked with *cis*-DDP. Immunopurification of NM23-bound DNA was obtained using anti-NDPK bound to rabbit IgG Dynabeads. The beads were eluted after incubation in the proper conditions, and the eluate was analyzed to determine the proteins and the DNA fragments isolated by immunoprecipitation. At the same time control tests were performed in which the only difference was the use of rabbit IgG instead of anti-NDPK.

In the DNA fraction of the eluate the promoter sequences of *P53* and *cMYC* were found enriched, as previously described [Cervoni et al., 2006].

The protein fraction was subjected to Western blotting with the specific antibodies. As shown in Figure 4, NM23 and IFI16 were present in the *cis*-DDP-cross-linked sample.

Since *cis*-DDP favors binding between DNA and proteins and has scarce cross-linking power between proteins, these results might indicate a direct binding of IFI16 to the DNA fragments cross-linked to NM23.

In order to verify the direct binding of IFI16 to the NM23 binding DNA sequence, ChIP was also performed on *cis*-DDP-cross-linked M14 cells using anti-IFI16. DNA fragments cross-linked to IFI16 were analyzed by PCR, using the same primers that amplified the 110 bp NM23 binding region of the *P53* promoter and the 213 bp NM23 binding region of the *cMYC* promoter, and two different exons as a negative control [Cervoni et al., 2006]. These exon fragments were located in the *P53* exon 2 and the *ING* exon 1a. There were no G-rich sequences inside these regions. Densitometric analysis of the amplified products shows the gene fragments enrichment in the immunoprecipitated DNA in respect to the control experiment (Fig. 5).

Re–ChIP (o SeqChIP)

ChIP assay demonstrate that both nuclear NM23 and IFI16 in vivo bind to *P53* and *cMYC* promoters. These results however do not reveal if there is co-occupancy of these two binding sites. To solve this problem we took advantage of the re-ChIP assay. In re-ChIP, protein–DNA complexes from the first immunoprecipitation with anti-NDPK were subjected to an additional immunoprecipitation with anti-IFI16. The resulting DNAs were analyzed by semi-quantitative PCR in an analogous manner to conventional ChIP samples.

The results of this experiment are shown in Figure 6. The sequential ChIP assay suggests that IFI16 and NM23 simultaneous bind the *P53* and *cMYC* promoters.

DECREASE OF IFI16 LEADS TO INCREASED P53 AND cMYC EXPRESSION

To determine the functional role of IFI16 in *P53* and *cMYC* regulation, we used IFI16 siRNA to knock down IFI16 expression in M14 cells, which endogenously express IFI16, P53, and MYC.

M14 cells were transfected with IFI16 siRNA (Qiagen), and the expression level of *P53* and *cMYC* were examined. The decrease of endogenous IFI16 over time resulted in a marked increase of *P53* and *cMYC* expression (Fig. 7), showing that IFI16 negatively affects *P53* and *cMYC* expression level.
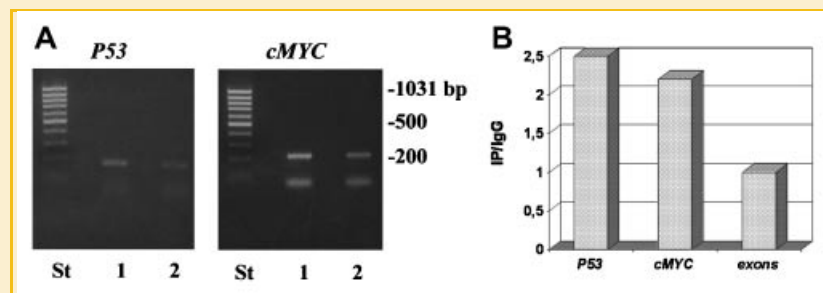

Fig. 5. Analysis of DNA immunoprecipitated by anti–IFI16 after cross-linking with *cis*-DDP. A: PCR amplified DNA. Lane 1: DNA obtained by ChIP with anti–IFI16; lane 2: control DNA obtained by ChIP with non–specific IgG; St: DNA markers. B: Densitometric analysis of PCR products: enrichment of genes in immunoprecipitated DNA. Results are expressed as ratio of immunoprecipitated DNA to control.
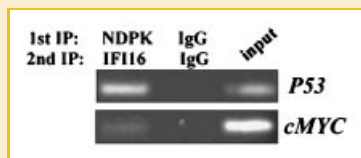
Fig. 6. Re–ChIP assay. PCR amplified DNA obtained using sequential ChIP. Following the first IP with anti–NDPK, NDPK–chromatin complex was eluted and subjected to second IP with anti IFI–16.

DISCUSSION

In a previous study [Cervoni et al., 2003] performed in vivo on M14 melanoma cells, we found that fragments of the *P53* and *cMYC* promoters are targets of the tumor suppressor protein NM23. Now by affinity purification we show that also IFI16 is bound to the same regions of the *P53* promoter and the *cMYC* promoter that interact with NM23 (Fig. 2).

The ChIP results obtained with the DNA–protein cross-linking agent *cis*-DDP confirm that IFI16 interacts directly in vivo with DNA at the level of the promoter region of the *P53* and the *cMYC* gene in M14 cells (Fig. 4). While the interaction of the protein IFI16 with the protein P53 has been described in other cultured cells [Kwak et al., 2003], the binding of IFI16 to the *P53* gene or to the *cMYC* gene, to our knowledge, has never before been mentioned in the literature.

The promoter fragments chosen contain G-rich sequences that may give rise to quadruplex structures [Mohanty and Bansal, 1993]. The unusual structures adopted by guanine rich DNA sequences have received wide attention in recent years. Such sequences constitute the telomeric ends of eukaryotic chromosomes and have also been found in the promoters or regulatory regions of important oncogenes such as *cMYC*, *bcl2*, *c-Fos*, and *c-ABL* [Liu et al., 2007].
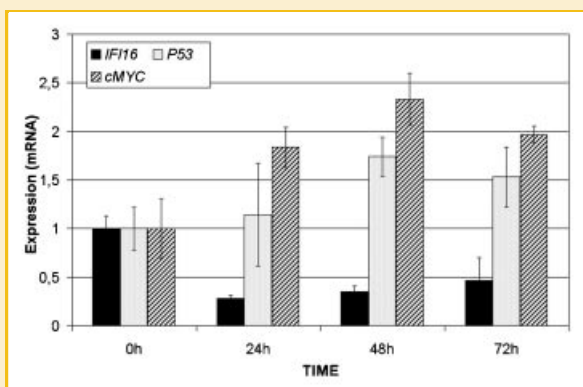

Fig. 7. Inhibition of IFI16 by RNA interference increases *P53* and *cMYC* expression. The histogram is a graphical display of the Real–time PCR expression profile of the two genes *P53* and *cMYC* at 0, 24, 48, and 72 h following silencing of the IFI16 (expression profile reported in the same graph). The trascriptional activity of the genes was normalized with the expression of two reference genes (*GADPH, RSP27A*) in the same sample. The relative expression in the sample at 0 h is given as 1.

We cannot rule out non-sequence specific or ssDNA preferential binding. In our case, however, G-rich sequences have been found to be good targets of the proteins under study. In particular, for *P53* we have found that shortening the promoter fragment to only 50 bp does not prevent the binding of NM23 and IFI16 in vitro (Fig. 3).

The *P53* tumor suppressor gene is important in the regulation of the cell cycle, and it is known that it plays a crucial role in the progression of cancer, as evidenced by the inactivation or loss of P53 in the majority of human tumors [Levine, 1997].

The *cMYC* oncogene has been found to be responsible of functions as different as proliferation and programmed cell death. According to Wagner et al. [1994] only the overexpression of both *P53* and *cMYC* could elicit MYC-mediated apoptosis.

Several studies suggest that NM23 interacts with some transcription factors (EBNA-3C, Menin, ROR/RZR) [Roymans et al., 2002]. However, it is not clear if these proteins contribute in identifying the target genes or if they are instrumental in the capacity of NM23 to activate or inhibit different sets of genes in different cell types. This question is relevant to understand the antimetastatic role of NM23, so that a more complete picture of the associated proteins is certainly desirable.

Here we have demonstrated the presence of IFI16 in NM23-binding *P53* and *cMYC* promoters. IFI16 (Interferon-inducible myeloid differentiation transcriptional activator) is a nuclear protein highly expressed by human monocytes (Dawson et al., 1998). This protein is capable to control cellular proliferation by interacting with several transcription factors, such as P53 and pRb, the product of the retinoblastoma gene [Johnstone et al., 2000]. Its nuclear localization and its ability to bind DNA with the highly basic residues at its N-terminal are typical of transcription factors [Kwak et al., 2003]. IFI16 has been described also as a potent transcriptional repressor [Johnstone et al., 2000]. This is here confirmed, since RNAi silencing of its transcription determines the overexpression of P53 and MYC, protein products of the *P53* and *cMYC* genes, respectively.

Taken together, the present results show that IFI16 interacts directly with DNA at the level of the NM23-binding promoter region of both *P53* and *cMYC* genes.

Kwak et al. [2003] suggest "that IFI16 binds to p53 and hinders p53 binding at the p21 promoter such that the removal of IFI16 results in increased p53 binding at the p21 promoter leading to increased transcription of p21" and that this determines cell cycle arrest. Our experiments show that IFI16 binds also to the *P53* promoter and causes transcriptional repression of *P53* and, consequently, of its downstream target genes involved in cell cycle regulation. A logic conclusion could be that the effect noticed by Kwak on the *p21* gene might be determined not only by IFI16 binding to the P53 protein, but also by the transcriptional repressor effect of IFI16 binding to the P53 gene.

Our results do not allow to establish if a direct interaction IFI16-NM23 occurs, but allow to establish that the two proteins bind to the same region at the same time. Since both IFI16 [Choubey et al., 2008] and NM23 [Kaetzel et al., 2006] appear to be involved in a reduced development of some tumors, their presence on the same DNA fragment, suggests a common intervention in regulation of cell growth and apoptosis in different phases of cellular cycle.

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