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p53-Mediated downregulation of H ferritin promoter transcriptional efficiency via NF-Y

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This paper is dedicated to the memory of one of the authors, Prof. S. Venuta, who recently passed away.

Abstract

The tumor suppressor protein p53 triggers many of the cellular responses to DNA damage by regulating the transcription of a series of downstream target genes. p53 acts on the promoter of the target genes by interacting with the trimeric transcription factor NF-Y. H ferritin promoter activity is tightly dependent on a multiprotein complex called Bbf; on this complex NF-Y plays a major role.

The aim of this work was to study the modulation of H ferritin expression levels by p53. CAT reporter assays indicate that: (i) p53 overexpression strongly downregulates the transcriptional efficiency driven by an H ferritin promoter construct containing only the NF-Y recognition sequence and that the phenomenon is reverted by p53 siRNA; (ii) the p53 C-terminal region is sufficient to elicitate this regulation and that a correct C-terminal acetylation is also required. The H ferritin promoter displays no p53-binding sites; chromatin immunoprecipitation assays indicate that p53 is recruited on this promoter by NF-Y. The p53–NF-Y interaction does not alter the NF-Y DNA-binding ability as indicated by electrophoretic mobility shift assay (EMSA) analysis.

These results demonstrate that the gene coding for the H ferritin protein belongs to the family of p53-regulated genes, therefore adding a new level of complexity to the regulation of the H ferritin transcription and delineate a role for this protein in a series of cellular events triggered by p53 activation.

Keywords: Ferritin gene; Transcriptional regulation; Transcriptional factor

1. Introduction

Ferritin, the major iron storage protein, is composed of 24 subunits of two types, named H and L for heavy and light, respectively (Arosio, Adelman, & Drysdale, 1978). Ferritin synthesis is regulated at both transcriptional and

Abbreviations: Bbf, B-box binding factor; CAT, chloramphenicol acetyltransferase; H, heavy; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; w.t., wild type.

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translational levels (Torti & Torti, 2002). The expression of the two ferritin genes varies among different cell types, during cell differentiation and neoplastic transformation as well as in response to some environmental stimuli (Bevilacqua et al., 1995; Bomford & Munro, 1992).

The human H ferritin promoter spans a length of 150 bp upstream from the transcriptional start, which includes two *cis*-acting elements (Bevilacqua et al., 1992). The distal element (A box) is a GC box and is recognized by the transcription factor Sp1. The proximal element (B box) is an inverted CCAAT box and is recognized by a protein complex termed B-box binding factor (Bbf) (Bevilacqua et al., 1992). Bbf is composed of the trimeric transcription factor NF-Y (Faniello et al., 1999) which directly contacts the DNA, the co-activator p300 (Bevilacqua, Faniello, Quaresima, et al., 1997; Faniello et al., 1999) and the histone acetylase pCAF (Bevilacqua, Faniello, Russo, Cimino, & Costanzo, 1998). Recently we have demonstrated that c-Jun may be recruited to the promoter by interacting with Bbf, where it stimulates the transcription efficiency, and that is able to potentiate the transcription of the H ferritin promoter without binding directly to the DNA (Faniello et al., 2002). Bbf is also able to bind E1A, which acts as a transcriptional repressor (Bevilacqua, Faniello, Quaresima, et al., 1997).

The NF-Y transcription factor consists of three subunits, NF-YA, B and C, all of which are required for binding to the CCAAT motif (Maity, Sinha, Ruteshouser, & de Crombrugghe, 1992). NF-Y is ubiquitously expressed and activates transcription from various promoters. It has been shown that the DNA-binding activity of NF-Y changes during different stages of B-cell development (Currie, 1998), senescence (Jung et al., 2001; Pang & Chen, 1993) and differentiation (Bevilacqua et al., 2002; Marziali et al., 1997, 1999). NF-Y is implicated in transcriptional repression of G2-specific genes after p53 induction and DNA damage (Ceribelli, Alcalay, Vigano, & Mantovani, 2006; Manni et al., 2001; Yun et al., 1999).

p53 directly activates the expression of genes that contain p53-binding sites within their regulatory regions. p53 also functions as a negative regulator of various genes, although the promoters of most of these genes do not contain a typical binding consensus for this protein (Ginsberg, Mechta, Yaniv, & Oren, 1991). The mechanism by which p53 exerts this inhibition is thought to involve physical interaction either with basal components of the transcriptional machinery (Mack, Vartikar, Pipas, & Laimins, 1993; Seto et al., 1992) or with other unidentified factors, but its molecular basis remains largely unclear (Kubicka et al., 1999; Yun et al., 1999). However, in at least one case, it has been proposed that p53 represses transcription through interaction with a transcription activator rather than the basal transcription machinery. To function efficiently as an activator of gene expression, p53 forms complexes with other transcriptional regulators, including acetyltransferases such as p300/CBP (Vousden & Lu, 2002).

In this study we have investigated the potential involvement of p53 in the control of the H ferritin gene transcription and demonstrate that its expression represses the H ferritin promoter through inhibition of the transcriptional effects of NF-Y.

2. Materials and methods

2.1. Northern blot analysis

Total RNA was extracted from HeLa and HCT116 p53−/− cells treated and untreated with doxorubicin using TRIzol reagent (Invitrogen). Northern blot analysis was performed as previously described (Bevilacqua, Faniello, Russo, Cimino, & Costanzo, 1994). The labelled probes were a 528-bp Pst fragment of the H ferritin cDNA (Costanzo et al., 1983) and a 1600-bp Bam HI fragment from the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Arcari, Martinelli, & Salvatore, 1984).

2.2. Cell culture

HeLa cells, HCT116 w.t. and HCT116 p53−/−, were cultured as monolayers in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies) and 100 units/l penicillin (Life Technologies). Cells were grown at 37 °C in a 5% CO_2 atmosphere. Doxorubicin was added at 0.5 μg/ml for 20 h (Fig. 1), or 0.5 μg/ml for 8, 24 and 48 h (Fig. 2).

2.3. DNA transfections and chloramphenicol acetyltransferase (CAT) assays

The H ferritin promoter/CAT (Bbf TATAA and Bbf_{mut} TATAA) have been described previously (Bevilacqua, Faniello, Quaresima, et al., 1997). The plasmid p53 (CMV p53), contains the full-length human w.t. p53 cDNA, p53 siRNA (Gostissa et al., 2004), p53-CT (the human p53 fragment from aa 294 to 393 was PCR amplified and cloned downstream of the HA epitope into pcDNA3), p53 9KR and p53 H175(Gadea, Lapasset, Gauthier-Rouviere, & Roux, 2002), was a gift from Prof. G. Del Sal. Transfections were carried out using the calcium-phosphate technique (Graham & van der Eb,

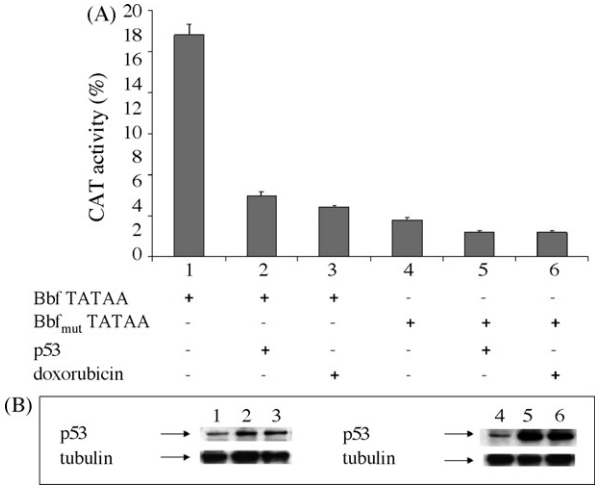

Fig. 1. p53 downregulates transcription of the H ferritin promoter. (A) CAT activity driven by H ferritin promoter/CAT construct (Bbf TATAA) which contains the inverted CCAAT box and its mutated version (Bbf$_{mut}$/TATAA), transiently transfected in HeLa cells with or without p53 expression vector and stimulated or not with doxorubicin. The values are the means ± S.E.M. of three independent transfection experiments and are expressed as the percentage of the acetylated form of [^{14}C]chloramphenicol. (B) Western blot analysis with anti-p53 antibody and anti-tubulin antibody of total extracts from HeLa cells transfected with or without p53 expression vector and stimulated or not with doxorubicin.

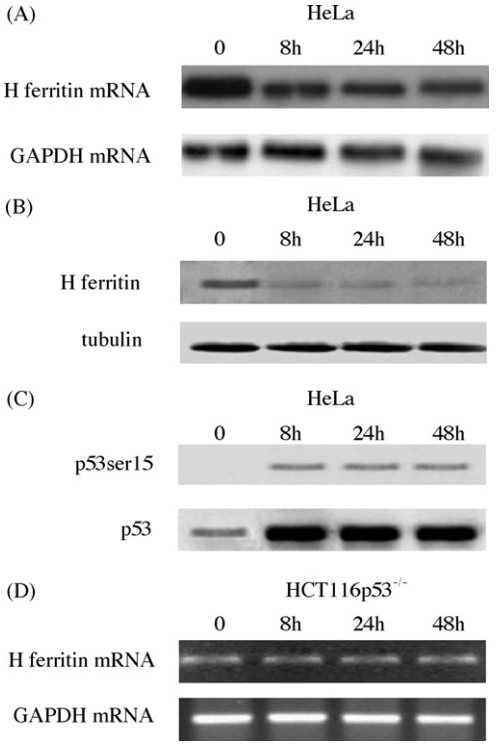

Fig. 2. Effect of doxorubicin on H ferritin gene expression. (A) Total RNA was isolated from HeLa cells treated with doxorubicin at different times (8, 24 and 48 h) and analyzed by hybridization with a radiolabelled H ferritin cDNA probe, and with a GAPDH probe as control. (B) Western blot analysis of H ferritin protein level after the treatment with doxorubicin. (C) Western blot analysis of p53 and p53 ser-15 after the treatment with doxorubicin. (D) Total RNA was isolated from HCT p53−/− cells treated with doxorubicin at different times (8, 24 and 48 h) and analyzed by hybridization with a radiolabelled H ferritin cDNA probe, and with a GAPDH probe as control.

1973) or the FuGENE-6 reagent (Roche). Variations in transfection efficiency were corrected by including the β-galactosidase gene as an internal control under the control of the Rous sarcoma virus (RSV) long terminal repeat in each experiment. Galactosidase activity was assayed as described previously (Oliner et al., 1993).

2.4. Western blot analysis

Protein lysates (50 μg) were separated by 10% SDS-PAGE, electro-transferred onto a nitrocellulose membrane and incubated with anti-p53 (sc-263, Santa Cruz Biotechnology), anti-p53 (ser-15) (9284, Cell Signaling), anti-H ferritin (sc-25617, Santa Cruz Biotechnology), or anti-tubulin antibody (sc-7396, Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit secondary antibody (sc-2005 and sc-2030, Santa Cruz Biotechnology). Protein–antibody complexes were revealed by enhanced chemiluminescence with the Santa Cruz Biotechnology ECL system.

2.5. Chromatin immunoprecipitation (ChIP)

HeLa cells (10^7) were treated with 1% formaldehyde directly into the media for 10 min at room temperature on a rocking platform. The cells were then washed and scraped with phosphate-buffered saline and collected by centrifugation at 700 × *g* for 4 min at 4 °C, resuspended in cell lysis buffer (10 mM Hepes pH 8.0, 85 mM KCl, 0.5% NP-40, protease inhibitor 1 μg/ml leupetin, 1 μg/ml aprotinin, 1 mM PMSF) and incubated on ice for 10 min. The pellet was resuspended in lysis buffer (50 mM Tris–HCl pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitor) and incubated on ice for 10 min. The DNA was sonicated to give fragments of approximately 500 bp. Initially, optimum conditions for sonication were determined by agarose gel analysis after reversing cross-links and precipitated DNA. The lysate was diluted 5-fold in ChIP IP buffer (0.01% SDS, 1% Triton-X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, protease inhibitor 1 μg/ml leupetin, 1 μg/ml aprotinin, 1 mM PMSF) and precleared with 100 μl of protein A beads, which has been pre-adsorbed

with sonicated salmon sperm DNA for 90 min at 4 °C on a rotary mixer. Beads were collected by centrifugation at 2000 × *g*, and chromatin solution was transferred to a fresh microcentrifuge tube. At this stage, the solution was split into microcentrifuge tubes and immunoprecipitated with 5 μg of NF-YB (sc-7711, Santa Cruz Biotechnology), p53 (sc-263, Santa Cruz Biotechnology) and p300 antibodies (05-257, Upstate) overnight at 4 °C on a rotating wheel. The immune complexes were then captured with 70 μl of protein A beads, prepared as described above, for 3 h at 4 °C on a rotating wheel. The beads were then washed with 1 ml of ChIP buffer 1 (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl, PMSF), ChIP buffer 2 (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl, PMSF), ChIP buffer 3 (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8, PMSF, DTT), and finally twice with TE 1× and protease inhibitor 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF. The protein–DNA complexes were then eluted by adding 250 μl of ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the beads and vortexing before incubating at room temperature for 15 min. After centrifugation, the eluate was transferred to a fresh tube and the elution process was repeated with the beads. The eluates were then combined, the cross-links were reversed by adding 1 mg/ml RNAse, 5 mM NaCl and incubating for 4 h at 65 °C. After centrifugation, the pellet was resuspended in 100 μl of H_2O and 2 μl of 0.5 mM EDTA, 4 μl of 1 M Tris pH 6.5 and 1 μl of proteinase K (20 mg/ml) were added and incubated for 1 h at 45 °C. The DNA was then recovered by phenol–chloroform extraction.

2.6. PCR

PCRs were carried out by using Taq DNA polymerase (Promega), 2 μl of template DNA and 10 pmol of each primer. An input DNA sample was also used as a control for the PCRs. The amplifications were performed with the following primers:

H ferritin promoter forward	5′ CGCTGTCCCAGAGGCAGTC 3′
H ferritin promoter reverse	5′ TGGTCTCTTATAGCCGCGTCGG 3′
MSH2 promoter forward	5′ GCTGAGTAAACACAGAAA 3′
MSH2 promoter reverse	5′ CTCCTGGTTGAAGAAAAT 3′

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from HeLa cells as described previously (Bevilacqua et al., 1994). The oligonucleotides used for EMSA were

forward 5′ CGGCGCTGATTGGCCGGGGCGGGC 3′;
reverse 5′ GCCCGCCCCGGCCAATCAGCGCCG 3′.

EMSA and competition assays were performed as described previously (Bevilacqua et al., 1995).

2.8. RT-PCR

Total RNA was prepared from HCT116 wild type and p53−/− cells. A total of 1 μg of RNA was used to generate cDNAs with random primers by SuperScript II reverse transcriptase (Invitrogen). PCR amplification was carried out under standard condition with Taq DNA polymerase (Promega). Primer sequences used for H ferritin gene were

forward 5′ CATCAACCGCCAGATCAAC 3′;
reverse 5′ GATGGCTTTCACCTGCTCAT 3′.

For GAPDH, the primers used were

forward 5′ TGATGACATCAAGAAGGTGGTGAAG 3′;
reverse 5′ TCCTTGGAGGCCATGTGGGCCAT 3′.

3. Results

3.1. p53 overexpression reduces transcription driven by H ferritin promoter

The human H ferritin promoter contains a CCAAT box recognized by the transcription factor NF-Y (Faniello et al., 1999). NF-Y was shown to be required for the p53-mediated inhibition of several genes (Imbriano et al., 2005; Joshi, Wu, Reed, & Suttle, 2003; Zhou, Mehta, Choi, Scolavino, & Zhang, 2003). To examine the effect of p53 on the H ferritin transcription, we transiently transfected HeLa cells with a construct bearing only the CCAAT sequence fused to the TATAA box of the H promoter gene (Bbf/TATAA) (Bevilacqua, Faniello, Quaresima, et al., 1997) upstream of the CAT reporter gene in the presence or absence of the p53 expression vector. As an internal control to monitor transfection efficiency, the plasmid RSV β-GAL carrying the β-galactosidase gene under the control of the RSV promoter was co-transfected. The results, shown in Fig. 1A, indicate that the transcription of the CAT gene driven by the H ferritin promoter is markedly inhibited by co-expression of p53 (lane 2). Since previous studies have shown that doxorubicin treatment stimu-

lates the nuclear accumulation and phosphorylation of p53, we also tested the activity of the H ferritin promoter in response to doxorubicin and found that activity of the construct was inhibited by doxorubicin treatment (Fig. 1A, lane 3). As a control, a construct carrying the mutated version of the NF-Y site (Bbf_{mut}/TATAA) was co-transfected. As expected, this construct showed low basal activity and did not respond to doxorubicin treatment (Fig. 1A, lane 6) and to p53 co-transfection (Fig. 1A, lane 5). The expression of exogenous p53 (Fig. 1B, lanes 2 and 5) and the induction of the expression of endogenous p53 in response to doxorubicin (Fig. 1B, lanes 3 and 6) were confirmed by Western blot analysis.

Next, we examined by Northern blot analysis the H ferritin mRNA levels in HeLa cells treated with doxorubicin at different times. As shown in Fig. 2A, lower amounts of transcripts were detected in HeLa cells treated with the drug. This effect was present after 8 h exposure and maintained throughout the experiment, whereas no changes were detected in the levels of GAPDH mRNA measured as an internal control. Protein expression levels were evaluated by Western blot analysis using anti-H ferritin antibody. As expected, doxorubicin significantly decreased the H ferritin levels, but not those of tubulin used as an internal control (Fig. 2B). Concomitantly with the decrease in H ferritin mRNA and protein expression levels, doxorubicin treatment induced accumulation of p53 as well as phosphorylation of p53 at ser-15 (Fig. 2C). To validate the p53-dependency of H ferritin downregulation upon doxorubicin treatment, H ferritin mRNA was measured in HCT p53−/− (Fig. 2D).

To confirm the role of p53 in the repression of the H ferritin gene, the H ferritin promoter (Bbf TATAA) was transfected in HCT116 cells, as well as in their p53−/− mutant counterpart. As shown in Fig. 3A, the activity of the H ferritin promoter was significantly higher in HCT116 p53−/− cells (Fig. 3A, lane 4) compared with wild type cells (Fig. 3A, lane 1). Transfection of wild type HCT116 with siRNA specific to the p53 (Fig. 3A, lane 3) resulted in increased activity of the Bbf TATAA promoter, similar to that observed in p53−/−cells (Fig. 3A, lane 4), whereas transfection of HCT116 p53−/− cells with a p53 expression vector caused a significant decrease in the activity of the promoter (Fig. 3A, lane 6). Transfection of both wild type

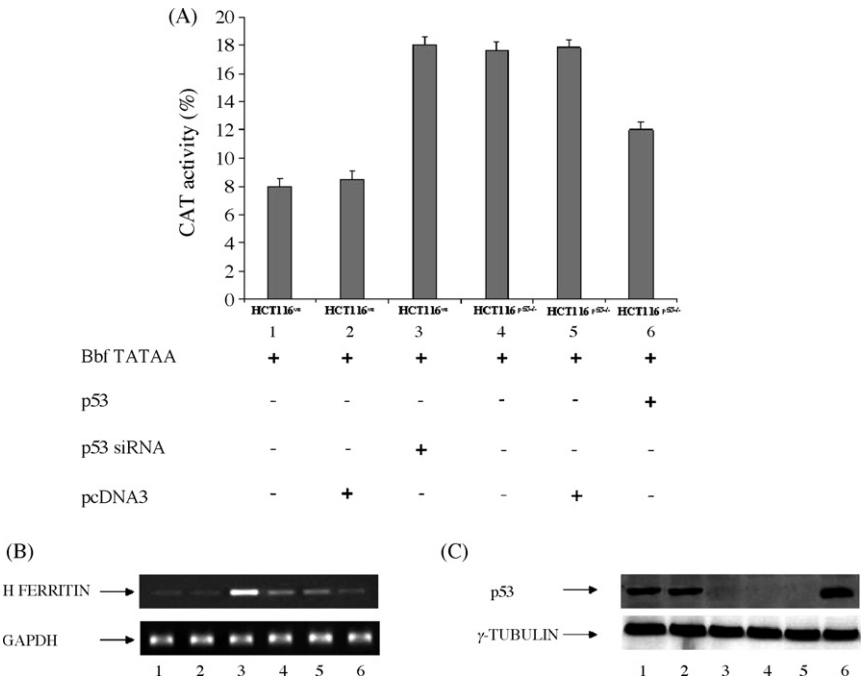

Fig. 3. Transcriptional activity of the H ferritin gene promoter in HCT116 wild type and p53−/− cells and effect of p53 modulation. (A) CAT activity driven by H ferritin promoter/CAT construct (Bbf TATAA) transiently transfected in HCT116 wild type and p53−/− cells with or without p53 expression vector, with or without siRNA p53 and with or without pcDNA3. The values are the means ± S.E.M. of three independent transfection experiments and are expressed as the percentage of [^{14}C]chloramphenicol acetylation. (B) RT-PCR of the H ferritin mRNA of total RNA from HCT116 wild type and p53−/− cells transfected with or without p53 expression vector, with or without siRNA and with or without pcDNA3. (C) Western blot analysis with anti-p53 and anti-anti-tubulin antibodies of total extracts from HCT116 wild type and p53−/− cells transfected with or without p53 expression vector, with or without p53 siRNA and with or without pcDNA3.

HCT116 and HCT116 p53−/− with the empty vector did not show significant changes in CAT activity (Fig. 3A, lanes 2 and 5, respectively). These results were substantiated by the analysis of the ferritin mRNA levels assayed by RT-PCR (Fig. 3B). The expression of p53 in the different samples was monitored by Western blot (Fig. 3C).

3.2. p53 is a member of the H ferritin promoter binding complex

To test whether p53 interacts *in vivo* with the transcriptional factor NF-Y on the H ferritin promoter, thereby modulating the transcription of this gene, we carried out a ChIP assay with antibodies to NF-Y, p53 and p300. Chromatin from HeLa cells was immunoprecipitated with anti-NF-YB, anti-p53 and anti-p300 antibodies, with non-immune antisera and the immunoprecipitated DNA fragments were analyzed by PCR using primers designed for the H ferritin promoter.

Amplified DNA fragments were detected in anti-NF-YB (α-YB), in anti-p53 (α-p53) and in anti-p300 (α-p300) immunoprecipitates but not in the control (α-Ctl) (Fig. 4A). As a further control, we used primers for the promoter of the *MSH2* gene, which does not contain sites for NF-Y and p53. As expected, no PCR product was detected using these primers (Fig. 4B).

3.3. Inhibition by p53 is dependent on NF-Y interaction

It has been shown that the carboxyl-terminal region of p53 is required for NF-Y interactions and the acetylation of the lysine residues within this domain is important for p53-mediated repression of CCAAT-containing promoters (Imbriano et al., 2005). We therefore asked whether the C-terminal region of p53 is necessary for the downregulation of the H ferritin promoter. To this end, HeLa cells were co-transfected with an expression plasmid (p53-CT) carrying the p53 C-terminal domain and with the H ferritin promoter–CAT reporter plasmid. As shown in Fig. 5, the expression of the p53 C-terminal portion reduced the H ferritin promoter basal activity. Co-transfection with a p53 mutant (p53-9KR), in which all lysine in the C-terminal are replaced with arginine, failed to repress the H ferritin promoter. A similar behaviour was observed upon co-transfection of the p53-H175, in which the mutation mapping to the DNA-binding domain of p53 results in the loss of the sequence-specific DNA-binding activity, therefore acting as dominant-negative mutant of the endogenous p53.

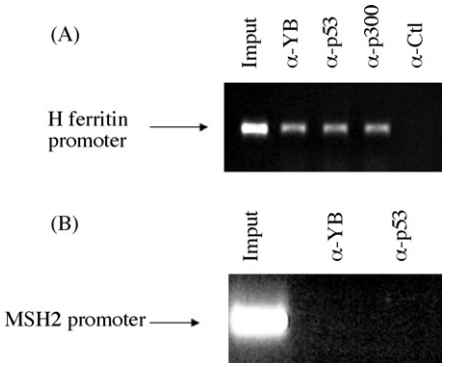

Fig. 4. ChIP analysis of NF-Y p53 and p300 on the H ferritin promoter. Chromatin immunoprecipitations were performed with HeLa cells using the p53, NF-Y, p300 antibodies and non-immune antisera. Immunoprecipitated DNA was amplified by PCR using primers designed for the H ferritin promoter (A) and MSH2 promoter as control (B).

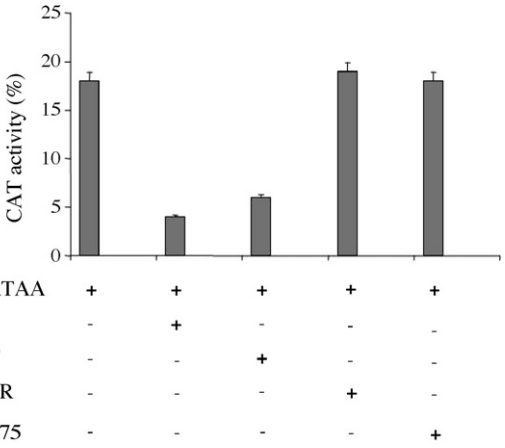

Fig. 5. The C-terminal region of p53 is necessary for the inhibition of the H ferritin promoter. CAT activity driven by H ferritin promoter/CAT construct (Bbf TATAA) transiently transfected in HeLa cells with p53, C-terminal p53 (p53-CT), p53 9KR and with p53 H175. The values are the means ± S.E.M. of three independent transfection experiments and are expressed as percentage of [^{14}C]chloramphenicol acetylation.

3.4. Binding of NF-Y transcription factor to the H ferritin promoter is not inhibited by expression of p53

The ChIP experiments suggest that p53 is bound *in vivo* to NF-Y on the H ferritin promoter. To analyze the role of p53 in the binding of NF-Y to the H ferritin promoter, EMSAs were performed. A synthetic oligonucleotide corresponding to the NF-Y recognition sequence on the H ferritin promoter was incubated with nuclear extracts from HeLa cells either mock-transfected (Fig. 6A, lane 1) or transfected with the p53 expression vector (lane 2). p53 overexpression did not affect the

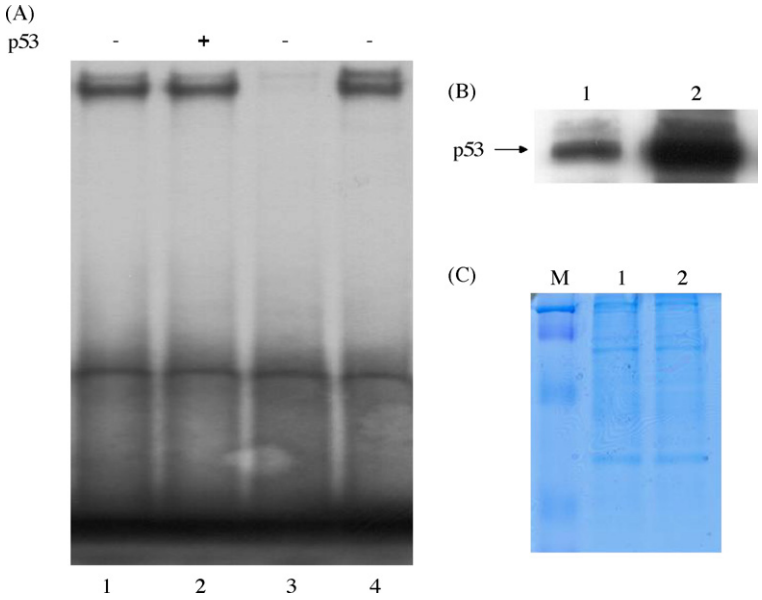

Fig. 6. p53 does not affect the binding of NF-Y to the H ferritin promoter. (A) The terminally labelled oligonucleotide corresponding to the NF-Y recognition sequence on the H ferritin promoter was incubated with 4 μg of nuclear extracts from HeLa cells mock-transfected (lane 1) and HeLa cells transfected with p53 expression vector (lane 2). Lane 3 as lane 2 plus 100-fold molar excess of unlabelled oligonucleotide, lane 4 as lane 2 plus 100-fold molar excess of unrelated competitor oligo. (B) Western blot analysis with anti-p53 antibody of nuclear extracts from HeLa cells mock-transfected (lane 1) and HeLa cells transfected with p53 expression vector (lane 2). (C) Coomassie blue-stained nuclear extracts from HeLa cells mock-transfected (lane 1) and HeLa cells transfected with p53 expression vector (lane 2).

binding of NF-Y to the H ferritin promoter (Fig. 6A, lane 2). Western blot analysis shows that p53 is overexpressed in p53-transfected HeLa cells (Fig. 6B, lane 2). Coomassie and silver staining confirmed that equal amounts of proteins were loaded (Fig. 6C).

4. Discussion

The expression of human ferritin genes is regulated at transcriptional (Bevilacqua et al., 1994; Bevilacqua, Faniello, Cimino, et al., 1997), post-transcriptional and translational level (Klausner, Rouault, & Harford, 1993). The multiplicity of the mechanisms governing the ferritin gene expression, together with the number of extra and intracellular stimuli, may explain the variety of isoferritins exploited by a single cell under different physiological and pathological conditions. The transcription driven by the H ferritin promoter is tightly dependent on the presence of the trimeric factor NF-Y (Faniello et al., 1999) on the inverted CCAAT box (B-box). NF-Y binds the central domain of the co-activator molecule p300 (Faniello et al., 1999) which, in turn, acts as a molecular "anchor" recruiting on the H ferritin gene promoter the oncoprotein E1A, a ferritin transcriptional repressor (Bevilacqua, Faniello, Quaresima, et al., 1997), or c-Jun, which acts as an activator (Faniello et al., 2002).

p53 is known to directly activate a subset of genes important for cell cycle arrest (el-Deiry et al., 1993; Kastan et al., 1992) or apoptosis (Nakano & Vousden, 2001; Okamura et al., 2001) by binding to specific nucleotide sequences located within the promoter region or the first intron of these genes (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992). In addition, p53 can function as a negative regulator of various genes, although the promoters of most of these genes do not contain a typical consensus binding site for this protein, therefore suggesting that the interaction of p53 with other transcription factors is essential for this effect (Mack et al., 1993; Yun et al., 1999). The transcription factor NF-Y has been shown to play an important role in regulating expression and mediating p53-depending repression of several cell cycle-regulated genes, such as cyclin A, cyclin B, cyclin B2, cdc2 and cdc25C (Bolognese et al., 1999; Manni et al., 2001; Yun et al., 1999). Interestingly, p53 can bind promoters lacking consensus p53-binding sites, but harbouring at least two CCAAT boxes.

In this study, we investigated a mechanism by which p53 negatively regulates transcription of the H ferritin gene in the presence of a single active CCAAT box. We demonstrated that p53, when overexpressed or activated by doxorubicin, strongly inhibits the expression of a reporter gene driven by the H ferritin gene

promoter. The inhibition of CAT expression was accompanied by repression of the H ferritin endogenous gene, as demonstrated by the strong decrease in H ferritin mRNA and protein levels in response to doxorubicin. Our findings are in apparent contrast with a recent study by Corna, Galy, Hentze, and Cairo (2006) in which the treatment with doxorubicin increases mRNA H ferritin levels in rat heart. Preliminary unpublished data from our group conducted on rat cardiomyocytes confirm this finding. We cannot provide a clear explanation for this discrepancy, but we hypothesize that a tissue-specific response might be taken into account. Moreover, it must be noticed that the concentrations of doxorubicin used in our experimental setting were different from those used by Corna et al. (2006) (1 μM vs. 5–10 μM). Also, even though rat and human H ferritin promoters are highly homologous, the iron responsive element (IRE), whose binding to iron responsive proteins (IRPs) is impaired by doxorubicin, share only a 40% of homology, and therefore this could account for the diverse behaviour between cardiomyocytes and HeLa cells.

Notably, p53 modulation of H ferritin expression is remarkably mimicked by two homologues, namely p63 and p73, that, albeit linked to different upstream pathways, nevertheless seem to share a regulatory effect on a common set of genes (Levrero et al., 2000) (Faniello et al., manuscript in preparation). Transfection of synthetic short interfering RNA (p53 siRNA) reversed the transcriptional block and restored reporter gene expression as well as ferritin mRNA levels.

Moreover, we have demonstrated by ChIP analysis that NF-Y and p53 can bind to the H ferritin promoter containing a single CCAAT site, although this promoter is lacking canonical p53-binding sequences. Electrophoresis mobility shift experiments showed that p53 does not reduce the binding of NF-Y to the H ferritin promoter, suggesting the possibility that p53 interferes with NF-Y recruitment of co-activators and/or general transcription factors. This hypothesis is supported by the evidence that p53 and NF-Y bind to overlapping domains of the p300 co-activator and both p53 and NF-Y bind to TATA-binding protein (Bellorini et al., 1997).

p53 post-translational modifications, in particular acetylation of the lysine residues at the C-terminus, are known to be essential for the activation of the protein (Joshi et al., 2003). Our data indicate that, when lysines are mutated into arginines, p53 fails to repress the transcriptional activity of the H ferritin promoter. Furthermore, co-transfection of a cDNA encoding the C-terminal portion of p53, required for NF-Y interaction (Imbriano et al., 2005), strongly reduced the ferritin promoter basal activity, indicating that the carboxyl-terminal portion of p53 is sufficient to inhibit the transcription of the H ferritin gene.

Our findings underscore the potential role of H ferritin in mediating p53 effects on cell cycle, DNA repair and apoptosis in response to a variety of environmental stress. Among them, oxidative stress, both endogenously and exogenously produced, is a major threat to organism survival. Among the multiple targets triggered by this injury, including transmembrane receptors and intracellular kinases, there is the H ferritin gene. Strong experimental evidence indicates that the H subunit plays an important role as a protectant against oxidative stress (Arosio & Levi, 2002). Hydrogen peroxide-induced cell toxicity is significantly reduced in cells overexpressing H ferritin (Cozzi et al., 2000). The molecular mechanisms underlying the protective effect of ferritin against oxidants are still under investigation. Ferritin is the major intracellular iron storage protein and can act in the free iron-reactive oxygen species (ROS) system either as source of iron, or as a cytoprotective agent towards ROS, by chelating the free iron in a non-toxic, rapidly available form, thanks also to the iron-oxidative activity of the H-chain (Tacchini, Recalcati, Bernelli-Zazzera, & Cairo, 1997). The increase in ferritin expression produces a reduction of the labile iron pool, as well as the degenerative and pro-apoptotic effects ROS-mediated (Epsztejn et al., 1999).

We propose that the inhibition of H ferritin expression by p53, which reduces the ROS-scavenging activity of this protein, might enhance the pro-apoptotic effects of p53, therefore contributing to the tumor suppressor activity of this major cell cycle regulator. Moreover, the mechanism by which H ferritin expression is repressed by p53, i.e. the binding to a single CCAAT site via NF-Y, with the contribution of p300 and pCAF, widens the scenario of physiopathological cell conditions to which ferritin is able to respond.

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References

Arcari, P., Martinelli, R., & Salvatore, F. (1984). The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: Evidence for multiple mRNA species. *Nucleic Acids Res.*, *12*, 9179–9189.

Arosio, P., Adelman, T. G., & Drysdale, J. W. (1978). On ferritin heterogeneity. *J. Biol. Chem.*, *253*, 4451–4458.

Arosio, P., & Levi, S. (2002). Ferritin, iron homeostasis, and oxidative damage. *Free Radical Biol. Med.*, *15*, 457–463.

Bellorini, M., Lee, D. K., Dantonel, J. C., Zemzoumi, K., Roeder, R. G., Tora, L., et al. (1997). CCAAT binding NF-Y–TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues. *Nucleic Acids Res.*, *25*, 2174–2181.

Bevilacqua, M. A., Faniello, M. C., Cimino, F., & Costanzo, F. (1997). Okadaic acid stimulates H ferritin transcription in HeLa cells by increasing the interaction between the p300 co-activator molecule and the transcription factor Bbf. *Biochem. Biophys. Res. Commun.*, *240*, 179–182.

Bevilacqua, M. A., Faniello, M. C., D'Agostino, P., Quaresima, B., Tiano, M. T., Pignata, S., et al. (1995). Transcriptional activation of the H-ferritin gene in differentiated Caco-2 cells parallels a change in the activity of the nuclear factor Bbf. *Biochem. J.*, *311*, 769–773.

Bevilacqua, M. A., Faniello, M. C., Iovine, B., Russo, T., Cimino, F., & Costanzo, F. (2002). Transcription factor NF-Y regulates differentiation of CaCo-2 cells. *Arch. Biochem. Biophys.*, *407*, 39–44.

Bevilacqua, M. A., Faniello, M. C., Quaresima, B., Tiano, M. T., Giuliano, P., Feliciello, A., et al. (1997). A common mechanism underlying the E1A repression and the cAMP stimulation of the H ferritin transcription. *J. Biol. Chem.*, *272*, 20736–20741.

Bevilacqua, M. A., Faniello, M. C., Russo, T., Cimino, F., & Costanzo, F. (1994). Transcriptional regulation of the human H ferritin-encoding gene (FERH) in G418-treated cells: Role of the B-box-binding factor. *Gene*, *141*, 287–291.

Bevilacqua, M. A., Faniello, M. C., Russo, T., Cimino, F., & Costanzo, F. (1998). P/CAF/p300 complex binds the promoter for the heavy subunit of ferritin and contributes to its tissue-specific expression. *Biochem. J.*, *335*, 521–525.

Bevilacqua, M. A., Giordano, M., D'Agostino, P., Santoro, C., Cimino, F., & Costanzo, F. (1992). Promoter for the human ferritin heavy chain-encoding gene (FERH): Structural and functional characterization. *Gene*, *111*, 255–260.

Bolognese, F., Wasner, M., Dohna, C. L., Gurtner, A., Ronchi, A., Muller, H., et al. (1999). The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated. *Oncogene*, *18*, 1845–1853.

Bomford, A. B., & Munro, H. N. (1992). Ferritin gene expression in health and malignancy. *Pathobiology*, *60*, 10–18.

Ceribelli, M., Alcalay, M., Vigano, M. A., & Mantovani, R. (2006). Repression of new p53 targets revealed by ChIP on chip experiments. *Cell Cycle*, *5*, 1102–1110.

Corna, G., Galy, B., Hentze, M. W., & Cairo, G. (2006). IRP1-independent alteration of cardiac iron metabolism in doxorubicin-treated mice. *J. Mol. Med.*, *84*, 551–560.

Costanzo, F., Castagnoli, L., Dente, L., Arcari, P., Smith, M., Costanzo, P., et al. (1983). Cloning of several cDNA segments coding for human liver proteins. *EMBO J.*, *2*, 57–61.

Cozzi, A., Corsi, B., Levi, S., Santambrogio, P., Albertini, A., & Arosio, P. (2000). Overexpression of wild type and mutated human ferritin H-chain in HeLa cells: *In vivo* role of ferritin ferroxidase activity. *J. Biol. Chem.*, *275*, 25122–25129.

Currie, R. A. (1998). Biochemical characterization of the NF-Y transcription factor complex during B lymphocyte development. *J. Biol. Chem.*, *273*, 18220–18229.

el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., & Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nat. Genet.*, *1*, 45–49.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., et al. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*, *75*, 817–825.

Epsztejn, S., Glickstein, H., Picard, V., Slotki, I. N., Breuer, W., Beaumont, C., et al. (1999). H-ferritin subunit overexpression in erythroid cells reduces the oxidative stress response and induces multidrug resistance properties. *Blood*, *94*, 3593–3603.

Faniello, M. C., Bevilacqua, M. A., Condorelli, G., de Crombrugghe, B., Maity, S. N., Avvedimento, V. E., et al. (1999). The B subunit of the CAAT-binding factor NFY binds the central segment of the co-activator p300. *J. Biol. Chem.*, *274*, 7623–7626.

Faniello, M. C., Chirico, G., Quaresima, B., Cuda, G., Allevato, G., Bevilacqua, M. A., et al. (2002). An alternative model of H ferritin promoter transactivation by c-Jun. *Biochem. J.*, *363*, 53–58.

Gadea, G., Lapasset, L., Gauthier-Rouviere, C., & Roux, P. (2002). Regulation of Cdc42-mediated morphological effect: A novel function for p53. *EMBO J.*, *21*, 2373–2382.

Ginsberg, D., Mechta, F., Yaniv, M., & Oren, M. (1991). Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. U.S.A.*, *88*, 9979–9983.

Gostissa, M., Morelli, M., Mantovani, F., Guida, E., Piazza, S., Collavin, L., et al. (2004). The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. *J. Biol. Chem.*, *279*, 48013–48023.

Graham, F. L., & van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, *52*, 536–539.

Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., et al. (2005). Direct p53 transcriptional repression: *In vivo* analysis of CCAAT containing G2/M promoters. *Mol. Cell. Biol.*, *25*, 3737–3751.

Joshi, A. A., Wu, Z., Reed, R. F., & Suttle, D. P. (2003). Nuclear factor-Y binding to the topoisomerase IIα promoter is inhibited by both the p53 tumor suppressor and anticancer drugs. *Mol. Pharmacol.*, *63*, 359–367.

Jung, M. S., Yun, J., Chae, H. D., Kim, J. M., Kim, S. C., Choi, T. S., et al. (2001). p53 and its homologues, p63 and p73, induce a replicative senescence through inactivation of NF-Y transcription factor. *Oncogene*, *20*, 5818–5825.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., et al. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, *71*, 587–597.

Klausner, R. D., Rouault, T. A., & Harford, J. B. (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell*, *72*, 19–28.

Kubicka, S., Kuhnel, F., Zender, L., Rudolph, K. L., Plumpe, J., Manns, M., et al. (1999). p53 represses CAAT enhancer-binding protein (C/EBP)-dependent transcription of the albumin gene. A molecular mechanism involved in viral liver infection with implications for hepatocarcinogenesis. *J. Biol. Chem.*, *274*, 32137–32144.

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J. Y., & Melino, G. (2000). The p53/p63/p73 family of transcription factors: Overlapping and distinct functions. *J. Cell Sci.*, *113*, 1661–1670.

Mack, D. H., Vartikar, J., Pipas, J. M., & Laimins, L. A. (1993). Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature*, *363*, 281–283.

Maity, S. N., Sinha, S., Ruteshouser, E. C., & de Crombrugghe, B. (1992). Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT binding factor. *J. Biol. Chem.*, *267*, 16574–16580.

Manni, I., Mazzaro, G., Gurtner, A., Mantovani, R., Haugwitz, U., Krause, K., et al. (2001). NF-Y mediates the transcriptional inhibition of the cyclin B1, cyclin B2, and cdc25C promoters upon induced G2 arrest. *J. Biol. Chem.*, *276*, 5570–5576.

Marziali, G., Perrotti, E., Ilari, R., Coccia, E. M., Mantovani, R., Testa, U., et al. (1999). The activity of the CCAAT-box binding factor NF-Y is modulated through the regulated expression of its A subunit during monocyte to macrophage differentiation: Regulation of tissue-specific genes through a ubiquitous transcription factor. *Blood*, *93*, 519–526.

Marziali, G., Perrotti, E., Ilari, R., Testa, U., Coccia, E. M., & Battistini, A. (1997). Transcriptional regulation of the ferritin heavy-chain gene: The activity of the CCAAT binding factor NF-Y is modulated in heme-treated Friend leukemia cells and during monocyte-to-macrophage differentiation. *Mol. Cell. Biol.*, *17*, 1387–1395.

Nakano, K., & Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell*, *7*, 683–694.

Okamura, S., Arakawa, H., Tanaka, T., Nakanishi, H., Ng, C. C., Taya, Y., et al. (2001). p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis. *Mol. Cell*, *8*, 85–94.

Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., & Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, *362*, 857–860.

Pang, J. H., & Chen, K. Y. (1993). A specific CCAAT-binding protein, CBP/tk, may be involved in the regulation of thymidine kinase gene expression in human IMR-90 diploid fibroblasts during senescence. *J. Biol. Chem.*, *268*, 2909–2916.

Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., et al. (1992). Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc. Natl. Acad. Sci. U.S.A.*, *89*, 12028–12032.

Tacchini, L., Recalcati, S., Bernelli-Zazzera, A., & Cairo, G. (1997). Induction of ferritin synthesis in ischemic-reperfused rat liver: Analysis of the molecular mechanisms. *Gastroenterology*, *113*, 946–953.

Torti, F. M., & Torti, S. V. (2002). Regulation of ferritin genes and protein. *Blood*, *99*, 3505–3516.

Vousden, K. H., & Lu, X. (2002). Live or let die: The cell's response to p53. *Nat. Rev. Cancer*, *2*, 594–604.

Yun, J., Chae, H. D., Choy, H. E., Chung, J., Yoo, H. S., Han, M. H., et al. (1999). p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. *J. Biol. Chem.*, *274*, 29677–29682.

Zhou, Y., Mehta, K. R., Choi, A. P., Scolavino, S., & Zhang, X. (2003). DNA damage-induced inhibition of securin expression is mediated by p53. *J. Biol. Chem.*, *278*, 462–470.