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Identification of p21 (CIP1/WAF1) as a direct target gene of HIC1 (Hypermethylated In Cancer 1)

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ABSTRACT

The tumor suppressor gene *HIC1* (*Hypermethylated In Cancer 1*) encodes a transcriptional repressor involved in the regulation of growth control and DNA damage response. We previously demonstrated that *p57Kip2*; a member of the CIP/KIP family of CDK (cyclin dependent kinase) inhibitors (CKI); is a direct target gene of HIC1 in quiescent cells. Here we show that ectopic expression of HIC1 in MDA-MB-231 cells or its overexpression in BJ-Tert fibroblasts induces decreased mRNA and protein expression of p21 (CIP1/WAF1) another member of this CKI family that plays essential roles in the p53-mediated DNA damage response. Conversely, knock-down of endogenous HIC1 in BJ-Tert through RNA interference up-regulates p21 in basal conditions and further potentiates this CKI in response to apoptotic etoposide-induced DNA damage. Through promoter luciferase activity and chromatin immunoprecipitation (ChIP), we demonstrate that HIC1 is a direct transcriptional repressor of *p21*. Thus, our results further demonstrate that HIC1 is a key player in the regulation of the DNA damage response.

1. Introduction

HIC1 (*Hypermethylated In Cancer 1*), a tumor suppressor gene frequently hypermethylated or deleted in numerous cancers, encodes a transcriptional repressor [1–3]. The HIC1 protein is composed of three main functional domains: a BTB/POZ protein–protein interaction domain (Broad complex, Tramtrack and Bric à brac/POx viruses and zinc finger) in the N-terminal part of the protein, a central region and a C-terminal domain containing five *Krüppel*-like C_2H_2 zinc fingers. These zinc fingers allow the specific binding of the protein to specific DNA sequences consisting of a 5′-(C/G)NG(C/G)GGGCA(C/A)CC-3′ centered on a GGCA motif and named HIC1 responsive elements (HiRE) [4]. The central region of HIC1 contains two short phylogenetically conserved motifs: (i) GLDLSKK, allowing the recruitment of the co-repressor CtBP (C-terminal Binding Protein) [5,6] and (ii) $MK^{314}HEP$, whose lysine is competitively acetylated or SUMOylated [7] to regulate the interaction between HIC1 and MTA1; a member of the NuRD (Nucleosome Remodeling and histone Deacetylase) complex [8]. To date only 11 direct target genes of HIC1 have been described including *p57kip2* [8] a cyclin-dependent kinase inhibitor belonging to the CIP/KIP family, which also includes p21(CIP1/WAF1) and p27(KIP1) [9]. Several studies have highlighted a central role for HIC1 in the regulation of the DNA damage response and more particularly through the existence of a complex regulatory loop between HIC1, p53 and the deacetylase SIRT1 [10]. First HIC1 is a direct target gene of p53 [1,11,12], the master regulator of the DNA damage-induced checkpoints which, depending on the damage intensity, transactivates various growth inhibitory or apoptotic genes. Among these are *p21* [13], promoting G1 growth arrest and *SIRT1* [14] that in a feed-back loop deacetylates and inactivates p53. HIC1 directly represses the transcription of SIRT1 [15] whereas SIRT1 by deacetylating the lysine 314 of HIC1 increases its transcriptional repression potential [7]. In this study, we show that *p21* is a new direct target gene of HIC1 thus adding a new step to the p53-HIC1-SIRT1 regulatory loop and further implicating HIC1 as an essential element in the regulation of the DNA damage response.

2. Materials and methods

2.1. Cell lines and plasmids

HEK 293T, U2OS, MDA-MB-231 and BJ-Tert cells were maintained in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum, non-essential amino acids and gentamycin. Cells were cultured at 37 °C in water-saturated 5% CO_2 atmosphere. The pcDNA3-FLAG-HIC1 has been previously described [6].

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2.2. Vector and retroviral infection

Retroviral infection of BJ-Tert fibroblasts with the pBABE-Puro-FLAG-HIC1 [16] were performed as previously described [17].

2.3. Small Interfering RNA

BJ-Tert fibroblasts were reverse-transfected with Lipofectamine RNAiMax (Invitrogen) according to manufacturer's instructions using 10 nM small interfering RNA targeting HIC1 (HIC1 siGENOME SMART Pool M-006532-01, Dharmacon), MTA1 (MTA1 siGENOME SMART Pool M-004127-02, Dharmacon), or a scrambled control sequence (si Ctrl; siGENOME RISC free control siRNA, Dharmacon) as previously described [17] 48 h later, cells were treated with 80 μM etoposide for 16 h and harvested for RNA/protein extraction [16,17].

2.4. Luciferase repression assays

The pGl3 p21 promoter construct has been kindly provided by Olivier Rohr (University of Strasbourg, France) and has been previously described [18].

HEK 293T or U2OS cells were transfected in OptiMEM (Invitrogen) by the PEI (Euromedex) method in 12-well plates with 500 ng of DNA. Cells were transfected for 6 h and then incubated in complete fresh medium. 48 h after transfection, cells were lysed in Luc assay buffer (25 mM glycyl glycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100). Luciferase and β-galactosidase activities were measured by using, respectively, beetle luciferine (Promega) and the Galacto-light kit (Tropix) with a Berthold chemiluminometer. After normalization to the β-galactosidase activity, the data were expressed as the Luc activity relative to the activity of pGl3-Luc with empty control vector, which was given an arbitrary value of 1. The results represent the mean values and standard deviations from 3 independent experiments.

2.5. Quantitative RT-PCR

Total RNA was reverse transcribed using random primers and MultiScribe™ reverse transcriptase (Applied Biosystems). Real-time PCR analysis was performed by Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cycler (Stratagene) according to the manufacturer's instructions. Results were normalized with respect to 18S RNA used as internal control. The primers used are as follows: HIC1 forward 5′-CGACGACTACAAG AGCAGCAGC-3′ and reverse 5′-CAGGTTGTCACCGAAGCTCTC -3′; p21 forward 5′-GACTCTCAGGGTCGAAAACG-3′ and reverse 5′-GGC TTCCTCTTGGAGAAGATCA-3′ and 18S forward 5′-GGCGCCCC CTCGATGCTCTTAG-3′ and reverse 5′-GCTCGGGCCTGCTTTGAACA CTCT-3′.

2.6. Chromatin immunoprecipitation

BJ-Tert cells were fixed by adding formaldehyde directly into the cell plate to a final concentration of 1% for 15 min at 37 °C. Adding glycine to a final concentration of 0.125 M stopped the cross-linking. After 5 min at 37 °C, cells were lysed directly in the plates by resuspension in cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP-40) for 5 min. Then, the samples were pelleted, resuspended in 100 μl of nuclei lysis buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 0.2% SDS), and sonicated to chromatins with an average size of 250 bp using a BioRuptor (Diagenode, Liege, Belgium). 20 μg of chromatin was transferred to a tube containing 4.8 μg of anti-HIC1 [19] antibody or IgG control coated to magnetic protein A beads (Millipore) and 900 μl of IP buffer (16.7 mM Tris–HCl pH 8, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100) and incubated for 6 h on a rotator at 4 °C. Immune complexes were washed once in IP buffer, once in TSE buffer (20 mM Tris pH 8, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), once in LiCl buffer (100 mM Tris pH 8, 500 mM LiCl, 1% deoxycholic acid, 1% NP40) and twice in TE buffer (10 mm Tris/HCl, pH 8, 10 mm EDTA). Each wash lasted for 3 min on a rotator at room temperature. ChIP complexes were eluted in 150 μl of elution buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1% SDS) containing 50 μg/ml of proteinase K and incubated 1 h at 55 °C. Then samples were treated with 133 μg/ml of RNase A for 30 min at 37 °C. Finally, the supernatant was recovered and incubated for 3 h at 68 °C. DNAs as well as 5% input were purified on Nucleobond Extract II (Macherey–Nagel) and eluted with 80 μl of H2O.

Immunoprecipitated DNA was analyzed in a MX3005P fluorescence temperature cycler (Stratagene) in triplicates by real time PCR starting from 3 μl of template DNA in a final volume of 20 μl containing power SYBR Green (Applied Biosystems) and primers at a final concentration of 0.5 μM. The primers used are summarized in Supplementary Table S1. According to a melting point analysis, only one PCR product was amplified under these conditions. An input control was used to generate a standard curve for each gene. Results were expressed as % input. The experiment was performed twice and a representative experiment is shown.

2.7. Western blotting and antibodies

Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes (GE healthcare). After 1 h of blocking in PBSM (PBS with 5% milk), the membranes were incubating overnight at 4 °C with specific primary antibodies in PBSTM (PBSM with 0.1% Tween) and washed three times with PBSN (PBS with 0.1% NP-40). The membranes were next incubated for 1 h at room temperature with secondary antibodies coupled to peroxydase (Amersham) in PBSM, washed three times in PBSN and revealed by chemiluminescence.

The anti-HIC1 antibody has been previously described [17]. Anti-p21, anti-p53 and anti-actin were purchased from Santa-cruz biotechnology and anti γ-H2AX from Abcam.

3. Results

3.1. Effects of modulating HIC1 expression on the levels of p21

To test whether p21 could be a new target gene of HIC1, we first investigated the effects of HIC1 ectopic expression in HIC1-deficient breast cancer cells, MDA-MB-231 or overexpression in BJ-Tert "normal" human fibroblasts on p21 mRNA (Fig. 1A) and protein levels (Fig. 1B). After retroviral infection of pBabe-HIC1 in these two cell lines, we observed a significant decrease in *p21* mRNA levels (Fig. 1A) correlated with a lower expression of the protein (Fig. 1B) in comparison with the cells infected with the empty vector. Conversely, inhibition of endogenous HIC1 expression in BJ-Tert fibroblasts by RNA interference [16,17] leads to a concomitant increase in *p21* mRNA and protein levels (Fig. 2). This increase was observed in basal conditions but more interestingly we demonstrated that knock-down of HIC1 super-induces *p21* in response to etoposide-induced DNA damages. In addition, inhibition of HIC1 leads to a modest increase of total p53 protein levels either in non-treated or in the etoposide-treated cells, reflecting the complexity of this regulatory loop. However, our results strongly suggests that the increased p21 expression observed in the cells transfected with the siRNA targeting HIC1 is not merely due to an increased expression of p53 resulting in an increased transcription of *p21*.

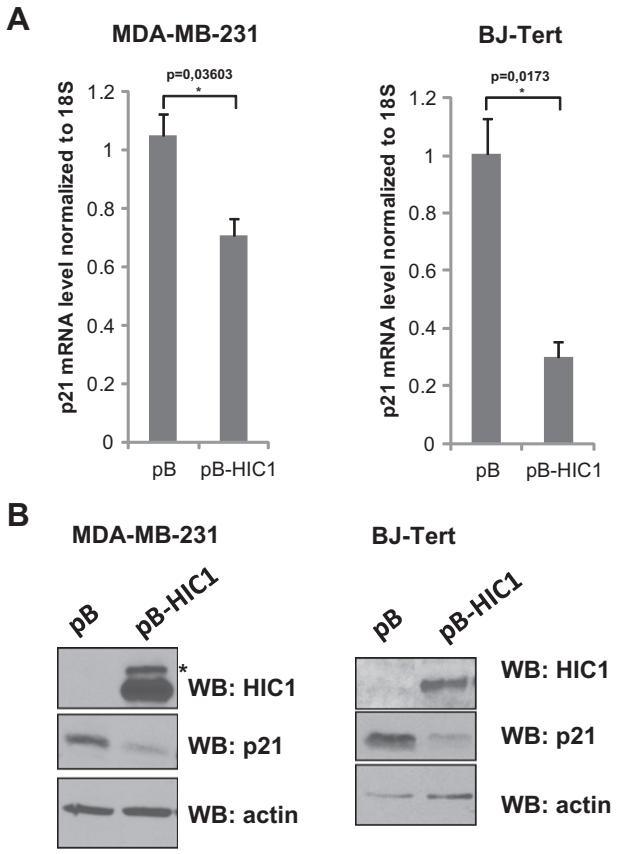

Fig. 1. p21 is down-regulated in pBabe-HIC1 infected cells. A. Quantitative real time RT-PCR were performed using total RNAs isolated from MDA-MB-231 and BJ-Tert cells infected with pBabe-FLAG (pB) or pBabe-FLAG-HIC1 (pB-HIC1) to assess the level of *p21* mRNA. Values were normalized to *18S*. B. Western blot (WB) analyses of HIC1 and p21 in lysates obtained from MDA-MB-231 and BJ-Tert cells infected with pBabe-FLAG (pB) or pBabe-FLAG-HIC1 (pB-HIC1). Equal loading was confirmed by immunoblotting for actin.* corresponds to SUMOylated form of HIC1 in MDA-MB-231 infected by pB-HIC1.

3.2. HIC1 directly represses the expression of p21

To determine whether *p21* is effectively a direct target gene of HIC1, we first scanned its promoter region for the presence of consensus HIC1-responsive elements (HiREs) that HIC1 could bind. Just based on the GGCA core motif, we identified several putative HiREs in the *p21* promoter (data not shown) but among them only one was highly homologous to the optimal consensus that we previously defined [4] and was located around 700 bp upstream of the transcription start codon (Fig. 3A bold and underlined sequence). Using the promoter region of *p21* cloned in the pGl3 basic reporter vector [18], we performed luciferase promoter-repressor assays in HEK 293T and in U2OS cells in the presence or absence of transiently transfected pcDNA3-FLAG-HIC1. As shown in Fig. 3B, transient transfection of HIC1 represses about 5 times the *p21* promoter activity in the two cell types. To definitively prove that HIC1 can bind to the promoter of *p21*, we designed a set of oligonucleotides to PCR amplify the region containing the putative HiRE (Fig. 3A, bold and underlined sequence) in DNA samples obtained from Chromatin immunoprecipitation (ChIP) of endogenous HIC1 in BJ-Tert fibroblasts. As shown in Fig. 3C, we were able to specifically amplify this region from chromatin immunoprecipitated with anti-HIC1 antibody but not with non-relevant rabbit IgG and with a same rate that several previously published direct target genes of HIC1: *CCND1* [8], *SIRT1* [15], *ADRB2* [17] and *EPHA2* [16]. Primers designed to amplify the *GAPDH* promoter were used as a negative control [16]. Taken together these results demonstrate that HIC1 is a direct repressor of *p21* transcription.

4. Discussion

In this study, we characterized the cyclin-dependent kinase inhibitor (CKI) *p21* as a new direct target gene of the HIC1 transcriptional repressor thus adding a new layer to the previously described p53-HIC1-SIRT1 regulatory loop that orchestrates the DNA damage response [10,15] (Fig. 4).

The correct response of the cell faced to DNA damages is crucial to maintain the genome integrity and avoid the transmission of genetic aberrations during cell division. The cell gatekeeper p53 is considered as the master regulator of the DNA damage response but besides p53, it becomes increasingly clear that HIC1 also plays a central role in this phenomenon. P53 is a transcription factor that is quickly stabilized and activated in response to DNA damages through the ATM/ATR pathway [20]. In its activated form, p53 transactivates many target genes implicated in DNA damage repair, reversible cell cycle arrest (as long as the repair is not

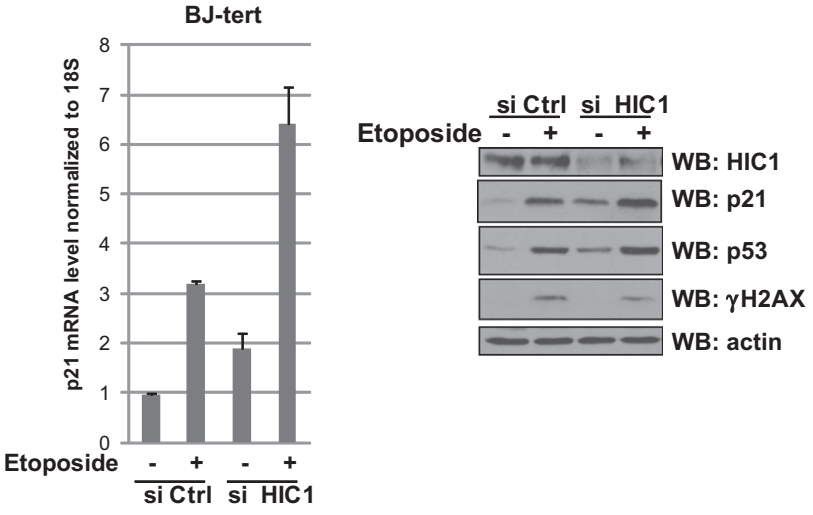

Fig. 2. Knock-down of endogenous HIC1 in BJ-Tert fibroblasts up-regulates p21 expression. BJ-Tert fibroblasts were transfected with non-target siRNA control (si Ctrl) or with HIC1 siRNA (si HIC1). 48 h later, cells were treated with 80 μM etoposide (Sigma–Aldrich) for 16 h and harvested for RNA/protein extraction. Left: Total RNAs were extracted and the mRNA expression levels of *p21* were assessed by quantitative RT-PCR. Values were normalized to *18S*. Right: Cell lysates were analyzed by Western blotting (WB) with the indicated antibodies.

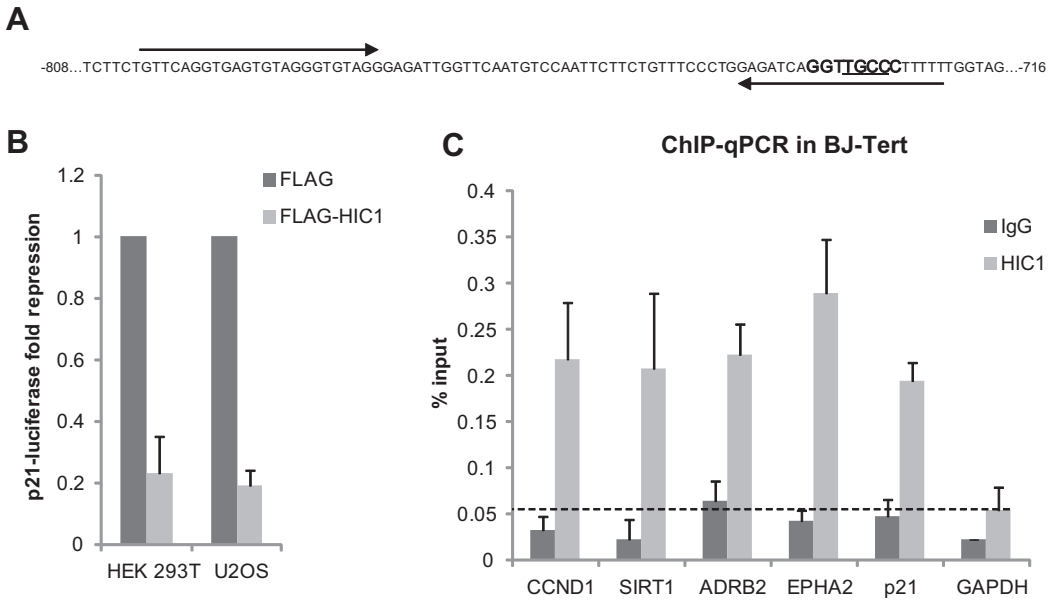

Fig. 3. HIC1 is a direct transcriptional repressor of *p21*. A. Nucleotide sequence of a part of the 5′ region of the human *p21* gene containing the potential HiRE shown in bold (optimal binding sequence) [4] with the core consensus 5′-GGCA-3′ (reverse complemented strand TGCC) underlined. Arrows indicate the position of the primers used in the ChIP experiment. B. HEK 293T and U2OS cells were transfected with 200 ng of p21 promoter reporter, 25 ng of β-galactosidase reporter and 275 ng of the plasmid encoding HIC1 or an empty vector. 48 h after transfection, p21-luciferase activity was calculated as described in the experimental procedures section. The results represent the mean values and standard deviations from 3 independent experiments. C. BJ-Tert fibroblasts chromatin was immunoprecipitated with anti-HIC1 antibody, as previously described [19]. The bound material was eluted and analyzed by quantitative PCR using primers flanking the putative HIC1 binding site in *p21* described above. The previously published HIC1 direct target genes *CCND1*, *SIRT1*, *ADRB2* and *EPHA2* were used as positive controls and *GAPDH* as an internal non-binding control.

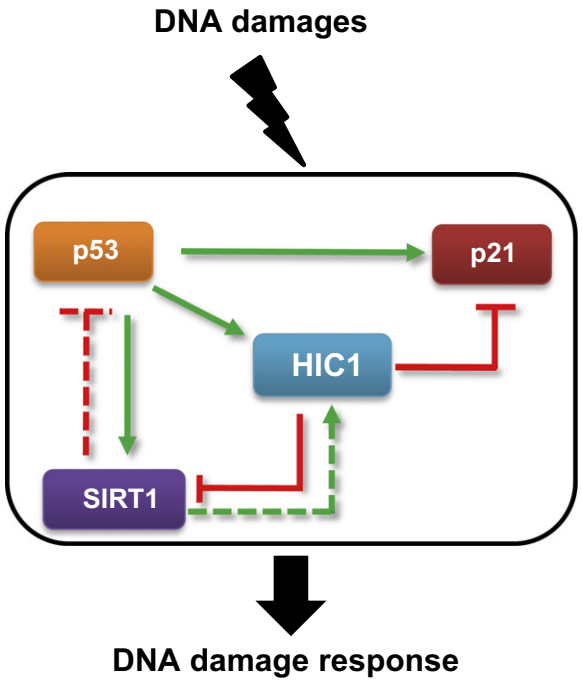

Fig. 4. A p53-HIC1-SIRT1-p21 regulatory loop orchestrates the DNA damage response. The induction of DNA damages activates a complex cellular signalization allowing the cell to repair the damaged DNA to stop the cell cycle as long as the repair is not ended or to lead to apoptosis if the damages are too important. This DNA damage response is under the control of p53 that promotes among others the transcription of the deacetylase *SIRT1*, the CKI *p21* and *HIC1*. HIC1 can repress the transcription of *SIRT1* and *p21* thus establishing a complex regulatory loop. Moreover, SIRT1 deacetylates P53 and HIC1 that leads to inactivation of p53 whereas it activates HIC1. Full arrows indicate direct transcriptional regulation, dotted arrows indicate post-traductional regulation.

completed) and apoptosis (in the case of irreparable damages). Among them *p21* (also known as WAF1 or CIP1) is probably the best known but *HIC1* is also a direct target gene of p53 [1,11,12]. A P53-dependent increase in HIC1 transcripts were observed upon irradiation of MCF7 cells (p53 +/+ breast cancer cells) but not in MCF7 cells in which endogenous p53 expression has been abolished by RNA interference [12]. Such an increase in *HIC1* mRNA has also been demonstrated in U87MG cells (p53+/+ glioblastoma cells) during the cisplatin-induced cell cycle arrest but not in U87MG cells expressing a "dominant-negative" form of p53 [21]. Taken together, these two studies suggest that p53 transactivates *HIC1* in response to DNA damages. The NAD^+-dependent class III HDAC *SIRT1* is both a target gene of P53 [14] and of HIC1 [15]. SIRT1 can deacetylate histones (H1K26; H3K9; H4K16) but also several non-histone proteins including p53 [22] and HIC1 [7]. Deacetylation of p53 on lysine 382 decreases the transcriptional activity of the protein towards its target genes whereas deacetylation of HIC1 on lysine 314 potentiates its repressor capacity notably by favoring its interaction with MTA1 one component of the nucleosome remodeling and deacetylase complex (NuRD). In normal WI38 fibroblasts, the knock-down of *HIC1* by RNAi provokes a decrease in etoposide-induced p53 acetylation in correlation with an increase in *SIRT1* expression demonstrating that HIC1 regulates the p53-dependent DNA damage response [15]. All together these studies suggest that a correct DNA damage response would require in part a kinetic of activation and repression of *SIRT1*: first of all, its transcription by p53 and its activity would be necessary to optimize the repressive capacity of HIC1 then its transcriptional repression would allow the activating acetylation of p53. The same hypothesis can be made for *p21* (also known as WAF1 or CIP1) as it is also both a direct target gene of p53 [13] and of HIC1 (this study). Indeed if the stimulation of *p21* transcription by p53 in response to DNA damages is necessary to stop the cell cycle by inactivating G1-phase cyclins/CDKs complexes and inhibits DNA replication through its interaction with PCNA more and more evidences tend to prove that p21 inhibits apoptosis notably by maintaining the caspases 3 and 2 in an inactive state [for review see [23,24]]. So one can easily suppose that *p21* needs first to be transcribed in

response to DNA damages to stop the progression of the cell cycle and allow the repair of the damaged DNA. Nevertheless, this cell cycle inhibitor needs to be repressed to allow the cell to resume cell cycle in case of repairable damages or to trigger apoptosis if the damages are too important, as for example with the etoposide treatment we (this study) and others [15] have used. Thus, by its ability to directly repress the transcription of *SIRT1* [8] [15] and *p21*, as shown in this study, HIC1 could be involved in various aspects of the DNA damage response.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.045.

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