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E2F1 activates p53 transcription through its distal site and participates in apoptosis induction in HPV-positive cells

Anthony Massip [1], Tania Arcondéguy [1], Christian Touriol, Céline Basset, Hervé Prats, Eric Lacazette *

INSERM UMR 1037, Cancer Research Center of Toulouse (CRCT), Cancer Department, 1 Avenue Jean Poulhes, Toulouse, France

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ABSTRACT

The p53 tumor suppressor protein, one of the most extensively studied proteins, plays a pivotal role in cellular checkpoints that respond to DNA damage to prevent tumorigenesis. However, the transcriptional control of the p53 gene has not been fully characterized. We report that the transcription factor E2F1 binds only to the E2F1 distal site of the *p53* promoter in the human papillomavirus positive carcinoma HeLa cell line. Moreover, we showed that etoposide, a DNA damaging agent, activates p53 transcription through the E2F1 pathway. This increase correlates with apoptosis induction as disruption of this pathway led to reduced apoptosis stimulation by the DNA damaging agent.

1. Introduction

The tumor suppressor p53 plays a crucial role to maintain genome integrity as it can: (i) induce cell growth arrest by holding cells at the G1/S checkpoint, (ii) activate DNA repair on damaged DNA, (iii) induce apoptosis if DNA damage is irreparable [1]. The exposure of a cell to a genotoxic stress leads to an increase of activated p53 protein levels [2]. Activated p53 works as a transcription factor that can bind DNA and induce the transcription of hundreds of downstream genes including cell cycle and apoptosis regulators [3].

It is well described that the increase and regulation of p53 level in DNA damaged cells occurs at the protein level. This includes translational control, post-translational modifications, protein–protein interactions and sub-cellular localization [4–6]. These mechanisms allow a rapid cellular response to the stress. Surprisingly, the transcriptional regulation of the p53 gene under genotoxic stress is poorly documented even if it has been demonstrated for a long time [7]. Only few studies have reported the identification of transcriptional factors involved in p53 regulation. Thus, p53 itself or p73 can bind the p53 promoter region (Fig. 1A) producing a positive feedback loop activated under stress conditions [8]. Another transcriptional factor involved in p53 cellular stress response is E2F1. Two E2F1 binding sites namely E2F1-BS-1 and E2F1-BS-2, have been reported within the p53 promoter (Fig. 1A). The proximal binding site (E2F1-BS-1) has been described by Choi et al. [9] and recently the second distal binding site (E2F1-BS-2) has been discovered by Bug and Dobbelstein [10]. In the latest work, the authors showed that E2F1 contributes to p53 transcriptional activation under anthracycline treatment through the binding at the proximal site in a Human Papillomavirus (HPV) negative cell line expressing a mutated p53 protein. However in this cell line, etoposide, another topoisomerase inhibitor failed to activate p53 transcription.

In this work, in a HPV positive model and in a wild type p53 background, we report that E2F1 distal site (E2F1-BS-2) accounted for p53 transcription. Moreover, its transcription was activated by etoposide treatment through E2F1 binding at the distal site (E2F1 BS-2). In this model, p73 another E2F1 target, slightly contributed to the increase of p53 mRNA level. Finally, we showed that activated E2F1 pathway upon etoposide treatment participate to the induction of apoptosis through the activation of p53 transcription.

2. Materials and methods

2.1. Cell line, drug treatments and transfections

HeLa (ATCC: CCL-2) cell line was grown in DMEM 1 g/l glucose supplemented with 10% fetal bovine serum, 1% glutamine and antibiotics, at 37 °C in a 5% CO_2 humidified atmosphere. When indicated, cells were incubated for 16 h in growth medium with 25 or 50 μM of etoposide (Biotrend).

* Corresponding author.
E-mail address: eric.lacazette@inserm.fr (E. Lacazette).
[1] These authors contributed equally to this work.

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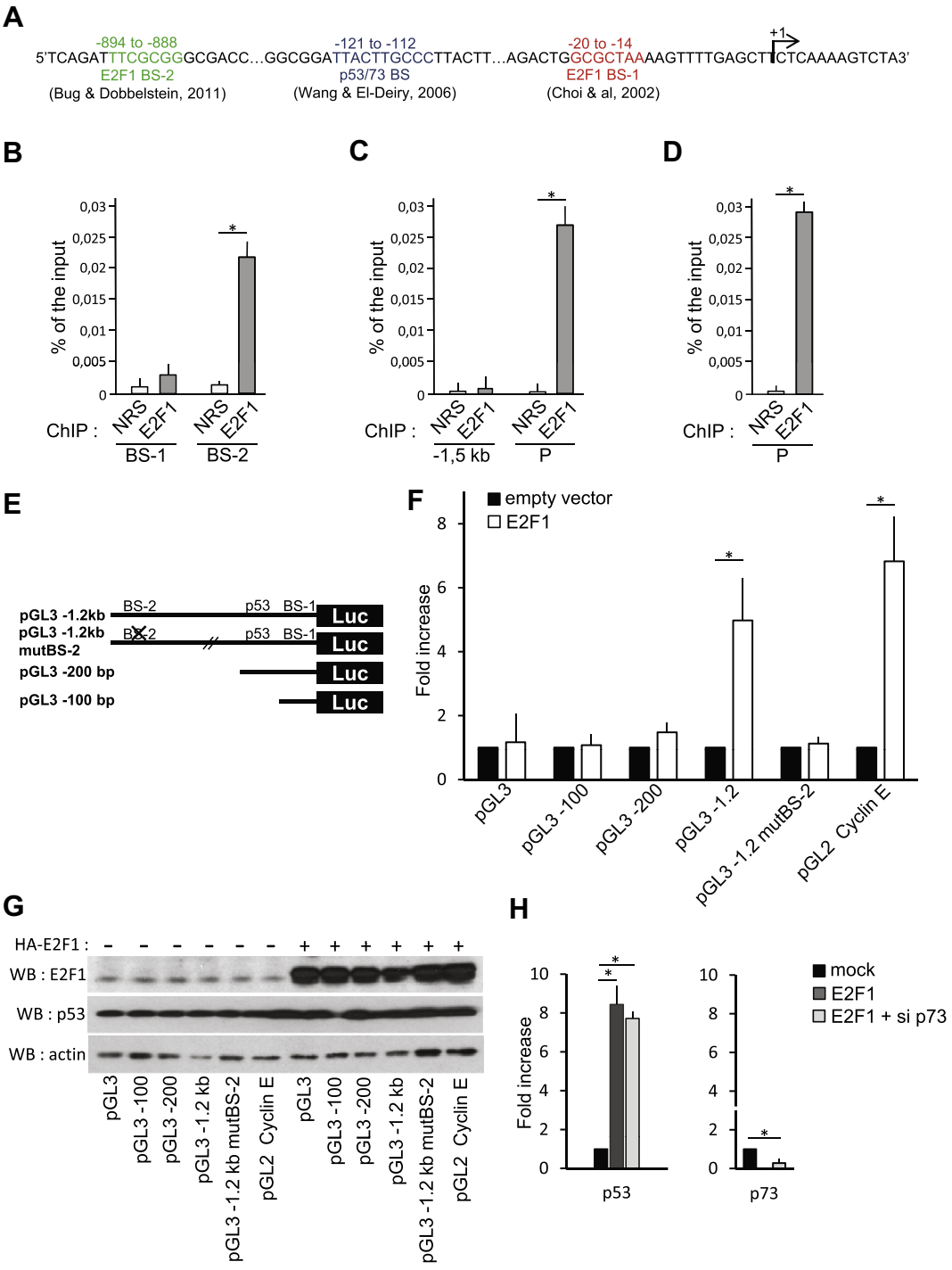

Fig. 1. E2F1 induces p53 transcription trough its distal binding site. (A) Partial representation of the p53 promoter sequence. The transcriptional start site (+1) is represented by the arrow. Two potential E2F1 binding sites are represented: E2F1 BS-1 (proximal), E2F1 BS-2 (distal) and a p53/p73 binding site. (B–D) ChIP experiments were performed using antibodies directed against E2F1, or IgG (NRS) as a negative control and quantitative PCR were performed using primers that amplify the potential indicated binding sites. (B) E2F1 binding on the p53 promoter. (C) E2F1 binding on the SKP2 promoter (P) as a positive control (right) and in a region 1.5 kb upstream of the SKP2 transcriptional start site (negative control). (D) E2F1 binding on the cyclin E promoter (P, positive control). (E) Schematic representation of the luciferase fusions including 5′ deletion mutants of the p53 promoter harboring the indicated binding sites for E2F1 and p53/p73. The cross indicates a disrupted BS-2 site. (F) Responsiveness of the luciferase fusions represented in (E) towards E2F1 overexpression. (G) Western blot experiments to assess E2F1 and p53 expression in Hela cells when pTTE2F1 is co-transfected with the indicated constructs. (H) RT-qPCR analysis of p53 and p73 mRNA levels. Experiments were done in triplicates (*$P < 0.05$).

Cells were transfected using JetPEI or InterferIN transfection reagents (Polyplus transfection) according to the manufacturer's instructions. siRNAs targeting sequences were as follow: E2F1 5′-GUCACGCUAUGAGACCUCAdTdT-3′; p53 5′-GGAAACUACUUCCUGAAAAdTdT-3′; p73 5′-GGGACUUCAAUGAAGGACAdTdT-3′; SCR 5′-CUACAAGAUUUGUGACGUAdTdT3′.

2.2. Luciferase reporter assay

Cells were co-transfected with the indicated p53 promoter constructs pGL3-100 bp; pGL3-200 bp; pGL3-1.2; pGL3-1.2 mutBS-2 (BS-2 was mutagenized with Quick Change XL using BS2 mut F and BS2 mut R as primers (primer sequences: Table 1) and pGL3-

1.2 as template; Ref. 200522; Agilent technologies); pGL2-CyclinE (300 ng) or empty plasmid (pGL3) and 300 ng of E2F1 expression vector (pTT-E2F1) or empty vector. 24 h after transfection, cells were lyzed in Passive Lysis Buffer and firefly luciferase activity was measured in a LB960 luminometer (Berthold). Light units were normalized to the amounts of transfected plasmids quantified by qPCR.

2.3. Western blot

Western blots were performed 48 h after transfection. Thirty micrograms of total extracts were resolved in 4–20% polyacrylamide gels and transferred onto a nitrocellulose membrane. Immunoblotting were performed using antip53 (DO-1):sc-126 antibody, E2F1 (C-20): sc-193 antibody (Santa Cruz), and monoclonal anti-β-actin antibody (Sigma). The signal was detected using enhanced chemiluminescence detection reagent.

2.4. RNA extraction and quantification using real-time PCR

Total RNA was extracted using the TriZol reagent protocol (Invitrogen). Reverse transcription was performed with 1 μg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) and oligo(dT) primer. Real-time PCR was performed with SsoFast EvaGreen Supermix with HPRT1 and GUSB mRNAs as endogenous references (for oligonucleotide sequences see Table 1). Assays were performed on 7500 Fast Real-Time PCR System (Applied Biosystems). Experiments were done in triplicate and calculations performed using the $\Delta\Delta C_q$ method.

2.5. Chromatin immuno-precipitation assay

Formaldehyde was added to the culture medium to a final concentration of 1% for 10 min and glycin was added to a final concentration of 0.125 M. Cells were washed twice with cold PBS and harvested by scraping. Cells were lysed (Pipes 5 mM pH 8, KCl 85 mM, NP-40 0.5%) and homogenized with a Dounce. Nuclei were harvested by centrifugation, incubated in a nuclear lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS), and sonicated (five times for 10 s at a power setting of 5% and 50% duty cycle; Branson Sonifier 250) to obtain DNA fragments of 500–1000 bp. Samples were diluted 10 times in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl) and pre-cleared with 140 μl of previously blocked EZview beads (Sigma). Blocking was achieved by incubating the agarose beads with 500 μg of BSA and 200 μg of herring sperm DNA for 3 h at 4 °C. Pre-cleared samples were incubated overnight at 4 °C with specific antibodies (1–2 μg) or with normal rabbit serum as negative control. Immune complexes were then recovered by incubating the samples with 140 μl of blocked EZview beads for 2 h at 4 °C on a rotating wheel. Beads were washed once in dialysis buffer (2 mM EDTA, 50 mM Tris pH 8, 0.2% Sarkosyl) and four times in wash buffer (100 mM Tris pH 8.8, 500 mM LiCl, 1% NP-40, 1% NaDoc). Elution was performed 15 min in elution buffer (1% SDS, 100 mM $NaHCO_3$). Crosslink was reversed by adding SDS and RNase A to the samples overnight at 70 °C. After a 1.5 h proteinase K treatment, DNA was purified with phenol/chloroform/isoamylalcool (25:24:1) and precipitated with ethanol and NaAc.

The immunoprecipitation of DNA–protein complexes was achieved with antibodies directed against E2F1 (sc-193, Santa Cruz) or phosphorylated polymerase II 8A7 (sc-13583, Santa Cruz) or with normal rabbit serum (NRS) for control experiments. Abundances of target sequences were analyzed by quantitative PCR (primer sequences: Table 1). Values reflecting chromatin enrichment are reported as the percent of input: immunoprecipitated with E2F1 or phosphorylated polymerase II antibodies after normalization to input levels.

2.6. Apoptosis assay

Apoptosis was measured with the Caspase Glo 3/7 reagent (Promega) according to the manufacturer protocol after treatment or not with etoposide at the indicated concentrations for 16 h and with a CytoGLO annexin V-FITC Apotosis detection kit (Ref. 10085 K) from IMGENEX after treatment (+) or not 12 h with 25 μM etoposide of Hela cells transfected with indicated siRNA. Analysis were performed on a FACS Verse (BD Biosciences).

Table 1
Oligonucleotide sequences.

−1200Fp53 −1.2kpb	5′-GGTACCACAGCAGTCCGGAGCTAACG-3′
−188F (p53 200)	5′-GAGCTCCGGCGAG AATCCTGACTCTG-3′
−99F (p53 100)	5′-GGTACCGCGACTGTCCAGCTTTGTGC-3′
+12R (p53)	5′-CCTGGACGGTGGCTCTAGAC-3′
E2F1 BS-1 F	5′-CCAGCTGAGAGCAAACGCAAAAGC-3′
E2F1 BS-1 R	5′-CTCGCCGACCTGGTGCCGTA-3′
E2F1 BS-2 F	5′-CCCGGATCAGATTTCGCGGGC-3′
E2F1 BS-2 R	5′-TACCGCGGGACTCGGTAGGG-3′
Cyclin E F	5′-GCCGCCCGCCGTGTTTACAT-3′
Cyclin E R	5′-GGCGCTGGAGC GGCAAAAAG-3′
SKP2 F	5′-CGGGACGGAAACTACAATTC-3′
SKP2 R	5′-AAGCCTAGCAACGTTCCATC-3′
SKP2-1500 F	5′-TCCCTCCTTAATAGCTCCCCATCCCC-3′
SKP2-1500 R	5′-CTGGGAGGCACTCGTTCGCC-3′
pGL2/3 F	5′-GAACTTCCCGCCGCCGTTGT-3′
pGL2/3 R	5′-TCCTCCGCGCAACTTTT TCGC-3′
RTqPCR HPRT1 F	5′-GGCTCCGTTATGGCGACCCG-3′
RTqPCR HPRT1 R	5′-AAACACCCTTTCCAAATCCTCAGCA-3′
RTqPCR GUSB F	5′-GATGACATCACCGTCACCACCAGC-3′
RTqPCR GUSB R	5′-CCCAGTCCCATTCGCCACGACT-3′
RTqPCR p73 F	5′-GGACGCAGCGAAACCGGGG-3′
RTqPCR p73 R	5′-CTGGGCCATCTTCCCCACGC-3′
RTqPCR p53 F	5′-CCAGGGAGCACTAAGCGAGCAC-3′
RTqPCR p53 R	5′-GAACATCTCGAAGCGCTCACGC-3′
BS2 mut F	5′-CCGCCGCATCCCGGATCAGATGATATCGGCGACCCACGGAACCCGCGG-3′
BS2 mut R	5′-CCGCGGGTTCCGTGGGTCGCCGATATCATCTGATCCGGGATGCGGCGG-3′

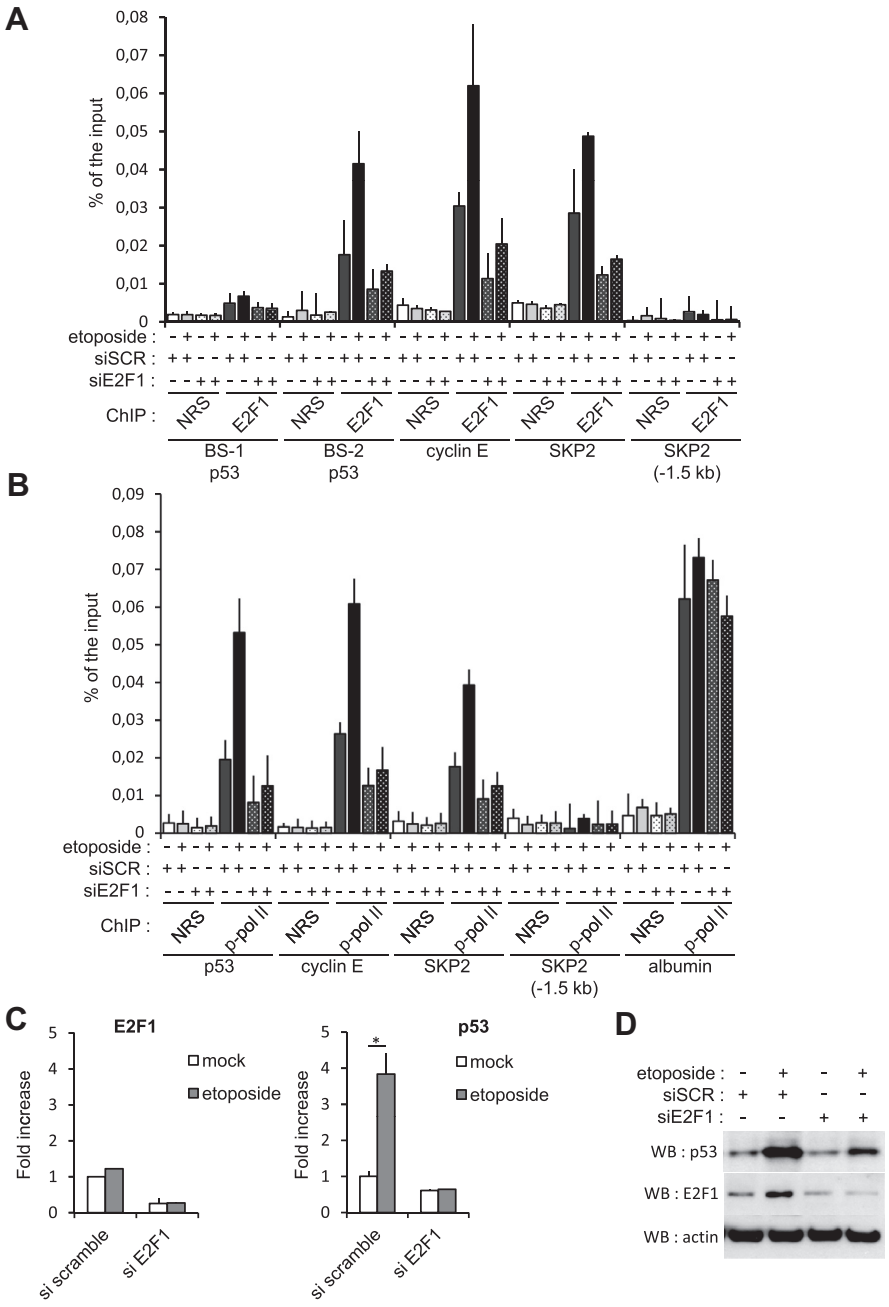

Fig. 2. Etoposide induces E2F1 dependent p53 transcription through its distal binding site and leads HeLa cells to apotosis. (A) Hela cells were transfected by either a siRNA against E2F1 (siE2F1) or a scrambled siRNA (siSCR) and treated or not with etoposide. ChIP experiments were performed using antibodies directed against E2F1, or IgG (NRS) as a negative control and quantitative PCR using primers that amplify the potential indicated binding sites. (B) Same conditions as in A, but the ChIP experiments were performed using antibodies directed against active phosphorylated polymerase II or IgG (NRS) as a negative control. (C) RT-qPCR analysis of E2F1 and p53 mRNA levels in HeLa cells transfected by scrambled or E2F1 siRNA and treated or not by etoposide. Experiments were done in triplicates ($^{*}P < 0.05$). (D) Western blot experiments corresponding to the same experimental conditions as described in C.

3. Results and discussion

It has been recently reported that the transcription factor E2F1 is able to bind the p53 promoter on the proximal E2F1 binding site (BS-1, Fig. 1A) in the human U251 glioblastoma cell line which harbors a mutated p53 [11]. We addressed the question if E2F1 can contribute to induce apoptosis under a genotoxic stress in a p53 wild type context by p53 transcriptional activation. To do this, we first investigated E2F1 binding on the p53 promoter in HeLa cells which possess low levels of wild type p53. The two previously described E2F1 binding sites E2F1 BS-1 and E2F1 BS-2 were subjected to chromatin immunoprecipitation (ChIP) to analyse E2F1 binding on the p53 promoter (Fig. 1B). E2F1 transcription factor was found associated with the distal binding site (E2F1-BS-2), but no binding was detected at the proximal binding site (E2F1-BS-1). As a positive control, E2F1 was associated with the SKP2 promoter, a known E2F1 target gene (Fig. 1C). Conversely, E2F1 was not found associated with the SKP2 genomic region located 1.5 kb upstream of the transcriptional start site (negative control). Additionally, E2F1 binding was observed for the cyclin E promoter another E2F1 target gene (Fig. 1D). To conclude, E2F1 is able to bind DNA at the distal site (E2F1 BS-2) of the p53 promoter in HeLa cells but not at the proximal site as previously observed in U251 cells [10]. Interestingly, HeLa cells are HPV18 positive cells and

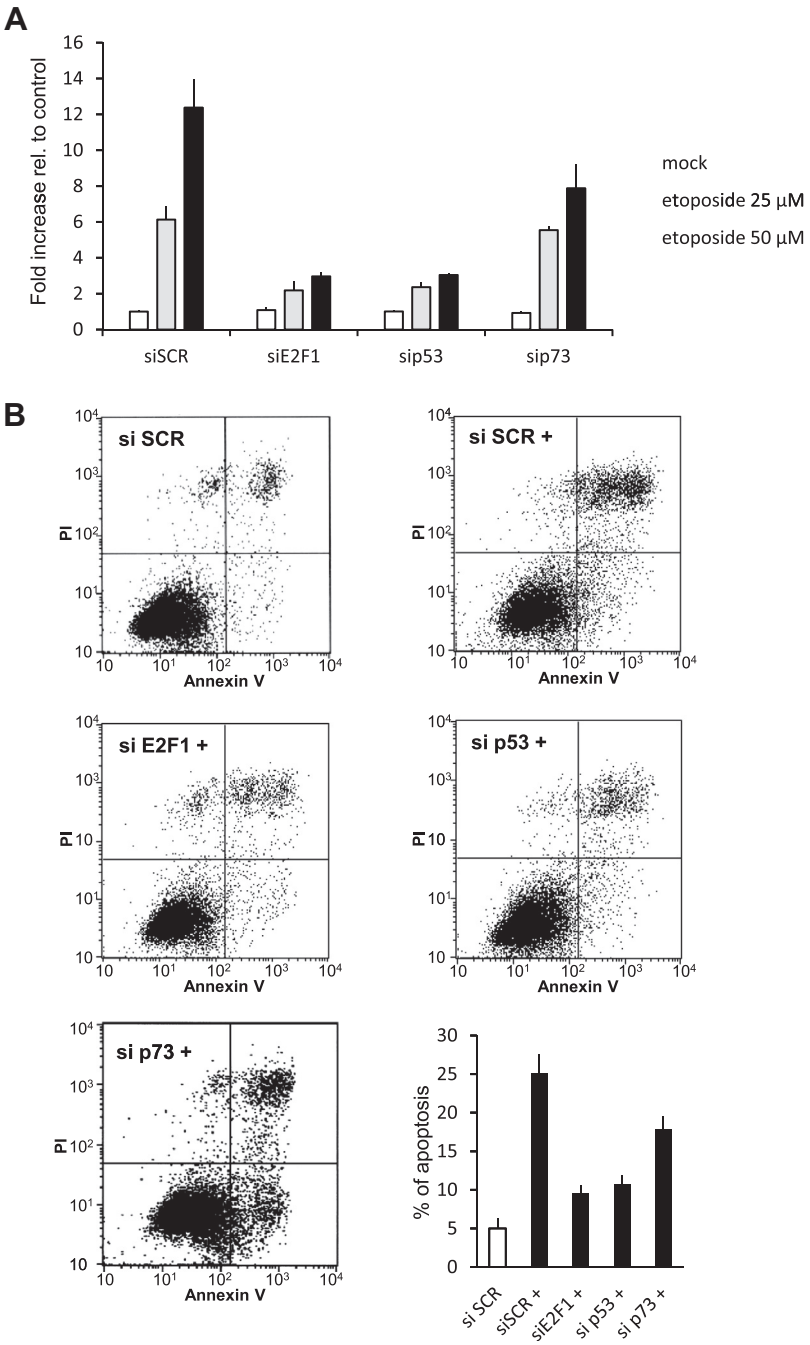

Fig. 3. Measure of apoptosis in HeLa cells. (A) Caspase 3 and 7 activity in HeLa cells transfected with the indicated siRNAs and treated or not by etoposide. (B) FACS analysis: evaluation of apoptosis induction in HeLa cells transfected with the indicated siRNA and treated (+) or not by etoposide (25 μM) for 12 h. Cells were stained with APC-Annexin V and PI followed by FACS analysis to determine the percentage of apoptotic cells. Graphs are the mean of three independent experiments. White bar represents untreated cells; black bars represent etoposide treated cells (+).

consequently express an HPV E7 protein of the high risk group HPVs, a protein that interacts with E2F1 and activates E2F1-driven transcription [12]. The impact of this interaction concerning the choice of the E2F1 binding sites on the p53 promoter region and the efficiency of these binding sites on p53 transcriptional activation will be the purpose of another investigation. This will evaluate the contribution of both HPV18 E6 or HPV18 E7 on p53 protein level in HeLa cells.

To further analyze the control of p53 transcription by E2F1, we searched for the presence of sequences that could regulate p53 transcription in a context where E2F1 was overexpressed. For this, four promoter constructs were generated to place different regions of the p53 promoter upstream of the luciferase reporter (Fig. 1E). The shortest construct (pGL3-100 bp) contains the minimal promoter [8] including the proximal E2F1 binding site; the pGL3-200 bp contains the E2F1 BS-1 and the p53/73 binding site, the pGL3-1.2 kb contains E2F1 BS-1, BS-2 and the p53/73 binding site and finally the pGL3-1.2 kb mutBS-2 exhibiting a mutagenized E2F1 BS-2 site to abolish E2F1 binding. Over-expression of E2F1 in transfected HeLa cells induced a 5-fold increase of the luciferase activity only for the −1.2 kb construct that possesses the intact E2F1-BS2 binding site. The −200 bp construct showed a slight, non-significant activation of transcription compared to the wild type −1.2 kb construct (Fig. 1F). The promoter fragment containing

the proximal BS-1 E2F1 binding site did not respond to E2F1 overexpression, thus confirming that this region of the promoter is not responsive to E2F1 in HeLa cells. As a positive control, a construct containing the luciferase reporter gene under the control of the cyclinE promoter [13] responded to E2F1 overexpression by a 6.8-fold increase (Fig. 1F). E2F1 and p53 expression levels were monitored by Western blot (Fig. 1G). These results indicate that E2F1 is able to activate p53 transcription in HeLa cells through the distal E2F1 BS-2 site in this context and that p53 and/or p73 do not contributed to p53 promoter activation under these conditions (1.7-fold increase).

We next investigated the effects of E2F1 over-expression on endogenous p53 mRNA levels. Ectopic expression of E2F1 in HeLa cells led to a significant increase (8-fold) in endogenous p53 mRNA level (Fig. 1H). This increase is likely due to a direct effect of E2F1 binding on the p53 promoter but could also be due, at least in part, to p53 itself on its own promoter. However p53 protein levels are low in HeLa cells due to the partial disruption of the p53 pathway by the HPV18 E6 protein that targets the p53 protein to the proteasome [14]. In addition, p73 did not significantly increased p53 mRNA levels. Effectively, a siRNA directed against p73 failed to modify p53 mRNA levels when E2F1 was overexpressed (Fig. 1H). The efficiency of the p73 siRNA was assessed by RT-qPCR and demonstrated a 83% decrease in p73 mRNA level (Fig. 1H, right) correlated with a low p73 protein level (data not shown). In order to determine the role of E2F1 under a genotoxic stress, binding of E2F1 on the p53 promoter upon etoposide treatment was analyzed (Fig. 2A). HeLa cells were transfected by siRNA (scrambled or directed against E2F1) and treated or not by etoposide (4 h at 50 μM). Cell extracts were then subjected to chromatin immunoprecipitation. Under control condition (siSCR, untreated cells) binding of E2F1 on the p53 promoter was observed only on the E2F1 BS-2 as presented in Fig. 1B. Etoposide treatment induced a strong recruitment of E2F1 on the E2F1 BS-2 binding site (~230% increase). However, no binding of E2F1 could be detected on the E2F1 BS-1 binding site even under genotoxic stress. HeLa cells transfected by a siRNA directed against E2F1 abolished most of the binding that was observed in untreated and etoposide treated cells demonstrating the specificity of the E2F1 immuno-precipitation. Similarly, an increase of E2F1 binding upon etoposide treatment compared to control conditions was observed for the cyclin E and SKP2 promoters and was impaired when the treated and non treated cells were transfected by the E2F1 siRNA. As a negative control, no E2F1 binding could be detected in a genomic region 1.5 kb upstream of the SKP2 transcription start site for all studied conditions.

Interestingly, the increase of enrichment for p53, cyclin E and SKP2 E2F1 dependent promoter regions coincides with an increased enrichment with phosphorylated RNA polymerase II (P-Pol II) immunoprecipitation (Fig. 2B). For untreated cells transfected with a scrambled siRNA, the presence of P-Pol II was detected at the E2F1 dependent promoter region of the p53, cyclin E, SKP2 promoters and for the albumin promoter. When the cells were transfected by the scrambled siRNA and treated by etoposide the recruitment of P-Pol II was strongly increased for E2F1 dependent promoters (e.g. a ~300% increase for p53) whereas no significant change was observed for the albumin promoter. Transfection by the siRNA directed against E2F1 reduced dramatically the presence of the P-Pol II under both conditions (untreated and etoposide treated cells), except for the E2F1 unresponsive albumin promoter.

Thus, dynamic recruitment of activated E2F1 after a genotoxic treatment is observed on the p53 E2F1 BS-2 distal site in HeLa cells and is able to initiate active transcription as revealed by the concomitant recruitment of active phosphorylated RNA polymerase II under these conditions.

These results were corroborated by the measurement of endogenous p53 mRNA levels that show a 3.9-fold increase under etoposide treatment (Fig. 2C, right). However this increase in p53 mRNA level upon etoposide treatment was not observed when the cells were depleted in E2F1 (Fig. 2C left and right) indicating that this mechanism is E2F1 dependent. At the protein level, etoposide treatment showed a strong increase in p53 protein level concomitantly with an increase in E2F1 level (Fig. 2D) when the cells where transfected by a scrambled siRNA (siSCR). It is well established that the increase in E2F1 protein level after etoposide treatment is due to protein stabilization by an ATM-induced phosphorylation [15,16]. However when E2F1 was knocked-down by a siRNA treatment, p53 protein levels increased but moderately (Fig. 2D). This remaining increase is likely due to p53 protein self induction due to its stabilization through the DNA damage pathway that disrupts the p53-mdm2 complex [17,18].

We finally assessed whether etoposide was functionally able to trigger apoptosis through the E2F1 pathway. For this, we measured caspase 3 and caspase 7 activation in HeLa cells transfected by a scrambled siRNA or a siRNA directed against E2F1 and treated or not by different concentrations of etoposide (Fig. 3A). In E2F1 depleted HeLa cells, a 3-fold decrease for a 25 μM etoposide treatment or a 4-fold decrease for a 50 μM treatment in caspase activity was observed. Similar results were obtained when a siRNA directed against p53 was transfected whereas a siRNA targeting p73 moderately contibuted to reduce the sensibility to etoposide. These results were confirmed by an annexin/propidium iodide (PI) staining experiment (Fig. 3B). Etoposide treatment induced a 5-fold increase in apoptotic cell number (PI and annexin positive cells) under control conditions (siSCR ± etoposide) whereas cells transfected by a siRNA against E2F1 or p53 displayed only a two fold increase in apoptotic cell number upon etoposide treatment. Again, depletion of p73 did not significantly protect cells from etoposide (3.8-fold increase of apoptosis). These results indicate that the E2F1/p53 pathway is still functional in HPV positive HeLa cells as a disruption of this pathway by E2F1 or p53 siRNA protects cells from apoptosis and that these tumor cells can be efficiently targeted by chemotherapeutic agents like etoposide.

To conclude, we showed that the DNA damaging chemotherapeutic agent etoposide induces p53 expression that, at least in part, occurs at the transcriptional level as demonstrated by the luciferase reporter, RTqPCR and ChIP experiments. Moreover in HeLa cells, this activation occurs mainly through the E2F1 pathway while the p53/p73 positive feedback loop slightly contributes to the regulation of the p53 gene transcription. Importantly, interference with the E2F1-mediated apoptotic pathway strongly impairs the p53-mediated apoptotic response to the DNA damaging agent etoposide. This study highlights the importance of the E2F1 pathway in mediating the apoptosis of Human papillomavirus positive carcinoma cells under genotoxic stress. Interestingly, HeLa cells are HPV-18 positive and thus produce E6 and E7 transforming proteins. E7 protein disrupts Rb/E2F repressor complexes and activates E2F1-driven transcription whereas E6 protein accelerates p53 ubiquitin-mediated degradation [19–23,12]. It appears that under a genotoxic stress, E6 activity is not sufficient to modulate the E2F1/p53 pathway leading to a strong E2F1 and RNA polymerase II recruitment and consequently a p53 level responsible of caspases activation and thus apoptosis. Interestingly, in HeLa cells, when E6 alone was repressed, apoptosis occurred [24,25]. Moreover, HPV 16 E6 and E7 proteins highly sensitize Human Keratinocytes to apoptosis (induction of caspase 3 activity) induced by chemotherapeutic agents: this increase in sensitivity may relate to both the higher level of p53 protein as well as the binding of E7 to the active form of pRb [26]. However, under normal conditions, one can imagine that the E6 protein lowers efficiently the p53 protein level whereas the E7 protein induces the E2F1-driven transcription leading for example to a cyclin E protein level able to disturb cell cycle regulation. In addition, it is interesting to note

that the high binding affinity to pRb for the E7 protein of the high risk group HPV correlates with the transforming potential of E7 [27].

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