

The Tumor Suppressor Gene *Hypermethylated in Cancer 1* Is Transcriptionally Regulated by E2F1

Mathias Jenal,¹ Emmanuelle Trinh,⁵ Christian Britschgi,^{1,3} Adrian Britschgi,¹ Vincent Roh,² Stephan A. Vorburger,² Andreas Tobler,^{1,4} Dominique Leprince,⁶ Martin F. Fey,^{1,3} Kristian Helin,⁵ and Mario P. Tschan^{1,3}

¹Experimental Oncology/Hematology and ²Visceral and Transplantation Surgery, Department of Clinical Research, University of Bern, Bern Switzerland; and Departments of ³Medical Oncology and ⁴Hematology, Inselspital, Bern University Hospital, Bern Switzerland; and ⁵Biotech Research and Innovation Centre and Centre for Epigenetics, University of Copenhagen, Copenhagen, Denmark; and ⁶CNRS UMR 8161 Institut de Biologie de Lille, Institut Pasteur de Lille, Lille Cedex, France

Abstract

The *Hypermethylated in Cancer 1 (HIC1)* gene encodes a zinc finger transcriptional repressor that cooperates with p53 to suppress cancer development. We and others recently showed that *HIC1* is a transcriptional target of p53. To identify additional transcriptional regulators of *HIC1*, we screened a set of transcription factors for regulation of a human *HIC1* promoter reporter. We found that E2F1 strongly activates the full-length *HIC1* promoter reporter. Promoter deletions and mutations identified two E2F responsive elements in the *HIC1* core promoter region. Moreover, *in vivo* binding of E2F1 to the *HIC1* promoter was shown by chromatin immunoprecipitation assays in human TIG3 fibroblasts expressing tamoxifen-activated E2F1. In agreement, activation of E2F1 in TIG3-E2F1 cells markedly increased *HIC1* expression. Interestingly, expression of E2F1 in the p53^{-/-} hepatocellular carcinoma cell line Hep3B led to an increase of endogenous *HIC1* mRNA, although bisulfite genomic sequencing of the *HIC1* promoter revealed that the region bearing the two E2F1 binding sites is hypermethylated. In addition, endogenous E2F1 induced by etoposide treatment bound to the *HIC1* promoter. Moreover,

inhibition of E2F1 strongly reduced the expression of etoposide-induced *HIC1*. In conclusion, we identified *HIC1* as novel E2F1 transcriptional target in DNA damage responses. (Mol Cancer Res 2009;7(6):916–22)

Introduction

HIC1 is a *bona fide* tumor suppressor as shown in *Hic1*^{+/-} mice that develop different spontaneous, age- and gender-specific malignant tumors (1). The *HIC1* gene is located on the short arm of human chromosome 17 (17p13.3) telomeric to the p53 tumor suppressor gene. Chromosome 17 is frequently altered in human cancers and its allelic loss at the p53 locus is often combined with mutations of the remaining p53 allele. Interestingly, the 17p13.3 region is frequently deleted or hypermethylated in human cancers, such as breast cancer (2), colorectal cancer (3), and hepatocellular carcinomas (4, 5). Moreover, hypermethylation in this chromosomal region often leads to inhibition of *HIC1* expression, and overexpression of *HIC1* in different cancer cell lines decreases their clonogenic survival (6).

The *HIC1* gene encodes a transcriptional repressor belonging to the broad complex, tramtrack, and bric à brac/poxviruses and zinc finger (BTB/POZ) transcription factor family. Currently, three transcriptionally repressed *HIC1* targets are known: (a) the type III NAD⁺-dependent histone/protein deacetylase Silent Information Regulator 2a homologue1 (SIRT1) is involved in regulating cellular senescence and longevity (7); (b) the fibroblast growth factor binding protein (8) enhances FGF-mediated biochemical and biological events specifically during blood vessel growth; and (c) the proneural transcription factor Atonal Homolog 1 (Atoh1) is essential for cerebellar growth and development (9).

Previous studies have shown, that *HIC1* plays a role in the p53-dependent apoptosis circuitry in response to DNA damage and p53 was identified as transcriptional inducer of *HIC1*. It has been reported that *HIC1* indirectly modulates p53-dependent DNA-damage responses by transcriptional repression of the p53 deacetylase SIRT1 (7). We showed that *HIC1* mRNA is induced by p53 on UV irradiation (10). These observations are supported by the fact that enforced *HIC1* expression leads to growth arrest and reduced survival of glioblastoma, breast cancer, and adenocarcinoma cell lines (6).

In this article, we show that *HIC1* is a new transcriptional target of the cell cycle and apoptosis regulator E2F1. E2F1 induces *HIC1* via two E2F DNA binding sites within the TATA-box containing *HIC1* P0 promoter. We further show that

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Note: Current address for E. Trinh: Novo Nordisk A/S, Biopharmaceutical Research Unit, Måløv.

Authors contributions: M. Jenal: project design, did experiments, collected and analyzed all data, manuscript writing; E. Trinh: did chromatin immunoprecipitation analysis and TIG3 expression analysis, reviewed manuscript; C. Britschgi: *HIC1* promoter cloning, reviewed manuscript; A. Britschgi and V. Roh: did and analyzed Hep3B expression experiments, reviewed manuscript; S.A. Vorburger, A. Tobler, D. Leprince, M.F. Fey, and K. Helin: project planning and design, reviewed manuscript; M.P. Tschan project design, data analysis, manuscript writing.

Requests for reprints: Mario P. Tschan, Experimental Oncology/Hematology, MEM E829, Department of Clinical Research, University of Bern, Murtenstrasse 35, Bern, CH-3010, Switzerland. Phone: 41-31-632-8780; Fax: 41-31-632-3297. E-mail: mtschan@dkf.unibe.ch

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E2F1 binds to this promoter region *in vivo* and induces endogenous *HIC1* mRNA expression. Furthermore, *HIC1* expression is induced by E2F1 regardless of *HIC1* P0 promoter hypermethylation. Moreover, using RNA interference to inhibit E2F1 expression, we show that E2F1 is necessary for *HIC1* mRNA induction on etoposide treatment. Lastly, we show that endogenous E2F1 protein directly binds to the *HIC1* promoter region in DNA damage responses to etoposide treatment.

Results

Two Functional E2F DNA Binding Sites Reside Within the HIC1 P0 Promoter

To date, p53 is the only known transcriptional regulator of *HIC1* (6, 10, 11). To find new transcriptional regulators of *HIC1*, we analyzed a 3.2-kb *HIC1* genomic fragment upstream

of exon 2 for putative transcription factor binding sites using MatInspector software (12). We found several putative E2F DNA binding sites spread over the whole genomic fragment analyzed (Fig. 1A, left). Cotransfection of the longest *HIC1* promoter construct (pGL3.HIC1prm.1) that comprises all three *HIC1* promoters (P2 GC-rich, P1 GC-rich, and P0 TATA) together with E2F1 into H1299 lung cancer cells led to a 20-fold induction of the *HIC1* reporter construct compared with the empty pcDNA3.1 control vector (Fig. 1A, right). Analysis of *HIC1* promoter deletion constructs (pGL3.HIC1prm2-pGL3.HIC1prm6), pointed to three putative E2F binding sites within the *HIC1* P0 promoter located 24, 126, and 235 bp downstream of the TATA-box. Individual mutations identified two active E2F responsive elements. Mutation of either active E2F-site was sufficient to markedly decrease E2F1-induced *HIC1* promoter activity. Additionally, mutations of both active E2F

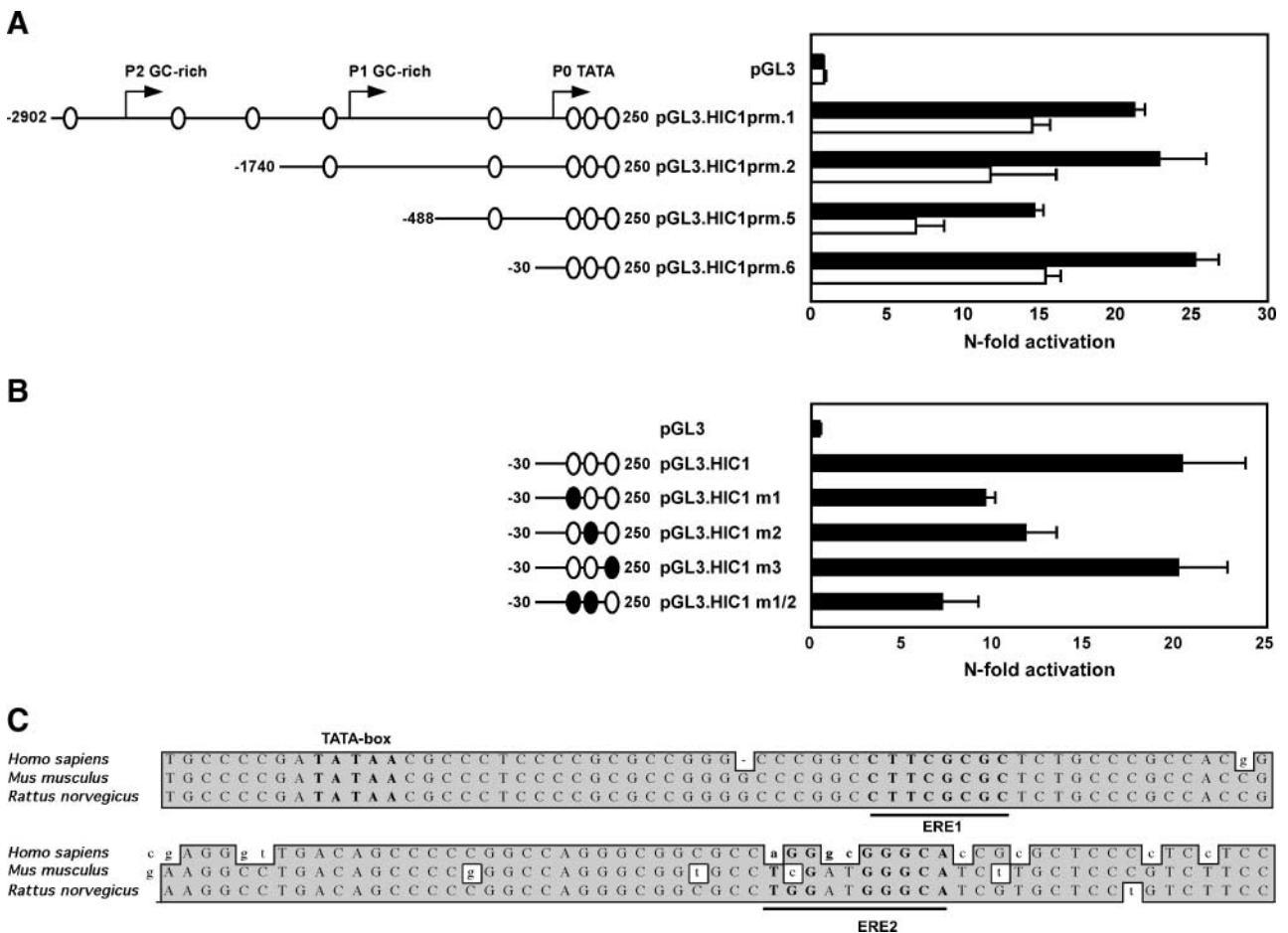


FIGURE 1. *HIC1* promoter activation via two proximal E2F binding sites. **A.** Left, schematic presentation of the *HIC1* promoter regions cloned into pGL3-Luciferase reporter plasmids. The three major promoter regions (P0, P1, and P2) as well as putative E2F binding sites (white ovals) are indicated. Right, H1299 non-small cell lung cancer cells were transfected with 400 ng *HIC1* promoter reporter, 400 ng E2F1 expression, and 10 ng of phRL-TK plasmid. Results from two independent experiments (black and white bars) are expressed relative to a value of 1.0 for cells transfected with empty vector. Experiments were done in duplicates; columns, mean; black and white bars, SD. **B.** Individual mutations of the three putative E2F binding sites within the *HIC1* P0 promoter identified two active E2F-sites. Mutating both active E2F binding sites further attenuated E2F1-dependent activation of *HIC1* P0 promoter. Left, schematic presentation of the *HIC1* promoter 6 or their mutated counterparts (black ovals), respectively, cloned into pGL3-Luciferase reporter plasmids. Right, wild-type and mutated pGL3. *HIC1* m1, m2, m3, and m1/2 reporter plasmids were cotransfected with E2F1 expression or empty control plasmids as described in **A**. Results, expressed relative to a value of 1.0 for cells transfected with empty vector, are the means of triplicates; bars, SD. **C.** Alignment of the two functional E2F binding sites (ERE1 and ERE2). ERE1 and ERE2 are conserved in the *HIC1* P0 promoter of human, mouse, and rat. The TATA-box and the newly identified E2F responsive elements (ERE1 and ERE2) are indicated.

binding sites further decreased *HIC1* promoter activity on E2F1 expression (Fig. 1B). We thus identified two new E2F responsive elements (ERE1 and ERE2). Interestingly, ERE1 and ERE2 are conserved among human, mouse, and rat genomic sequences, as shown by BLAST sequence comparisons (Fig. 1C).

In vivo Binding of E2F1 to the *HIC1* Promoter

To verify if the transcription factor E2F1 would bind *in vivo* to the identified putative E2F binding sites within the *HIC1* P0 promoter, a chromatin immunoprecipitation (ChIP) assay in TIG3 cells stably expressing the estrogen receptor (ER) or a ER-E2F1 fusion protein was done. TIG3-ER-E2F1 cells were treated with 4-hydroxytamoxifen (OHT) to induce translocation of the ER-E2F1 fusion protein to the nucleus. The cells were then cross-linked and the DNA-protein complexes immunoprecipitated with an anti-E2F1 antibody or an unrelated anti-Flag antibody as negative control. The precipitated DNA was amplified by PCR using primers in the E2F1-responsive region. Significant enrichment of E2F1 on the *HIC1* P0 promoter was seen (Fig. 2A). As a control for the binding of E2F1, its association with the E2F target gene, *CDC6* (13), was measured (Fig. 2B).

Induction of *HIC1* mRNA by E2F1

Using promoter reporter and ChIP assays, we identified two E2F binding sites placed within the *HIC1* P0 promoter region, which bind to E2F1 *in vivo*. We next asked whether activation of E2F1 in TIG3-ER-E2F1 fibroblasts would increase endogenous *HIC1* mRNA levels. To this end, TIG3-ER-E2F1 fibroblast cells were incubated with 600 nmol/L OHT for 4 hours. Activation of E2F1 resulted in a 12-fold induction of endogenous *HIC1* mRNA (Fig. 3A, left). Successful activation of E2F1 was confirmed by measuring mRNA expression of the E2F1 target gene *Cyclin E1* (*CCNE1*; Fig. 3A, right; ref. 14) that was induced 17-fold. Similarly, activation of endogenous E2F in TIG3-ER-E1A fibroblasts resulted in a 6- and 4.5-fold induction of *HIC1* and *CCNE1* mRNA, respectively (Fig. 3B). Expression of E1A competes with endogenous E2F for binding

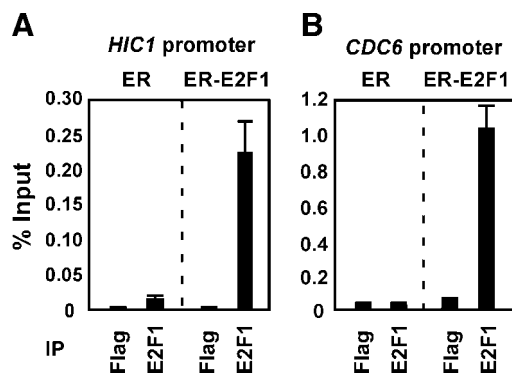


FIGURE 2. *In vivo* binding of E2F1 to the *HIC1* promoter. **A.** ChIP assays using OHT-treated TIG3-ER and TIG3-ER-E2F1 cells. Enrichment of E2F1 protein on the *HIC1* promoter is shown. Immunoprecipitation (IP) using the anti-E2F1 or an unrelated antibody (anti-Flag) were done in duplicates. Results are presented as percentage of the input DNA. **B.** *In vivo* binding of E2F1 to the *CDC6* promoter, a known E2F1 target gene, is shown as a control. Analysis as in **A.**

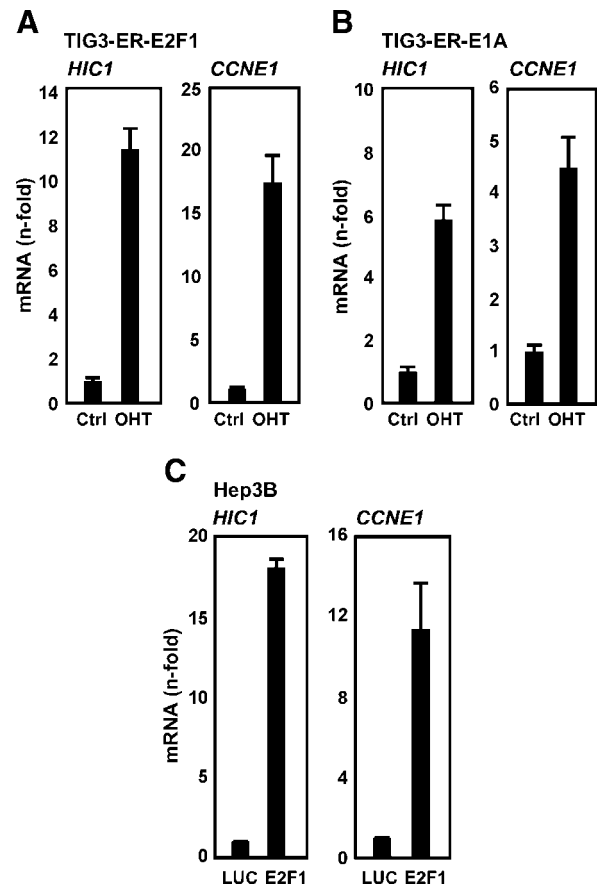


FIGURE 3. E2F1 induces endogenous *HIC1* mRNA. **A** and **B.** Activation of E2F1 in TIG3 human fibroblast cells induces endogenous *HIC1* mRNA. TIG3-ER-E2F1 or -E1A human fibroblast cells expressing ER fusion proteins were incubated with 600 nmol/L OHT for 4 h. *HIC1* mRNA levels were measured by real-time quantitative PCR. *CCNE1* (*Cyclin E1*) mRNA induction was measured as a control for E2F1 activation. *HIC1* and *CCNE1* mRNA regulation are shown as n-fold changes compared with untreated cells using *ABL* mRNA expression as a reference gene. Experiments were done in duplicates; columns, mean; bars, SD. **C.** Hep3B hepatocellular carcinoma cells were infected with adenovirus expressing Luciferase control (AdLuc) or E2F1 (AdE2F1) for 24 h. *HIC1* and *CCNE1* mRNA were determined as in **A.** Results are given as n-fold changes compared with AdLuc infected Hep3B cells using *GAPDH* as reference gene. Experiments were done in duplicates; columns, mean; bars, SD.

to the retinoblastoma protein and thus derepresses E2F target genes.

Moreover, expression of E2F1 in Hep3B hepatocellular carcinoma cells using adenoviral vectors resulted in an 18-fold induction of endogenous *HIC1* mRNA levels compared with Hep3B cells infected with a control adenovirus expressing the luciferase gene (Fig. 3C, left). We used p53-negative Hep3B cells to exclude that induction of *HIC1* by E2F1 occurs indirectly via the E2F1-ARF-p53 pathway. Activation of *CCNE1* mRNA after adenoviral expression of E2F1 in Hep3B cells was measured as a control (Fig. 3C, right).

HIC1 mRNA Is Induced On E2F1 Expression Despite Dense Methylation of the P0 Promoter

Different reports indicate that the *HIC1* promoter is epigenetically silenced in a wide variety of human cancers, as for

example in primary human hepatocellular carcinomas (4, 5). Of note, it was shown that in most cell lines, dense methylation of the *HIC1* P0 promoter was sufficient to completely silence *HIC1* mRNA expression. Treatment of Hep3B hepatocellular carcinoma cells with 0.5 or 3.0 $\mu\text{mol/L}$ 5'-aza-2'-deoxycytidine methyltransferase inhibitor for 2 days increased *HIC1* mRNA 1.6- and 2.3-fold, respectively (Fig. 4A). With the technique of bisulfite genomic sequencing, we confirmed that the *HIC1* P0 promoter is indeed hypermethylated in Hep3B cells including CpGs within the two E2F binding sites (Fig. 4B).

E2F1 Is Critically Involved in Etoposide-Induced Up-Regulation of HIC1

Chen et al. (7) showed that *HIC1* plays a role in etoposide-induced DNA damage responses. Furthermore, it has been reported that E2F1 is stabilized in response to DNA damage and this stabilization would lead to the activation of a subset of E2F1 target genes (15, 16). Treating Hep3B cells with 40, 80, and 120 $\mu\text{mol/L}$ etoposide for 24 hours leads to a 2.7-, 4.8-, and 8-fold induction of *HIC1* mRNA levels, respectively (Fig. 5A). To examine whether *HIC1* mRNA is induced via E2F1 in Hep3B cells on exposure to etoposide, we treated two different Hep3B cell lines expressing small hairpin (sh) RNAs targeting E2F1 and a nontargeting shRNA control cell line with 80 $\mu\text{mol/L}$ etoposide. Inhibition of E2F1 in the two Hep3B E2F1 knock-down cell lines diminished induction of *HIC1* mRNA on etoposide treatment by 40% and 60%, respectively, compared with a nontargeting shRNA control cell line. Efficient E2F1 knockdown in Hep3B cells was shown by Western blotting (Fig. 5B). To investigate if endogenous E2F1 directly activates *HIC1* expression on etoposide treatment, we did a ChIP assay in Hep3B cells treated with 80 $\mu\text{mol/L}$

etoposide for 24 hours. Cross-linked DNA-protein complexes were immunoprecipitated with an anti-E2F1 antibody, or with an unrelated anti-IgG antibody as negative or with an anti-acetyl-histone H3 antibody as positive control. Precipitated DNA was PCR amplified with primers in the E2F-responsive region. Significant enrichment of the endogenous E2F1 protein on the *HIC1* P0 promoter was seen (Fig. 5C). As an E2F1 binding control, promoter association with *CDC6* (13), a well-known E2F target, was measured.

These observations further support our finding that the tumor suppressor gene *HIC1* is a direct transcriptional target of E2F1.

Discussion

In this article, we identified the cell cycle and apoptosis gene E2F1 as a new transcriptional regulator of *HIC1*. E2F1 is a member of the E2F transcription factor family involved in different cellular processes such as cell cycle progression, DNA replication, oncogenic transformation, and in apoptosis responses. E2F1 can promote cell growth or induces apoptosis dependent on the amount of active E2F1 in the cell. For example, E2F1 is activated in response to oncogenic hyperproliferation and can induce apoptosis in a p53-dependent or p53-independent manner as part of a failsafe antiproliferative mechanism (17-19).

Our results show that E2F1 directly activates the transcription of *HIC1* mRNA as shown with promoter deletion, E2F binding site mutation, and *in vivo* binding experiments. Individual mutations of the two functional E2F-binding sites, ERE1 and 2, in the *HIC1* P0 promoter led to a 53% and 42% reduction of reporter activity, respectively. Mutating both functional E2F binding sites further reduced promoter activity. These

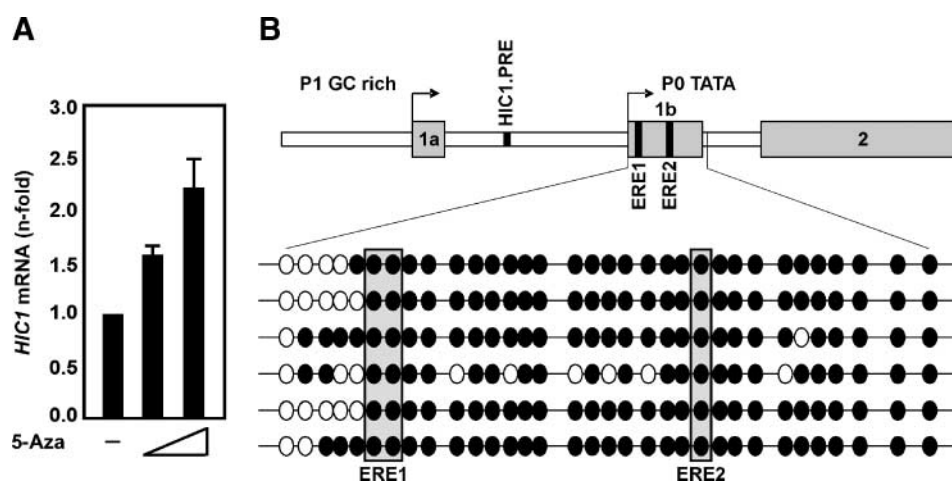


FIGURE 4. E2F1-induced up-regulation of *HIC1* mRNA despite *HIC1* P0 promoter hypermethylation. **A.** Hep3B cells were treated with 0.5 or 3.0 $\mu\text{mol/L}$ 5'-aza-2'-deoxycytidine (5-Aza) for 2 d. Total RNA was extracted and *HIC1* mRNA levels were measured by real-time quantitative PCR. Results are depicted as n-fold up-regulation compared with DMSO-treated cells and *HMBS* as reference gene. Experiments were done in triplicates; columns, mean; bars, SD. **B.** Top, schematic representation of the *HIC1* P0 promoter (adapted from ref. 36). ERE1 and ERE2, E2F responsive elements; HIC1.PRE, p53 responsive element. Bottom, the methylation status from multiple individual clones derived by PCR on bisulfite-treated genomic DNA from Hep3B cells was investigated by bisulfite genomic sequencing. Black circles, methylated CpG sites; white circles, unmethylated CpG sites; each horizontal line, an individual allele. CpGs within the EREs are boxed.

results are comparable with results obtained from other E2F1 target genes such as *CDC25A* or *DNMT1* (20, 21), but we cannot completely exclude that other E2F binding sites further upstream might contribute to *HIC1* regulation.

Interestingly, Zhang et al. (22) recently published that HIC1 directly binds to the *E2F1* promoter and inhibits its expression. The HIC1-mediated transcriptional inhibition of E2F1 and consequently of E2F-responsive genes led to growth suppression. The observation of HIC1-mediated E2F1 repression together with our results showing activation of *HIC1* by E2F1 might point to a novel negative feedback mechanism.

Recent studies showed that ectopic expression of E2F1 can induce apoptosis in different tumors (23-25). Additionally, it was clearly shown that adenoviral expression of E2F1 results in growth suppression and increased responsiveness of tumor cells to chemotherapy (23, 26). Moreover, different studies suggest that overexpression of E2F1 could be a valuable tool for cancer therapy in tumor cells (reviewed in ref. 27). It was also shown that E2F1 exerts its apoptotic effects through p53-dependent and p53-independent mechanisms (17, 18). In addition, *HIC1* expression leads to reduced survival of different cancer cell lines (6). We now show that expression of E2F1 in p53^{-/-} Hep3B cells using adenoviral vectors results in induction of endogenous *HIC1* mRNA level. We speculate that E2F1 could exert its apoptotic function partially through the activation of *HIC1*, in a p53-independent manner. Moreover, *HIC1* mRNA is inducible in Hep3B cells on E2F1 expression, although methylation analysis revealed that the *HIC1* P0 promoter bearing the E2F binding sites is hypermethylated. This fact indicates that E2F1 induces *HIC1* mRNA levels in Hep3B cells independent on the methylation status of the *HIC1* P0 promoter. Importantly, hypermethylation of the P0 promoter is sufficient to silence *HIC1* expression (28). In line with our methylation results, two studies measured *HIC1* promoter hypermethylation in different morphologic grades of the liver and found increasing *HIC1* promoter methylation from normal liver tissue, to precancerous liver tissue (showing chronic hepatitis or cirrhosis), to primary hepatocellular carcinoma, but no significant correlation between hypermethylation and *HIC1* mRNA expression levels was seen (4, 5). These results indicate that mechanisms other than hypermethylation contribute to the low *HIC1* expression seen in hepatocellular carcinoma.

We further show that *HIC1* mRNA levels are induced in Hep3B cells on etoposide treatment. Previously, Chen et al. (7) pointed out a certain role of HIC1 in the p53-dependent apoptosis response to DNA-damage. The fact that E2F1 is involved in p53-dependent and p53-independent DNA-damage responses (29-32) raises the possibility that *HIC1* mRNA induction is a direct E2F1 effect on etoposide induced DNA damage. Indeed, silencing of E2F1 with the help of two different lentivector delivered shRNAs diminished induction of *HIC1* mRNA by 40% and 60%, respectively, in p53^{-/-} Hep3B cells on etoposide treatment. Further investigations revealed direct binding of endogenous E2F1 protein to the *HIC1* P0 promoter after etoposide treatment of Hep3B cells. We show for the first time that E2F1 activates *HIC1* expression on etoposide induced DNA damage in a p53-independent fashion.

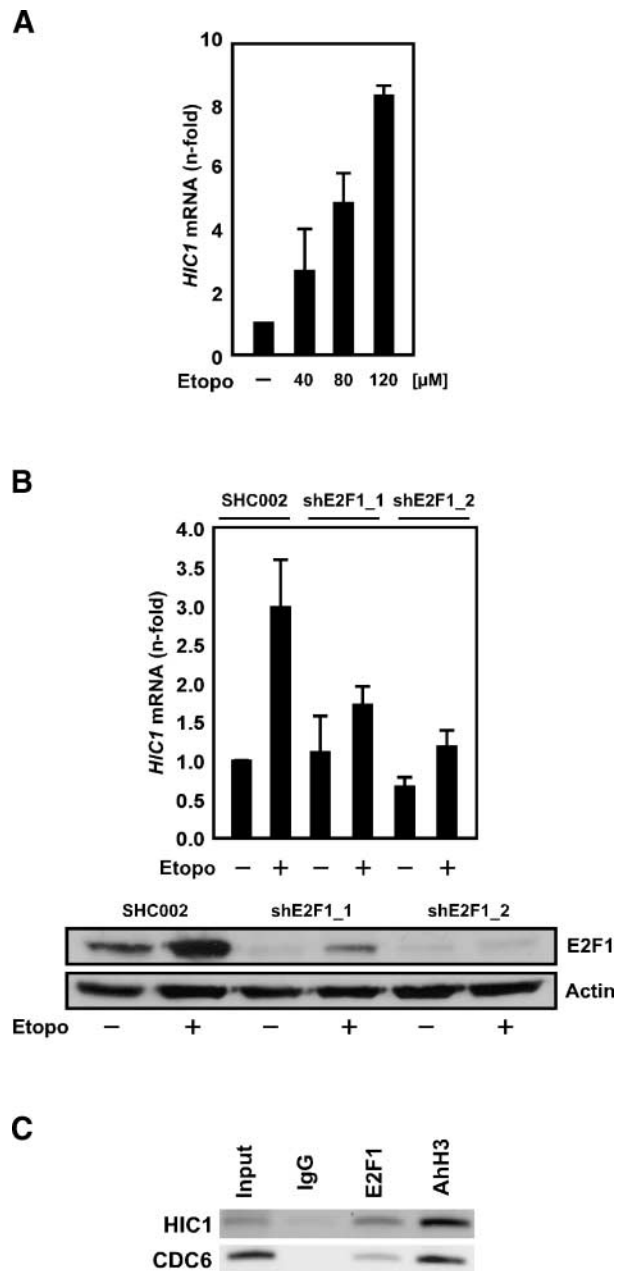


FIGURE 5. E2F1 knockdown inhibits up-regulation of *HIC1* mRNA after etoposide treatment. **A.** Hep3B cells were treated either with DMSO or with 40, 80, and 120 μmol/L etoposide for 24 h. Total RNA was extracted and *HIC1* mRNA levels were measured by real-time quantitative PCR. *HIC1* mRNA expression levels are given as n-fold changes compared with DMSO-treated cells and *GAPDH* mRNA as control gene. Experiments were done in duplicates; columns, mean; bars, SD. **B.** Hep3B cells were stably transduced with nontargeting shRNA (*SHC002*) or shRNAs targeting E2F1 (*shE2F1_1* and *shE2F1_2*). The 3 cell lines were treated with DMSO or 80 μmol/L etoposide for 24 h, respectively. Top, *HIC1* mRNA levels were measured as described in **A**. Experiments were done in duplicates; columns, mean; bars, SD. Bottom, E2F1 Western blot analysis of *SHC002* control and E2F1 knockdown Hep3B cells treated with DMSO and 80 μmol/L etoposide for 24 h, respectively. Actin expression is shown as loading control. **C.** ChIP assays were done in Hep3B cells that were treated with 80 μmol/L etoposide for 24 h. Binding of E2F1 protein to the *HIC1* P0 promoter is shown. Immunoprecipitation was done by using the anti-E2F1 antibody, an unrelated anti-IgG antibody as negative control, and an anti-acetyl histone H3 (*AhH3*) antibody as positive control. Binding of E2F1 to the *CDC6* promoter is shown as control.

Taken together, we identified *HIC1* as a new transcriptional target of E2F1. We found two functional E2F responsive elements within the TATA-box containing *HIC1* P0 promoter that mediate *HIC1* transcription. Using Hep3B E2F1 knockdown cells, we further show, that E2F1 is required for etoposide-induced up-regulation of *HIC1* expression. Additionally, we show that E2F1 directly binds to the *HIC1* P0 promoter region on etoposide treatment. These findings indicate that HIC1 is involved in the p53-independent DNA-damage response of E2F1. Lastly, because *HIC1* promoter hypermethylation cannot prevent induction of *HIC1* by E2F1, HIC1 might represent an important effector of E2F1-mediated cancer therapy.

Materials and Methods

Cell Culture and Chemicals

H1299 non-small lung cancer and Hep3B hepatocellular carcinoma cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Tamoxifen-inducible TIG3-ER-E2F1 and -E1A cells were generated as described elsewhere (33). Cells were maintained in RPMI 1640 or DMEM supplemented with 10% FCS, 50 U/mL penicillin, and 50 µg/mL streptomycin in a 5% air humidified atmosphere at 37°C. OHT was dissolved in ethanol at 1mg/mL and stored protected from light at -20°C (Sigma-Aldrich). The human hepatocellular carcinoma cell line Hep3B was treated with 5'-aza-2'-deoxycytidine (Sigma-Aldrich) in DMSO for 2 d.

HIC1 Promoter Constructs

The *HIC1* promoter deletion constructs pGL3.HIC1prm 1, 2, 5, and 6 were generated as described (10). HIC1prm.6 m1, 2, 3, and 1/2 constructs with mutated E2F-binding sites were generated using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene) and the following oligonucleotides: m1, 5'-CGCGCCGGCCCGGCCTTAAAAATCTGCCCGC-CACGGCAGC-3'; m2, 5'-GGCCAGGGCGGCAGGATATCCACCGCGCTCCCCTCCT'-3; m3, 5'-CCCTACTTGGGTAAAGTTCGATATCGGGCTCGA-GATCTGCGATC'-3. The site-specific mutations were confirmed by DNA sequencing.

Luciferase Reporter Assays

H1299 non-small cell lung cancer cells were transfected with 400 ng *HIC1* promoter reporter, 400 ng E2F1 expression, and 10 ng of pRL-TK plasmid using Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h after transfection and Luciferase activity was measured using the Dual-Luciferase Reporter Plasmid System (Promega). Results, expressed relative to a value of 1.0 for cells transfected with empty vector, are the means of 2 replications, and error bars represent SDs.

ChIP

For ChIP assays, OHT-treated TIG3-ER and TIG3-ER-E2F1 cells or Hep3B cells treated for 24 h with 80 µmol/L etoposide, and an antibody against E2F1 (Sc-193) were used. An unrelated anti-Flag antibody (F3165; Sigma) or an unrelated anti-IgG antibody (Stratagene) were used as negative controls and an anti-acetyl-histone H3 antibody (Stratagene) as a positive control. ChIP assays were done and analyzed as described previously (34). Conventional and quantitative PCR were done

using the following primers: *HIC1* promoter, forward 5'-ACCGAGGGTTGACAGCCC-3' and reverse 5'-AGTAGGGA-GAAAGGGAGCCG-3'; *CDC6* promoter, forward 5'-AAAGGCTCTGTGACTACAGCCA-3' and reverse 5'-GATCCTTCTCACGTCTCTCACA-3'.

Real-time Quantitative-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen), and total RNA was reverse transcribed using random primers (Roche Diagnostics) and M-MLV reverse transcriptase (Promega). PCR and fluorescence detection were done using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

For quantification of *HIC1* mRNA, Taqman Gene Expression Assay Hs00359611_s1 (Applied Biosystems) or SYBR Green and the following primers fw 5'-CGACGACTACAA-GAGCAGCAGC-3' and rev 5'-CAGGTTGTCAACGAAGCTCTC-3' were used. *Cyclin E1* mRNA was quantified using Taqman Gene Expression Assay Hs01026536_m1 (Applied Biosystems) or SYBR Green and the following primers: fw 5'-TGCAGAGCTGTTG-GATCTCTGTG-3' and rev 5'-GGCCGAAGCAGCAAGTA-TACC-3'. For the housekeeping gene *GAPDH*, we used the following primers and probe: 5'-GAAGGTGAAGGTCG-GAGT-3' and 5'-GAAGATGGTATGGGGATTT-3' at 900 nmol/L and 5'-FAM-CAAGCTTCCCGTCTCAGCC-TAM-RA-3' at 200 nmol/L. Primers and probe for the housekeeping genes *ABL* and *HMBS* have been described (10). All measurements were done in duplicates, and the arithmetic mean of the Ct-values was used for calculations: target gene mean Ct-values were normalized to the respective housekeeping gene, and then to the experimental control. Obtained values were exponentiated $2^{-\Delta\Delta Ct}$ to be expressed as n-fold changes in regulation compared with the experimental control (35).

Bisulfite Genomic Sequencing

Genomic DNA was extracted and bisulfite converted using the Methylamp™ DNA modification kit (Epigentek). Primers for PCR amplification, cloning, and sequencing have been described elsewhere (36).

Lentiviral and Adenoviral Vectors

pLKO.1-puro lentiviral vectors expressing shRNAs targeting E2F1 (shE2F1_1: NM_005225.1-502s1c1 and shE2F1_2: NM_005225.1-948s1c1) and the nontargeting control shRNA vector (SHC002) were purchased from Sigma-Aldrich. These vectors contain a puromycin antibiotic resistance gene for selection of transduced mammalian cells. Lentivirus production and transduction were done as described (10, 37). AdCMV-E2F1 and AdCMV-Luc vectors were used as described (25).

Western Blot Analysis

Immunoblotting and protein extraction have been described previously (38, 39). Anti-E2F1 (sc-251; Santa Cruz Biotechnology) was used as primary and donkey anti-rabbit horseradish peroxidase-conjugated IgG (Amersham) as secondary antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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