

E2F1 represses β -catenin/TCF activity by direct up-regulation of Siah1

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Abstract

Transcription factor E2F1 is a key regulator of cell proliferation and apoptosis. Its activity is strictly controlled by the pRB/E2F pathway. In the majority of cancer cells, however, this pathway is frequently found deregulated, and the underlying mechanism involving transcriptional control by E2F1 has not yet been fully elucidated. Here we report the identification of two putative E2F1-binding sites located upstream from Siah1 transcription start site (+1). Chromatin immunoprecipitation assay reveals that transcription factor E2F1 is capable of binding to the putative sites, and luciferase reporter assay shows that E2F1 can activate transcription from the Siah1 promoter. Ectopic expression of E2F1 elevates the Siah1 level, hence suppressing the β -catenin/TCF activity. Consistently, knock-down of endogenous E2F1 by a shRNA strategy results in reduced expression of Siah1. Moreover, repression of β -catenin/TCF activity by E2F1 can be attenuated by shRNA-based repression of endogenous Siah1, implying that Siah1 is a *bona fide* E2F1 target gene, which at least partly, mediates the suppression of β -catenin/TCF signalling pathway.

Keywords: E2F1 • Siah1 • transcription • β -catenin/TCF

Introduction

The E2F1, a member of the E2F transcription factor family, is a regulator of cell proliferation and apoptosis and was first identified as a factor bound to the promoter of adenovirus E2 gene. E2Fs control the cell cycle by regulating the expression of a number of genes, whose products are required for the S-phase entry and cell cycle progression [1]. Most of the E2F family proteins can associate with and are regulated by pocket protein family proteins in a cell cycle progression-dependent manner. Among eight E2F family members identified so far, E2F1 is best characterized. It strongly promotes cell cycle progression by regulating critical regulator genes involved in the DNA replication and G1/S transition. In addition, numerous studies have suggested that ectopic expression of E2F1 induces apoptosis through different

mechanisms [2–4]. Many important genes involved in apoptosis have been identified as E2F1 targets and in addition, E2F1 has been reported to induce apoptosis by stabilizing and activating p53 activity. Moreover, E2F1-deficient mice exhibit a defect in thymocyte apoptosis and an increasing susceptibility to the development of tumours [5, 6], which implies the ability of E2F1 to suppress tumorigenesis under physiological circumstances.

Seven in absentia homologue 1 (Siah-1), a member of the RING-finger-containing E3 ubiquitin ligases, is a highly related mammalian homologue of *Drosophila* Sina, which targets the transcriptional repressor Tramtrack for ubiquitination and proteasome-mediated degradation [7, 8]. Like their *Drosophila* homologue, mammalian Siah proteins can bind E2 conjugating enzyme (E2s) via an amino-terminal ring domain and promotes the targets for proteasomal degradation [9, 10]. Siah has two human homologues, Siah1 and Siah2, which share significant amino acid sequence homology. They have been implicated in the ubiquitination and proteasome-dependent degradation for many important protein targets such as OBF1 [11, 12], Kid [13], DCC [14], Bag-1 [15], NcoR [16], Numb [17] and α -synuclein [18]. Recently, Siah1 was reported to potentiate the proteasomal degradation of β -catenin through Siah-1 interacting protein (SIP)/Skp1/Ebi [19] or APC pathway [20], and then inhibits cell proliferation by repressing

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the activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors.

Here we show that E2F1 can repress TCF/LEF activity by directly transactivating the expression of Siah1. This pathway suggests a novel effect of E2F1 on suppressing cell cycle progression *via* a E2F1-Siah1- β -catenin/TCF pathway.

Materials and methods

Reagents and antibodies

The following antibodies were used in this study: Siah1 goat polyclonal antibody P-18 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); E2F1 rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Inc.); β -actin mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.); the majority of reagents used for this study were purchased from Sigma-Aldrich, Inc (St Louis, MO, USA). Restriction enzymes were ordered from New England Biolabs (Beverly, MA, USA).

Plasmids

The UCSC Genome Browser Database and the Genomatix Suite of sequence analysis tools MatInspector (professional version 6.2.2) were used for analysing the Siah1 promoter. Siah1 promoter constructs were described previously [21]. A primer pair used for pSiah-BgIII was: 5' > GAA GAT CTC TAG CTT CTC GCG GGC CAG AG <3' and 5' > CCA AGC TTC TCG AGA GCG CGC CCC GCA <3'. The promoter fragments were sub-cloned into the XhoI/HindIII sites of pGL3-Basic plasmid (Promega Corporation, Madison, WI, USA). The primers used for site-specific deletion of E2F-binding sites are: 5' > CGC CTG GCG GCG CCC CGC CCC AGG CTC <3' and 5' > GGG GGG GCG GGG GCG GCG CAG GCG CAC AGG CGT TG <3' for E2F-BS1; 5' > GGC GGG CTC GAG GAC CGG CGG CGG CGG CGG CCA GGG <3' and 5' > CCC TGG CCG CCG CCG CCG CCG GTC CTC GAG CCC GCC GCG C <3' for E2F-BS2. The detailed procedure of PCR-mediated mutagenesis, E2F expression plasmids and luciferase reporter plasmid pApaf1-Luc were described previously [22, 23]. pU6-E2F1 shRNA and control vector were gifted by Dr. W. Douglas Cress [24]; pSuper-Siah1 shRNA and control vector were kindly provided by Dr. Solomon H. Snyder (Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA) [25].

Cell culture, transfection and RNAi

Human non-small cell lung carcinoma cells H1299 were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate. Culture medium was purchased from Invitrogen Corporation (GIBCO, Grand Island, NY, USA). Cells were maintained at 37°C in a humidified 5% CO₂-containing atmosphere. Transfection of cells with various mammalian expression constructs by LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) was performed according to the methods provided by manufacturer's specification. For RNAi assay, H1299 cells were transfected with appropriate shRNA or control vector and cultured for 48 hrs before cells were harvested to examine the effectiveness of RNA interference.

Semi-quantitative RT-PCR and Western blot analysis

For semi-quantitative RT-PCR, H1299 cells were first transfected with E2F1 expression plasmid or empty vector and were cultured for additional 24 hrs. Total RNA was then isolated using SV Total RNA Isolation System (Promega Corporation) and 0.1 μ g RNA from each preparation was used as PCR template. RT-PCR was performed by TaKaRa One-step RNA PCR Kit (AMV) (TaKaRa Bio, Inc., Otsu, Shiga, Japan) using the following specific primer pairs: Siah-1: 5' > GC TCC ATT CGC AAC TTG GCT ATG and Siah-2: 5' > GC TCA ACA CAT GGA AAT AGT TAC <3'; β -actin: 5' > GAC CTG ACT GAC TAC CTC ATG AAG AT-3' and 5' > GTC ACA CTT CAT GAT GGA GTT AAG G -3'; Western blotting was performed according to the protocol described previously [22]. Protein concentration in each sample was measured by BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described previously [23]. Briefly, H1299 cells (2×10^7 cells) were fixed with 1% formaldehyde and then neutralized by adding 0.125 M glycine. Cells were collected and lysed in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP40, protease inhibitor cocktail). Nuclei pellet was obtained by centrifuge and subsequently lysed in Nuclei lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% SDS, protease inhibitor cocktail). The nuclei lysate was sonicated to obtain soluble chromatin with average length of 1000 bp. After 1:10 dilution by dilution buffer (10 mM Tris-Cl, pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100), chromatin solutions were pre-cleared and then incubated with or without anti-E2F1 antibody (sc-193X, Santa Cruz Biotechnology, Inc.) and the mixture was incubated at 4°C overnight on a rotating platform. The same immunocomplexes were recovered with protein A-Sepharose beads. After extensive washing, the bound DNA fragments were eluted, and the resulting DNAs were subjected to PCR reactions using the following primer pair: 5' > CGC TCG AGC AGC AAC GGT AGC CGA GTA G <3' and 5' > GCA GAT CTT GGC CGC CGC CGC CGC CGT TTC GC <3'. PCR products were separated by gel electrophoresis on 2% agarose gel and visualized.

FACS cell cycle analysis

H1299 cells were harvested and washed with phosphate buffered saline (PBS). After brief centrifugation ($200 \times g$), cell were collected and fixed overnight in 70% ethanol at 4°. Then cells were collected by centrifugation for 5 min. at $200 \times g$ and then incubated in ice-cold PBS for 30 min. After incubation, cells were collected, washed and then resuspended in PBS containing 5 mg/ml propidium iodide and 50 μ g/ml RNase A. The cells were incubated at room temperature for 20 min., and then applied to measure fluorescence intensity using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Dual-luciferase reporter assays

H1299 cells were seeded into 12-well plates (5×10^5 cells per well) and cultured for 20 hrs in RPMI-1640 medium plus 10% FBS before transfection. E2F1 expression plasmid (50–200 ng) or empty vector was individually cotransfected into H1299 cells, together with appropriate Siah1

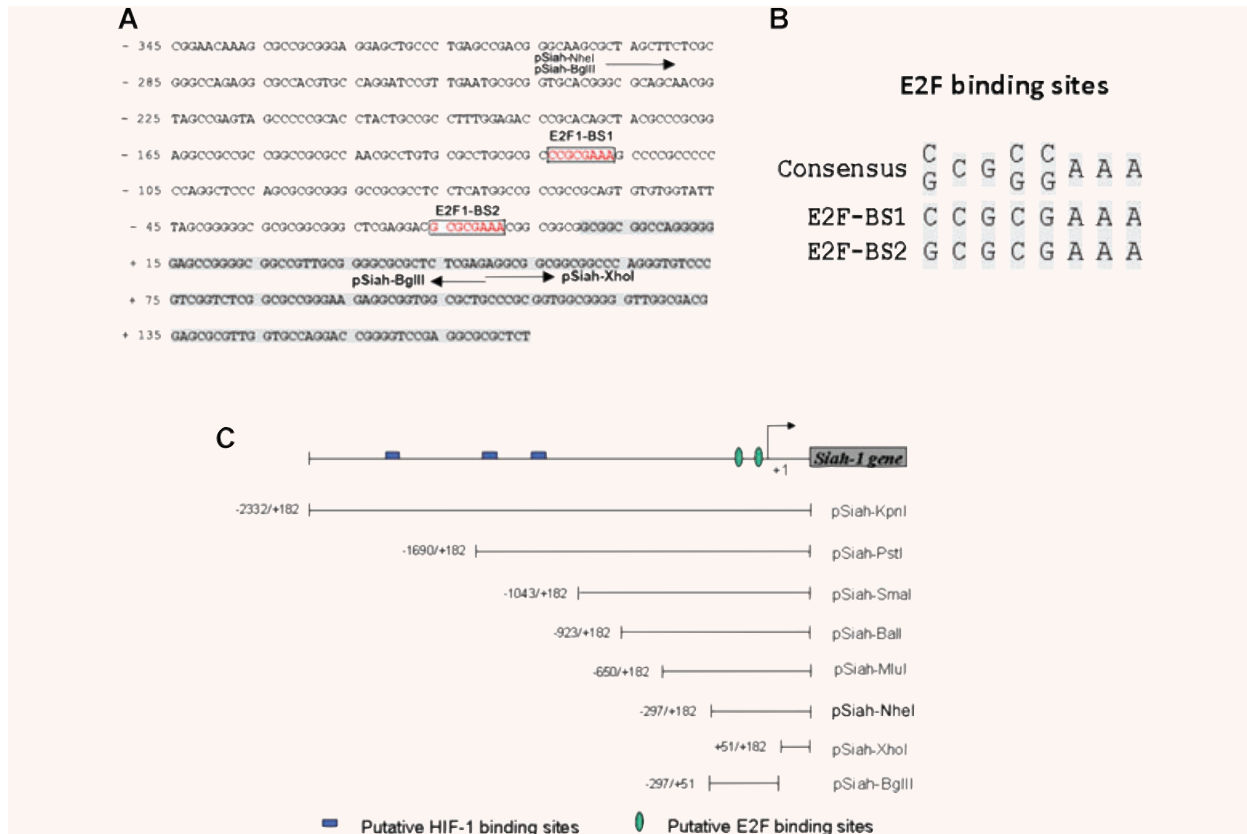


Fig. 1 Putative E2F1-binding sites are located in *Siah1* promoter (**A**) Human genomic sequence of *Siah1* gene from -345 to +174 is shown. Shaded part represents the first exon of *Siah1*. The boxed are the predicted E2F1-binding sites. The arrow under the DNA sequence represents the start/end position of some indicated promoters. (**B**) Alignment of the putative E2F-binding sites with the consensus sequence. (**C**) Schematic representation of various *Siah1* promoter deletion constructs. Putative E2F-binding sites are indicated. The constructs harbouring different fragment of *Siah1* promoter were cloned into pGL3-Basic reporter vector (Promega Corporation).

promoter reporter plasmids (200 ng) using Lipofectamine™ 2000 (Invitrogen). In each transfection, cells were also cotransfected with *Renilla* luciferase reporter plasmid. For TCF activity assay, equal amounts of pTop-flash reporter plasmid (100 ng) and pRL-CMV (5 ng) were cotransfected into each cell sample. Firefly and *Renilla* luciferase activity were assayed using Dual-Luciferase Reporter Assay System according to manufacturer's instructions (Promega Corporation). Fold-activation values were measured relative to the levels of luciferase activity in cells transfected with empty vectors and normalized by *Renilla* luciferase activities.

Results

Identification of E2F1-binding sites at *Siah1* gene locus

To explore how *Siah1* gene is regulated in p53-independent manners, which may reflect the real situation for most cancer types

with null or mutated p53, We used MatInspector (professional version 6.2.2), which is designed to inspect possible transcription-factor-binding sites in a given sequence, to scan the genomic sequence upstream of *Siah1* coding region. Two putative E2F-binding sites (BS) were found within the near upstream region, one (BS1) is at -124 bp to -117 bp, the other (BS2) is at -15 bp to -8 bp upstream of *Siah1* transcription initiation site, respectively (Fig. 1A). Sequence alignment between the consensus E2F1-binding site (C/G)CG(C/G)(C/G)AAA and the two predicted E2F1-binding sites shows very high identity with each other (Fig. 1B).

E2F1 activates *Siah1* promoter

To examine whether *Siah1* promoter could be activated by E2F1, luciferase reporter assays were performed with a series of *Siah1* promoter deletion mutants. The schematic representation of the constructs used in this assay is shown in Fig. 1C. A p53-null cell

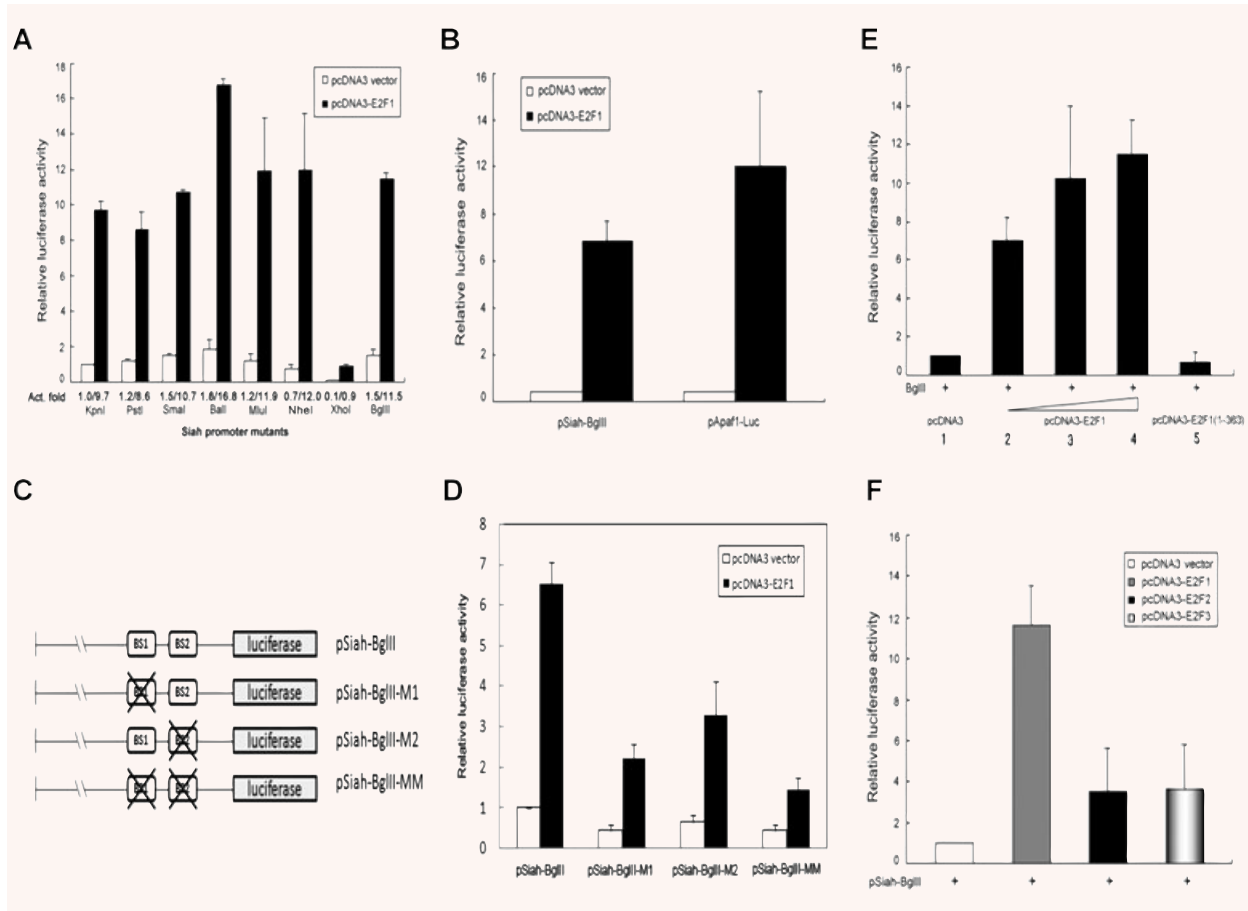


Fig. 2 Activation of *Siah1* promoter by E2F1. **(A)** Determination of minimal promoter region of *Siah1* that can be activated by E2F1. H1299 cells were individually cotransfected with equal amount (100 ng) of eight indicated *Siah1* promoter deletion constructs of Luc-reporter, together with either pcDNA3 empty vector (200 ng) or E2F1 expressing vector pcDNA3-E2F1 (200 ng). *Renilla* luciferase plasmid pRL-CMV was also introduced into each transfected cell samples as internal control. Cells were harvested 24 hrs after transfection and luciferase activities were assayed using the dual luciferase assay system. Fold activation values were measured relative to the levels of *Renilla* luciferase activity and were listed under their respective bars. **(B)** pSiah-BgIII is sufficient to activate *Siah1* promoter activity in response to E2F1. H1299 cells transfected with either reporter plasmids pSiah-BgIII (100 ng) or pApaf-Luc (pGL3-Apaf-1[−396/+208])(100 ng) were further cotransfected in combination with pcDNA3 expression vectors (200 ng) or pcDNA3-E2F1 (200 ng). *Renilla* luciferase plasmid pRL-CMV was also introduced into each transfected cells as internal control. Fold activation values were measured relative to the levels of *Renilla* activity. **(C)** Schematic diagrams of E2F1 deletion mutants of *Siah1* promoter. **(D)** H1299 cells were transfected with either reporter plasmids pSiah-BgIII (100ng) or one of E2F1 deletion mutants (pSiah-BgIII-M1, pSiah-BgIII-M2 and pSiah-BgIII-MM, 100 ng) along with pcDNA3 expression vectors (200 ng) or pcDNA3-E2F1 (200 ng). *Renilla* luciferase plasmid pRL-CMV was also introduced into each transfected cells as internal control. Fold activation values were measured relative to the levels of *Renilla* activity. **(E)** Activation of *Siah1* promoter by E2F1 is dose-dependent. H1299 cells were cotransfected with pSiah-BgIII (200 ng), together with either pcDNA3 vector (200 ng), pcDNA3-E2F1(1–363) (200 ng, an E2F1 deletion mutant) or increasing concentrations of pcDNA3-E2F1 (from 50 ng, 100 ng to 200 ng) separately. *Renilla* luciferase plasmid pRL-CMV was introduced into each cotransfection cell sample as internal control. Luciferase activity was measured and plotted. **(F)** *Siah1* promoter specifically responds to E2F1. H1299 cells were cotransfected with reporter plasmid pSiah-BgIII (200 ng), together with one of the indicated activator plasmids: pcDNA3, pcDNA3-E2F1, pcDNA3-E2F2 and pcDNA3-E2F3 at equal amount. *Renilla* luciferase plasmid pRL-CMV was introduced into each cotransfected cell samples as internal control. Luciferase activity was measured and plotted after normalizing with respect to *Renilla* luciferase activity. In **(A)–(F)**, all cells were harvested 24 hrs after transfection and Luciferase and *Renilla* activity assayed using the Dual-Luciferase Reporter Assay System (Promega Corporation). Luciferase values were corrected for transfection efficiency with *Renilla* activity. All experiments were independently performed in triplicate. Vertical error bars are the standard deviations from the mean of the values within three standard deviations.

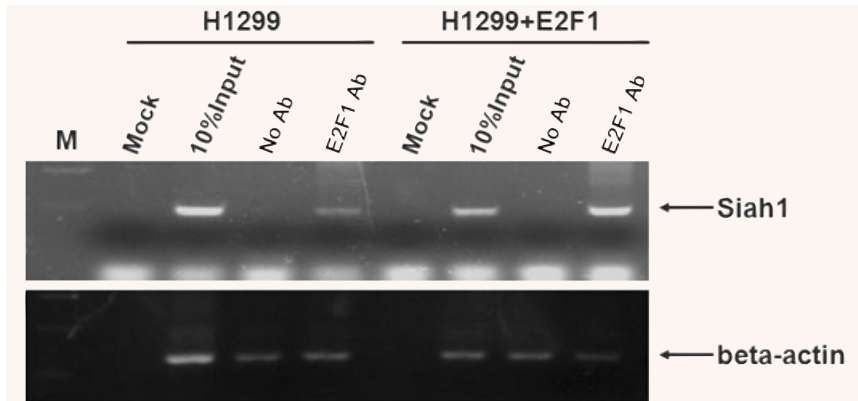


Fig. 3 E2F1 binds to *Siah1* promoter *in vivo*. H1299 cells were transfected with or without pcDNA-HA-E2F1 expression vector. Twenty-four hours after transfection, cells were harvested and subjected to ChIP assay. ChIP products were amplified by PCR reaction. β -actin was used as a negative control. M: DNA marker; Mock: control IP without nuclear lysate; No Ab: IP without E2F1 antibody; E2F1 Ab: IP with E2F1 antibody. For the right four lanes, cells were expressing with pcDNA3-E2F1.

line H1299 was employed to exclude the unanticipated influence directly or indirectly due to p53. E2F1 expression plasmid and empty vector were transfected into H1299 cells together with each *Siah1* promoter reporter plasmid indicated in Fig. 2A, respectively. Equal concentrations of *Renilla* reporter plasmid pRL-CMV were also cotransfected as an internal control. Luciferase activity was measured and the relative activation fold of each *Siah1* promoter normalized to the pRL-CMV internal standard was presented in the histogram with arbitrary units. As shown in Fig. 2A, all *Siah1* promoter fragments containing the two putative E2F1-binding sites displayed significant activation fold under ectopic E2F1 expression. Moreover, as the *Siah1* promoter undergoes continuous deduction from 5' to 3', the responsiveness to E2F1 did not display significant reduction until the two putative E2F1-binding sites has been deleted (lane: XhoI), suggesting that the *Siah1* promoter region pSiah-BgIII (-297 - +51) may represent the *Siah1* minimal response element to E2F1 (lane: BgIII). Then we compared the responsiveness of the pSiah-BgIII to pApaf1-Luc (-396/+208), one of the known E2F1-responsive promoters described elsewhere [22]. As shown in Fig. 2B, two promoters exhibited similar patterns and comparable fold increases upon E2F1 activation.

Because there exist putative-binding motifs of other transcriptional factors in this minimal E2F1-responsive promoter, we generated three mutant promoter constructs with deletion of either E2F1-binding site (pSiah-BgIII-M1 and pSiah-BgIII-M2) or double deletion of E2F1-binding sites (E2F1-BS1 and E2F1-BS2) to examine the importance of the two putative E2F-binding sites. The schematic representations are presented in Fig. 2C. As shown in Fig. 2D, the deletion of either or both E2F1-binding sites significantly impaired the responsiveness of *Siah1* promoter to E2F1, which demonstrates that both E2F1-binding sites are crucial for the activation of *Siah1* promoter.

Next, H1299 cells were cotransfected with pSiah-BgIII, together with either pcDNA3 empty vector, pcDNA3-E2F1 (1-363) (an E2F1 transactivation domain-deficient variant) [26] or increasing amount of pcDNA3-E2F1 (50-200 ng). As shown in Fig. 2E, increasing amount of wild-type E2F1 resulted in correspondingly

augmented activation fold of pSiah-BgIII (lanes 2, 3 and 4), whereas the mutated E2F1 (lane 5) or the empty vector (lane 1) failed to do so. To examine whether the responsiveness of *Siah1* promoter is limited to E2F1, E2F family members E2F2 and E2F3 were tested along with E2F1 under the same condition. As shown in Fig. 2F, E2F2 and E2F3 exhibited much less activation than E2F1, suggesting that *Siah1* promoter responds specifically and significantly to E2F1.

E2F1 binds to *Siah1* promoter *in vivo*

To determine if endogenous E2F1 protein directly interacts with *Siah1* promoter, we performed the ChIP assay, which allows the detection of proteins bound to specific regions of DNA *in vivo*. We designed a pair of primers flanking *Siah1* promoter region -293 ~ +51bp harbouring the two putative E2F1-binding sites. H1299 cells were transfected with or without pcDNA-HA-E2F1 prior to ChIP assay. As shown in Fig. 3, the promoter regions were found enriched by antibody against E2F1 in H1299 cells both transfected or untransfected with pcDNA-HA-E2F1, (the upper panel, lanes 4 and 8). The enrichment was not detected after the amplification of an unrelated genomic DNA fragment β -actin (the lower panel, lane 3 *versus* lane 4; lane 7 *versus* lane 8). This result implied that E2F1 could interact with *Siah1* promoter directly *in vivo*. Taken together, our results demonstrated that E2F1 indeed interacts with and activates *Siah1* promoter.

E2F1 induces *Siah1* expression

To examine whether E2F1 could regulate expression of *Siah1*, H1299 cells were starved in 0.5% serum for 48 hrs, which allows the cells to be synchronized at G0/G1-phase and keeps endogenous E2F1 activity at a low level. Starved H1299 cells were then transfected with E2F1 expression plasmid or empty vector. Twenty-four hours after transfection, cells were harvested and subject to Western analysis using antibodies specific to E2F1,

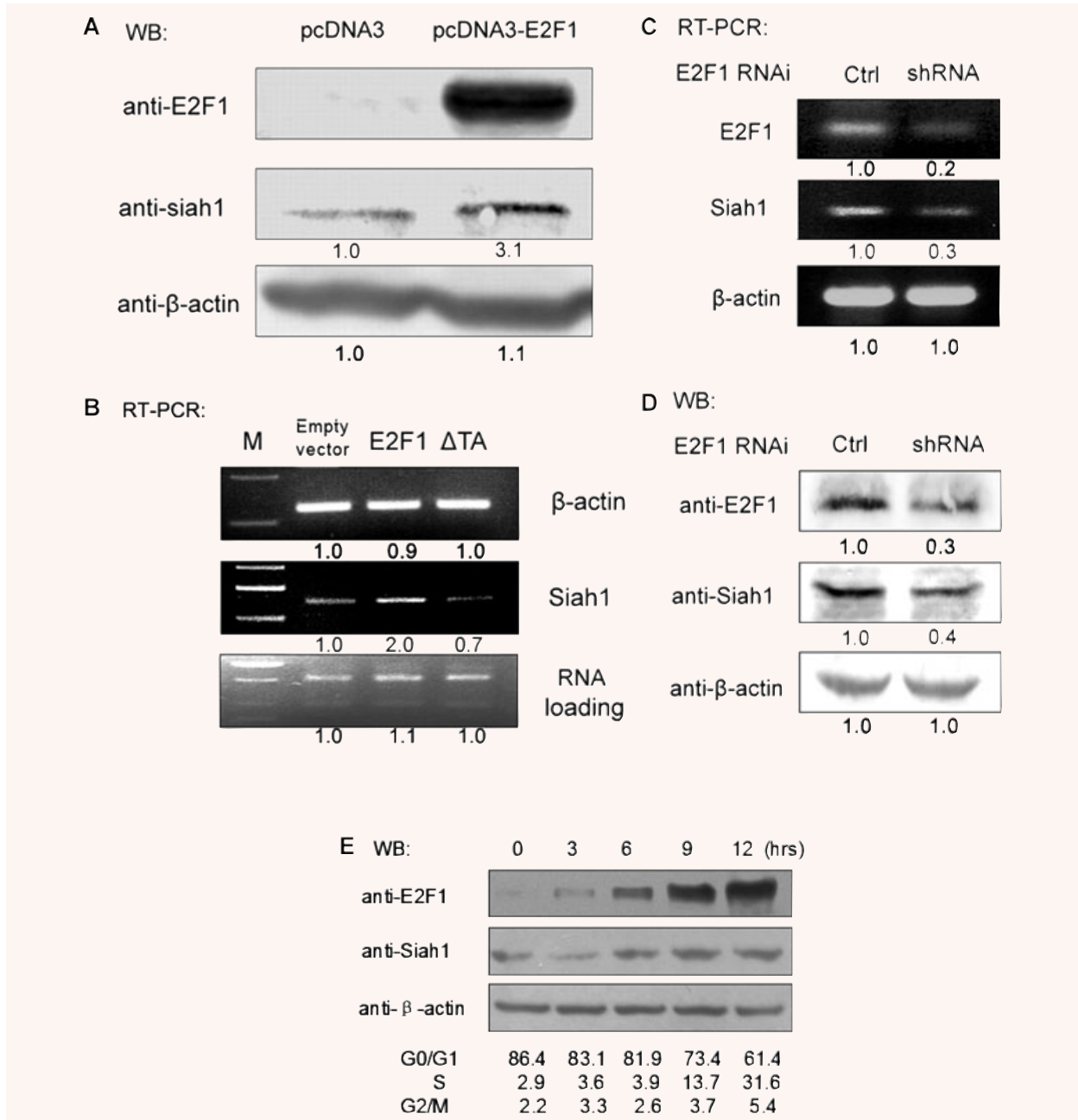


Fig. 4 E2F1 up-regulates the expression of endogenous Siah1. **(A)** H1299 cells were incubated in medium containing 0.5% serum for 48 hrs. Treated cells were then transfected with either pcDNA3 (200 ng) or pcDNA3-E2F1 (200 ng). Thirty-six hours after transfection, cells were harvested and subjected to Western analysis with indicated antibodies. **(B)** H1299 cells were treated under same conditions as described in **(A)**. Thirty-six hours after transfection, cells were harvested for RNA isolation and equal amounts of total RNA were reverse transcribed and amplified. PCR amplification of β-actin was performed simultaneously as negative control. Total RNA used for each PCR reaction was shown as loading control (bottom panel). **(C)** H1299 cells were transfected with 1 μg of E2F1 shRNA construct pU6-E2F1 or pU6 empty vector. Forty-eight hours after transfection, cells were harvested for preparation of total RNA and followed by semi-quantitative RT-PCR analysis with primer pairs specific to E2F1, Siah1 and β-actin. **(D)** H1299 cells were treated the same way as described in **(C)** and then subject to Western analysis using antibodies against E2F1, Siah1 and β-actin, respectively. **(E)** H1299 cells were incubated in medium containing 0.5% serum for 48 hrs and then released in medium containing 20% serum. Cells were harvested at the indicated time-points and applied to Western blots and FACS analysis.

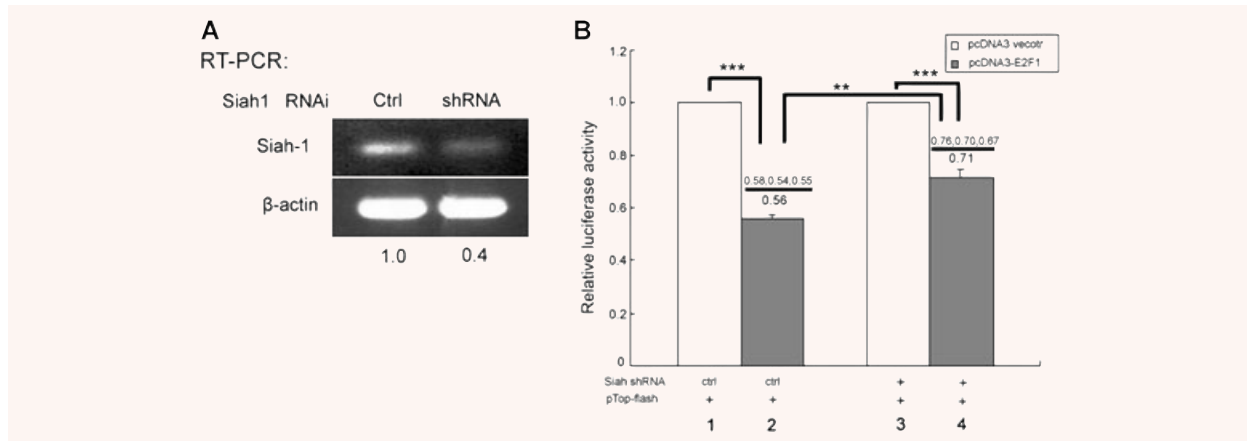


Fig. 5 Repression of *Siah1* expression attenuates E2F1-repressed β -catenin/TCF activity. **(A)** H1299 cells were transfected with gene-specific shRNA construct pSuper-Siah1 (1 μ g) or pSuper empty vector (1 μ g). Forty-eight hours after transfection, cells were harvested for preparation of total RNA and semi-quantitative RT-PCR analysis with primer pairs specific to *Siah1* and β -actin. Actin (control) was then performed. **(B)** H1299 cells were transfected with either *Siah1* shRNA construct pSuper-Siah1 (200 ng) or pSuper empty vector (200 ng). Forty-eight hours after transfection, 200 ng of pTop-flash and 5 ng of pRL-CMV were further introduced into each transfected cells. An additional 24 hrs later, cells were harvested for measuring dual luciferase activity as described in Fig. 2. Luciferase values were corrected for transfection efficiency with *Renilla* activity. All the experiments were independently performed in triplicate. The data of triplicate experiments and their average were indicated in the figure. Error bars indicate S.D. (standard deviation). *** $P < 0.001$; ** $P < 0.01$.

Siah1 and β -actin, respectively. As shown in Fig. 4A, the *Siah1* level was up-regulated in pcDNA-E2F1-transfected cells compared to that in empty vector-transfected cells.

To confirm the Western analysis results (Fig. 4A), total RNA was prepared from cells pre-treated under the same conditions as described above. This time we included a sample of cells expressing E2F1deltaTA, an E2F1 dominant negative construct deficient for the C-terminal transactivation (TA) domain. Equal amounts of total RNA were used to perform semi-quantitative RT-PCR with primer pairs specific to *Siah1* and β -actin (control). As shown in Fig. 4B, up-regulation of *Siah1* mRNA level in H1299 expressing ectopic E2F1 was observed (lane 2). As expected, cells expressing either E2F1deltaTA (lane 3) or empty vector (lane 1) did not show significant elevation of *Siah1* expression.

To further validate that the endogenous E2F1 regulates *Siah1* expression, H1299 cells were transfected with either E2F1 shRNA or empty control construct. After 48 hrs, cells were harvested for semi-quantitative RT-PCR and Western analysis. As shown in Fig. 4, repression of endogenous E2F1 effectively reduced both the endogenous *Siah1* mRNA (Fig. 4C) and protein level (Fig. 4D). Taken together, we demonstrated that E2F1 is able to transcriptionally up-regulate the expression of *Siah1*.

To examine the relationship between *Siah1* expression and cell cycle progression, we starved H1299 cells in medium containing 0.5% serum for 48 hrs and then released in medium containing 20% serum. Cells were harvested at different time-points and applied to FACS cell cycle analysis and Western blots using antibodies against E2F1 and *Siah1*. As shown in Fig. 4E, under 20% serum stimulation, synchronized cells re-entered cell cycle as

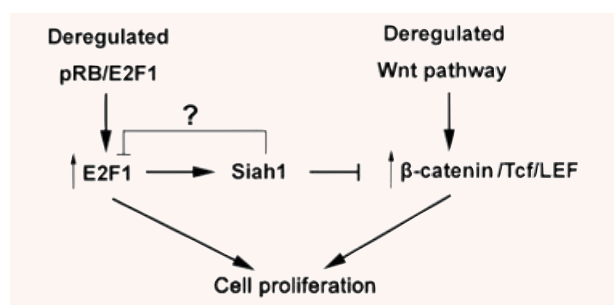


Fig. 6 Hypothetical model for illustrating the proposed pathway through which E2F1 represses β -catenin/TCF activity by elevating the level of *Siah1*.

demonstrated by the fact that more than 30% of cells entered S-phase after 12 hrs. Correspondingly, levels of *Siah1* were clearly up-regulated along with the increase in E2F1, indicating that the expression of *Siah1* was cell cycle-dependent and *Siah1* is likely to be regulated by E2F1.

Siah1 mediates the repression of β -catenin/TCF activity by E2F1

Siah1 was reported to mediate the proteasomal degradation of β -catenin and accordingly represses the activation of downstream transcription factor TCF/LEF. We then examined whether E2F1 could interfere with the TCF/LEF activity through *Siah1*.

H1299 cells were transfected with either pSuper-Siah1 shRNA or pSuper empty vector. Forty-eight hours after transfection, both shRNA and mock treated cells were harvested for preparation of total RNA and analysed by semi-quantitative RT-PCR to examine Siah1 expression. As shown in Fig. 5A, the pSuper-Siah1 shRNA significantly repressed the expression of the endogenous Siah1 at its mRNA level. To evaluate the effect of E2F1 on β -catenin/TCF activity by elimination of Siah1, H1299 cells were transfected with either pSuper-Siah1 shRNA or pSuper empty vector. Forty-eight hours later, shRNA- or mock-treated cells were further cotransfected with E2F1 expression vector or control vector, together with equal amounts of pRL-CMV and pTop-flash plasmid, which contains the wild-type binding site of TCF that serves as a reporter for endogenous TCF Activity. An additional 24 hrs later, cells were harvested to measure the luciferase activity. The relative luciferase activity of pTop-flash promoter normalized to the pRL-CMV internal standard was presented in the histogram with arbitrary units (Fig. 5B). Comparing the two columns both transfected with pSuper vector (control), the TCF activity was obviously repressed by ectopically expressed E2F1 (lane 1 *versus* lane 2). From the two columns both transfected with E2F1 expression vector, the suppressed TCF activity by ectopic E2F1 expression was significantly derepressed through the repression of Siah1 (lane 2 [0.56] *versus* lane 4 [0.71]). Taken together, these results demonstrated that Siah1 mediated the repression of TCF activity by E2F1.

Discussion

In this study, we found two highly conserved E2F1-binding sites existing in the promoter region of Siah1 gene. E2F1 was found to bind to and activate Siah1 promoter directly. Semi-quantitative RT-PCR and Western analysis demonstrated that ectopic E2F1 expression up-regulated endogenous Siah1 at both the mRNA and protein levels. Repression of endogenous E2F1 by E2F1-specific shRNA led to a reduction of Siah1 expression. Finally we found that although ectopic E2F1 expression could suppress the activity of β -catenin/TCF, this suppression, however, could be attenuated by knocking-down of endogenous Siah1. Based on these results, we conclude that E2F1 represses the β -catenin/TCF activity by transcriptionally up-regulating the expression of Siah1.

E2F1 is a key transcription factor that effectively drives cell cycle progression from G0/G1 into S-phase. Many E2F1 target genes are known to be involved in cell cycle progression and DNA replication [1]. In normal cells, activity of E2F1 is strictly controlled by pRB/E2F1 pathway and kept at a relatively low level [27–30]; however, in most tumour cells, pRB/E2F1 pathway is usually disrupted at multiple points and leads to unrestrained activity of E2F1, which keeps the cells in a proliferative state and is thought to be one of the important reason for the carcinogenic effects. Recently cumulative evidence suggests that ectopic expression of E2F1 could lead to cell apoptosis and knockout of endogenous E2F1

makes mice vulnerable to tumorigenesis [3, 5, 6]. Therefore, in healthy cells, E2F1 activity must be kept at a low level. E2F1 was known to be ubiquitinated by an E3 ligase complex Skp, Cullin, F-box containing complex (SCF complex)^{skp2} and degraded *via* the ubiquitin-mediated proteasome pathway [31]. Our finding in this study demonstrated that E2F1 could elevate Siah1 level. The latter was reported to degrade β -catenin *via* a proteasome-mediated proteolysis by forming a complex with SIP and SCF^{skp2} [19]. Thus, one needs to test a hypothesis whether there exists a negative feedback loop involving E2F1-up-regulated Siah1, by forming a ubiquitination complex with SIP/SCF^{skp2}, which in turn causes E2F1 degradation to maintain a relatively lower level of E2F1 within the cell. Furthermore, β -catenin/TCF is implicated in the downstream of Wnt signalling pathway, and its deregulation is thought to be related to tumorigenesis in many cancers [32, 33]. Our finding also suggests a novel tumour suppression function of E2F1 in Wnt signalling pathway, in which E2F1 up-regulates Siah1 expression to restrict the β -catenin/TCF activity. A proposed model is shown in Fig. 6, in which Siah1 serves as the mediator between pRB/E2F1 and Wnt/ β -catenin pathways. A recent study also reported that E2F1 can up-regulate protein Axin2 to suppress Wnt/ β -catenin/TCF activity [34]. Whether there exists some other hierarchical regulation in these signalling pathways remains to be investigated.

Because hypoxia circumstances exist in most tumours, and under this condition, transcription factor HIF-1 α (hypoxia inducible factor-1 α) is stabilized for the inhibition of oxygen-dependent degradation. The targets of HIF-1 α include the genes involved in cell cycle control, cell proliferation, metabolism and angiogenesis [35–40]. Recently, Siah1 has been reported to enhance HIF- α stability by promoting the degradation of two E3 ligases (FIH and PHD1/3) targeted for HIF- α [41, 42], implying that E2F1 may have promoting effect on HIF- α *via* un-regulated Siah1; meanwhile, there is a report that β -catenin could associate with HIF-1 α to potentiate its stabilization and transcriptional activity [43], this suggests that E2F1 may have an inhibitory effect on HIF-1- α *via* suppressing β -catenin/TCF/LEF activity. In summary, our finding that E2F1 can suppress β -catenin/TCF/LEF activity *via* up-regulation of Siah1 may shed new light on the relationship between two important transcription factors E2F1 and HIF-1 α .

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