Novel link between E2F1 and Smac/DIABLO: proapoptotic Smac/DIABLO is transcriptionally upregulated by E2F1

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ABSTRACT

Deregulated expression of E2F1 not only promotes S-phase entry but also induces apoptosis. Although it has been well documented that E2F1 is able to induce p53-dependent apoptosis via raising ARF activity, the mechanism by which E2F induces p53-independent apoptosis remains unclear. Here we report that E2F1 can directly bind to and activate the promoter of Smac/DIABLO, a mitochondrial pro-apoptotic gene, through the E2F1-binding sites BS2 ($-542 \sim -535$ bp) and BS3 ($-200 \sim -193$ bp). BS2 and BS3 appear to be utilized in combination rather than singly by E2F1 in activation of Smac/DIABLO. Activation of BS2 and BS3 are E2F1-specific, since neither E2F2 nor E2F3 is able to activate BS2 or BS3. Using the H1299 ER-E2F1 cell line where E2F1 activity can be conditionally induced, E2F1 has been shown to upregulate the Smac/DIABLO expression at both mRNA and protein levels upon 4-hydroxytamoxifen treatment, resulting in an enhanced mitochondria-mediated apoptosis. Reversely, reducing the Smac/DIABLO expression by RNA interference significantly diminishes apoptosis induced by E2F1. These results may suggest a novel mechanism by which E2F1 promotes p53-independent apoptosis through directly regulating its downstream mitochondrial apoptosis-inducing factors, such as Smac/DIABLO.

INTRODUCTION

The E2F transcription factor family is the key regulators of cell proliferation, which were first described for their necessity by adenovirus E1A protein for transactivating the adenovirus E2 promoter (1). 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 (2). The E2F proteins themselves can be negatively regulated by the retinoblastoma tumor suppressor RB, which exhibits the growth suppression activity by interacting with E2Fs to shield their transactivation domain (3). Of the eight E2F proteins identified thus far, E2F1 is the best-characterized member. It promotes cell cycle by regulating critical regulator genes involved in the DNA replication and G_1/S transition (4). In addition, numerous studies have suggested that ectopic expression of E2F1 induces apoptosis by different mechanisms (5–13) and consistently, E2F1-deficient mice exhibit a defect in thymocyte apoptosis and an increasing susceptibility to the development of tumors (14,15). The E2F1-p14ARF-p53 cascade is the most important p53-dependent apoptotic pathway for E2F1. During this signaling, E2F1 upregulates p14ARF, which in turn stabilizes p53 and promotes p53-induced apoptosis by alleviating the proteosome degradation of p53 by Mdm2 (16,17). Recently, it has been shown that ARF directly binds to DP1 (a DNA-binding partner of E2Fs) to inhibit its transcriptional activity, which implies a novel negative feedback loop between ARF and E2F1 (18–20). In addition to the p53-dependent pathway, many genes involved in p53-independent apoptotic regulation have also been demonstrated as E2F1 targets (4), such as p73 (21,22), Apaf1 (23,24), caspase-3, -7, -8, -9 genes (25,26), BH3-only genes noxa, puma, bim (27) and akt (28).

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Smac (the second mitochondria-derived activator of caspase), also known as DIABLO (direct IAP-binding protein with low pI), is normally compartmentalized and stored in mitochondria after protein translation (29–32). Upon receiving apoptotic stimuli, Smac/DIABLO is released into cytosol, where it binds to IAPs and allows the activation of caspases by eradicating IAP's caspase-binding capability or enhancing the proteosome-mediated degradation of IAPs (33,34). Altered expression of Smac/DIABLO has been reported in some cancer cells, e.g. downregulation of Smac/DIABLO has been observed in renal cell carcinoma (35) and lung cancers (36), and Smac/DIABLO upregulation was detected in Folic acid-induced acute renal failure (37). However, the detailed molecular mechanism underlying regulation of Smac/DIABLO remains uncharacterized.

In this report, we present the first evidence that E2F1 can bind to the Smac/DIABLO promoter and transactivate its expression. Two putative E2F1-binding elements BS2 and BS3 were located respectively within the regions $-542/-535$ and $-200/-193$ relative to the transcriptional initiation site (+1) of Smac/Diablo gene were characterized. Transactivation of Smac/DIABLO promoter by E2F1 can achieve its maximal induction only when BS2 and BS3 are jointly utilized. Repression of Smac/DIABLO by RNA interference (RNAi) technique attenuates the E2F1-induced apoptosis, indicating Smac/DIABLO is positioned downstream of this E2F1-mediated apoptotic signaling pathway. Similarly, enhanced accumulation of nuclear E2F1 induced by 4-hydroxytamoxifen (4-OHT) upregulates Smac/DIABLO, resulting into an augmented mitochondria-mediated apoptosis. These data suggest that Smac/DIABLO may act as a target gene for E2F1 and contribute to a novel E2F1-induced apoptosis via p53-independent pathway.

MATERIALS AND METHODS

Reagents and antibodies

The following antibodies were used in this study: Smac/DIABLO rabbit polyclonal antibody (Calbiochem, LA Jolla, CA); E2F1 rabbit polyclonal antibody H-137 and C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); HA mouse monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA); Noxa mouse monoclonal antibody (Imgenex Corp., San Diego, CA); Cytochrome C mouse monoclonal antibody (R&D Systems Inc., Minneapolis, USA); β-actin goat polyclonal antibody (Santa Cruz Biotechnology); Rhodamine-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc). The majority of reagents used for this study including 4-OHT and Hoechst 33342 were purchased from Sigma-Aldrich, Inc. Restriction enzymes were ordered from New England Biolabs (Beverly, MA).

Plasmids construction and the isolation of the Smac/Diablo promoter

The UCSC Genome Browser Database, the Genomatix Suite of sequence analysis tools MatInspector (professional version 6.2.2) and PromoterInspector Release 1.0 were used for analyzing the Smac/Diablo promoter. Primer pairs flanking single or combinations of putative E2F-binding sites used for PCR were synthesized and listed below. Genomic DNA isolated from human lung carcinoma H1299 cells by Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) was used as a template for PCR to obtain a 1.5 kb ($-1000 \sim +500$) genomic fragment. Various promoter deletion fragments were obtained with primer pairs indicated below. Briefly, Pa and Pii are used for PCR amplification of P_{1234}, Pa and Pi for P_{123}, Pb and Pii for P_{234}, Pb and Pi for P_{23}, Pc and Pii for P_{34}, Pc and Pi for P_3, Pb and Piii for P_2, Pd and Pii for P_0. The promoter fragments were subcloned into the XhoI/HindIII sites of pGL3-Basic plasmid (Promega Corporation). Pa, 5′-CCG CTC GAG CAC AGA AGA GCA GGT TTG GGC CTG-3′; Pb, 5′-CCG CTC GAG CCG TCC GCC CCT CTG GGA CGG CGC-3′; Pc, 5′-CCG CTC GAG AGC TCC GCG CCG GAC CCG CCT-3′; Pd, 5′-CCG CTC GAG GTA CCG CTG CGG CCG CGT-3′; Pi, 5′-CGC AAG CTT AGC AGT CGG GAT TGG GCA GGC-3′; Pii, 5′-CGC AAG CTT CTG CAG CGC TAG GTA GAG AGC-3′; Piii, 5′-CGC AAG CTT TGA GTC GGG CGC CGT GAC-3′.

The E2F genes are kindly gifted from Dr Stefan Gaubatz and Dr Doron Ginsberg and subcloned into pcDNA3 or pEGFP-C1 vector by standard techniques. The plasmids pGL3-Apaf1 ($-396/+208$) (23) and pBabeHAERE2F1 (38) are kind gifts from Dr Kristian Helin.

Cell culture and transfection

Human H1299 nonsmall lung carcinoma cells 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, and a culture of HeLa cervical carcinoma cells was grown in DMEM supplemented with 10% (v/v) FBS, 1× nonessential amino acid, 100 μg/ml penicillin, 1× MEM sodium pyruvate, 100 μg/ml streptomycin. Culture medium was purchased from Invitrogen Corporation (Gibco, Grand Island, NY). Cells were maintained at 37°C in a humidified 5% CO_2-containing atmosphere. Tranfection of cells with various mammalian expression constructs by Lipofectamine™ 2000 (Invitrogen, Carsbad, CA) was performed according to the methods provided by manufacturer's specification. Transfection of Smac/DIABLO siRNA oligo (sc-36505; Santa Cruz) and irrelevant control siRNA oligo (sc-37007; Santa Cruz) was carried out by Oligofectamine™ reagent (Invitrogen).

ER-E2F1-expressing H1299 cell lines were derived from H1299 cells as described previously (23). Briefly, H1299 cells were infected by retroviruses produced in Phoenix cells transfected with pBabeHAER-E2F1. Infected H1299 cells were selected by puromycin (2 μg/ml) for 10 days. Expression of ER-E2F1 proteins in the selected cells was confirmed by western blotting with E2F1-specific antibodies C-20 (Santa Cruz) and HA-specific antibodies (Cell Signaling Technology, Inc.), respectively. Immunofluorescence with specific anti-E2F1 antibodies (H-137; Santa Cruz) in absence or presence of OHT (1 μM) was carried out to examine the translocation of the ER-E2F1 from the cytoplasm into the nucleus and hence their activation.

Semiquantitative RT–PCR

For semiquantitative RT–PCR, H1299 cells stably expressing ER-E2F1 were first incubated in RPMI-1640 medium

containing 0.1% FBS for 48 h and then induced for desired length of time with 1 μM 4-OHT. Total RNA was isolated from the 4-OHT treated H1299 cells by SV Total RNA Isolation System (Promega Corporation) and 0.1 μg RNA of each sample was used as template. RT–PCR was performed by TaKaRa One-step RNA PCR Kit (AMV) (TaKaRa Bio Inc., Japan) using the following specific primer pairs: Smac/DIABLO, 5′-ATG GCG GCT CTG AAG AGT TGG CTG

and 5′-TCA ATC CTC AAC GCA GGT AGG TCC-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′; Cyclin A, 5′-ATG AGA CCC TGC ATT GGG CTG-3′ and 5′-TTG AGG TAG GTC TGG TGA AGG-3′.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described previously (27,39). Briefly, HeLa cells (5×10^6 cells) were fixed with formaldehyde and then sonicated to obtain soluble chromatin. After dilution, the chromatin solutions were incubated with and without anti-E2F1 antibody (sc-193X; Santa Cruz) and placed on a rotating platform at 4°C overnight. Immunocomplexes were recovered with preblocked protein A–Sepharose beads. After extensive washing, the bound DNA fragments were eluted. The resulting DNAs were subjected to PCRs using primer sets denoted in the Figure 1a and the detailed sequences are listed as follows: P1u, 5′-GAG CAG GTT TGG GCC TGT GCC TTC-3′ and P1d, 5′-AAC TGC CCT CGT TCT TCG GCT CTG-3′ for BS1; P2u, 5′-GCT GTT GGG GAG GTC GGC ACT GTG-3′ and P2d, 5′-GCC CCG ATG AGC ACC GTG TAG CTG-3′ for BS2; P3u, 5′-TTC CCT TCA AGC CCT GGC CCG AAC-3′ and P3d, 5′-ACG CCC CCA CCC AAG GAA GCA GTC-3′ for BS3; P4u, 5′-TCC TTG GGT GGG GGC GTG GCT ATG-3′ and P4d, 5′-CGT CGG TCC CTC CCT CTG GTC CTG-3′ for BS4. PCR products were separated by gel electrophoresis on a 2% agarose gel and visualized.

Assessment of apoptosis by Annexin V staining

Annexin V-FITC Apoptosis Detection kit (PharMingen, San Diego, CA) was used in this assay. Cells were harvested and resuspended in 100 μl binding buffer (0.01 M HEPES/NaOH, pH7.4; 0.14 mM NaCl, 2.5 mM $CaCl_2$) at the concentration of 1×10^6 cells/ml. After incubation with 5 μl of Annexin V-FITC and 10 μl of PI (50 μg/ml) at room temperature for 15 min in the dark, cells were analyzed by flow cytometry FACS Calibar using the Cell Quest software system (Becton Dickinson, San Diego, USA) (40). The percentages of apoptosis of the cells were calculated by data from FACS analysis and the result (% Apoptosis) is presented in the bar graph relative to the apoptosis in cells without treatment with small interfering RNA of Smac, which is depicted as 100%.
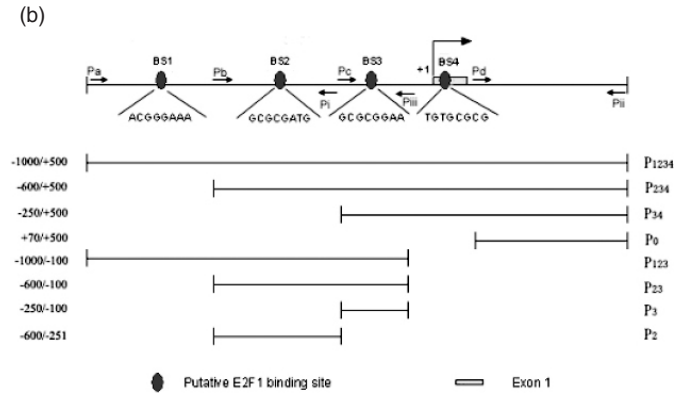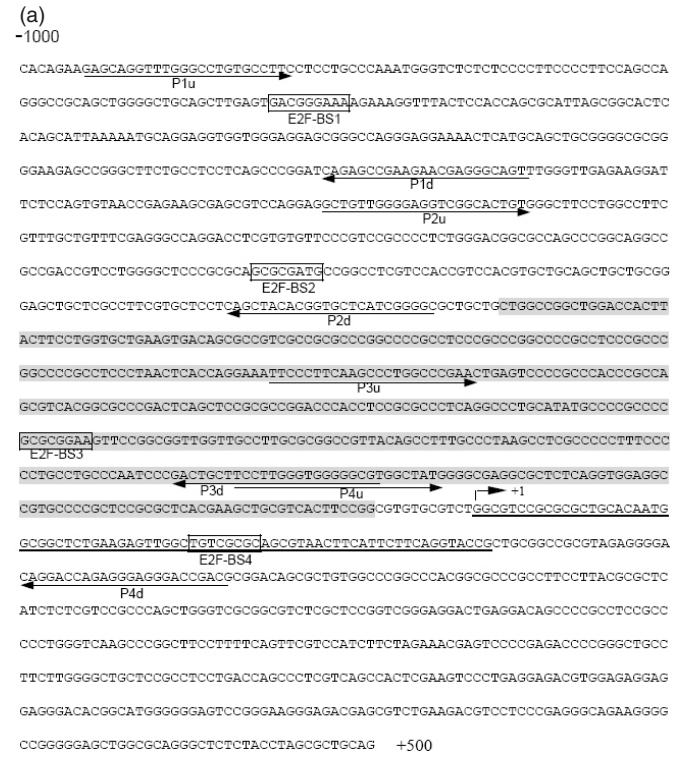

Figure 1. Putative E2F1-binding sites are located in *Smac/Diablo* genomic locus. (**a**) Human genomic sequence of *Smac/Diablo* gene from −1000 to +500 upstream is shown. The first exon is underlined and the transcription initiation site (+1) is indicated. The boxed are the putative E2F1-binding sites and the shaded region is the predicted Smac/Diablo promoter. The arrow represents the primers used in ChIP assay, its length demarked the numbers of bases for primer and its orientation is 5′–3′. P1u, primer 1 upstream, P1d, primer 1 downstream, same as the rest of the primers. (**b**) Schematic representation of various Smac/Diablo promoter deletion constructs. Putative E2F-binding sites are indicated. The Promoter constructs (P) harboring various E2F-binding sites were cloned into pGL3-Basic reporter vector (Promega). Primers used to subclone all these deletion constructs are indicated. The subscript numbers in letter P represent the number of E2F1-binding sites included in each construct. (**c**) Alignment of the putative E2F-binding sites with the consensus sequence. In the consensus sequence, S can be C or G. All the E2F1-binding sites (BS) are in 5′ to 3′, the plus indicates the reading is in the downstream direction relative to Smac/Diablo ORF, and (minus) indicates the use of complimentary stand.

Immunofluorescence and western blot analysis

ER-E2F1-expressing H1299 cells were grown on coverslips, and treated with 1 μM 4-OHT for 24 h. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, and blocked with 1% BSA in PBS for 30 min. Afterward, cells were incubated with E2F1 rabbit polyclonal antibody (H-137; Santa Cruz) at 4°C overnight. Rhodamine-conjugated anti-rabbit IgG secondary antibody was used to detect E2F1. Hoechst 33342 (5 μg/ml) was used to stain cell nucleus for 5 min. The cells were visualized under fluorescence microscope using standard red and blue filter sets. Images were
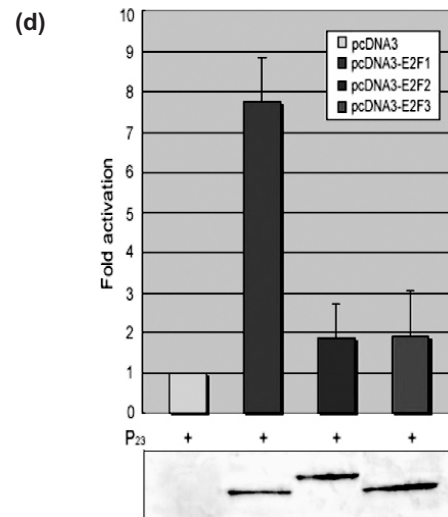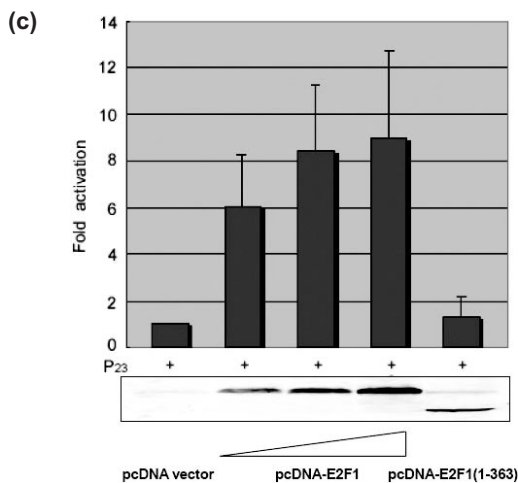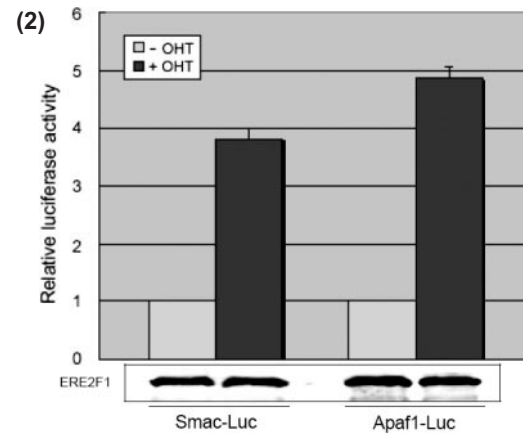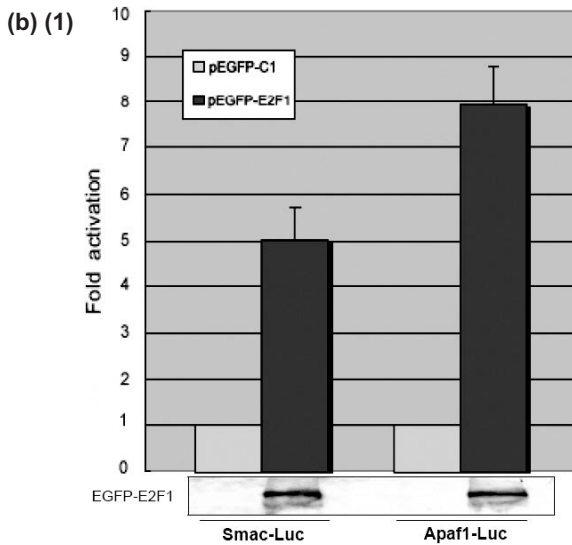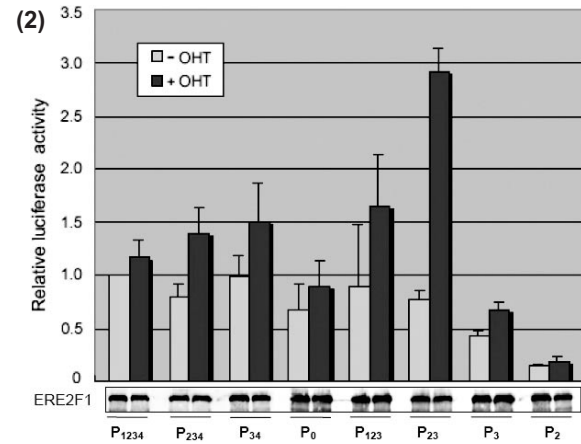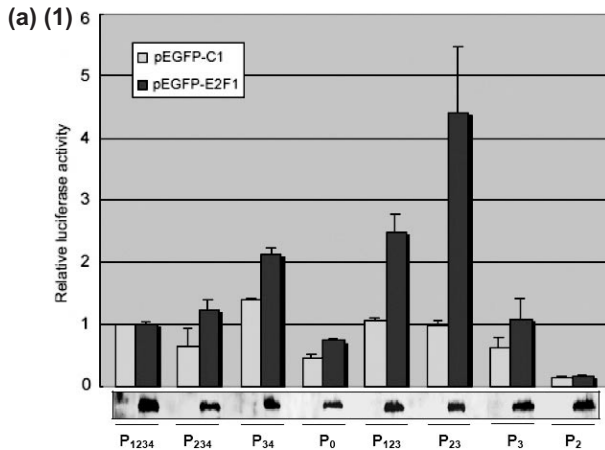
captured with a Zeiss Axioskop2 plus fluorescence microscope (magnification 400×) controlled by image processing software named *in situ* imaging system (ISIS). Western blotting was performed according to the protocol described by Song *et al.* (40).

Dual-luciferase reporter assays

H1299 cells were seeded into 12-well (5×10^5 cells per well) plates and cultured for 20 h in RPMI-1640 medium plus 10% FBS before transfection. H1299 cells were transiently cotransfected with appropriate Smac/Diablo luciferase reporter plasmids (200 ng) and either empty vector or E2F1 transactivator vector ($50 \sim 200$ ng) using Lipofectamine™ 2000 (Invitrogen). In each transfection, cells were also cotransfected with *Renilla* luciferase reporter plasmid. Firefly and *Renilla* luciferase activity were assayed using Dual-Luciferase Reporter Assay System according to manufacturer's instructions (Promega). 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 *Smac/DIABLO* gene locus

To explore whether potential E2F1-binding elements residing in the genomic locus of Smac/Diablo promoter region exist, we employed computer softwares PromoterInspector Release 1.0 and MatInspector (professional version 6.2.2), of which the former was used to inspect potential promoter regions and the latter for possible transcription factor-binding sites. Four putative E2F1-binding sites (BS) were found within the region from 1000 to +500 bp upstream of transcription initiation site of *Smac/Diablo* gene (Figure 1a). Among the four putative E2F1-binding sites, only BS3 is accurately located within the predicted *Smac/Diablo* promoter region (highlighted with gray shading, $-435 \sim -12$ bp), whereas BS2 is next to BS3 and BS1 is further upstream. It is interesting to note that BS4 located downstream of BS3 is found to be within

the first exon of *Smac/Diablo* gene (Figure 1b). This is not unusual, E2F1-binding site for *Cyclin A*, *myc* and the *hox3D* genes, for example, are also reported to be located within its exon (25,41,42). The E2F-binding consensus sequence is 5′-TTTSSCGC-3′ or 5′-GCGSSAAA-3′, where S can be C or G. Except for BS1, which contains a T instead of C at its 3′ end, the remaining three putative E2F1-binding sites exhibit high homology to consensus E2F1-binding sequence (Figure 1c).

E2F1 activates Smac/DIABLO promoter

To verify whether these potential E2F1-binding sites truly associate with E2F1 and how effective do they work in transactivating Smac/DIABLO expression. A series of Smac/DIABLO promoter deletion mutants were constructed as shown in Figure 1b. A p53-null cell line H1299 was chosen for transfection in order to exclude the unanticipated influence directly or indirectly from p53. An equal amount of reporter plasmids containing different E2F1-binding sites indicated in Figure 2a was individually cotransfected into H1299 cells with the same amount of either empty pEGFP-C1 or pEGFP-E2F1 transactivator plasmid. Equal concentrations of *Renilla* reporter plasmid pRL-CMV were also cointroduced into cells as an internal control. Luciferase activities were measured and pGL3-Smac-P-Luc reporter activities normalized to the pRL-CMV internal standard were presented in the histogram with arbitrary units. As shown in Figure 2a (panel 1), the Smac/Diablo promoter fragment P_{23} ($-600/-100$) harboring the BS2 and BS3 showed the strongest response to E2F1. Removal of either binding site P_2 or P_3 drastically reduced the activity of promoter, indicating that P_{23} ($-600/-100$) may be the minimal promoter of Smac in response to E2F1 and maximal transactivation of Smac/Diablo may require the combined use of BS2 and BS3. Similarly, by adding the BS1 or BS4 on P_{23}, the yielding P_{123} and P_{234} both exhibit significantly reduced activity compared with P_{23}. To validate this conclusion in a more physiological setting, we also compared the responsiveness of various constructs to ER-E2F1 activation in H1299ER-E2F1 cells, where ERE2F1 can be conditionally induced upon 4-OHT

Figure 2. Activation of *Smac/Diablo* promoter by E2F1. (**a**) Panel 1: determination of active E2F1-binding sites in *Smac/Diablo* promoter region. H1299 cells were individually cotransfected with equal amount (100 ng) of eight luciferase reporter deletion constructs including pGL3-Smac-P_{1234}, pGL3-Smac-P_{234}, pGL3-Smac-P_{34}, pGL3-Smac-P_0, pGL3-Smac-P_{123}, pGL3-Smac-P_{23}, pGL3-Smac-P_3 and pGL3-Smac-P_2 and either empty vector pEGFP-C1 (200 ng) or E2F1 expressing vector pEGFP-E2F1 (200 ng). *Renilla* luciferase plasmid pRL-CMV was also introduced into cotransfected cells as internal control plasmid. Cells were harvested 24 h 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. (a) Panel 2: H1299 ER-E2F1 cells were transfected with eight luciferase reporter deletion constructs under the same conditions described in panel 1 of (a). Twenty hours later, 1 μM 4-OHT was added to incubate for another 24 h. Results from the dual luciferase assay were shown in panel 2 of (a). (**b**) Panel 1: P_{23} is sufficient to activate Smac/Diablo promoter activity in response to E2F1. H1299 cells were cotransfected with luciferase reporter plasmids pGL3-Smac-P_{23} (100 ng) or pGL3-Apaf-1($-396/+208$) (100 ng) and in combination with expression vectors pEGFP-C1 (200 ng) or pEGFP-E2F1 (200 ng). *Renilla* luciferase plasmid pRL-CMV was also introduced into cotransfected cells as internal control. Fold activation values were measured relative to the levels of *Renilla* activity. (b) Panel 2: H1299 ER-E2F1 cells were transfected with luciferase reporter plasmids pGL3-Smac-P_{23} (100 ng) and pGL3-Apaf-1($-396/+208$) (100 ng) separately. Twenty hours post-transfection, 1 μM 4-OHT was added, and cells were incubated for another 24 h. Results from the dual luciferase assay were shown in panel 2 of (b). (**c**) Activation of Smac/Diablo promoter by E2F1 is dose-dependent. H1299 cells were cotransfected with pGL3-Smac-P_{23} (200 μg) and either pcDNA3 vector (200 μg), pcDNA3-E2F1(1-363) (200 μg, an E2F1 deletion mutant) or increasing concentrations of pcDNA3-E2F1 separately. Increased amount of plasmid pcDNA-E2F1 from 50, 100 and 200 μg is indicated as the triangle pointing up. For each cotransfection, *Renilla* luciferase plasmid pRL-CMV was introduced into the cells as internal control plasmid. Luciferase activity was measured and plotted. (**d**) Smac/Diablo promoter specifically responds to E2F1. H1299 cells were cotransfected with reporter plasmid pGL3-Smac-P_{23} (200 μg) and equal concentration of activator plasmid pcDNA3, pcDNA3-E2F1, pcDNA3-E2F2 or pcDNA3-E2F3. Luciferase activity was measured and plotted after normalizing with respect to *Renilla* luciferase activity. *Renilla* luciferase plasmid pRL-CMV was introduced into each cotransfected cells as internal control plasmid. In (a–d), the western blottings indicating E2Fs expression levels are shown below their respective histogram plots of luciferase assay. Luciferase and *Renilla* activity assayed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase values were corrected for transfection efficiency with *Renilla* activity. All experiments were independently performed in triplicate. Vertical error bars are the SDs from the mean of the values within three SDs.

treatment. A similar activation pattern was observed in ER-E2F1 stably transfected H1299 cells, and the result was shown in Figure 2a (panel 2). These data imply that under *in vivo* situations, BS1 and BS4 may individually exhibit inhibitory effect on E2F1 activation, or alternatively, some of the putative binding sites are not bona fide E2F1-binding site. P_0, which does not contain any predicted E2F1-binding sites, was included in this study in order for excluding the possibility that the promoter region without E2F1 binding can respond to it.

To confirm whether Smac/Diablo promoter can be activated by E2F1, We compared the responsiveness of the most active promoter region P_{23} to that of the Apaf1 promoter Apaf1-Luc(−396/+208) described elsewhere (23). As shown in Figure 2b (panel 1), two promoters exhibit the similar pattern and comparable fold increase under E2F1 activation. This conclusion was further validated in H1299ER-E2F1 cells and the result was shown in Figure 2b (panel 2). This result further demonstrated that the Smac/Diablo was a bona fide E2F target gene. Next, H1299 cells were cotransfected with pGL3-Smac-P_{23} and either pcDNA3 empty vector, pcDNA3-E2F1(1-363) (an E2F1 transactivation domain-deficient variant) or increasing amounts of pcDNA3-E2F1 (from 50 to 200 ng). As shown in Figure 2c, increasing amount of wild-type E2F1 resulted in correspondingly augmented activation of Smac/Diablo P_{23}, whereas the mutant E2F1 or the empty vector failed to give rise to noticeable activation of P_{23}. To examine whether the responsiveness of Smac/Diablo promoter is limited to E2F1, the other two E2F family members E2F2 and E2F3 were tested along with E2F1 in the same condition. As shown in Figure 2d, E2F2 and E2F3 exhibited much less activation than E2F1, suggesting that Smac/Diablo promoter responds specifically and significantly to E2F1.

E2F1 binds to Smac/Diablo promoter *in vivo*

To determine if endogenous E2F1 protein directly interacts with Smac/Diablo promoter, we performed the ChIP assay, which allows the detection of the interaction between protein and specific region of DNA *in vivo*. We designed four pairs of oligonucleotide primers flanking four putative E2F1-binding sites as denoted in Figure 1a. HeLa cells were transfected with or without pcDNA-HA-E2F1 prior to ChIP assay, and as shown in Figure 3, in HeLa cells without transfection of pcDNA-HA-E2F1, only BS3 fragment can be enriched by antibody against E2F1 (the third panel, lane 4). However, in HeLa cells ectopicly expressing E2F1, besides the more enrichment of BS3, we also observed the relatively faint band of BS2 (the second upper panel, lane 8), and the similar enrichment was not detected after the amplification of an unrelated genomic DNA fragment β-actin (the lower panel, lanes 4 and 8). It may imply that (i) endogenous E2F1 binds to BS3 or weakly binds to BS2, and (ii) when E2F1 is upregulated or overexpressed, it binds to both BS2 and BS3 to result in a maximal activation of Smac/DIABLO. These results are in good agreement with data obtained from the above-mentioned reporter assay, in which P_{23} exhibits the highest activation of Smac/Diablo promoter upon E2F1 binding. Taken together, our results demonstrated the E2F1 interacts with Smac/Diablo promoter, mainly through BS2 and BS3 regions.
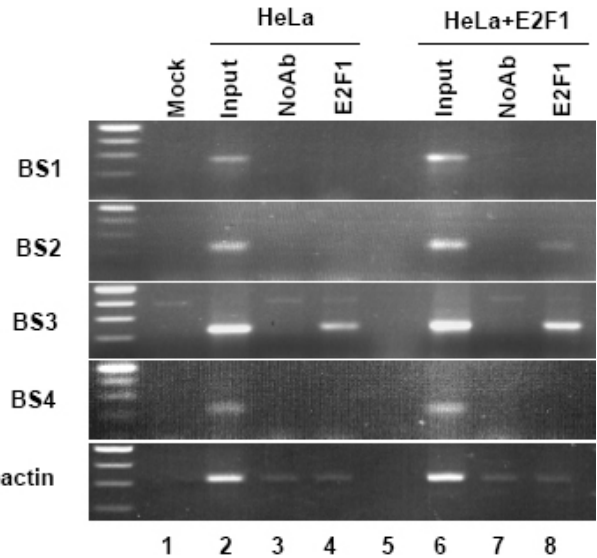

Figure 3. E2F1 binds to *Smac/Diablo* promoter *in vivo*. HeLa cells were transfected with or without pcDNA-HA-E2F1 expression vector and 24 h later, cell lysates were prepared and analyzed by ChIP in the presence and absence of E2F1 specific antibody (Santa Cruz). Products of ChIP and PCR amplification were analyzed by 2% agarose gel electrophoresis and β-actin was performed as a negative control. Primer pairs flanking indicated E2F1-binding sites used for PCR were indicated in Figure 1a.

E2F-1 induces Smac/DIABLO expression

To study the transcriptional regulation of Smac/DIABLO by E2F1, we constructed a stable H1299 cell line, which constitutively expresses an estrogen receptor-fused E2F1 protein ER-E2F1 (48). In this cell line, E2F1 is fused to a modified version of the estrogen receptor ligand-binding domain and thus unable to bind estrogen yet retains high affinity for 4-OHT and full transactivation activity. In the absence of OHT, the ER-E2F1 fusion protein is detained in the cytoplasm and remains transcriptionally inactive. Upon the addition of OHT, however, the fusion protein rapidly enters the nucleus and induces transcription of E2F1 target genes. As shown in Figure 4a, immunofluorescence of the stable H1299 cell line with anti-E2F1 antibody displayed an apparent nuclear translocation of ER-E2F1 in the presence of 4-OHT, demonstrating that the stable cell line we constructed was functionally validated. To test if Smac/DIABLO expression is regulated by E2F1, the stable H1299 cells were treated by 4-OHT for the indicated periods of time. Total RNA was prepared and semi-quantitative RT–PCR was performed using primer pair specific to gene Smac/Diablo. As shown in Figure 4b, 6 h after addition of 4-OHT, similar to positive control Cyclin A, which is known to be transcriptionally upregulated by E2F1 (40), Smac/DIABLO mRNA tended to increase and peaked at 8 h. As 4-OHT treatment time prolonged, the Smac/DIABLO mRNA level was kept relatively steady. Western blotting was also performed for validating the upregulation of Smac/DIABLO protein level. As shown in Figure 4c, Smac/DIABLO exhibited an apparent increase upon E2F1 activation. And Noxa, which is reported to be upregulated by E2F1 (27), was used as a positive control (Figure 4c, middle panel).
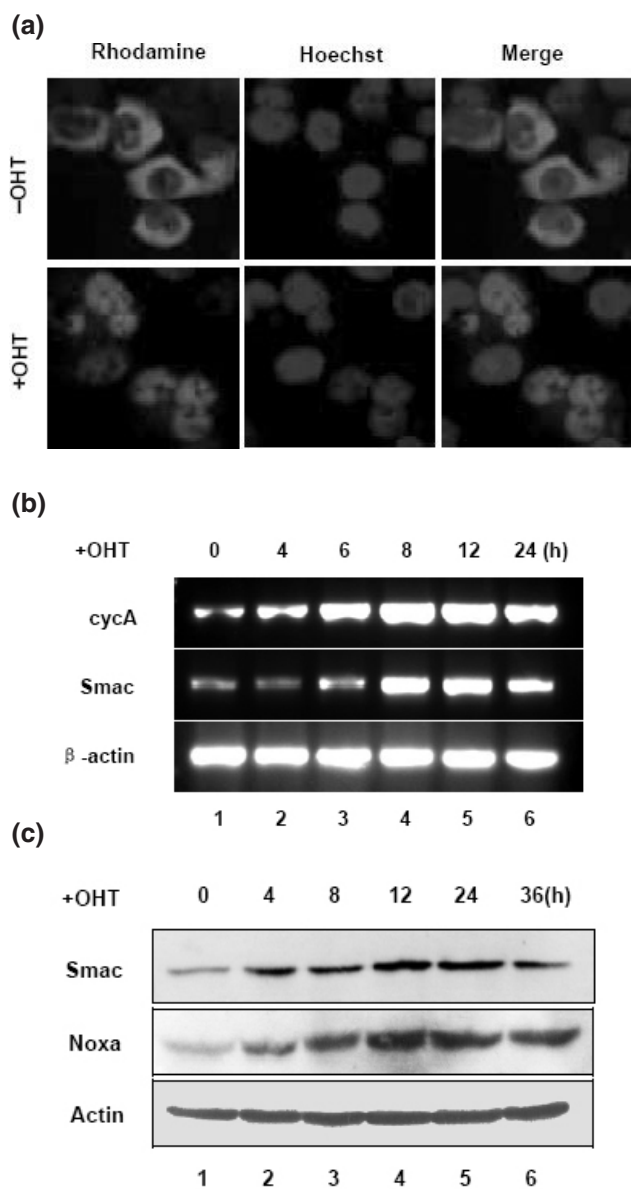
(a)

(b)

(c)

Figure 4. Activated E2F1 upregulates the expression of Smac/DIABLO. (**a**) Subcellular localization of ERE2F1. H1299 cells stably expressing ER-E2F1 fusion protein were examined by immunofluorescence staining using an E2F1 specific polyclonal antibody (H-137; Santa Cruz) in the absence (upper panels) or in the presence (lower panels) of 1 μM 4-OHT. The nuclei were stained with Hoechst 33342. (**b**) H1299 cells stably expressing ER-E2F1 were incubated in medium containing 0.1% serum for 48 h and then treated with 1 μM 4-OHT for the indicated periods of time. Total RNA was prepared from each treated samples and the equal amounts of RNA were employed in all the RT–PCR experiments. Smac/Diablo mRNA level was compared on 2% agarose gel. Cyclin A and β-actin mRNA levels were used as positive and negative controls, respectively. (**c**) H1299 cells stably expressing ER-E2F1 were treated similarly as in (b), and treated cells were harvested and lysed in RIPA buffer. An equal amount of total proteins from each time point indicated was employed for western blotting. The Smac/DIABLO protein levels were detected using a Smac-specific polyclonal antibody. Noxa and β-Actin protein levels were served as positive and negative controls, respectively.

E2F1-induced apoptosis is mediated by Smac/DIABLO

Large numbers of reports have demonstrated that unrestrained E2F1 could strongly induce apoptosis (13). To examine the
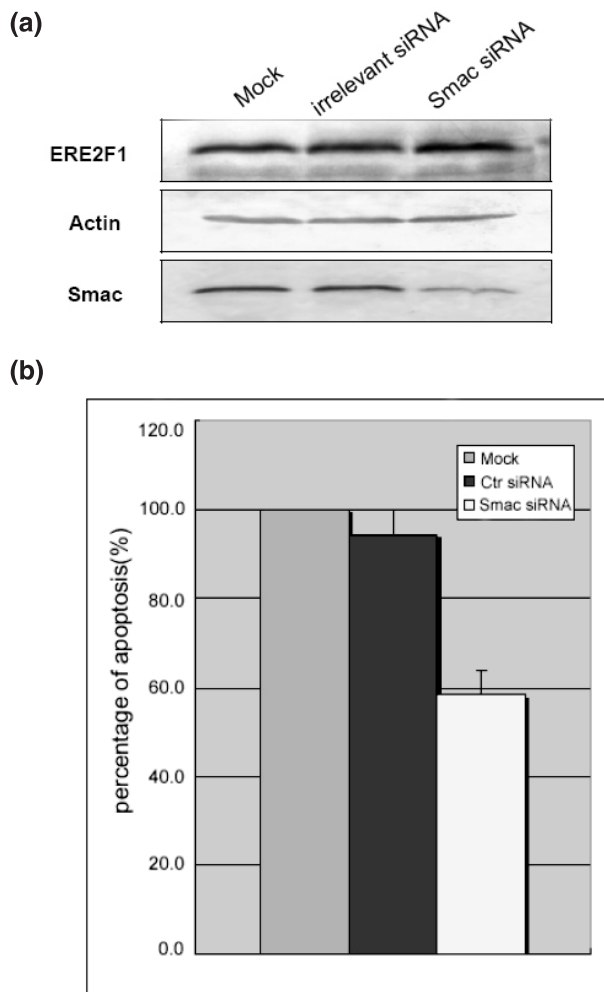
(a)

(b)

Figure 5. Repression of *Smac*/*DIABLO* expression attenuates E2F1-induced apoptosis. (**a**) H1299 cells stably expressing ERE2F1 were transfected with either Smac/DIABLO specific siRNA (sc-36505; Santa Cruz), irrelevant control siRNA (sc-37007; Santa Crutz) or Mock (water), and at 48 h post-transfection, cells were harvested for western analysis and for further FACS analysis to examine cell apoptosis. (**b**) H1299 cells stably expressing ERE2F1 were transfected with either Smac/DIABLO specific siRNA (sc-36505; Santa Cruz), irrelevant control siRNA (sc-37007; Santa Cruz) or Mock (water), and 48 h later, cells were added with 1 μM 4-OHT for another 24 h incubation. The percentages of apoptosis of the cells were examined by FACS analysis and the result (% Apoptosis) is presented in the bar graph relative to the apoptosis in the mock-transfected cells, which is depicted as 100%. All the experiments were independently performed in triplicate. Error bars indicate SD.

role of Smac/DIABLO in E2F1-induced apoptosis, we studied the effect of reducing Smac/DIABLO expression on E2F1-induced apoptosis. To achieve this, H1299 cell line stably expressing ER-E2F1 was transfected with either Smac/DIABLO specific siRNA, irrelevant control siRNA, or no siRNA (Mock). Cells were collected for assessing the degree of silencing of Smac/DIABLO expression by western analysis using anti-Smac/DIABLO antibody, 48 h post-transfection. As shown in Figure 5a, ~70% of Smac/DIABLO expression had been suppressed compared with controlled transfected cells. To examine whether the suppression of Smac/DIABLO affects E2F1-induced apoptosis, sample pairs were treated with or without equal concentration of 4-OHT (1 μM) 48 h

post transfection of siRNA. Cells were harvested and subjected for FACS analysis 24 h later. Comparing with the mock and the irrelevant control, suppression of Smac/DIABLO expression greatly attenuated the E2F1-induced apoptosis (Figure 5b). These data suggested that Smac/DIABLO is involved in the E2F1-induced apoptotic signaling pathway, and blocking Smac/DIABLO expression significantly diminishes E2F1-induced and Smac/DIABLO-involved apoptosis.

DISCUSSION

Smac/DIABLO was first identified as a proapoptotic factor for its release from mitochondria to cytosol upon apoptotic stimuli and initiating apoptosis by binding the IAP (Inhibitor of Apoptosis Proteins), thereby relieving the inhibition on caspases (29,30). Compartmentally stored in mitochondria in the absence of apoptotic stimuli can be thought to be a primary regulation of Smac/DIABLO protein level. Recent studies have demonstrated that Smac/DIABLO is also subject to polyubiquitination-mediated degradation through the interaction with IAPs (43), which could be thought as a secondary control of the cellular level of Smac/DIABLO. The data presented in this study suggest an additional control: Smac/DIABLO is transcriptionally upregulated by E2F1 transcription factor. However, the detailed mechanism underlying the transcriptional or translational regulation by E2F1 on Smac/DIABLO and how the balance of Smac/DIABLO is maintained in the physiological circumstances still remain to be elucidated.

E2F1 is best known for its role in regulating the timely expression of genes required for DNA replication and cell cycle progression (2). With growing number of novel target genes involved in E2F1-induced apoptosis identified, it has additionally been found that E2F1 exhibits functions in DNA damage response and apoptosis. Apoptosis-related E2F1 target genes are involved in both extrinsic and intrinsic pathways and are regulated by p53-dependent as well as p53-independent mechanisms (4,13,44). This information emphasizes its role as a key mediator of apoptosis and reminds us the complexities of E2F1 in inducing apoptosis. Pützer and coworker recently reported a novel mitochondrial protein DIP (Death Inducing Protein) as an E2F1 target gene (45) and not accidentally, the other mitochondrial proteins, such as cytochrome c and AIF (Apoptosis Inducing Protein) are also transcriptionally regulated by E2F1 (46,47). Data from present study added still more evidence for the assumption that E2F1 promotes p53-independent apoptosis may through activating downstream mitochondrial factor. We have also analyzed the promoter region of another proapoptotic mitochondrial protein Omi/HtrA2, and interestingly enough, highly conserved E2F1-binding sites were identified (W. Xie and M. Wu, unpublished data). Although data has not yet accumulated enough to conclude that the proapoptotic role of E2F1 for p53-independent apoptosis is through modulating mitochondrial apoptotic factors, yet it opens up the possibility for exploring the correlation between E2F1 and mitochondrial proapoptotic factors.

Examination of the Smac/DIABLO promoter region revealed four putative E2F1-binding sites with high homology to the consensus E2F1-binding sequence 5′-TTTSSCGC-3′, where S can be C or G. BS3 contains one C/T base substitution in TTT, whereas BS2 has C/T and A/T two substitutions, this may explain why BS3 exhibits stronger affinity than BS2. We noticed there is a base variation T/C in 3′ CGC of BS1, which could eliminate E2F1-binding affinity to that site. In luciferase and ChIP assays, BS2 and BS3 exhibit the evidential promoter activation and E2F1-binding activity, as a result of that, the responsiveness of Smac/Diablo promoter towards E2F1 appears to require the simultaneous binding to both sites. This is because neither BS2 nor BS3 alone is shown to confer sufficient contribution to activating Smac/DIABLO expression. Data from luciferase assay imply that BS1 and BS4 may display inhibitory effects on transactivation of Smac/DIABLO; however, we can not exclude the possibility that the inhibitory effect may come from a different sequence(s) near BS1 and BS4 and not from BS1 and BS4 *per se*. Data from ChIP assay revealed that neither BS1 nor BS4-binding site can be detected even in the presence of E2F1 overexpression. Based on these observations, we can conclude that BS1 and BS4 are unlikely to be the true E2F1-binding sites, despite their extensive sequence homology to E2F1 consensus binding sites. From ChIP assay, we demonstrated that BS3 has stronger affinity to endogenous E2F1 than BS2, which could be the underlying mechanism for differentially using these two binding sites and making cell-fate decisions: to enter cell cycle or to go for apoptosis. In healthy cells, E2F1 is at a physiological level and only BS3 is activated, thus the Smac/DIABLO is well below the threshold required to undergo apoptosis. Once E2F1 is unrestrainedly expressed (overexpression), both BS2 and BS3 are employed, resulting in an enhanced production of Smac/DIABLO. The detailed mechanism for cells to make a choice between life and death still await further investigation. Nonetheless, we propose a hypothetical model to illustrate the possible mechanism by which E2F1 activates Smac/DIABLO expression by differential binding to E2F1 responsive elements located at Smac/Diablo upstream regulatory region, and the detailed descriptions are outlined in Figure 6.

It is interesting to note that when the newly-released version of MatInspector (professional version 7.4) was used to analyze the Smac/Diablo promoter, a few uncharacterized putative E2F1-binding sites were disclosed, Most of which are located between BS2 and BS3 (data not shown), which may partially explain why the promoter region P_{23} containing BS2 and BS3 displays the highest activation by E2F1.

In summary, our data show that E2F1 can bind to the Smac/Diablo promoter, and thereby activate its expression at mRNA and protein levels. Using the H1299/ER-E2F1 stable cell line where E2F1 activity can be conditionally induced, we demonstrated that upon 4-OHT treatment, upregulated E2F1 results in an enhanced mitochondria-mediated apoptosis with an increased expression of Smac/DIABLO. Consistently, diminution of Smac/DIABLO expression by RNAi significantly attenuates apoptosis induced by E2F1. Our present data may add further evidence to a growing list of mitochondrial factors which are under E2F1 regulation. To gain a further understanding of the molecular mechanism by which E2F1 promotes p53-independent apoptosis could shed light on the gene therapy for tumors, of which majority are p53 defective.
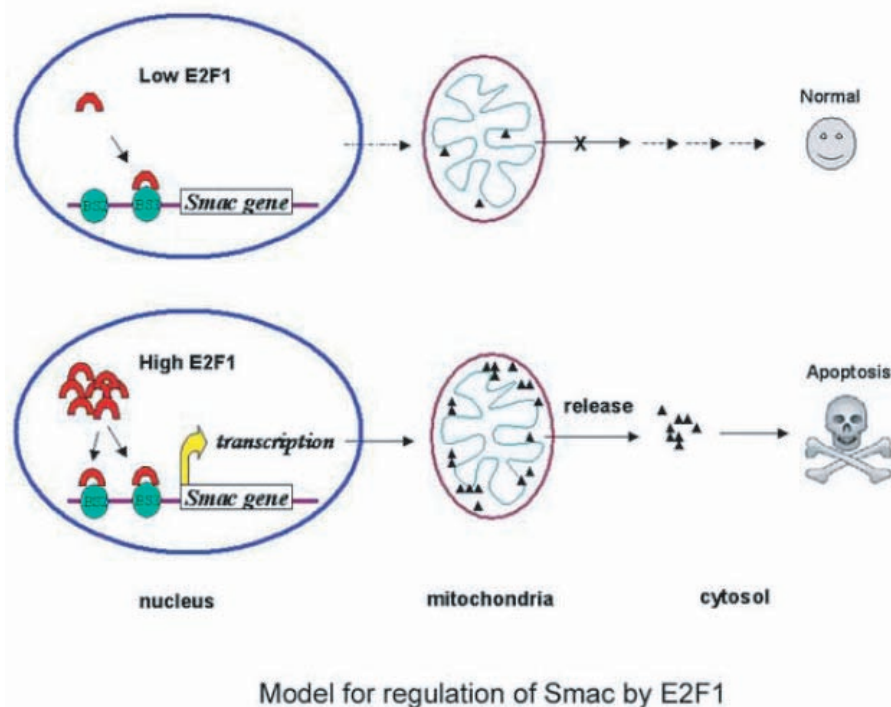
Figure 6. Hypothetical model for illustrating the proposed mechanism by which E2F1-induced apoptosis is differentially regulated. Blue circle represents nucleus and purple mitochondria; arch is symbolized as E2F1 and triangle as Smac/DIABLO. BS is simplified for E2F1-binding site. When E2F1 is present in low level, only BS3 is bound by E2F1. The minimal transctivation of Smac/DIABLO by E2F1resulted from binding to single BS3 is unable to produce enough Smac/DIABLO to induce apoptosis. When E2F1 is expressed at a high level, it binds to both BS2 and BS3, transactivation of Smac reaches its maximal activity and a great number of Smac/DIABLO molecules are produced and cells undergo apoptosis.

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