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Nuclear Matrix Protein SMAR1 Represses c-Fos-mediated HPV18 E6 Transcription through Alteration of Chromatin Histone Deacetylation*

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Background: HPV18 E6 oncogene represents one of the most promising therapeutic targets for the treatment of HPV-positive tumors.
Results: Curcumin-induced SMAR1-HDAC1 recruitment at *LCR* and *E6* region on *E6* promoter deacetylates chromatin histones to attenuate c-Fos-mediated *E6* transcription to reinstall p53-mediated apoptosis in HPV18-infected cervical cancer.
Conclusion: SMAR1 induces *E6* repression.
Significance: SMAR1 is a repressor of E6-mediated anti-apoptotic network in HPV18-infected cervical cancers.

Matrix attachment region (MAR)-binding proteins have been implicated in the transcriptional regulation of host as well as viral genes, but their precise role in HPV-infected cervical cancer remains unclear. Here we show that *HPV18* promoter contains consensus MAR element in the *LCR* and *E6* sequences where SMAR1 binds and reinforces HPV18 *E6* transcriptional silencing. In fact, curcumin-induced up-regulation of SMAR1 ensures recruitment of SMAR1-HDAC1 repressor complex at the *LCR* and *E6* MAR sequences, thereby decreasing histone acetylation at H3K9 and H3K18, leading to reorientation of the chromatin. As a consequence, c-Fos binding at the putative AP-1 sites on *E6* promoter is inhibited. E6 depletion interrupts degradation of E6-mediated p53 and lysine acetyl transferase, Tip60. Tip60, in turn, acetylates p53, thereby restoring p53-mediated transactivation of proapoptotic genes to ensure apoptosis. This hitherto unexplained function of SMAR1 signifies the potential of this unique scaffold matrix-associated region-binding protein as a critical regulator of E6-mediated anti-apoptotic network in HPV18-infected cervical adenocarcinoma. These results also justify the candidature of curcumin for the treatment of HPV18-infected cervical carcinoma.

Cellular pathogens and their hosts have co-evolved with mechanisms to counter and survive with each other. In most virus borne cancers, synergistic cellular changes must take place for malignant progression to occur. Development of cervical cancer, which is the second most common cause of cancer death in women worldwide (1), is highly associated with infection by high risk human papilloma viruses (HPVs)[2] (2). HPVs are nonenveloped DNA viruses that infect mucosal or cutaneous squamous epithelium and result in a global change in cellular gene expression that facilitates cellular hyperproliferation. Adenocarcinomas of the cervix account for ~10–30% of cervical carcinomas, and their incidence is increasing, especially among young women (3). Similar to cervical squamous cell carcinomas, the high risk HPV types 16 and 18 are the most important types associated with cervical adenocarcinomas (4). However, in contrast to cervical squamous cell carcinomas with prevalence of HPV16, a predominance of HPV18 infection has been described in cervical adenocarcinomas (5, 6), particularly in invasive ones.

The high risk HPV encodes two transforming genes: *E6* and *E7*, both of which interfere with the key elements in the cell cycle control machinery. The constitutive expression of *E6* and *E7* is mainly dependent on the availability of host cell transcription factor, activator protein-1 (AP-1) that is formed by either homodimerization of Jun proteins (c-Jun, JunB, and JunD) or heterodimerization of Jun and Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) through the "leucine zipper." It was reported that JunB constitutes the major dimerization partner of c-Fos, which increases with increased severity of cervical cancer (7), at the active AP-1 complex during HPV oncogene expression in cervical cancers (7–9). It has also been reported that CBP/p300 acts as a co-activator of c-Fos during HPV oncogene expression (9, 10). The known transforming functions of E6 include accelerated proteosomal degradation of tumor suppressor p53 (11, 12), as well as activation of telomerase (13). In fact, E6 alters the substrate specificity of a cellular ubiquitin ligase, E6AP, so that it stably associates with and polyubiquitinylates tumor suppres-

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[2] The abbreviations used are: HPV, human papilloma virus; MAR, matrix attachment region; AP-1, activator protein-1; HDAC, histone deacetylases; Tip60, Tat-interacting protein 60 kDa.

sor p53, thereby degrading it via 26 S proteasome (1). The resultant effect counteracts the normal apoptotic and cell cycle arrest responses of HPV-positive cells, thereby ultimately resulting in deregulated cell proliferation.

The above discussion reveals that E6, contributing effectively in the antiapoptosis network, represents one of the most promising therapeutic targets for the treatment of HPV-positive tumors and dysplasias because its repression may result in reactivation of tumor suppressor pathways in cancer cells. Although prophylactic vaccines are currently available and show high efficacy against the establishment of HPV infection, low rates of initiation and lower rates of completion of the vaccination regimen, as well as the lack of an opportunity to be vaccinated prior to infection, has led to the development of a patient population for whom no therapy for infection is available.

Increasing evidence suggests that epigenetic alterations are essential in establishing the transformed phenotype in addition to the genetic changes associated with the transformation of a normal cell into a cancer cell. In this relation, acetylation of histone, as well as other transcription regulatory non-histone factors by lysine acetyltransferases, *e.g.* Tip60 (14, 15), commonly correlates with the open chromatin structures required for the binding of multiple transcription factors and leads to transcriptional activation correlated with an increase in gene expression, whereas removal of acetyl groups by histone deacetylases (HDACs) accompanies with transcriptional repression. Lysine acetyltransferases and HDACs have been shown to play a critical role in transcriptional regulation in eukaryotic cells. HPV18 E6 protein has been observed to induce the degradation of the tumor suppressor lysine acetyltransferase, Tip60 (Tat-interacting protein 60 kDa), which is involved in transcriptional regulation, checkpoint activation, and p53-directed proapoptotic pathways (14, 16). On the other hand, nuclear matrix protein SMAR1 interacts with HDAC1-associated repressor complex at cyclin D1 promoter and allows histone deacetylation and transcriptional repression (17). SMAR1 also stabilizes p53 via post-translational modification (18) and inhibits tumor growth through cell cycle arrest (19). Further, SMAR1-derived p44 peptide is shown to actively inhibit tumor growth *in vivo* (20). SMAR1 has also been implicated in the transcriptional regulation of viral genes in which it regulates viral transcription by alternative compartmentalization of LTR, resulting in a decreased virion production of HIV-1 (21). All of this information leads to the possibility of reversing the key alterations in the apoptotic machinery in HPV18-infected cervical adenocarcinoma by modulating SMAR1 that may alter the status and/or function of E6, Tip60, p53, and HDACs. However, there is no report on this critical function of SMAR1, if any, in reinstalling the "missing" apoptotic program in HPV18-infected cervical cancer cells.

Recently, curcumin-induced up-regulation of SMAR1 and the contribution of this MAR-binding protein in sensitizing breast cancer cells toward doxorubicin have been reported from our laboratory (22). Recent reports have also suggested the HPV16 E6 protein as a target for curcuminoids, curcumin conjugates, and congeners for chemoprevention of cervical cancers (23). In this regard, curcumin-induced suppression of STAT3 activation has been reported to be associated with the gradual loss of HPV16 E6 and E7 expression and cell viability (24). According to Maher *et al.* (25), curcumin restores p53, Rb, and PTPN13 proteins to induce apoptosis in HPV16-infected cervical cancer cells. However, there are hardly any reports describing the involvement of SMAR1 in curcumin-induced HPV18-infected cervical adenocarcinoma cell apoptosis.

Here, for the first time to our knowledge, we elucidate the role of SMAR1 as the suppressor of HPV18 E6 to refurbish the lost apoptotic program of the cells. Mechanistically, curcumin installs a proapoptotic cycle in HPV18-infected cervical adenocarcinoma cells. Curcumin up-regulates SMAR1 to potentiate recruitment of SMAR1-HDAC1 repressor complex at the *LCR* and *E6* MAR sequences, thereby decreasing histone acetylation at Lys-9 and Lys-18 leading to reorientation of the chromatin. As a consequence, c-Fos binding at the putative AP-1 sites on *E6* promoter is inhibited. E6 depletion interrupts degradation of E6-mediated p53 and lysine acetyl transferase, Tip60. Tumor suppressor p53, being stabilized by SMAR1 and acetylated by Tip60, in turn induces SMAR1 to orchestrate the cycle that leads to HPV18-infected cervical adenocarcinoma cell apoptosis via p53-mediated transactivation of proapoptotic genes. In fact, earlier reports have shown that Tip60 also promotes repression of E6 after it was rescued from E6-mediated degradation (15). Cumulatively, restoration of SMAR1 by curcumin effectively warrants apoptosis in cervical cancer cells. This hitherto unappreciated but novel function of SMAR1 highlights the potential of this protein in regulating the E6-mediated antiapoptotic network in HPV18-infected cervical adenocarcinoma.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The cervical cancer cell line HeLa (p53 degraded/HPV18-E6-positive) were obtained from National Centre for Cell Science (Pune, India). The cells were routinely maintained in complete Dulbecco's modified Eagle's medium at 37 °C in a humidified incubator containing 5% CO_2 (26). Cells were allowed to reach confluence before use. Viable cell numbers were determined by trypan blue exclusion test (26). Cells were treated with different concentrations of curcumin (Sigma) for different time points to select the optimum dose and time required for cancer cell apoptosis. An equivalent amount of carrier (dimethyl sulfoxide) was added to untreated cells. To inhibit HDAC1 activity, cells were preincubated with broad HDAC inhibitor, trichostatin A (0.5 μM; Sigma-Aldrich) for 6 h prior to curcumin treatment. To inhibit proteasome activity, cells were preincubated with MG-132 (10 μM; Sigma).

Flow Cytometry—To assess cell death, cells were stained with propidium iodide and annexin V-FITC (BD Pharmingen) and analyzed on a flow cytometer (FACSCalibur; Becton Dickinson). Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. Total 10,000 events were acquired for analysis using CellQuest software (Becton Dickinson). Annexin V-positive cells were regarded as apoptotic cells (27).

Co-immunoprecipitation and Immunoblotting—To obtain whole cell lysates, cells were homogenized in lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, and 1 mM DTT) supplemented with protease and phosphatase inhibitor cocktails (28). For direct Western blot analysis, a total of 50 μg of protein was resolved using SDS-PAGE and transferred to nitrocellulose membrane and

probed with specific antibodies, for example, anti-p53, -Bax, -Puma, -Caspase-3, -Caspase-9, -E6, -c-Fos, -SMAR1, -Tip60, and -p300 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Ser(P)-15-p53 (Cell Signaling Technology, Danvers, MA); thereafter, the immunoblots were visualized by chemiluminescence (GE Healthcare). Equal protein loading was confirmed with anti-α-actin antibodies (Santa Cruz Biotechnology). For the determination of direct interaction between two proteins, a co-immunoprecipitation technique was employed (28). p53-ubiquitin or SMAR1-HDAC1 interactions were performed using cell lysates prepared in Nonidet P-40 (1%) lysis buffer containing protease inhibitors. Samples (300 μg of protein from the total lysate) were incubated at 4 °C overnight with anti-p53/SMAR1 antibody and then incubated for 2h at 4 °C with protein G-Sepharose (Invitrogen). Immunocomplexes were washed of unbound proteins with cold Tris-buffered saline with protease inhibitors, and pelleted beads were boiled for 5min in SDS-polyacrylamide gel electrophoresis sample buffer. The immunoprecipitated proteins were resolved on SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting for detection of Ub/p53/SMAR1/HDAC1. The input protein used in immunoprecipitation was confirmed by Western blotting with anti-α-actin.

RT-PCR—Two μg of the total RNA extracted from cells with TRIzol Q16 reagent (Invitrogen) was reverse transcribed and then subjected to PCR with enzymes and reagents of the RTplusPCR system (Eppendorf, Hamburg, Germany) using GeneAmpPCR 2720 (Applied Biosystems) (26). The cDNAs were amplified with primers specific for *BAX* (5′-TTTGCTTCAGGGTTTCATCC-3′/5′-CAGTTGAAGTTGCCGTCAGA-3′), *E6* (5′-AAGCTACCTGATCTGTGCACGG-3′/5′-GCTGGATTCAACGGTT-TCTGG-3′), c-*FOS* (5′-AGACAGACCAACTAGAAGATGA-3′/5′-AGCTCTGTGGCCATGGGCCCC-3′), *SMAR1* (5′-GCATTGAGGCCAAGCTGAAAGCTC-3′/5′-CGGAGTTCAGGGTGATGAGTGTGAC-3′), *PUMA* (5′-CCACCACCATCTCAGGAAAG-3′/5′-ACGTTTGGCTCATTTGCTCT-3′), and *GAPDH* (internal control) (5′-CAG AACATCATCCTGCCTCT-3′/5′-GCTTGACAAAGTGGTCGTTGAG-3′).

Plasmids, siRNA, and Transfections—The expression constructs pBK-CMV-*SMAR1*-cDNA and control pcDNA3.0 vectors (2 μg/million cells) were introduced into exponentially growing cancer cells using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer. Stably expressing clones were isolated by limiting dilution and selection with G418 sulfate (1 mg/ml; Cellgro), and G418-resistant cells were cloned and screened by immunofluorescence or Western blotting with specific antibodies. For endogenous silencing of specific genes, cells were transfected with 300 pmol of Tip60 siRNA/p53 shRNA/SMAR1shRNA using Lipofectamine 2000 separately for 12 h. The mRNA and protein levels were determined by RT-PCR and Western blotting, respectively.

Chromatin Immunoprecipitation—ChIP assays were performed using a ChIP assay kit (Millipore) according to the manufacturer's instructions. PCR assay for identification of SMAR1, HDAC1, and c-Fos binding regions on *E6* promoter was performed using the nine different primer sets: set 1, 5′-GACCGTTTTCGGTTACTCCC-3′/5′-CTACCTGATCTGTGCACGG-3′; set 2, 5′-GGATCCTCAAAGCGCGCC-3′/5′-AGTATACCCATGCTGCA-3′; set 3, 5′-GCAGCATGGGGTATACTG-3′/5′-GCCTGCGATGTCAGAAAC-3′; set 4, 5′-GTGTCTCCATACACAGAGTC-3′/5′-GAGCACGACAGGAACGAC-3′; set 5, 5′-CCTGGCACGTACACGCACACGC-3′/5′-CTGTGTGTTATGTGGTTGCGCCC-3′; set 6, 5′-GGGCGCAACCACATAACACACAG-3′/5′-GCACAATACAGTACGCTGGC-3′; set 7, 5′-CCACAGACATAAGCCAAAGGCAAC-CG-3′/5′-CCTGTCCAGGTGCGCTAC-3′; set 8, 5′-GTAGCGCACCTGGACAGG-3′/5′-GCAACCGAAATAGGTTGGGCAGC-3′; and set 9, 5′-CCCGACCGTTTTCGGTTACTCCC-3′/5′-GGCGCGCTTTGAGGATCCAAC-3′. PCR amplifications of AP-1-1 and AP-1-2 response elements on the *E6* promoter region were performed using the following primers: AP-1-1, 5′-TGCTTGCATAACTATATC-CACTCCC-3′ and 5′-TATGTGCTGCCCAACCTATTTCGG-3′; and AP-1-2, 5′-TGCTTGCATAACTATATCCACTCCC-3′ and 5′-GAAAAGTATAGTATGTGCTGCCCAACCTA-3′. Extracted DNA (2 μl) was used for 45 cycles of amplification in 5 μl of reaction mixture under the following conditions: 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s. The PCR products were analyzed by 2% agarose gel electrophoresis.

Electrophoretic Mobility Shift Assay—For EMSA, nuclear extracts from HeLa cells were prepared according to standard protocol, and 30 μg was used for gel shift assays. Oligonucleotides corresponding to the SMAR1 binding sites 1 and 5 or the AP-1 binding sites were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Probe purification was done using Probequant G-50 column (Amersham Biosciences). Binding reactions were performed in a 10 μl of total volume containing 10 mM HEPES (pH 7.9), 1 mM dithiothreitol, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.5–1 μg of double-stranded poly(dI-dC), 10 μg of BSA, and 1 μg of SMAR1 recombinant protein or 30 μg of nuclear lysate. For cold competitor, 100 times the unlabeled probe was used. Samples were incubated for 5 min at room temperature prior to addition of radiolabeled probe. The samples were then incubated for 15 min at room temperature, and the products of binding reactions were resolved using 5% native polyacrylamide gel electrophoresis. The gels were dried under vacuum and processed for autoradiography.

Statistical Analysis—The values are shown as standard error of mean, unless otherwise indicated. The data were analyzed and, where appropriate, significance ($p < 0.05$) of the differences between mean values was determined by a Student's t test.

RESULTS

SMAR1 Represses HPV18 E6 Oncogene Expression and Restores the Lost Apoptotic Program in HPV18-infected HeLa Cells—Nuclear matrix and matrix attachment regions (MARs) have been implicated in the transcriptional regulation of host, as well as viral genes, but their precise role in HPV or human papillomavirus transcription remains unclear. Recently the contribution of SMAR1 in regulating transcription of HIV genes has been accredited (21, 29), but its effect on HPV18 oncogenes is not yet known. To address the role of SMAR1 in regulation of HPV18 E6, we first deciphered their relationship, if any. Our results depicted that the increase in SMAR1 by 25 μM curcumin (according to our earlier reports (27) down-regulated E6 expression both at mRNA

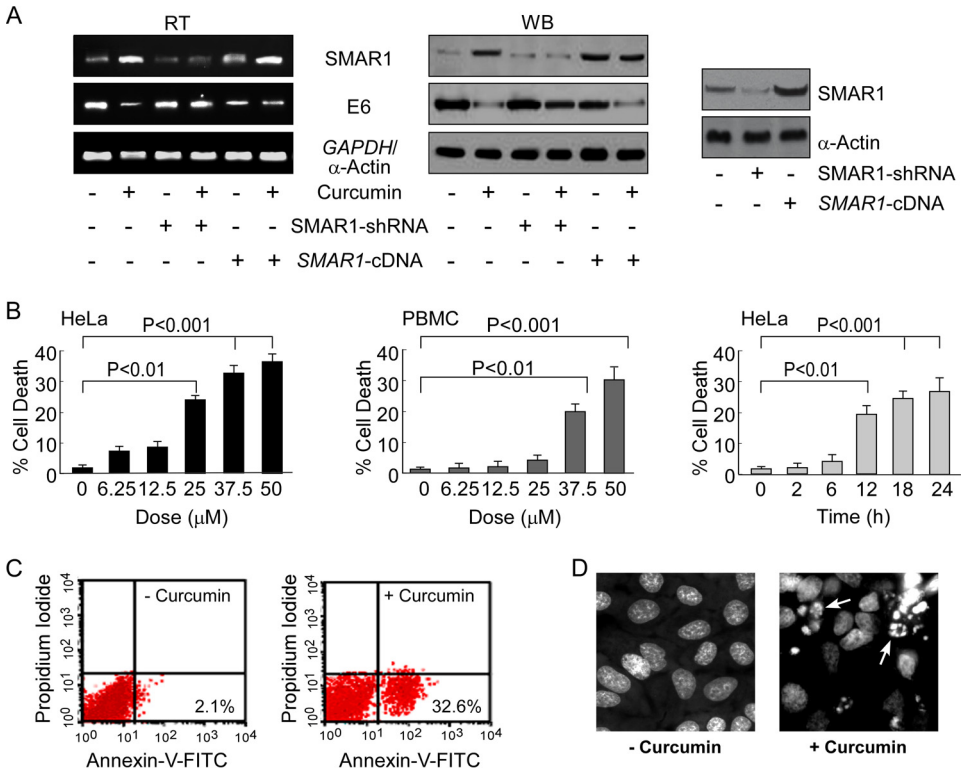

FIGURE 1. **SMAR1-mediated transcriptional repression of HPV18-E6 oncogene.** *A*, contribution of curcumin (25 μM)-induced up-regulation of SMAR1 in manipulating E6 expression both at transcriptional (*left panel*) and translational (*middle panel*) levels was determined in *SMAR1*-cDNA- or SMAR1-shRNA-transfected functional p53-deficient HPV18-positive HeLa cells. Transfection efficiencies of SMAR1-cDNA and SMAR1-shRNA were evaluated by Western blot (*WB*) analysis (*right panel*). *B*, HeLa cells (*left panel*) and normal peripheral blood mononuclear cells (*PBMC*) (*middle panel*) were treated with a dose range (0–50 μM) of curcumin for 24 h, and the percentage of cell death was scored by trypan blue dye exclusion assay. The nontoxic dose of curcumin (25 μM) was administered to HeLa cells for varied time points, and the percentage of cell death was determined (*right panel*). *C*, HeLa cells in the presence or absence of 25 μM curcumin were subjected to flow cytometric analysis for annexin V-FITC/PI staining, and annexin V-FITC-positive HeLa cells were regarded as apoptotic cells. *D*, DAPI staining revealed nuclear morphology of apoptotic cells. Apoptotic nuclei are marked with *arrows*. α-Actin/*GAPDH* was used as internal loading control. The values are the means ± S.E. of three independent experiments in each case or representative images of typical experiment.

(Fig. 1*A*, *left panel*) and protein levels (Fig. 1*A*, *middle panel*) in HPV18-infected HeLa cells. Similar results were obtained by transfecting HeLa cells with *SMAR1*-cDNA (Fig. 1*A*). In contrast, down-regulation of *SMAR1* transcript by SMAR1-specific shRNA was correlated with the increased E6 expression both at transcriptional (Fig. 1*A*, *left panel*) and translational levels (Fig. 1*A*, *middle panel*) in HeLa cells even in the presence of curcumin. The results shown in the *right panel* of Fig. 1*A* confirmed the transcription efficiency of the experiment. Because HPV18 E6 disrupts the normal apoptotic machinery of the noncancerous cells, our next approach was to understand the effect of SMAR1-induced E6 down-regulation in HeLa cells. To this end, up-regulation of SMAR1 by curcumin in a dose-dependent manner in wild-type p53-expressing HPV-infected cervical cancer cells resulted in significant cell death at concentrations starting from 25 μM (Fig. 1*B*, *left panel*). Because concentrations beyond 25 μM were found to be toxic to normal cells (peripheral blood mononuclear cells) (Fig. 1*B*, *middle panel*), we restricted our future experiments to this dose. Next, we performed cell death analyses of HeLa cells using 25 μM curcumin in a time-dependent manner. Because maximum cell death was obtained at 24 h (Fig. 1*B*, *right panel*), beyond which no significant change in cell death was observed (data not shown), our subsequent experiments were performed using a 25 μM dose of curcumin for 24 h. In fact, curcumin-treated HeLa cells furnished an increased number of annexin V-positive cells (Fig. 1*C*) and DAPI-stained nuclear blebbing per visual field (Fig. 1*D*), thereby confirming induction of apoptosis in these HPV18-infected cervical carcinoma cells.

SMAR1 Up-regulates p53-SMAR1 Feedback Loop to Suppress E6 and Mediate Apoptosis—Because E6 mediates the accelerated proteosomal degradation of p53 tumor suppressor (12, 26), our next attempt was to check the status of p53 in these HPV18-infected cervical carcinoma cells. Our results indicate that with curcumin-induced up-regulation of SMAR1 (Fig. 1*A*), endogenous p53 protein expression also increased in a time-dependent manner (Fig. 2*A*). Interestingly, in SMAR1 cDNA-transfected HeLa cells, E6-mediated p53 ubiquitination was decreased (Fig. 2*B*). In addition, whereas curcumin increased phosphorylation of p53 at residue Ser-15, the same was decreased in SMAR1-silenced cells, even in the presence of curcumin (Fig. 2*C*). Because it has been reported that SMAR1 stabilizes p53 at the Ser-15 residue (18), our results validated the role of SMAR1 in p53 stabilization and activation.

Being a multifunctional protein, p53 forms molecular complexes with different DNA targets and interacts with a number of cellular proteins including SMAR1 (30). Interestingly, whereas curcumin treatment led to an increase in SMAR1 both at protein, as well as mRNA levels in HeLa cells (Fig. 1*A*), it failed to do so in cells stably transfected with p53-shRNA (Fig. 2*D*, *left* and *middle panels*). The results shown in the *right panel*

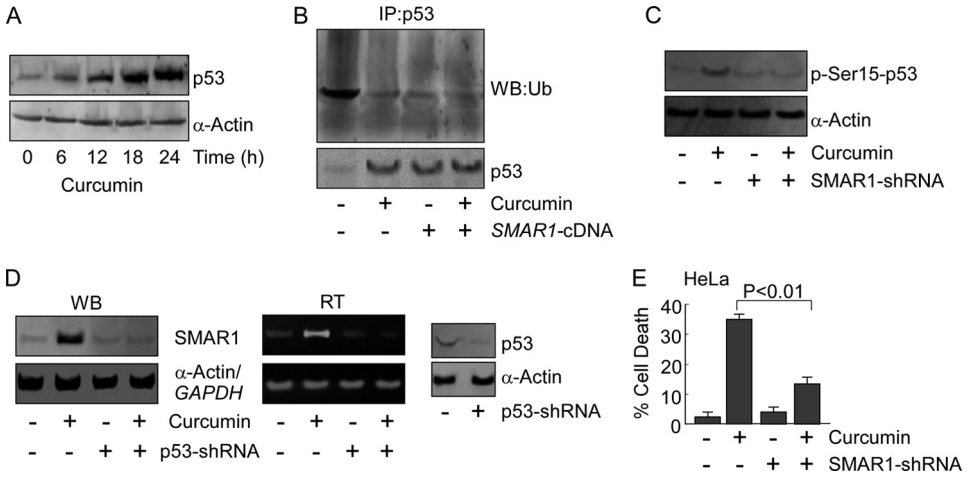

FIGURE 2. **Up-regulation of SMAR1 by p53-SMAR1 feedback loop.** *A*, expression levels of p53 protein were determined by Western blotting from untreated and curcumin-treated HeLa cells for the indicated time points. *B*, p53 was immunoprecipitated (*IP*) by anti-p53 antibody from the lysates of *SMAR1*-cDNA-transfected HeLa cells pretreated with curcumin and immunoblotted with anti-ubiquitin antibody to monitor p53 ubiquitination. The ladder of bands represents ubiquitinated p53. The immunoprecipitates were further assayed for p53 protein expression level by Western blot (*WB*) analysis. *C*, untransfected/SMAR1-shRNA-transfected HeLa cells were subjected to Western blot analysis for determination of the levels of Ser(P)-15-p53 in the presence or absence of curcumin. *D*, protein and mRNA expression levels of *SMAR1* were determined in HeLa cells transfected with p53-shRNA in absence and presence of curcumin by Western blot (*left panel*) and RT-PCR (*middle panel*) analyses. The efficiency of p53-shRNA transfection was also verified by Western blot analysis (*right panel*). *E*, HeLa cells transfected with SMAR1-shRNA were subjected to curcumin treatment and were scored for percent cell death by trypan blue dye exclusion assay and represented graphically. α-Actin/*GAPDH* was used as internal loading control. The values are the means ± S.E. of three independent experiments in each case or representative images of typical experiment.

of Fig. 2*D* confirmed the transcription efficiency. In addition, SMAR1 ablated E6-expressing HeLa cells significantly resisted curcumin-induced apoptosis (Fig. 2*E*). These results therefore suggest the existence of positive interdependence between p53 and SMAR1 in which p53 activates *SMAR1* transcription and SMAR1 in turn (i) up-regulates p53 *via* E6 down-regulation and (ii) stabilizes p53 to ensure its function in curcumin-treated HeLa cells. Culminating the results above, it can be suggested that proper functioning of p53-SMAR1 loop is indispensable for *E6* repression and reinstalling apoptosis in HPV18-infected cervical cancer cells.

SMAR1 Binds to Conserved MAR Sequence within HPV LCR and E6—Several reports highlight the importance of MARs in viral integration and their role in viral transcription (29), but the mechanism by which they elicit these effects is not yet understood. In the previous sections, we observed SMAR1-mediated suppression of the HPV18 oncogene *E6* and restoration of the apoptotic machinery. It is known that the promoter region of *E6* is divided into several regions *L1*, *LCR*, origin of replication/promoter, and *E6* itself (Fig. 3*A*) (31). To explore the SMAR1-binding site(s), if any, on the *E6* promoter, different sets of overlapping primers of the total promoter region were designed for DNA ChIP experiment (overlapping primer sets 1–9; Fig. 3*A*). Fig. 3*B* depicted the recruitment of SMAR1 on the *LCR* (7110–7299 bp; site 5) and *E6* coding region (128–162 bp; site 1) of HPV18 *E6* promoter. We validated those results by EMSA using radiolabeled oligomers from the E6 promoter region containing SMAR1 binding sites 1 and 5. The results shown in Fig. 3*C* (*lane 2* of *left* and *right panels*) indicate direct interaction between the *E6* promoter region and SMAR1. Furthermore supershift EMSA using SMAR1 specific antibody validated the same. (Fig. 3*C*, *third lanes* of *left* and *right panels*). These results not only corroborate the direct recruitment of SMAR1 on *E6* promoter sequence but also identify SMAR1 as the molecule responsible for repression of *E6* transcription.

SMAR1 Restrains E6 Expression in HPV18-infected Cervical Adenocarcinoma Cells by Inhibiting the Recruitment of c-Fos to E6 Promoter—We next assessed whether binding of SMAR1 with the *E6* promoter leads to inhibition of any other transcriptional activator(s) of E6 because, being a nuclear matrix attachment protein, SMAR1 can modulate the binding of other transcriptional activators (30, 32). It is known that among the various members of the AP-1 family, c-Fos acts as a tumor promoting factor, up-regulation of which causes cellular transformation (7–9). Moreover, during HPV-infected tumor development, a shift in the composition of AP-1 from Fra-1/c-Jun to c-Fos/c-Jun heterodimers has also been documented (7–9). In fact, various c-Fos target genes are reported to be expressed at higher levels in cervical cancer cells in comparison with normal cervical epithelial cells (33), thereby highlighting the importance of c-Fos in cervical carcinoma. To this end, we employed bioinformatics matrix/motif finding tool and identified AP-1 binding sites on *E6* promoter. The results shown in Fig. 3*D* (*top panel*) indicate that the probability of transcription factor-binding matrix was highest on bp 34–41 and 82–89 regions (Ori/pram and *E6* sequence junction; AP-1-1) and bp 7164–7175 (LCR region; AP-1-2) of *E6* promoter (cutoff score, >5). Putative AP-1-binding sites, as deduced by *in silico* exploration, were validated by ChIP analysis using antibodies against AP-1 binding factors, c-Fos and Fra-1, in HeLa cells. It was observed that whereas c-Fos significantly occupied both the sites (AP-1-1: 34–41 and 82–89; AP-1-2: 7164–7175) on *E6* promoter (Fig. 3*D*, *middle panel*), negligible binding was observed for Fra-1 (Fig. 3*D*, *bottom panel*). Previously our results depicted that SMAR1 binds at the *LCR* (7110–7299 bp), a portion of which overlaps with an AP-1-binding site (7164–7175 bp).

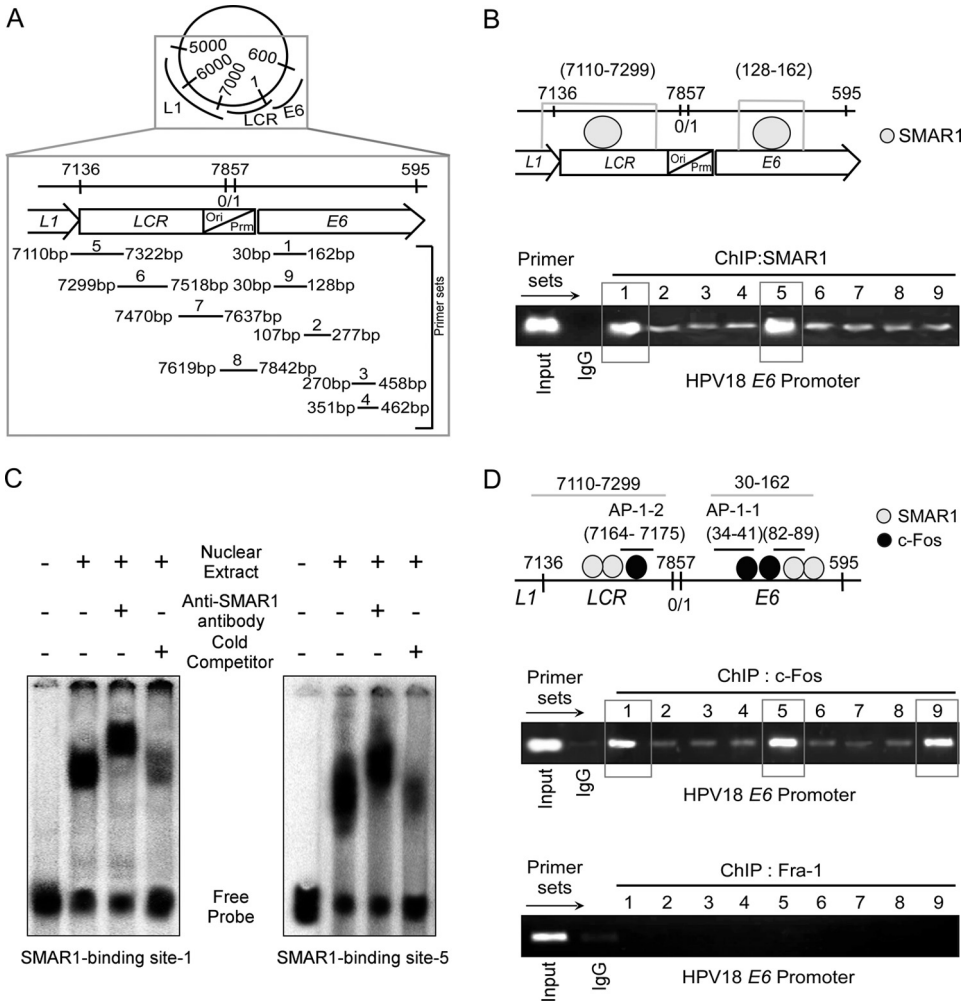

FIGURE 3. **SMAR1 binds to conserved MAR sequence within HPV LCR and E6.** *A*, schematic diagram representing different regions of E6 promoter: *L1*, *LCR*, *Ori* (origin of replication), and *E6* coding region, and the sequential order of primer sets (sets 1–9) was designed to identify SMAR1 binding regions on HPV18-*E6* promoter by ChIP analysis. *B*, schematic representation of SMAR1-occupied region on *E6* promoter (*upper panel*). ChIP assay for SMAR1 binding on the HPV18-*E6* promoter in curcumin-treated HeLa cells. Binding site numbers correspond to primer set numbers. Positive bands for the SMAR1-binding sites on *E6* promoter were shown in *lanes 3* and 7 (binding sites 1 and 5, *lower panel*). *C*, EMSA was done using the radiolabeled probes for the binding sites 1 and 5 along with nuclear extract of HeLa cells. There was significant complex formation between nuclear extract with the probes (*second lanes* in both *left* and *right panels*). Incubation with SMAR1 antibody induced supershift of the band (*third lanes* in both *left* and *right panels*). Addition of the cold competitor (*fourth lanes* in both *left* and *right panels*) showed reduced complex formation. *D*, schematic representation of c-Fos-binding sites adjacent to SMAR1-binding sites, on *E6* promoter (*top panel*). ChIP assay with anti-c-Fos (*middle panel*) and anti-Fra-1 (*bottom panel*) was performed on *E6* promoter. Positive bands for c-Fos-binding sites (AP-1 sites: AP-1-1 and AP-1-2) on *E6* promoter are shown in *lanes 3*, *7*, and *11* (binding sites 1, 5, and 9, *bottom panel*).

Considering the findings from our *in silico* analysis that the binding probability of SMAR1 is highest around c-Fos binding sites at *LCR* (Fig. 3*D*, *top panel*), it was logical to hypothesize that in the presence of SMAR1, c-Fos might be failing to bind to its specific binding sites (AP-1-1 and AP-1-2) on HPV18 *E6* promoter. Our chromatin immunoprecipitation results indeed demonstrated that in HeLa cells in which SMAR1 was up-regulated by curcumin, whereas SMAR1 was recruited to the *LCR* and *E6* region of *E6* gene, the recruitment of c-Fos to AP-1-1 (Fig. 4*A*, *left panel*) and AP-1-2 (Fig. 4*A*, *right panel*) sites, respectively, was reduced. However, such a decrease in the promoter occupancy was considerably rescued in SMAR1-silenced HeLa cells where significant c-Fos recruitment was observed (Fig. 4*A*, both *left and right panels*). These results were corroborated by EMSA, in which bindings of SMAR1 and c-Fos to the radiolabeled probes of AP-1-binding sites were monitored. The results shown in Fig. 4*B* (*lane 2* of *left* and *right panels*) depicted the recruitment of rSMAR1 at both the AP-1-binding sites on the *E6* promoter. Supershift assay using SMAR1 antibody validated these results (*lane 3* of *left* and *right panels*). In contrast, in the presence of rSMAR1 (as in *lane 2*), c-Fos antibody failed to furnish any supershift of the bands (*lane 4* of *left* and *right panels*), thereby negating the possibility of binding of c-Fos with the AP-1-binding sites on the *E6* promoter once SMAR1 is bound to the same. All these findings together reinforced that SMAR1 functions as a transcriptional repressor of HPV18 *E6* gene.

SMAR1 Associates with HDAC1 to Repress E6 Transcription—Recent studies have shown that SMAR1 recruits HDAC1/Sin3A co-repressor complex to various promoters and repress gene expression (17, 18) and that the presence of HDACs at the promoter is strongly correlated with transcriptional repression (34). Therefore, we next tested whether both SMAR1 and HDAC1 are co-recruited to the SMAR1-binding sites identified above, *i.e. E6*

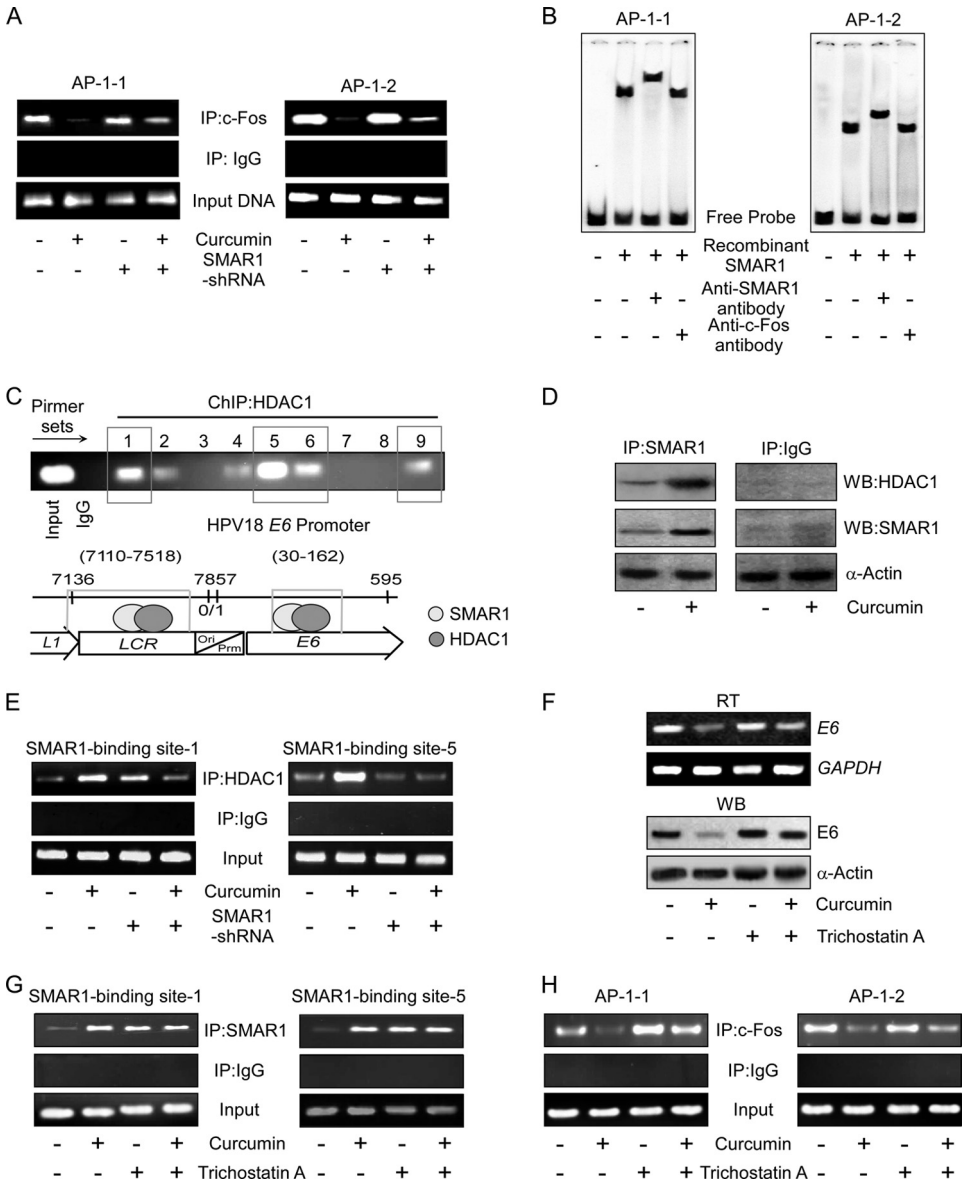

FIGURE 4. **Curcumin-induced recruitment of SMAR1/HDAC1 complex at E6 promoter inhibits E6 transcription.** *A*, SMAR1-shRNA transfected HeLa cells in absence and presence of curcumin were analyzed for c-Fos binding (AP-1 sites: AP-1-1 and AP-1-2) on *E6* promoter by ChIP assay. *B*, EMSA showing binding of recombinant SMAR1 protein to AP-1 sites: AP-1-1 and AP-1-2 (*left* and *right panels*). The binding specificity was verified by supershift assay using antibodies against SMAR1 (*third lane* of both *left and right panels*). Preincubation with recombinant SMAR1 prevents supershift with c-Fos antibody (*fourth lane* of both *left and right panels*). *C*, HDAC1 binding on the *E6* promoter in HeLa cells in presence of curcumin was analyzed by ChIP assay. Positive bands for HDAC1-binding sites in *E6* promoter were shown in *lanes 3*, *7*, *8*, and *11* (binding sites 1, 5, 6, and 9, *upper panel*). Illustration demonstrating sites on *E6* promoter at which both SMAR1 and HDAC1 were recruited (*lower panel*). *D*, co-immunoprecipitation experiment was employed to study the physical interaction of SMAR1 and HDAC1 in HeLa cells in the absence or presence of curcumin. *E*, in SMAR1-shRNA-transfected HeLa cells, binding of HDAC1 to the SMAR1-binding sites 1 and 5 on the *E6* promoter in the absence and presence of curcumin was determined by ChIP assay. *F*, *E6* expression both at transcriptional and translational levels was determined in untreated/curcumin-treated HeLa cells in the presence and absence of broad HDAC inhibitor, trichostatin A (0.5 μM) employing RT-PCR (*upper panel*) and Western blot (*lower panel*) analyses. *G*, SMAR1 binding on the *E6* promoter (binding site 1 and 5) in untreated/curcumin-treated HeLa cells in the presence and absence of broad HDAC inhibitor, trichostatin A, was analyzed by ChIP assay. *H*, in similar conditions, c-Fos binding to AP-1 sites (AP-1-1 and AP-1-2) on the *E6* promoter was assessed by ChIP assay. α-Actin/*GAPDH* was used as internal loading control. *IP*, immunoprecipitation; *WB*, Western blot.

coding regions: 30–162 bp and LCR: 7110–7299 bp, by verifying HDAC1 occupancy on HPV18 *E6* promoter (Fig. 4*C*, *upper panel*). ChIP experiment was performed (using primer sets 1–9) in chromatin fractions pulled with anti-HDAC1 antibody from HeLa cells in which SMAR1 was up-regulated by curcumin. The results shown in Fig. 4*C* (*lower panel*) confirmed the recruitment of HDAC1 to the SMAR1-binding sites on *E6 promoter*. Moreover, our co-immunoprecipitation studies validating the direct association of SMAR1-HDAC1 in curcumin-treated SMAR1-up-regulated HeLa cells (Fig. 4*D*, *left panel*) indicated the possibility of the involvement of HDAC1 in transcriptional repression of *E6* gene by SMAR1.

It is acknowledged that the transcription factors, which are the components of the chromatin remodeling complex, can affect transcription in two ways: one by recruiting repressor complexes, other by modifying the chromatin structure through direct binding. We therefore next assessed the role of SMAR1 in the SMAR1-HDAC1 repressor complex. ChIP assay

of chromatin extracts from SMAR1-shRNA-transfected HeLa cells showed a reduced association of HDAC1 with *LCR* and *E6* regions even when the transfectants were treated with curcumin (Fig. 4*E*). These results validate the indispensible role of SMAR1 for promoting association of HDAC1 with *LCR* and *E6* regions of the HPV18 genome. To further confirm the involvement of HDAC1 in SMAR1-induced repression of *E6*, HeLa cells were preincubated with broad HDAC inhibitor trichostatin A prior to SMAR1 up-regulation by curcumin. The results revealed that perturbing HDAC1 activity attenuated SMAR1-mediated *E6* transcriptional repression, even in the presence of curcumin (Fig. 4*F*). In fact, although these cells demonstrated SMAR1 recruitment on *E6* promoter (Fig. 4*G*), they failed to inhibit c-Fos binding to its cognate sites (AP-1-1 and AP-1-2) (Fig. 4*H*) as assessed by ChIP analysis. Together, these findings conclusively substantiated the involvement of SMAR1-HDAC1 co-repressor complex in perturbing c-Fos-regulated *E6* transcription in HeLa cells.

SMAR1 Binding to HPV18 E6 Promoter Causes Local Chromatin Condensation—It has been reported that significant deacetylations at H3K9 and H3K18 are specifically regulated by HDAC1 (35, 36). Therefore, the effect of SMAR1-HDAC1 co-repressor complex recruitment on chromatin condensation was verified by analyzing the histone modifications at c-Fos-binding regions on *E6* promoter. Our results indicated significant acetylations of H3K9 (Fig. 5*A*, *upper panel*) and H3K18 (Fig. 5*A*, *lower panel*) on *E6* promoter in untreated HeLa cells. However, the same was significantly reduced under curcumin-induced SMAR1 up-regulated condition (Fig. 5*B*, *upper* and *lower panels*). In fact, modulation in chromatin observed above was rescued in SMAR1-shRNA-transfected cells as manifested by increased histone acetylation at H3K9 (Fig. 5*B*, *upper panel*) and H3K18 (Fig. 5*B*, *lower panel*), which even curcumin treatment failed to reverse (Fig. 5*B*). Increased acetylation was also observed when HeLa cells, in which SMAR1 was up-regulated by curcumin, were pre-exposed to broad HDAC inhibitor trichostatin A (Fig. 5*B*). All these results together confirm that SMAR1-HDAC1 repressor complex binds to *LCR* and *E6* coding region and deacetylates histones to repress c-Fos-mediated *E6* transcription. SMAR1, therefore, plays a major role in modulating chromatin structure at HPV18 *E6* promoter.

Inhibition of E6 Reinstalls Apoptotic Program in HPV18-infected Cervical Carcinoma Cells in p53-Tip60-dependent Manner—We next sought to identify the ultimate effect of SMAR1-induced E6 down-regulation in HeLa cells. For the purpose, we explored the possibility of p53-mediated apoptosis in cervical adenocarcinoma cells because curcumin-induced SMAR1 up-regulation resulted in E6 down-regulation and subsequent restoration of p53. Earlier reports stated curcumin as an inhibitor of the acetyl transferase CBP/p300, which is a co-activator of p53 apoptotic machinery (37). Fig. 5*C* furnishing similar results not only ruled out the involvement of CBP/p300 in curcumin-mediated p53-dependent apoptosis of HeLa cells but also indicated the involvement of other acetyl transferase proteins. Previous reports (14, 38) have shown that p53 acts as a substrate for the proapoptotic acetyl transferase Tip60 that catalyzes acetylation of lysine 120 of the DNA binding domain of p53. Importantly, Lys-120 acetylation is crucial for p53 to transactivate proapoptotic genes, *e.g. PUMA*, *BAX*, etc. (38, 39). However, Tip60 has been shown to be down-regulated in HPV18-infected cervical adenocarcinoma cells in which HPV 18 E6 degrades it in a proteosome-dependent pathway (15). Our results depicted a significant restoration and increase in Tip60 expression in HeLa cells after curcumin treatment in a time-dependent manner (Fig. 5*D*). Next our attempt to confirm the involvement of SMAR1 in the restoration of Tip60 furnished above revealed down-regulation of this lysine acetyltransferase by SMAR1 ablation (Fig. 5*E*). Cumulatively, down-regulation of E6 by SMAR1 might have resulted in Tip60 accumulation in HPV-18-infected cervical cancer cells. Finally, addition of the proteasome blocker, MG-132, restored the expression of Tip60 (Fig. 5*F*), thereby not only validating the role of E6 in degradation of Tip60 but also SMAR1-induced E6 down-regulation as the reason behind curcumin-induced up-regulation of Tip60 protein. In the next experiment, Tip60 ablation by siRNA decreased endogenous Lys-120 acetylation of p53 (Fig. 5*G*), which curcumin treatment failed to restore. In line with these results, whereas curcumin treatment increased Puma and Bax at both mRNA and protein levels, Tip60 silencing abrogated both in HeLa cells (Fig. 5*H*). Finally, downstream, loss of mitochondrial transmembrane potential (Fig. 5*I*, *left panel*) and activation of caspases-9 and -3 (Fig. 5*I*, *right panel*) were observed. These results implicate the existence of the mitochondrial pathway of apoptosis in these SMAR1 up-regulated HeLa cells.

SMAR1-p53-Tip60 Network Ensures the Fine Tuning of E6 Abrogation and Apoptosis—To further validate that SMAR1-mediated E6 down-regulation was required for restoring p53 protein levels, a battery of HPV-DNA-negative cancer cells, MCF-7, HCT-116, A549, and H460, was ectopically expressed with HPV18 *E6*-cDNA. The results depicted in Fig. 5*I* demonstrated that there was significant E6 expression with low levels of p53 and SMAR1 in these transfectants, whereas curcumin effectively reversed the situation (Fig. 5*J*). These results validated that E6 suppression was indeed inevitable for preventing p53 protein degradation.

All these results together underscore the role of SMAR1 in down-regulating E6 and relieving p53 and Tip60 from E6-mediated degradation. In turn, p53 and Tip60 activate the downstream apoptotic machinery. SMAR1, therefore, might be acting as a double-edged sword by (i) suppressing E6 through SMAR1-HDAC1 repressor complex and (ii) restoring the long lost apoptotic program through p53-Tip60 interplay.

DISCUSSION

The etiology, pathogenesis, and prophylaxis of poorly differentiated cervical adenocarcinoma exclusively expressing HPV18 oncogenes are feebly recognized despite its topical prevalence worldwide. Although HPV types 16 and 18 remain the most common in cervical lesions, causing 60 – 80% of all cervical cancers, it is known that HPV18 behaves more aggressively than HPV16, and the transcriptional regulatory regions of HPV16 and HPV18, upstream of the E6 and E7 genes, are the major determinants that discriminate between the biological activities of the respective viruses (40). Because the capability of transcriptional activity is higher for HPV18 than HPV16, HPV18 is more aggressive in

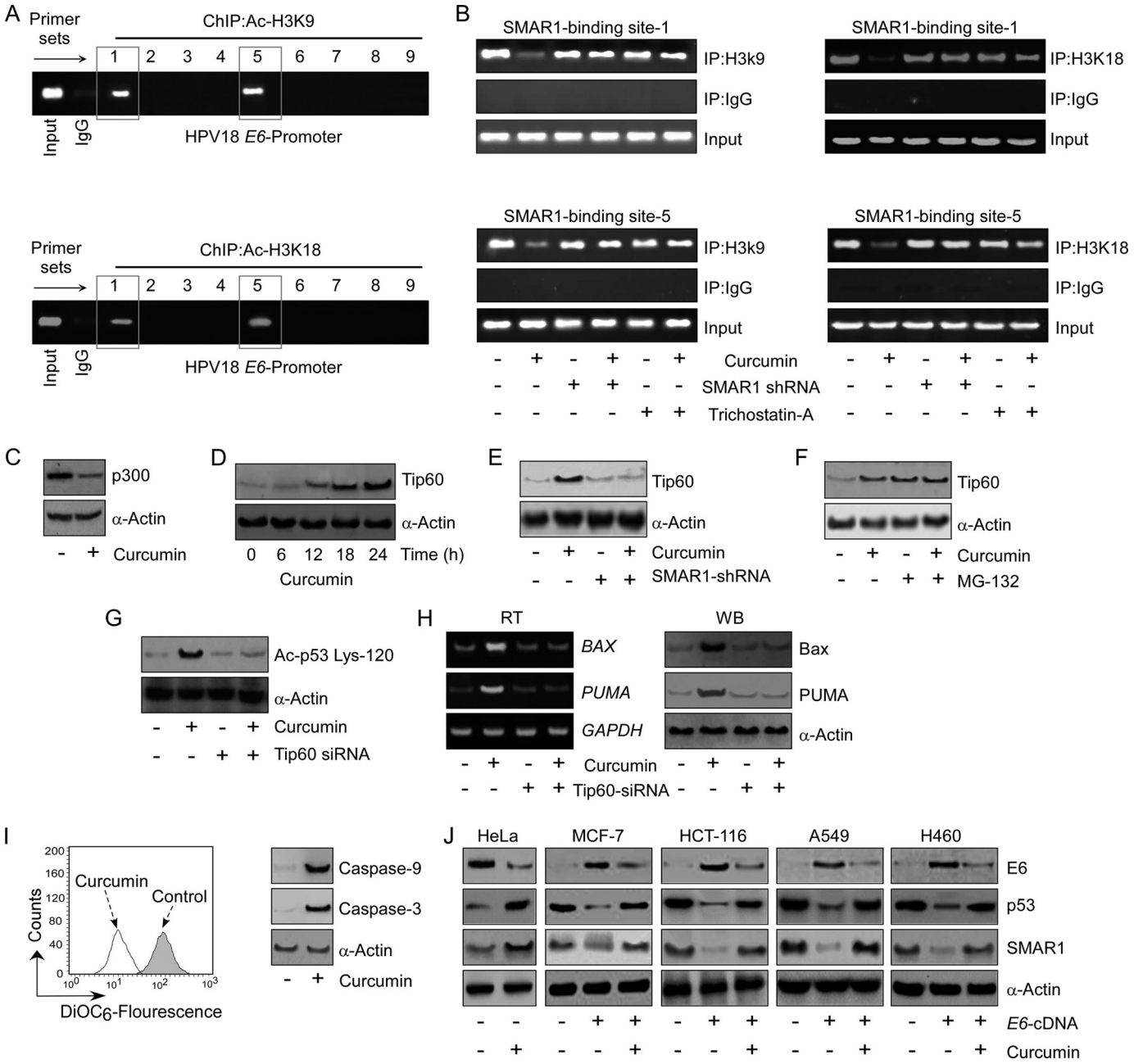

FIGURE 5. **Curcumin promotes histone deacetylation and restores Tip60 expression to promote p53-mediated proapoptotic transactivation.** *A*, histone acetylations (H3K9 and H3K18 positions) at *E6* promoter of HeLa cell were confirmed by ChIP assay. *B*, histone acetylation at H3K9 and H3K18 of *E6* promoter were assayed in presence of curcumin and broad HDAC inhibitor (trichostatin A), as well as under SMAR1 silenced conditions by ChIP assay. *C*, immunoblots showing expression profile of p300 in untreated/curcumin-treated HeLa cells. *D*, time-dependent expression profiles of Tip60 protein were determined by Western blot in curcumin-treated HeLa cells. *E*, expression of Tip60 protein in untransfected/SMAR1-shRNA-transfected HeLa cells in absence and presence of curcumin was analyzed by Western blot analysis. *F*, immunoblots showing expression profile of Tip60 in untreated/curcumin-treated HeLa cells in MG-132 (10 μM)-pretreated HeLa cells. *G*, expression of p53 acetylation at the Lys-120 residue was analyzed by Western blot analysis in Tip60-siRNA-transfected HeLa cells in the absence and presence of curcumin. *H*, Bax and Puma expressions both at transcriptional and translational levels were determined in untransfected or Tip60-siRNA-transfected HeLa cells in the absence and presence of curcumin employing RT-PCR (*left panel*) and Western blot (*right panel*) analyses. *I*, HeLa cells were incubated with or without curcumin and were monitored for mitochondrial transmembrane potential loss by $DiOC_6$ fluorescence measurement using flow cytometry (*left panel*). Immunoblots showing expression profiles of Caspase-9 and -3 in untreated and curcumin-treated HeLa cells (*right panel*). *J*, lysates of untreated or curcumin-treated HeLa cells were subjected to Western blot analysis for the determination of changes in p53, SMAR1, and E6 expressions, to use these parameters as controls for the following experiment. Inverse relationship between E6 and p53, as obtained in the case of HeLa cells, was reconfirmed in wild-type p53-expressing and HPV-18 E6-negative cancer cells, *i.e.* MCF-7, HBL-100, A549, and H460 cells, transfected with *E6*-cDNA. α-Actin was used as internal control. The values are the means ± S.E. or the representative of three independent experiments. *IP*, immunoprecipitation; *WB*, Western blot.

nature, and therefore detailed study on HPV18 is of the utmost necessity for betterment of screening and treatment of women progressing higher grade lesions or invasive carcinoma. It is acknowledged that the prominent role of HPV oncogene *E6* is to inhibit p53 function, thus impairing the cell cycle or inhibiting the cells to enter the apoptotic pathway in response to DNA damage (41). Therefore, HPV E6 seems to be a potential therapeutic target for regression of cervical cancer.

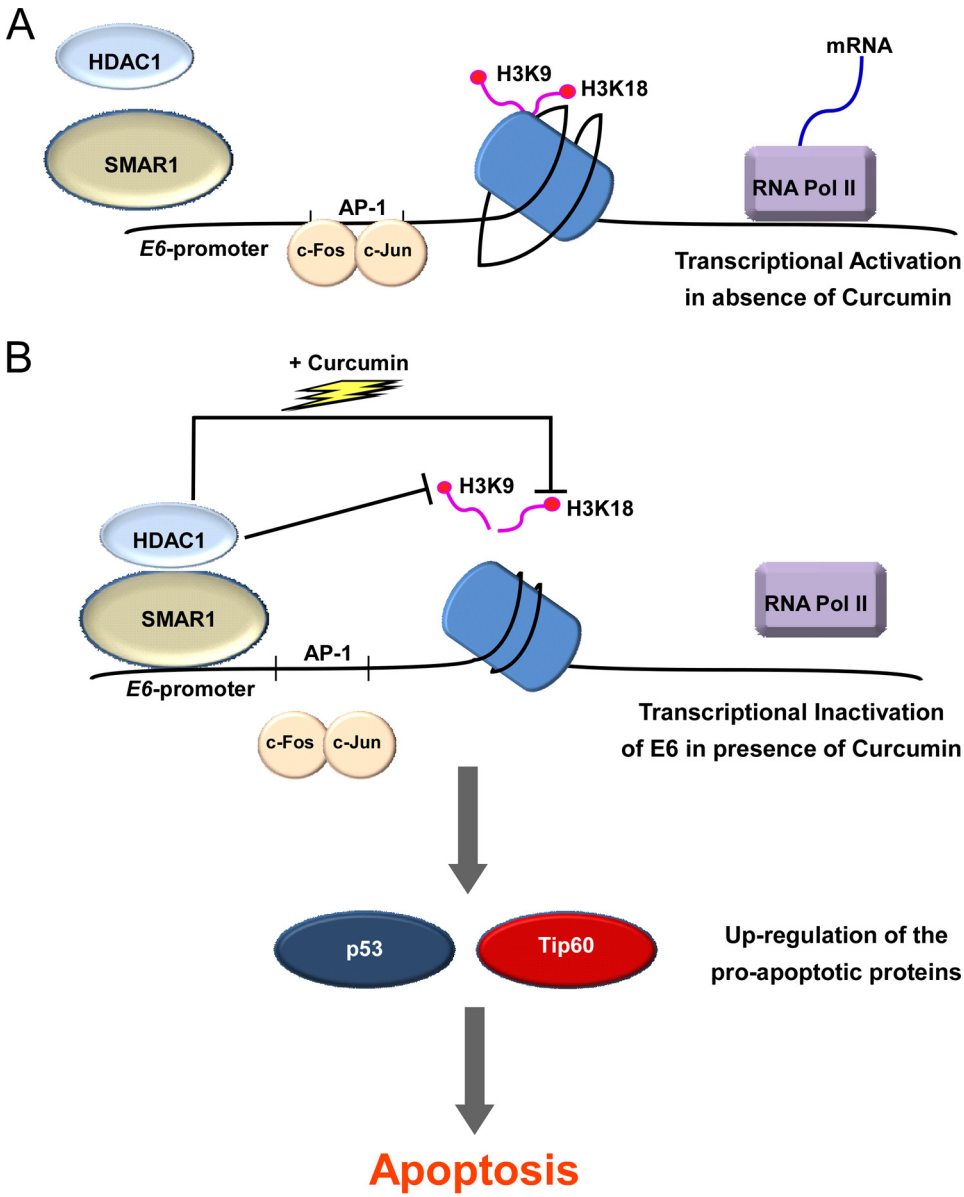

FIGURE 6. **Schematic representation of turn-on of *E6* promoter in absence of curcumin (*A*) and curcumin-induced up-regulation of SMAR1 (B) and recruitment of HDAC1-SMAR1 repressor complex to the *E6* promoter to turn off the same culminating in p53-Tip60-mediated HPV18-infected cervical adenocarcinoma cell apoptosis.**

In the present study, using the natural plant polyphenol curcumin, a potent anticancer agent (22, 23, 25), we have demonstrated the restoration of the apoptotic program in HPV18-infected cervical adenocarcinoma cells. Curcumin-mediated apoptosis of cervical adenocarcinoma cells relies on its ability to up-regulate SMAR1, which in turn causes transcriptional repression of HPV18 *E6*. In general, SMAR1 ensures the recruitment of HDAC1-dependent repressor complex at the LCR and E6 coding regions of the *E6* promoter that deacetylates chromatin histones to restrict binding of its transcriptional activator, c-Fos to its putative AP-1-binding sites. As a result, *E6* transcription is repressed, thereby restoring p53. On the other hand, E6 depletion stalls degradation of the acetyl transferase Tip60 to reinstall p53-mediated apoptotic program in HPV18-infected tumors. Cumulatively, we establish a critical role of curcumin-induced SMAR1 in repressing the viral oncogene E6 and thereby inducing apoptosis in HPV18-infected cervical adenocarcinoma.

Because *E6*-suppression is dependent on SMAR1, loss of SMAR1 in these HPV18 cells is possibly linked with the up-regulated levels of E6 in executing cervical tumor progression. We observed that curcumin re-establishes the p53-SMAR1-positive feedback tumor suppressor loop in these cervical adenocarcinoma cells. Although on one hand, curcumin-induced SMAR1depletes E6, thereby rescuing p53 from E6-mediated degradation, on the other hand, it stabilizes and activates p53 by phosphorylation at Ser-15 as reported previously (17, 18). Activated p53 in turn augments SMAR1 transcriptionally. This p53-SMAR1-positive feedback loop therefore helps maintaining SMAR1 expression continually, thereby leading to repression of *E6* and p53-mediated apoptosis of HPV-infected cervical adenocarcinoma cells.

To understand the complete mechanism of SMAR1-induced repression of E6 transcription, we considered the possible role of the transcription factor, AP-1, because it transcriptionally activates both the viral proto-oncogenes, E6 and E7 (8, 9). The present study illustrates that SMAR1 escalation by curcumin treatment or SMAR1-cDNA transfection down-regulated E6 by inhibiting c-Fos recruitment to AP-1 sites on *E6* promoter. Further search for the detailed mechanism revealed the presence of overlapping SMAR1- and c-Fos-binding sites on the *E6* promoter, as a result of which curcumin-induced SMAR1-HDAC1 complex recruitment to the *E6* promoter hindered the binding of c-Fos to its AP-1-binding sites on the *E6* promoter, thereby causing transcriptional repression of *E6*. In addition, SMAR1-mediated recruitment of HDAC1 to the *E6* promoter led to the deacetylation of local histones. The resultant change in chromatin structure might have hampered any further association of c-Fos to its specific binding sites, thereby enforcing transcriptional repression of the HPV18 *E6*. Our results not only verified the association of SMAR1 and HDAC1 but also binding of HDAC1 exactly on the SMAR1-binding site on *E6* promoter. It was also perceived that SMAR1-HDAC1 recruitment to these SMAR-binding sites modulates the chromatin structure to finally hinder the access of transcription factors like c-Fos on *E6* promoter.

In keeping with previous reports (42), our results revealed curcumin-mediated down-regulation of the transcription coactivator p300, which is required for both c-Fos- and p53-dependent transcription (9, 43). These results not only ruled out the involvement of CBP/p300 in p53-dependent apoptosis pathways in HeLa cells in which curcumin up-regulated SMAR1 to ensure p53 functionality but also demanded justification for the counteracting activity of curcumin in c-Fos and p53 functions. At this juncture, our search for other probable acetyl transferases revealed an increment in the endogenous tumor suppressor acetyl transferase Tip60 protein expression in the HPV18-infected cervical carcinoma cells under curcumin-treated conditions. It has been reported that whereas Tip60 promotes the proapoptotic Lys-120 acetylation of p53, thereby executing its anti-tumor function (14), it is degraded by the proto-oncogene *E6* in the cervical adenocarcinoma cells (15). In our experimental sets, curcumin-induced repression of *E6* via SMAR1 rescues Tip60 from E6-mediated degradation and promotes acetylation and activation of p53 to initiate downstream apoptotic pathways. These results suggest the presence of a SMAR1-p53-Tip60 synergistic network in repression of *E6* and successive apoptosis of cervical adenocarcinoma.

SMAR1 therefore appears to exert transcriptional repression of HPV promoter through bimodal mechanisms: (i) SMAR1 recruits HDAC1 to its binding sites within *LCR* and *E6* coding region of *E6* promoter to coordinate histone deacetylation and condensation of chromatin, and (ii) such chromatin modulation restricts binding of c-Fos to its putative AP-1-binding site on HPV18 *E6* promoter because the binding probability of SMAR1 is highest around c-Fos-binding sites. These bimodal actions of SMAR1 make it a strong negative regulator of HPV18 *E6* transcription. E6 depletion, in turn, leads to restoration of p53 and Tip60, which act together to reactivate the apoptotic pathways in these cervical adenocarcinoma cells to eventually lead to apoptosis (Fig. 6).

Our studies presented here strongly suggest p53-SMAR1-positive feedback loop as the likely targeting candidate that may inhibit the viral transcription as well as the aggressiveness of HPV18-infected cervical adenocarcinoma. However, further studies are needed to determine the complete cellular network that coordinates SMAR1-induced transcriptional repression of the HPV oncogenic and malignancy networks.

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