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Curcumin Differentially Regulates Endoplasmic Reticulum Stress through Transcriptional Corepressor SMILE (Small Heterodimer Partner-interacting Leucine Zipper Protein)-mediated Inhibition of CREBH (cAMP Responsive Element-binding Protein H)*

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Background: Curcumin has been reported to play an important role in ER stress.
Results: Curcumin blocks the CREBH-mediated transactivation of target gene, whereas it has no such effect on ATF6-mediated transactivation.
Conclusion: Curcumin differentially regulates ER stress-induced genes.
Significance: Curcumin may provide a way to ameliorate ER stress.

Curcumin (diferuloylmethane), a major active component of turmeric (*Curcuma longa*), is a natural polyphenolic compound. Herein the effect of curcumin on endoplasmic reticulum (ER) stress responsive gene expression was investigated. We report that curcumin induces transcriptional corepressor small heterodimer partner-interacting leucine zipper protein (SMILE) gene expression through liver kinase B1 (LKB1)/adenosine monophosphate-activated kinase (AMPK) signaling pathway and represses ER stress-responsive gene transcription in an ER-bound transcription factor specific manner. cAMP responsive element-binding protein H (CREBH) and activating transcription factor 6 (ATF6) are both ER-bound bZIP family transcription factors that are activated upon ER stress. Of interest, we observed that both curcumin treatment and SMILE overexpression only represses CREBH-mediated transactivation of the target gene but not ATF6-mediated transactivation. Knockdown of endogenous SMILE significantly releases the inhibitory effect of curcumin on CREBH transactivation. Intrinsic repressive activity of SMILE is observed in the Gal4 fusion system, and the intrinsic repressive domain is mapped to the C terminus of SMILE spanning amino acid residues 203–269, corresponding to the basic region leucine zipper (bZIP) domain. *In vivo* interaction assay revealed that through its bZIP domain, SMILE interacts with CREBH and inhibits its transcriptional activity. Interestingly, we observed that SMILE does not interact with ATF6. Furthermore, competition between SMILE and the coactivator peroxisome proliferator-activated receptor α (PGC-1α) on CREBH transactivation has been demonstrated *in vitro* and *in vivo*. Finally, chromatin immunoprecipitation assays revealed that curcumin decreases the binding of PGC-1α and CREBH on target gene promoter in a SMILE-dependent manner. Overall, for the first time we suggest a novel phenomenon that the curcumin/LKB1/AMPK/SMILE/PGC1α pathway differentially regulates ER stress-mediated gene transcription.

Curcumin, a polyphenol (diferuloylmethane), possesses a diverse range of molecular targets; among them are transcription and growth factors, cytokines, enzymes and genes regulating cell proliferation, and apoptosis (1, 2). The pharmacological safety of curcumin is well demonstrated by the fact that people in certain countries have consumed curcumin as a dietary spice for centuries in excess of 100 mg/day without any side effects (1). Ample evidence exists to support its use in cancer prevention for its antiproliferative and anticarcinogenic properties or as an adjunct in overall cancer treatment (1). Curcumin is a potent inhibitor of the initiation and promotion of chemical carcinogen-induced skin carcinogenesis in mice (1). Curcumin blocks the transformation, proliferation, and invasion of tumor

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cells (2). Curcumin suppresses the growth of several tumor cell lines, including drug-resistant lines (2). It suppresses the expression of cyclin D1, which is deregulated in a wide variety of tumors (2). Various transcription factors are strongly inhibited by curcumin, including nuclear factor κB, signal transducer and activator of transcription proteins, activated protein-1 (1–3), β-catenin, and peroxisome proliferator-activated receptor-γ (4).

AMPK[2] is an important integrator of signals that control cellular energy balance through the regulation of multiple biochemical pathways. The activation of AMPK requires phosphorylation of Thr-172 in the activation loop of the catalytic α-subunit (5). Recent studies have identified LKB1 (serine/threonine kinase 11) and Ca^{2+}/calmodulin-dependent protein kinase kinase-β as two kinases that phosphorylate Thr-172 (6). In resting cells, LKB1 is reported to be predominantly located in the nucleus (7). Recent evidence suggests that phosphorylation of LKB1 at Ser-428 increases export of LKB1 from the nucleus and influences the ability of LKB1 to bind and phosphorylate AMPK at Thr-172 (8).

SMILE belongs to the bZIP family (9, 10). SMILE gene produces two isoforms, SMILE-L (long isoform of SMILE, also known as CREBZF) and SMILE-S (short isoform of SMILE, previously known as Zhangfei), from alternative usage of initiation codons (10). Although SMILE has the ability to homodimerize like other bZIP proteins, it cannot bind to DNA as a homodimer (9–11). SMILE has been reported as a coactivator of ATF4 and as a corepressor of CREB3, a cellular host cell factor-binding transcription factor (12). Previously, we have reported that SMILE can act as a transcriptional co-repressor of nuclear receptors glucocorticoid receptor, constitutive androstane receptor, and hepatocyte nuclear factor 4α (13). SMILE recruits SIRT1, a class III histone deacetylase, to inhibit nuclear receptor ERRγ transactivity (14).

ER membrane-bound bZIP transcription factors, such as ATF6, Luman, OASIS, and CREBH constitute a novel class of factors that are regulated by ER stress-dependent mechanisms (15–18). Activated ATF6 and CREBH are responsible for unfolded protein response-mediated activation of target genes such as glucose-regulated protein 78 (GRP78), CHOP, XBP-1 and serum amyloid P-component (SAP), C-reactive protein (CRP), and hepcidin (19–23). CREBH plays an important role in controlling iron metabolism in liver (23). Both CREBH and ATF6 have been reported to regulate glucose metabolism by regulating phosphoenolpyruvate carboxykinase and glucose 6-phosphatase expression (24, 25). Tunicamycin (Tm) is a well known ER stress inducer that has been used to activate CREBH, ATF6, and other ER stress-regulated chaperon proteins (22, 23, 26).

In this current study we demonstrate that curcumin activates LKB1 and decreases the cellular ATP level that activates AMPK. Activation of AMPK in turn leads to the induction of SMILE gene expression. Through its bZIP domain, SMILE specifically interacts with CREBH and not with ATF6 and inhibits CREBH transcriptional activity via competition with co-activator PGC1α, thereby highlighting a novel mechanism of differential repressive action of curcumin on ER stress-regulated genes.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Curcumin, (1*E*,6*E*)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, was obtained from Sigma. Tunicamycin was obtained from Calbiochem. Wortmannin and U0126 were from Sigma; SB203580, SP600125, and compound C were from Calbiochem. The reporter plasmids hSMILE-Luc, Gal4-tk -Luc (Gal4-DBD (UAS)-TK-Luc vector for mammalian one-hybrid assay), and 5XATF6-Luc (14, 28, 29) were described previously. Mouse hepcidin promoter (−982/+84) was constructed by PCR amplification from mouse genomic DNA (Novagen) and inserted into the pGL3 basic vector (Promega) using MluI and HindIII restriction enzyme sites. pcDNA3-dnAMPKα (30) and Gal4 DBD (28), expression vector for CREBH (24), FLAG SMILE, HA-PGC1α, pcDNA3-ERRγ, pSUPER, and pSUPER-siSMILE (14), the SMILE leucine zipper region mutant SMILE-L(239–267)V (13), SMILE deletion constructs SMILE-L, SMILE-S, SMILE-(203–354), and SMILE-(269–354), were described previously (13). To generate deletion constructs CREBH-(1–270), CREBH-(1–195), CREBH-(240–320), the fragments were generated by PCR and subcloned into the EcoR1 and Xba1 sites of the FLAG-tagged pcDNA3. All plasmids were confirmed by DNA sequencing analysis.

Cell Culture and Transient Transfection Assay—HepG2 (human hepatoma cell line), H4IIE (rat hepatoma cell line), and AML12 (mouse hepatoma cell line) cells were obtained from the American Type Culture Collection. Maintenance of cell lines and transient transfections were performed as described previously (30).

Preparation of Recombinant Adenovirus—For ectopic expression of the genes, the adenoviral delivery system was used. Adenoviruses (Ad) encoding c-Myc-tagged DN-AMPK and constitutively active-AMPK (30), adenovirus-encoding human SMILE (10), and adenovirus-encoding CREBH (24) were described previously, and adenovirus-encoding shSMILE was prepared as follows. Briefly, the shSMILE (AAGGCGTCGTCGTCTCTTAAA) constructs were constructed with 21-mer double-stranded oligonucleotide containing +1053 to +1074 of the SMILE cDNA sequence into the pBS/U6 vector. The cDNA-encoding shSMILE was cloned into the pAdTrack-CMV vector. The recombination of the pAdTrack-CMV-shSMILE with adenoviral gene carrier vector was performed by transformation to pretransformed adEasy-BJ21-competent cells. The primers used for shSMILE construction are as follows: h/mSMILE forward (5′-AAGGCGTCGTCGTCTCTTAAAAAGCTTTTTAAGAGACGACGACGCCTTTTTTTGC-3′) and reverse (5′-GGCCGCAAAAAAAGGCGTCGTCGTCTCTTAAAAAGCTTTTTAAGAGACGACGACGCCTT-3′).

[2] The abbreviations used are: AMPK, AMP-activated kinase; ER, endoplasmic reticulum; SMILE, small hetero dimer partner interacting leucine zipper protein; LKB1, liver kinase B1; CREBH, cAMP responsive element-binding protein H; ATF6, activating transcription factor 6; bZIP, basic region leucine zipper; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1 α; GRP78, glucose-regulated protein 78; Tm, tunicamycin; DBD, DNA binding domain; Ad, adenovirus; DN, dominant negative; m.o.i., multiplicity of infection; RLU, relative light units; ERRγ, estrogen-related receptor gamma.

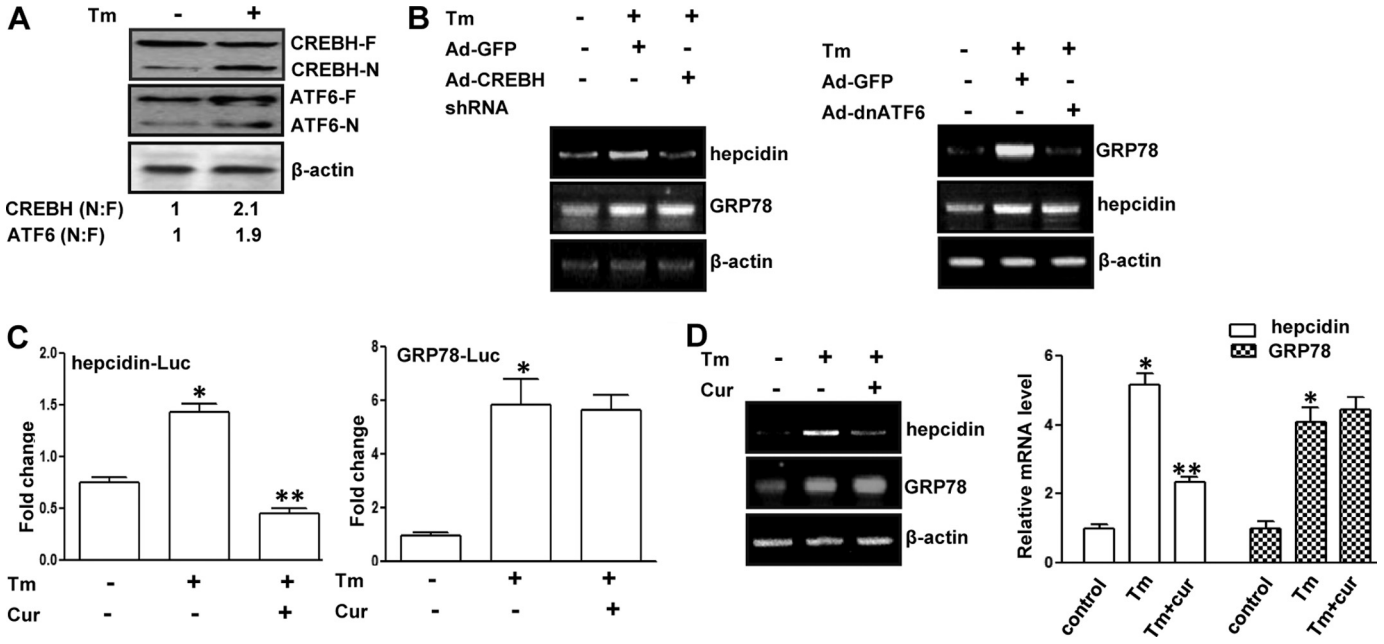

FIGURE 1. **Curcumin differentially inhibits ER stress-regulated gene expression.** *A*, HepG2 cells were treated with tunicamycin (5 μg/ml) for 12 h. Protein was isolated for Western blot. CREBH and ATF6 full-length (*CREBH-F* and *ATF6-F*, respectively) and CREBH and ATF6 active form (*CREBH-N* and *ATF6-N*, respectively) expression was checked. *B*, HepG2 cells were infected with Ad-CREBHshRNA (50 m.o.i., *left panel*) and Ad-dnATF6(50 m.o.i., *right panel*) for 48 h followed by tunicamycin treatment (5 μg/ml). RNA was isolated from cells to perform semiquantitative RT-PCR. *C*, HepG2 cells were transfected with mouse hepcidin-Luc (200 ng, *left panel*) and rat GRP78-Luc(200 ng, *right panel*). 24 h after transfection, cells were serum-starved for further 24 h followed by tunicamycin treatments (5 μg/ml) for 12 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$, compared with untreated control and tunicamycin-treated cells, respectively. *D*, HepG2 cells were serum-starved for a further 24 h followed by tunicamycin treatment (5 μg/ml) for 12 h followed by curcumin treatment (10 μM) for 12 h. Total RNA was isolated for RT-PCR analysis of hepcidin and GRP78 mRNA expression and was normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$, and **, $p < 0.05$ compared with untreated control and tunicamycin-treated cells, respectively.

Adenovirus wild type LKB1 (Ad-LKB1) encoding mouse LKB1 gene and mutant LKB1 (Ad-dnLKB1) were generated with the pAd-easy system as described previously (38). All viruses were purified using $CsCl_2$ or Adeno-X maxi purification kit (Clontech).

RNA Isolation and Analysis—Total RNA was isolated and analyzed by semiquantitative PCR and real time PCR using probes for SMILE, hepcidine, GRP78, and β-actin as described previously (28).

Western Blot Analysis—Cell lysate preparation and Western blot analysis in different cell lines using different antibodies were previously described (28). Antibodies used in this work were as follows: rabbit monoclonal AMPKα (Cell Signaling), rabbit monoclonal phospho-AMPKα(Thr-172) (Cell Signaling), rabbit monoclonal LKB1(Cell Signaling), rabbit monoclonal p-LKB1(S-428) (Cell Signaling), rabbit polyclonal zhangfei (Abcam), anti-HA (12CA5, Roche Applied Science), anti-FLAG M2, anti-GST (Santa Cruz Biotechnology), anti-CREB3L3 (Santa Cruz Biotechnology), and β-tubulin antibodies (Santa Cruz Biotechnology). The primary antibodies were used at a dilution ranging from 1:200 to 1:1000 in Western blot analysis and at a dilution of 1:200 in immunoprecipitation.

ChIP Assay—The ChIP assay was performed according to the manufacturer's protocol (Upstate Biotechnology). Briefly, HepG2 cells were transfected with reporter plasmids and treated as indicated. Cells were then fixed with 1% formaldehyde and harvested. Soluble chromatin was immunoprecipitated with goat polyclonal CREB3L3 (Santa Cruz) and rabbit polyclonal PGC1α antibodies (Santa Cruz). Upon DNA recovery, real-time quantitative PCR was performed using primers encompassing the mouse hepcidin promoter, forward (5′-CCAACATGACAGCTACATC-3′) and reverse (5′-CCTTTACCCCAGAACTGTA-3′).

Statistical Analysis—Data are expressed as the means ± S.D. Statistical analysis was performed using Student's *t* test. All experiments were performed at least three times. Differences were considered significant at $p < 0.05$.

RESULTS

Curcumin Differentially Inhibits ER Stress-regulated Gene Expression—It was previously reported that tunicamycin activates both CREBH and ATF6 protein (21, 22). HepG2 (human hepatoma cell line) cells were treated with tunicamycin followed by Western blot analysis. A significant increase of the active form of both CREBH and ATF6 protein level was observed after tunicamycin treatment (Fig. 1*A*). It was previously reported that tunicamycin, through CREBH and ATF6, regulates hepcidin and GRP78 gene transcription, respectively (23, 26). HepG2 cells were infected with adenovirus CREBH shRNA (Ad-shCREBH) and adenovirus dominant negative ATF6 cDNA (Ad-dnATF6) to knock down endogenous CREBH and to block endogenous ATF6, respectively. Tunicamycin-mediated induction of both hepcidin and GRP78 gene transcription was significantly diminished after CREBH and ATF6 knockdown, respectively (Fig. 1*B*). According to previous reports, tunicamycin activated hepcidin promoter (23) and

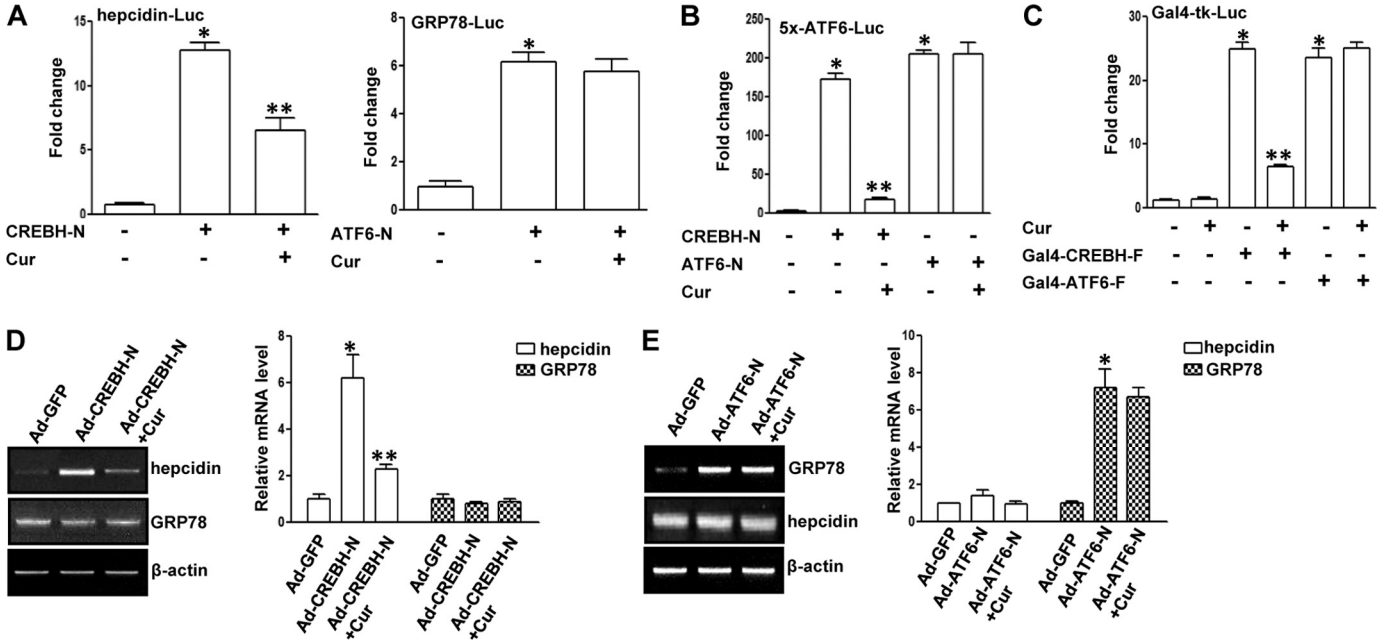

FIGURE 2. **Curcumin inhibits transcriptional activity of CREBH and not ATF6.** *A*, HepG2 cells were transfected with hepcidin-Luc (200 ng, *left panel*) or GRP78-Luc (200 ng, *right panel*) along with CREBH (200 ng, *left panel*) or ATF6 (200 ng, *right panel*), respectively. 24 h after transfection cells were serum-starved for a further 24 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ compared with untreated control and CREBH-N (*left panel*)/ATF6-N (*right panel*)-treated cells, respectively. *B*, HepG2 cells were transfected with 5xATF6-Luc (200 ng) along with CREBH (200 ng) or ATF6 (200 ng). 24 h after transfection, cells were serum-starved for a further 24 h followed by curcumin treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ compared with untreated control and CREBH-N-treated cells, respectively. *C*, HepG2 cells were transfected with pFR-Luc (200 ng) along with Gal4-CREBH (200 ng) or Gal4-ATF6 (200 ng). 24 h after transfection cells were serum-starved for a further 24 h followed by curcumin treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ compared with untreated control and Gal4-CREBH-F-treated cells, respectively. *D*, HepG2 cells were infected with Ad-CREBH-N (50 m.o.i.) for 48 h followed by curcumin treatment (10 μM) for 12 h. RNA was isolated from cells to perform semiquantitative RT-PCR analysis of hepcidin and GRP78 mRNA expression and was normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$, and **, $p < 0.05$ compared with Ad-GFP and Ad CREBH-N-treated cells, respectively. *E,* HepG2 cells were infected with Ad-ATF6-N (50 m.o.i.) for 48 h followed by curcumin treatment (10 μM) for 12 h. RNA was isolated from cells to perform semiquantitative RT-PCR analysis of hepcidin and GRP78 mRNA expression and was normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$ compared with Ad-GFP treated cells, respectively.

GRP78 promoter (26).To evaluate the potential role of curcumin on hepcidin and GRP78 promoter activity, a transient transfection assay was performed in HepG2 cells with hepcidin and GRP78 promoter. Curcumin treatment significantly decreased tunicamycin-stimulated hepcidin promoter activity, although no significant change was observed for GRP78 promoter activity after curcumin treatment (Fig. 1*C*). To further elucidate the role of curcumin in hepcidin and GRP78 gene transcription, HepG2 cells were treated with tunicamycin followed by curcumin treatment. Curcumin significantly decreased tunicamycin-stimulated hepcidin expression at the mRNA level, whereas no significant change in GRP78 mRNA level was observed after curcumin treatment (Fig. 1*D*). Taken together, these results clearly indicate that curcumin represses CREBH-stimulated gene transcription but not ATF6-mediated gene transcription.

Curcumin Inhibits Transcriptional Activity of CREBH and Not ATF6—In the aforementioned results, it was clear that curcumin inhibits CREBH target gene transcription but not ATF6 target gene transcription. To assess whether curcumin has any role in controlling the transactivation of the transcription factors, CREBH and ATF6, a transient transfection assay was performed in HepG2 cells with hepcidin or GRP78 promoter along with CREBH or ATF6 expression vector followed by curcumin treatment. Curcumin significantly inhibited transactivation of CREBH on hepcidin promoter, although no significant inhibition of ATF6 transactivation on GRP78 promoter was observed (Fig. 2*A*). To confirm this differential regulatory effect of curcumin on CREBH and ATF6 transactivation, a reporter gene containing multiple copies of the an ATF6 binding site, 5X-ATF6-Luc (activated by both CREBH and ATF6), was used along with CREBH and ATF6 expression vector in a transient transfection assay in HepG2 cells. As expected from the previous results, curcumin significantly inhibited CREBH-mediated transactivation of the reporter gene, whereas no inhibition was noted for ATF6 transactivation (Fig. 2*B*).

To further confirm these results, transient transfection was performed with Gal4-tk-Luc in HepG2 cells. Consistent with the previous findings, activation of the reporter gene by CREBH, but not by ATF6, was significantly repressed by curcumin, and no such inhibitory effect of curcumin was observed for ATF6 transactivation of the reporter gene (Fig. 2*C*). Next, to determine whether curcumin controls the hepcidin and GRP78 gene transcription, HepG2 cells were infected with adenovirus CREBH (Ad-CREBH) and adenovirus ATF6 (Ad-ATF6) followed by curcumin treatment. Ad-CREBH-mediated overexpression of hepcidin gene was significantly inhibited by curcumin, whereas curcumin treatment had no significant effect on Ad-ATF6-mediated overexpression of

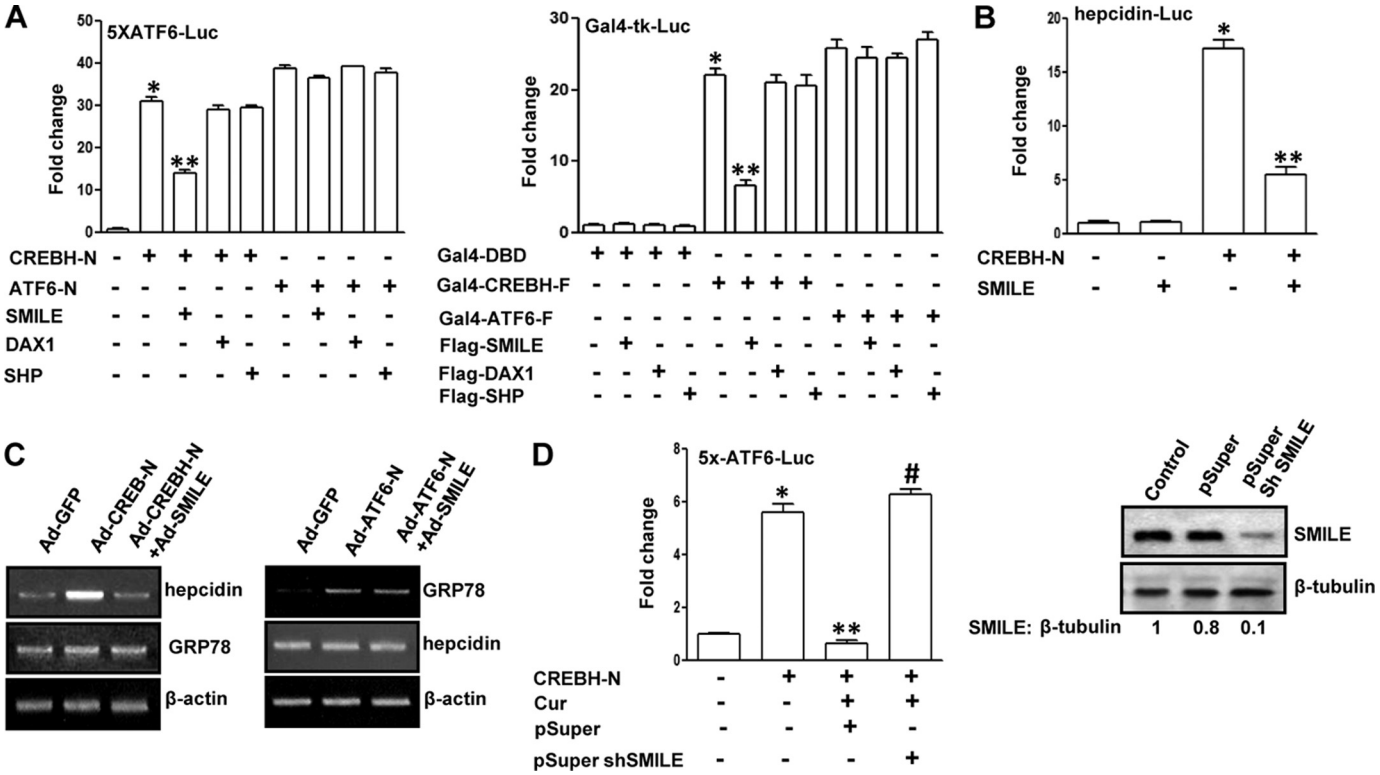

FIGURE 3. **Transcriptional co-repressor SMILE inhibits CREBH but not ATF6.** *A*, HepG2 cells were transfected with 5xATF6-Luc (200 ng) along with CREBH (200 ng) or ATF6 (200 ng) and SMILE (200 ng) or DAX1 (200 ng) or SHP (200 ng) (*left panel*), and HepG2 cells were transfected with pFR-Luc (200 ng) along with Gal4-DBD (200 ng) or Gal4-CREBH (200 ng) or Gal4- ATF6 (200 ng) and FLAG SMILE or FLAG DAX1 or FLAG SHP (*right panel*). Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing the mean ± S.D. of three individual experiments. *, $p <$ 0.05 and **, $p < 0.05$ compared with untreated control and CREBH-N (*left panel*)-treated cells, respectively, and *, $p < 0.05$ and **, $p < 0.05$ compared with Gal4-DBD-treated cells and Gal4-CREBH-F (*right panel*)-treated cells, respectively. *B*, HepG2 cells were co-transfected with mouse hepcidin-luc (200 ng) and CREBH (300 ng) or/and SMILE (300 ng). Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ compared with untreated control and CREBH-N-treated cells, respectively. *C*, HepG2 cells were infected with Ad-CREBH (50 m.o.i., *left panel*) or Ad-ATF6 (50 m.o.i, *right panel*) along with Ad-SMILE (50 m.o.i) for 48 h. RNA was isolated from cells to perform semiquantitative RT-PCR. *D*, *left panel*, HepG2 cells were transfected with 5xATF6-Luc (200 ng) along with CREBH (200 ng) and or shSMILE (200 ng). 24 h after transfection, cells were serum-starved for a further 24 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control representing the mean ± S.D. of three individual experiments. *, $p < 0.05$; **, $p < 0.05$; #, $p < 0.05$ compared with untreated control and CREBH-N-treated and CREBH-N plus cur plus pSuper-treated cells, respectively. *Right panel*, HepG2 cells were transfected with pSuper only or pSuper shSMILE, and after 72 h total protein was isolated. The protein expression of SMILE was detected by Western blot analysis. Results are representative of three experiments.

GRP78 gene transcription (Fig. 2*D*). Overall, these results suggest that curcumin specifically inhibits ER-bound transcription factor CREBH-mediated transactivation of its target gene but not ATF6-mediated transactivation.

Transcriptional Co-repressor SMILE Inhibits CREBH and Not ATF6—The inhibition of transcription factors by transcriptional co-repressors is a well known phenomenon in cellular systems. DAX1, SMILE, and SHP are notable transcriptional co-repressors that were previously reported to be involved in the cellular repression of transcription factors (13, 27, 28). To assess the role of these transcriptional co- repressors in our current study, a reporter assay was performed with the reporter gene, 5X-ATF6-Luc, and vectors expressing CREBH or ATF6 along with the co-repressor expression vectors listed above. We observed that among DAX1, SMILE, and SHP, only SMILE could significantly inhibit transcriptional activity of CREBH, and of great interest, SMILE did not repress ATF6 transactivity in a similar way as curcumin (Fig. 3*A*, *left panel*). To confirm this result, the transcriptional activities of SMILE, DAX1, and SHP were further investigated (Fig. 3*A*, *right panel*). The reporter plasmid Gal4-tk-Luc and indicated expression vectors encoding Gal4-DBD alone, Gal4-CREBH, or Gal4-ATF6 were cotransfected with the expression vectors of SMILE, DAX1, and SHP into HepG2 cells.

As expected, only SMILE significantly repressed CREBH transactivity. As SMILE was found to be involved in the repression of CREBH transactivity, we next investigated whether SMILE could repress CREBH transactivity on the hepcidin promoter. A transient transfection assay was performed with hepcidin promoter along with CREBH and SMILE expression vector in HepG2 cells. Results clearly demonstrated that SMILE significantly inhibits CREBH transactivation on hepcidin promoter (Fig. 3*B*).

To investigate the effect of SMILE overexpression on CREBH and ATF6 target gene transcription, HepG2 cells were infected with Ad-CREBH or Ad-ATF6 alone or with adenovirus expressing SMILE, Ad-SMILE. Interestingly Ad-SMILE significantly repressed Ad-CREBH-induced hepcidin mRNA levels (Fig. 3*C*, *left panel*), whereas Ad-SMILE had no significant effect on Ad-ATF6-induced GRP78 mRNA levels (Fig. 3*C*, *right panel*). As the results clearly indicated that SMILE could be involved in the inhibitory effect of curcumin on CREBH, we

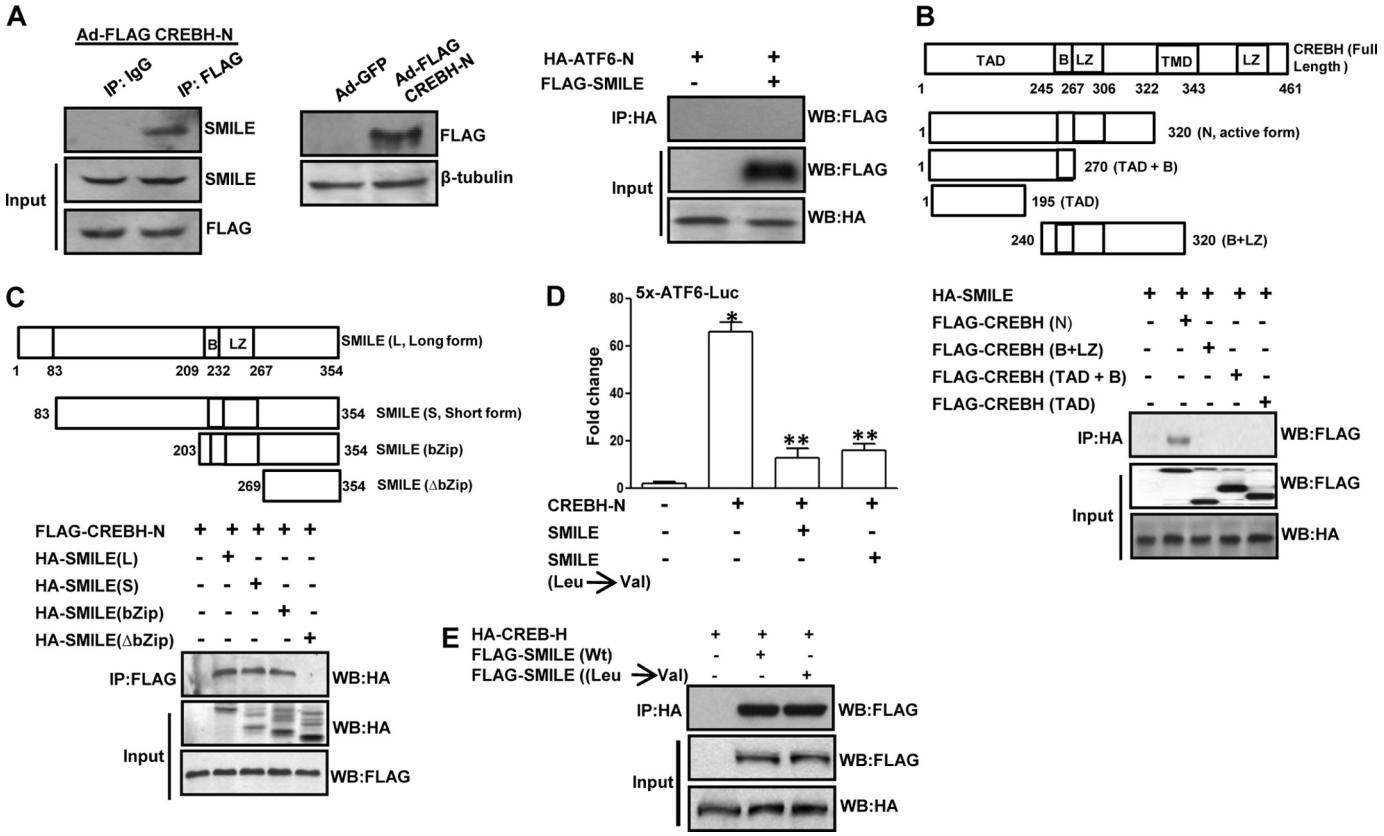

FIGURE 4. **SMILE interacts and inhibits CREBH.** *A*, *left panel*, co-immunoprecipitation assays were performed with cell extracts from HepG2 cells after Ad-FLAG CREBH infection. Endogenous SMILE was immunoprecipitated (*IP*) with CREBH and analyzed by Western blot (*WB*) using indicated antibodies. *Middle panel*, shown is a Western blot analysis after Ad-GFP and Ad-FLAG CREBH infection in HepG2 cells. *Right panel*, 293T cells were co-transfected with the mammalian expression vector encoding either HA-ATF6 alone or with FLAG-SMILE. Lysates were immunoprecipitated with HA antibody, and we performed Western blotting using indicated antibodies. *B*, shown is a schematic representation of wild type and deletion constructs of CREBH (*upper panel*). *TAD*, transactivation domain; *B*, basic domain; *LZ*, leucine zipper domain; *TMD*, transmembrane domain. *Lower panel*, 293T cells were cotransfected with the mammalian expression vector encoding either HA-SMILE alone or with indicated construct of CREBH. Lysates were immunoprecipitated with HA antibody and Western blotting was performed using indicated antibodies. *C*, shown is a schematic representation of wild type and deletion constructs of SMILE (*upper panel*), and (*lower panel*) 293T cells were cotransfected with the mammalian expression vector encoding either FLAG-CREBH alone or with indicated construct of HA-SMILE. Lysates were immunoprecipitated with FLAG antibody, and we performed Western blotting using indicated antibodies. *D*, 293T cells were transfected with 5xATF6-Luc (200 ng) along with CREBH (200 ng) and SMILE (200 ng) or mutant form (leucine to valine). Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$, compared with untreated control and CREBH-N-treated cells, respectively. *E*, 293T cells were cotransfected with the mammalian expression vector encoding HA-CREBH alone or with FLAG-SMILE (wild type) or with FLAG-SMILE mutant (Leu to Val). Lysates were immunoprecipitated with HA antibody and were analyzed by Western blot using indicated antibodies.

investigated the effect of curcumin on CREBH transactivation after knocking down the endogenous SMILE by siRNA (siSMILE). A transient transfection assay was performed in HepG2 cells with the reporter gene, 5X-ATF6-Luc, along with CREBH and siSMILE expression vectors. As expected, after knocking down endogenous SMILE, curcumin no longer inhibited the transcriptional activity of CREBH on the reporter gene (Fig. 3*D*). These results clearly demonstrate the involvement of SMILE as a co-repressor in curcumin-mediated repression of the transcription factor CREBH.

SMILE Interacts and Inhibits CREBH—To further verify the involvement of SMILE in the repression of CREBH by curcumin, Ad-FLAG CREBH-N (N, active form of CREBH) was overexpressed in HepG2 cells (Fig. 4*A*, *left* and *middle panel*). Lysates were immunoprecipitated with FLAG antibody followed by Western blot analysis with indicated antibodies. We observed that CREBH interacted with endogenous SMILE. Our previous results described that SMILE did not repress ATF6 transactivity. To examine whether SMILE interacts with ATF6, we overexpressed SMILE and ATF6 in 293T cells followed by immunoprecipitation and Western blot analysis with the indicated antibodies. As expected, no significant interaction was observed between SMILE and ATF6 (Fig. 4*A*, *right panel*). Next, to determine the domains of CREBH (Fig. 4*B*) and SMILE (Fig. 4*C*) involved in this interaction, several deletion constructs of CREBH and SMILE, along with the wild type forms, were transfected in 293T cells. Interestingly we found that the bZIP domain of SMILE interacted with the whole active form of CREBH, which clarified the involvement of bZIP domain of SMILE in the interaction with CREBH. To determine whether SMILE homodimerization is required for this function, a transient transfection assay was performed in 293T cells with 5XATF6-Luc along with CREBH and wild type or mutant forms of SMILE, where all the leucine residues in the bZIP domain were converted to valine, thus rendering this mutant form of SMILE incapable of dimerization. Of interest, the mutant form was equally capable of inhibiting CREBH transcriptional activity as the wild type SMILE (Fig. 4*D*). As the SMILE mutant

form was capable of inhibiting CREBH transactivity like the wild type SMILE, we overexpressed CREBH along with the wild type SMILE or mutant SMILE in 293T cells followed by immunoprecipitation and Western blot analysis. As expected (from Fig. 4*D*), both the wild type and the mutant SMILE almost equally interacted with CREBH (Fig. 4*E*). These results clearly indicated that SMILE, without being homodimerized, interacts with CREBH through its bZIP domain and represses it.

Competition between SMILE and PGC-1α on CREBH Transactivation—Previously our laboratory demonstrated that SMILE inhibited the transactivation of another well known transcription factor, ERRγ, via competition with transcriptional co-activator PGC1α (14). To determine whether PGC1α is a main target for SMILE-dependent repression of CREBH activity, an interaction study of PGC1α with CREBH was performed. The results clearly indicated that CREBH interacted with PGC1α both *in vitro* (Fig. 5*A*) and *in vivo* (Fig. 5*B*) conditions. To confirm this interaction, expression vectors for PGC-1α, SMILE, and CREBH active form were introduced into 293T cells along with 5xATF6-Luc reporter (Fig. 5*C*). As expected, PGC-1α co-expression further stimulated CREBH transactivation, and overexpression of SMILE repressed this induction in a dose-dependent manner. In a reciprocal experiment, overexpression of PGC-1α released the inhibitory effect of SMILE on CREBH in a dose-dependent manner. To confirm if SMILE inhibits the interaction between PGC1α and CREBH, a transient transfection assay was performed with FLAG-CREBH, HA-PGC1α, and GST-SMILE expression vectors in 293T cells followed by immunoprecipitation with FLAG antibody and Western blot. SMILE dose-dependently decreased the interaction between CREBH and PGC-1α (Fig. 5*D*). Taken together, our results reveal that SMILE interacted with CREBH via competition with PGC1α to inhibit CREBH transcriptional activity, but it did not interact with ATF6.

Curcumin Induces SMILE Gene Expression—From the previous results it was clear that SMILE was involved in the inhibition of CREBH transactivation by curcumin. To elucidate the role of curcumin on SMILE gene expression, HepG2, AML12 (mouse hepatoma cell line), and H4IIE (rat hepatoma cell line) cells were treated with curcumin in a dose- and time-dependent manner. Both SMILE mRNA (data not shown) and protein levels were significantly increased by curcumin treatment in both a dose- and time-dependent manner (Fig. 6*A*). In an attempt to determine whether the increase of SMILE mRNA level by curcumin treatment was attributable to the increase in transcription or protein synthesis, HepG2 cells were pretreated with the transcription inhibitor actinomycin D preceding curcumin treatment. This resulted in a drastic decrease in SMILE mRNA levels. However, the protein synthesis inhibitor cycloheximide showed no significant effect on curcumin-induced SMILE mRNA levels, thereby suggesting that curcumin induces SMILE gene expression at the transcriptional level and does not require *de novo* protein synthesis (Fig. 6*B*). It has previously been reported that nuclear receptor ERRγ activates SMILE promoter (14). To determine whether curcumin regulates SMILE gene promoter activity, a transient transfection assay was performed in 293T cells with SMILE promoter using ERR γ as a positive control. Curcumin treatment resulted in an increase in SMILE gene promoter activity in a dose-dependent manner (Fig. 6*C*). Overall, these results clearly demonstrate that curcumin activates SMILE promoter and induces SMILE gene expression.

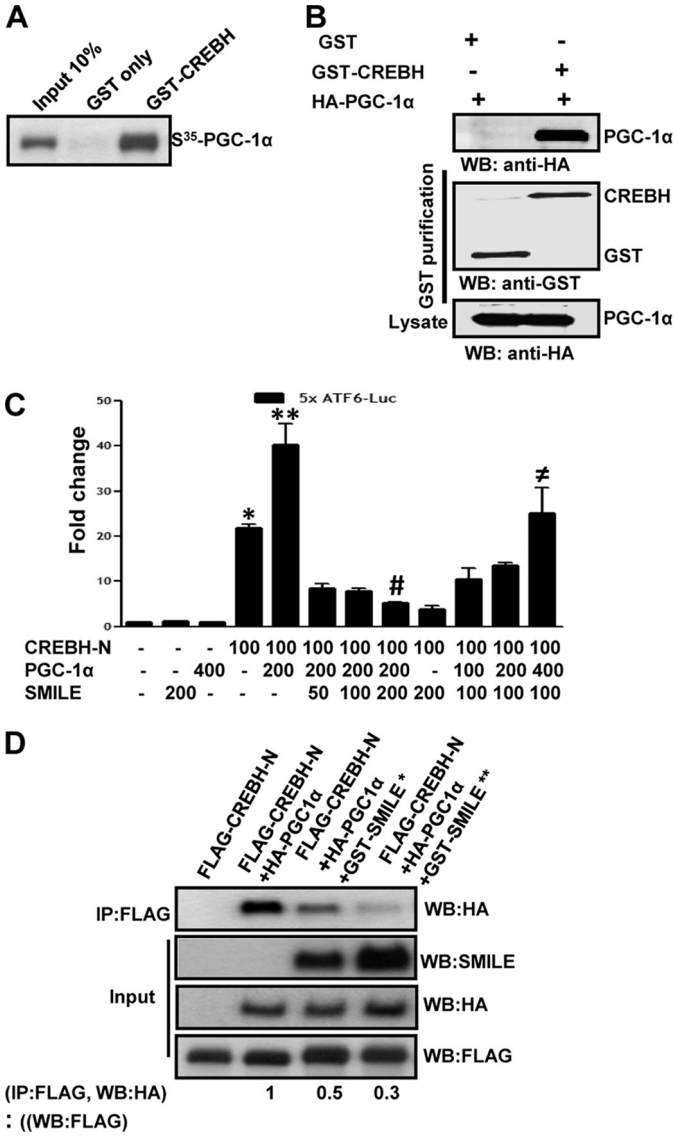

FIGURE 5. **Competition between SMILE and PGC-1α on CREBH transactivation.** *A*, ^{35}S-radiolabeled PGC1α protein was incubated with GST or GST-CREBH fusion proteins. The *input lane* represents 10% of the total volume of *in vitro* translated proteins used for binding assay. Protein interactions were detected via autoradiography. *B*, *in vivo* interactions of exogenous PGC1α with exogenous CREBH are shown. Cells were cotransfected with expression vectors for HA- PGC1α with GST-CREBH or GST alone. The complex formation (*top panel*) and the amount of HA- PGC1α, used for the *in vivo* binding assay (*bottom panel*, lysate), were determined via Western blot (*WB*) using an anti-HA antibody. The same blot was stripped and reprobed with an anti-GST antibody (*middle panel*) to confirm the expression levels of the GST fusion protein (GST-CREBH) and the GST control. *C*, 293T cells were co-transfected with 5XATF6-Luc (200 ng) and CREBH or PGC1α or SMILE. Experiments were performed in triplicate and represent the mean ± S.D. of three individual experiments. *, $p < 0.001$; **, $p < 0.001$; #, $p < 0.001$; ≠, $p < 0.05$ compared with untreated control, CREBH-N treated, CREBH-N+PGC1α treated, and CREBH-N (100) + PGC1α (200) + SMILE (200)-treated cells, respectively. *D*, 293T cells were co-transfected with the mammalian expression vector encoding FLAG-CREBH, HA- PGC1α, and GST-SMILE in the indicated manner. Lysates were immunoprecipitated (*IP*) with FLAG antibody and Western blot analysis was performed using indicated antibodies.

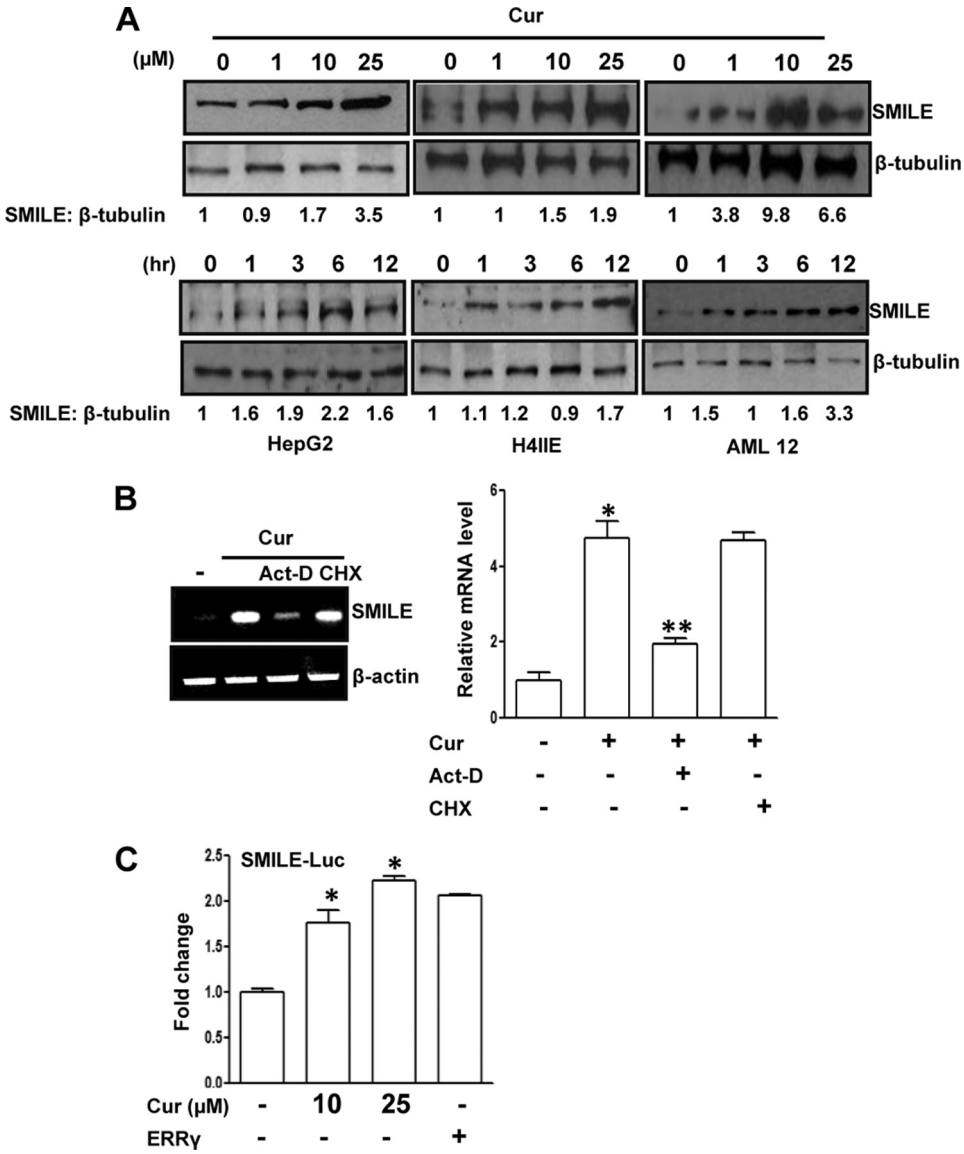

FIGURE 6. **Curcumin induces SMILE gene expression.** *A*, HepG2, H4IIE, and AML12 cells were treated with curcumin (0, 1, 10, 25 μM) for dose course (*upper panel*) and treated with (25 μM) curcumin for 1–24 h for time course (*lower panel*). Protein was isolated for Western blotting. SMILE protein expression was determined. *B*, HepG2 cells were treated with actinomycin D (*Act-D*) or cycloheximide (*CHX*) for 1 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Total RNA was isolated for semiquantitative RT-PCR analysis of SMILE mRNA expression and was normalized to β-actin expression. Data represent the mean $\pm$ S.D. of three individual experiments. *, $p < 0.05$, and **, $p < 0.05$ compared with untreated control and curcumin-treated cells, respectively. *C*, 293T cells were transfected with human SMILE-Luc (200 ng). After 24 h, cells were serum-starved for 24 h followed by curcumin treatment (10 μM) or co-transfection with ERRγ. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing mean $\pm$ S.D. of three individual experiments. *, $p < 0.05$ compared with untreated control cells.

Curcumin Induces SMILE via AMPK Signaling—To evaluate the potential signaling pathways involved in the induction of SMILE gene expression by curcumin, HepG2 cells were pretreated with several specific protein kinase inhibitors followed by curcumin treatment. Semiquantitative PCR analysis indicated that pretreatment of compound C (an AMPK inhibitor) significantly abolished curcumin-mediated SMILE induction. However, no significant effect was observed for SP600125 (a JNK inhibitor), SB203580 (a p38 kinase inhibitor) or U0126 (an ERK inhibitor) on SMILE mRNA expression, although there was a decrease in the case of wortmannin (a PI3 kinase inhibitor), but it was not very significant (Fig. 7*A*). Next, using a transient transfection assay with SMILE-Luc in HepG2 cells, we demonstrated that only compound C pretreatment significantly inhibited curcumin-mediated increase of SMILE promoter activity (Fig. 7*B*), suggesting that AMPK signaling pathway mediates the curcumin effect on SMILE gene expression.

To confirm the involvement of the AMPK signaling pathway in curcumin-mediated SMILE gene regulation, the phosphorylation levels of LKB1 and its direct downstream target AMPK was assessed using immunoblot analysis with antibodies specifically detecting the phosphorylated as well as the total LKB1 and AMPK levels in HepG2 cells (Fig. 7*C*). Curcumin treatment phosphorylated both LKB1 (p-LKB1) and its downstream substrate AMPK (p-AMPKα), confirming that curcumin activates the LKB1/AMPK signaling pathway. To further confirm the role of LKB1 and AMPK in curcumin-mediated induction of SMILE gene expression, we overexpressed LKB1 (*Ad-LKB1*)

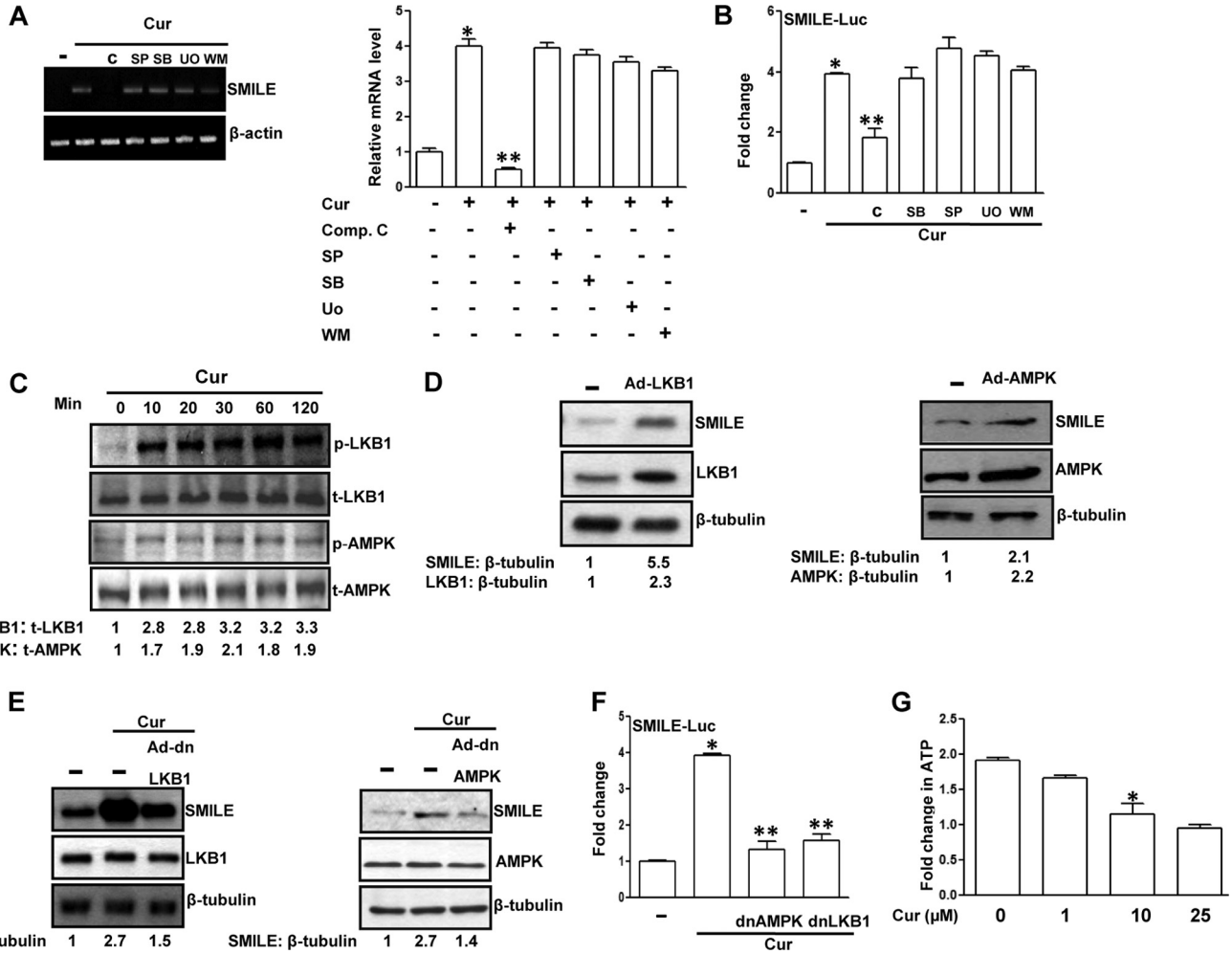

FIGURE 7. **Curcumin induces SMILE via AMPK signaling.** *A*, HepG2 cells were pretreated with the protein kinase inhibitors compound C (*C*, 10 μM), SP600125 (*SP*, 25 μM), SB203580 (*SB*, 25 μM), U0126 (*U0*, 10 μM), and wortmannin (*WM*, 0.1 μM) for 1 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Total RNA was isolated for semiquantitative RT-PCR analysis of SMILE mRNA expression and normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$, and **, $p < 0.05$ compared with untreated control and only curcumin-treated cells, respectively. *B*, HepG2 cells were transfected with SMILE-Luc (200 ng). 24 h after transfection, cells were serum-starved for a further 24 h followed by pretreatment of inhibitors for 1 h preceding curcumin treatments (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing mean ± S.D. of three individual experiments. *, $p < 0.001$ and **, $p < 0.05$ compared with untreated control and curcumin-treated cells, respectively. *C*, HepG2 cells were treated with curcumin (10 μM) and harvested for Western blot analysis using indicated antibodies. Result shown is representative of three independently performed experiments. *D,* HepG2 cells were infected with Ad-LKB1 (50 m.o.i.) (*left panel*) and Ad-AMPKα (50 m.o.i.) (*right panel*) for 48 h and harvested for Western blot analysis using indicated antibodies. Result shown is representative of three independently performed experiments. *E,* HepG2 cells were infected with Ad-dnLKB1 (50 m.o.i.) (*left panel*) and Ad-dnAMPKα (50 m.o.i.) (*right panel*) for 48 h followed by curcumin (10 μM) and harvested for Western blot analysis using indicated antibodies. Result shown is representative of three independently performed experiments. *F*, HepG2 cells were transfected with SMILE-Luc (200 ng) along with dnAMPKα (200 ng) or dnLKB1 (200 ng). 24 h after transfection, cells were serum-starved for a further 24 h, followed by curcumin treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the-fold activation relative to the control, representing mean ± S.D. of three individual experiments. *, $p < 0.001$ and **, $p < 0.05$ compared with untreated control and curcumin-treated cells, respectively. *G*, HepG2 cells were treated with curcumin at indicated concentration for 12 h and harvested to measure the cellular ATP level. Data represent mean ± S.D. of three individual experiments. *, $p < 0.05$ compared with untreated control cells.

(Fig. 7*D*, *left panel*) and constitutively active AMPKα (*Ad-AMPKα*) (Fig. 7*D*, *right panel*) in HepG2 cells, which resulted in an increase of SMILE protein level. These results clearly demonstrated that the LKB1 and AMPK signaling pathway was involved in curcumin-mediated induction of SMILE. To further confirm the role of LKB1 and AMPK, we used adenovirus-mediated overexpression of a dominant negative form of LKB1 (*Ad-dnLKB1*) (Fig. 7*E*, *left panel*) and AMPKα (*Ad-dnAMPKα*) (Fig. 7*E*, *right panel*) in HepG2 cells preceding curcumin treatment. We found that the increase in SMILE protein level by curcumin treatment was significantly decreased upon pretreatment with both Ad-dnLKB1 and Ad-dnAMPKα. Next, we performed a transient transfection assay with SMILE promoter along with dnAMPK or dnLKB1expression vector in HepG2 cells followed by curcumin treatment and found that both significantly inhibited curcumin-mediated activation of the SMILE promoter (Fig. 7*F*). It was previously reported that depletion of cellular ATP levels also leads to the AMPK activation and that curcumin decreases cellular ATP levels, thus changing ATP/AMP ratios (31). Consistent with this previous finding, we observed that curcumin treatment significantly decreased cellular ATP levels (Fig. 7*G*). Collectively, these results suggest that curcumin activates LKB1 and decreases cellular

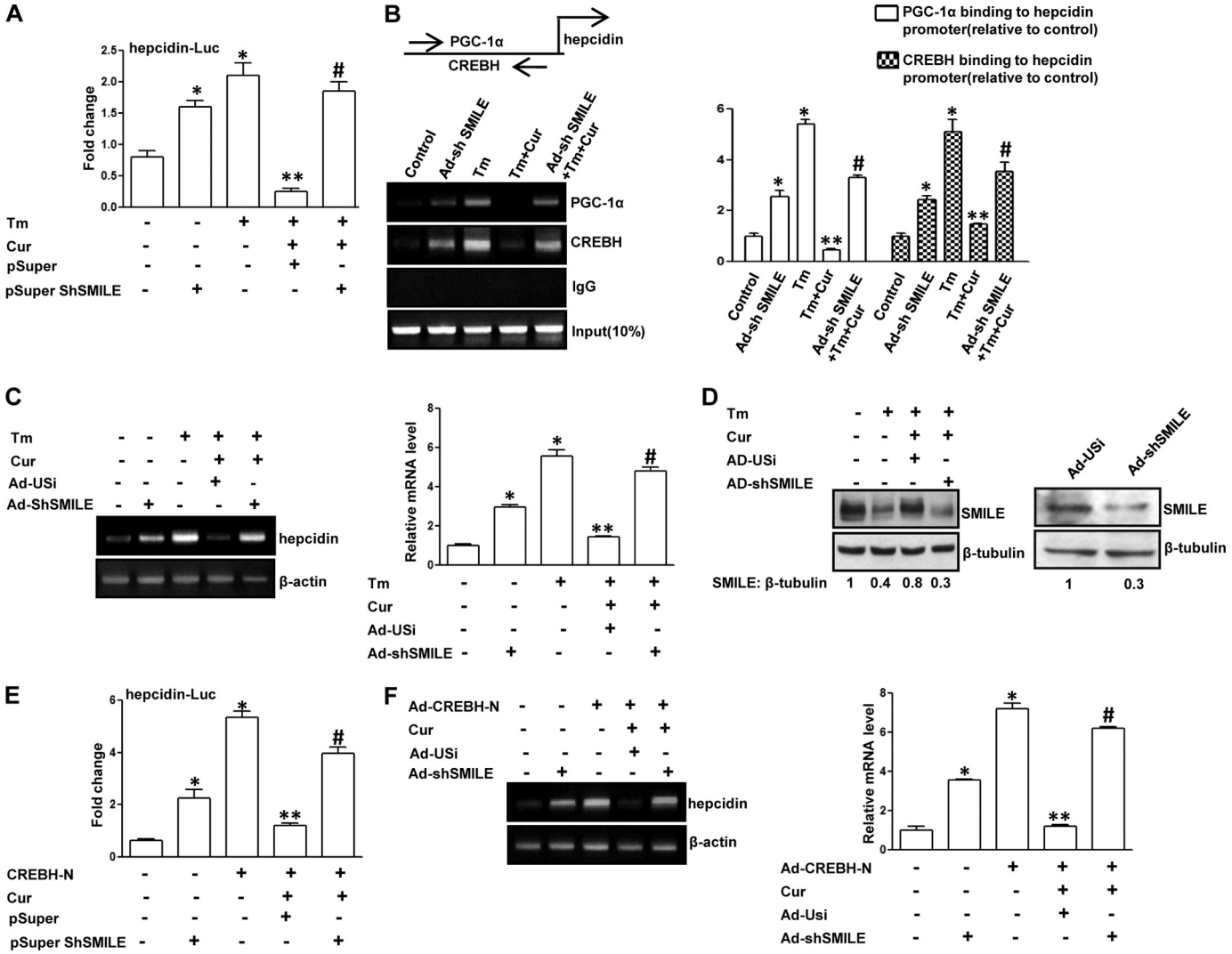

FIGURE 8. **Effect of SMILE knockdown on CREBH-mediated regulation of hepcidin.** *A*, mouse hepcidin-Luc was co-transfected with pSuper only or pSuper siSMILE into HepG2 cell. After 24 h cells were serum-starved for 24 h followed by tunicamycin treatment (5 μg/ml) for 12 h followed by curcumin (*Cur*) treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ and #, $p < 0.05$ compared with untreated control, tunicamycin-treated and tunicamycin plus curcumin plus pSuper-treated cells, respectively. *B*, a ChIP assay is shown. HepG2 cells were infected with Ad-shSMILE (m.o.i. 50) for 48 h and then serum-starved for 24 h followed by tunicamycin (5 μg/ml) and curcumin treatment (10 μM) for 12 h as indicated. Soluble chromatin was prepared and immunoprecipitated with monoclonal antibody against PGC1α, CREBH, or IgG only as indicated. 10% of the soluble chromatin was used as input. Real-time quantitative PCR was performed to determine and quantify the binding of PGC1α and CREBH to endogenous hepcidin promoter and was normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ and #, $p < 0.05$ compared with untreated control, tunicamycin (5 μg/ml), and tunicamycin (5 μg/ml) plus curcumin (10 μM)-treated cells respectively. *C*, HepG2 cells were infected with Ad-unspecific siSMILE (Ad-USi) (50 m.o.i.) or Ad-ShSMILE (50 m.o.i) for 48 h followed by tunicamycin (5 μg/ml) and curcumin (10 μM) treatment. Total RNA was isolated for semiquantitative RT-PCR analysis of hepcidin mRNA expression and was normalized to β-actin expression. Data represent mean ± S.D. of three individual experiments. *, $p < 0.05$, and **, $p < 0.05$ and $^{\#}$, $p < 0.05$ compared with untreated control, tunicamycin (5 μg/ml) and tunicamycin plus curcumin (10 μM) plus Ad-USi-treated cells, respectively. *D*, HepG2 cells were infected with Ad-USiSMILE (50 m.o.i.) or Ad-ShSMILE(50 m.o.i) for 48 h followed by tunicamycin (5 μg/ml) and curcumin (10 μM) treatment. Western blot was performed to determine SMILE protein level. *E*, hepcidin-Luc was co-transfected with CREBH (300 ng) or pSuper only (300 ng) or pSuper siSMILE(300 ng) in HepG2 cell. After 24 h cells were serum-starved for 24 h followed by curcumin treatment (10 μM) for 12 h. Experiments were performed in triplicate, and data are expressed in RLU and as the -fold activation relative to the control, representing the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ and #, $p < 0.05$ compared with untreated control, CREBH and CREBH plus curcumin plus pSuper-treated cells, respectively. *F*, HepG2 cells were infected with Ad-CREBH (50 m.o.i.) and Ad-USiSMILE (50 m.o.i.) or Ad-ShSMILE (50 m.o.i) for 48 h followed by curcumin (10 μM) treatment. Total RNA was isolated for semiquantitative RT-PCR analysis of hepcidin mRNA expression and was normalized to β-actin expression. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$ and **, $p < 0.05$ and #, $p < 0.05$ compared with untreated control, Ad-CREBH-N, and Ad-CREBH +curcumin (10 μM) +Ad-USi-treated cells, respectively.

ATP levels, subsequently activating the AMPK signaling pathway to induce SMILE gene expression.

Effect of SMILE Knockdown on CREBH-mediated Regulation of Hepcidin Gene Expression—To verify the effect of SMILE on tunicamycin-induced ER stress-regulated gene, we examined the effect of SMILE knockdown on the tunicamycin-mediated hepcidin promoter activation by transient transfection assay in HepG2 cells (Fig. 8*A*). Curcumin treatment significantly decreased the Tm-induced promoter activation. Knock-down of the endogenous SMILE (pSuper siSMILE), however, blunted the decrease significantly. This result clearly demonstrated that the inhibitory effect of curcumin was due to SMILE. To further

confirm this SMILE-mediated effect, a ChIP assay was performed to determine the change in binding of CREBH and PGC1α on hepcidin promoter by curcumin treatment in HepG2 cells. ChIP assay results demonstrated that PGC1α and CREBH were present in hepcidin promoter, and Tm treatment further induced their binding to hepcidin chromatin, and Tm treatment along with curcumin drastically abolished that binding. Interestingly, knockdown of endogenous SMILE significantly increased the binding of CREBH and PGC1α even in the presence of curcumin, suggesting that curcumin through SMILE decreases the hepcidin gene transcription via decreasing the binding of CREBH and PGC1α to the promoter and ChIP assay results provide critical *in vivo* evidence of the effect of the curcumin/AMPK/SMILE signaling cascade, resulting in decreased CREBH transcriptional activity (Fig. 8*B*).

To further verify the effect of SMILE, HepG2 cells were treated with Tm to induce hepcidin gene expression. Curcumin significantly repressed hepcidin gene expression, and knockdown of endogenous SMILE by adenoviral shRNA specifically targeting SMILE (Ad-shSMILE) dramatically reversed the inhibitory effects of curcumin on tunicamycin-induced hepcidin mRNA expression (Fig. 8*C*). As the repression of hepcidin gene expression by curcumin was clear and as our results from the previous section clearly demonstrated that curcumin increased the SMILE protein level, our results demonstrating both repression of hepcidin mRNA and increase in SMILE protein by curcumin led us to speculate that tunicamycin might decrease the SMILE protein level. To assess this, the expression of SMILE was checked in the presence of tunicamycin in HepG2 cells. As expected, tunicamycin reduced SMILE protein levels compared with basal levels, and it was recovered significantly by curcumin treatment (Fig. 8*D*). Next, the effect of SMILE knockdown was examined on hepcidin promoter activity, which was significantly induced by adenovirus CREBH. Curcumin decreased the hepcidin promoter activity, and after SMILE knockdown, curcumin was unable to show its inhibitory effect in HepG2 cells (Fig. 8*E*). Similar results were obtained for human hepcidin mRNA where blocking of endogenous SMILE diminished the curcumin effect significantly in HepG2 cells (Fig. 8*F*). As a whole, these results indicate that SMILE is responsible for the differential inhibitory effect of curcumin on ER stress-regulated genes.

DISCUSSION

The antioxidant effect of curcumin is well known. It has many beneficial roles such as anti-inflammatory, anti-oxidant, antifungal, antibacterial, and anticancer activities (32). Here in our study we demonstrate that curcumin differentially regulates ER stress. Curcumin induces transcriptional co-repressor SMILE gene expression by an LKB1/AMPK-dependent signaling pathway and SMILE inhibits CREBH. CREBH and ATF6, two important members of bZIP family transcription factors residing on ER, are activated upon tunicamycin treatment and act as an ER stress generator. SMILE, through its bZIP domain, only interacts with CREBH to down-regulate its transcriptional activity. Although ATF6 is a similar ER-bound transcription factor like CREBH, neither curcumin nor SMILE plays a role in its transcriptional activity. We found that both curcumin and SMILE decrease the transcription of the CREBH target gene hepcidin, whereas induction of ATF6 target gene, GRP78, is not hampered by curcumin treatment or SMILE overexpression. This specific inhibition of CREBH by curcumin is significant, as the role of CREBH in different critical metabolic pathway has already been reported (23, 24). Overall, our results depict a novel mechanistic pathway of differential regulation of ER stress-regulated transcription factors by curcumin, which could provide a therapeutic method of controlling ER stress.

Several previous studies show that the working concentration of curcumin varies significantly depending upon the nature of the study and the cell line. The anticancer effects of curcumin have been demonstrated in multiple cell types at concentrations between 5 and 50 μmol/liter (33). It has been reported that *in vitro* studies with curcumin in the 10 μmol/liter range or below might have human physiological relevance (34). The acceleration of oxidative protein folding by curcumin (10–50 μM) has already been reported (32). Previously, it was reported that curcumin plays an important role in ER stress-mediated cellular apoptosis processes, whereby curcumin induces ER stress (35, 36). It has also been reported that curcumin (5–10 μM) induces ER stress response and protects against oxidative stress in the myogenic C2C12 cell line by increasing levels of the ER chaperon protein, GRP94, which acts as a regulator of calcium homeostasis (36). From these previous findings the effect of curcumin on ER stress is prominent, but whether curcumin, being an antioxidant, can ameliorate ER stress is still not well understood. Of most interest, in our study we observed curcumin significantly blocks CREBH-mediated transactivation of its target gene, whereas it has no such repressive effect on ATF6-mediated transactivation. The most interesting aspect from this study was the specific inhibition of CREBH by curcumin. We speculated that there might be some other factors involved in curcumin-mediated repression as curcumin only represses CREBH. Eventually, we observed that this inhibitory effect of curcumin is solely dependent on a transcriptional co-repressor, SMILE. Curcumin induces SMILE gene expression, and SMILE in turn inhibits CREBH and not ATF6.

Here we identified that SMILE inhibits transcriptional activity of CREBH and not ATF6, mediated through an interaction between SMILE and CREBH. We investigated the potential functional domain of SMILE responsible for its repressive action on CREBH. Our results (Fig. 4) describe that only the bZIP domain of SMILE is essential for interaction and through this domain SMILE interacts with CREBH. We also examined the potential functional domain of CREBH responsible for the interaction with SMILE. Interestingly, the full-length CREBH was found to be involved. It has previously been reported that the bZIP region of SMILE is essential for the dimerization and function of bZIP proteins, although homodimerization of SMILE is not required for its repressive action (13). Consistent with this previous finding, we observed that the homodimerization of SMILE is not required for its repressive effect on CREBH (Fig. 4*D*). According to the previous findings, SMILE undergoes competition with transcriptional co-activators to inhibit transcription factors (14). We observed that there is, in fact, competition between SMILE and the co-activator PGC1α that regulates the transactivity of CREBH (Fig. 5). Chromatin

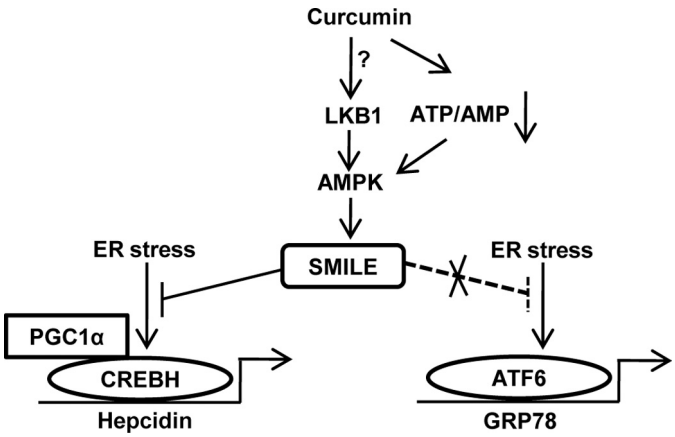

FIGURE 9. **Schematic diagram of SMILE-mediated inhibition of hepcidin.** Curcumin decreases cellular ATP levels and activates LKB1 that in turn activates AMPK signaling, leading to SMILE activation. SMILE then inhibits CREBH-mediated activation of hepcidin promoter via competition with PGC1α, whereas SMILE does not inhibit ATF transactivation.

immunoprecipitation results (Fig. 8*B*) clearly demonstrate that binding of CREBH and PGC1α on the CREBH target gene promoter is significantly inhibited by curcumin treatment, and this inhibition is significantly released by SMILE knockdown. Most strikingly, unlike CREBH, there is no such repression of ATF6 either by curcumin or by SMILE. This partial repression of ER-bound transcription factors by curcumin and SMILE is the most important finding of this study, as both CREBH and ATF6 are members of the same bZIP family. We further speculate that the reason behind this differential inhibition could be due to the structural difference that CREBH and ATF6 share despite being family members, and therefore, it would be necessary to further study these novel phenomena in more detail.

It has been previously reported that curcumin activates LKB1 and its downstream kinase AMPK signaling pathway by phosphorylating both proteins (37). A previous report also describes that curcumin decreases cellular ATP levels to activate AMPK (31). Consistent with these previous results, our results demonstrate that curcumin decreases cellular ATP levels as well as phosphorylates LKB1 within 10 min to activate AMPK. We found that this activation of AMPK by curcumin leads to the increase of SMILE protein level in both a time- and dose-dependent manner. Over expression of either LKB1 or constitutively active AMPK leads to the increase of SMILE protein level, whereas blocking endogenous LKB1 or AMPK by adenovirus overexpression of DN-LKB1 or DN-AMPK inhibits the increase of SMILE protein level by curcumin. Therefore, we suggest that curcumin through the LKB1/AMPK signaling pathway increases the level of SMILE protein, which in turn interacts with CREBH and inhibits its transcriptional activity.

Many physiological and pathological processes that induce ER stress, such as gene mutations that disturb protein folding, cholesterol or lipid overloading, hyperhomo-cysteinemia, nutrient deprivation, or infection with pathogenic organisms, can induce an inflammatory response through ER stress-mediated CREBH cleavage (22). Therefore, any exogenous substance that could control the ER stress-mediated induction of CREBH or its transactivation may generate novel therapeutics that suppress or promote activation of the acute phase response in different disease states. From our study it is quite clear that curcumin is a potential candidate for controlling the CREBH-mediated transactivation. These notions merit future detailed research efforts on the role of curcumin in ER stress.

Overall, from these observations we provide a previously unknown effect of curcumin on ER stress. As depicted in Fig. 9, curcumin activates LKB1 and decreases cellular ATP levels that in turn phosphorylates and activates the AMPK signaling pathway. This AMPK activation leads to the activation of the SMILE gene promoter. SMILE then undergoes competition with the co-activator PGC1α and inhibits CREBH transactivity by directly interacting with CREBH and inhibiting the binding of CREBH and PGC1α on CREBH target gene promoter, whereas neither curcumin nor SMILE has any effect on the ATF6-mediated pathway of ER stress.

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