6066–6079 *Nucleic Acids Research, 2008, Vol. 36, No. 19* *Published online 27 September 2008*
doi:10.1093/nar/gkn607

Sumoylation of LAP1 is involved in the HDAC4-mediated repression of *COX-2* transcription

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Received March 1, 2008; Revised September 4, 2008; Accepted September 8, 2008

ABSTRACT

CEBPB, one of the CEBP family members, is a crucial regulator of gene expression during innate immunity, inflammatory responses and adipogenesis. In this study, the EGF-induced increase of CEBPB mRNA is shown to be coincident with the decrease of COX-2 mRNA. We identified that all of the individual CEBPB isoforms, LAP1, LAP2 and LIP, attenuate EGF-induced COX-2 promoter activity. Although increased sumoylation of both LAP1 and LAP2 is observed during the lagging stage of EGF treatment, only the sumoylated LAP1, but not the sumoylated LAP2, is responsible for COX-2 gene repression. In addition, EGF treatment can regulate the nucleocytoplasmic redistribution of HDAC4 and SUMO1. We further demonstrated by loss-of- and gain-of-function approaches that HDAC4 can be a negative regulator while inactivating COX-2 transcription. The sumoylation mutant LAP1, LAP1K174A, exhibits an attenuated ability to interact with HDAC4, and increased COX-2 promoter activity. Furthermore, the *in vivo* DNA binding assay demonstrated that LAP1K174A and CEBPDK120A, sumoylation-defective CEBPD mutants, attenuate the binding of HDAC4 on the COX-2 promoter. In light of the above, our data suggest that the suCEBPD and suLAP1 are involved in the repression of COX-2 transcription through the recruitment of HDAC4.

INTRODUCTION

There are two known isoforms of cyclooxygenase (COX), which is also known as prostaglandin H synthase and prostaglandin endoperoxide synthase, COX-1 and COX-2 (1,2). COX-1 functions as a housekeeping gene and is constitutively expressed in most tissues. Conversely, COX-2 is an inducible enzyme that is induced by cytokines (3), growth factors (4), phorbol esters (5), endotoxins (6) and oncogenes (7,8) in different cell types. Previous studies have shown that COX-2 is expressed in a large number of human cancers and is involved in cancer development and progression (9,10). Overexpression of COX-2 plays important roles in hyperproliferation, transformation, cell growth, invasion and metastasis of tumor cells. For the transcriptional activation of the *COX-2* gene, several transcriptional activators, including nuclear factor-kappa B (NF-κB) (11), CCAAT/enhancer-binding protein β (CEBPB) (12), CEBP delta (CEBPD) (13,14), cyclic AMP-responsive element binding protein (CREB) (15) and activating protein 1 (AP1) (16), have been reported. However, the participating components in the repression of the *COX-2* gene and mechanism have been less studied.

The CEBPs belong to a subfamily of the basic region of leucine zipper (bZIP) transcription factors. Six members have been identified in mammalian cells, including CEBP alpha (CEBPA), CEBPB, CEBPD, CEBP epsilon (CEBPE), CEBP gamma (CEBPG) and CEBP zeta (CEBPZ). All CEBPs, except for CEBPZ, consist of three structural domains: an N-terminal domain containing both positive and negative regulatory regions, a canonical basic domain and a C-terminal leucine zipper domain. The basic region binds to specific CCAAT motifs located in CEBPs targeted gene promoter, whereas the leucine zipper domain is responsible for heterodimer/homodimer formation between various CEBP members (17). CEBPB and CEBPD are involved in the regulation of *COX-2* transcription (12–14). The binding of CEBPB or CEBPD on the CEBP or cyclic AMP-responsive element (CRE) motifs of the human *COX-2* promoter is increased by inflammatory stimulation (18,19). Three variants of CEBPBs have been detected in many cell types: a 46-kDa full-length liver-enriched transcription-activating protein (LAP1), a 42-kDa LAP2 and a 20-kDa

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liver-enriched transcription-inhibitory protein (LIP). These variants are the result of an alternative translation initiation due to a leaky ribosomal scanning mechanism (20,21). LAP1 and LAP2 contain an N-terminal regulatory domain that is able to regulate the transcriptional transactivation; however, their behavior is thought to be different within cells (22,23). Moreover, LIP is considered to be a dominant-negative regulator of both LAP1/2 and the remainder of the CEBP family members, because of the lack of a transactivation domain. Recent studies have shown that LAP1 not only functions as a transcriptional activator but can also act as a transcriptional repressor to inhibit gene transcription, such as *peroxisome proliferator-activated receptor beta /delta (PPARβ/δ)* (24) and *cyclin D1* (25). Modification of LAP1 by the small ubiquitin modifier (SUMO) family members, SUMO1 (26), SUMO2 and SUMO3 (23), has been recently reported, and this modification has been proposed as important for its inhibitory function. However, the chromatin-remodeling enzymes involved in the sumoylated LAP1 (suLAP1)-mediated transcriptional repression and target genes are unclear.

Histone deacetylases (HDACs) participate in transcriptional repression through the recruitment/interaction with repressor/corepressor and the deacetylation of histones, resulting in local modification of chromatin structures. In mammalian cells, there are two major categories of HDACs classified according to the structures and homology of the yeast counterparts (27). The widely expressed class I HDACs, including HDAC1, 2, 3 and 8, are similar to yeast RPD3 and are exclusively localized in the nuclei. Class II HDACs are composed of HDAC4, 5, 6, 7, 9 and 10, which are expressed in a tissue-specific manner and shuttle between the cytoplasm and nuclei in response to external signaling (28). Both classes of HDACs can interact with mSin3 and NuRD to form a large repressor complex (29), which can be recruited by specific DNA-binding factors to bind to the gene promoters (30). In addition, other studies indicate that class II HDACs participate in repression of gene expression in cell-specific development not only through their deacetylase activity but also by interacting with proteins and corepressors, such as CtBP (31), NCoR/SMRT (32,33) and HP-1 (34). HDAC4 plays a pivotal role in cell development through posttranslational modification of sumoylation. For example, HDAC4 can interact with the myocyte enhancer factor-2 (MEF2) family, enhancing their sumoylation, then subsequently repressing MEF2-dependent transcription (35,36).

We previously reported that CEBPD modulates the basal and epidermal growth factor (EGF)-induced *COX-2* transcription (14). Herein, we further demonstrated that the increases of LAP1 and its sumoylation form are coincident with the re-inactivation of the *COX-2* gene expression upon long-term EGF treatment. Therefore, we suggest that not only sumoylated CEBPD (suCEBPD) but also suLAP1 may collaboratively participate in the repression of *COX-2* transcription. In addition, we showed that EGF can regulate the nucleocytoplasmic shuttling of HDAC4 and SUMO1 by immunofluorescence assay, and the time frame of CEBPBs induction coincides with this reimporting of HDAC4 and SUMO1 into the nuclei. Furthermore, the LAP1K174A shows a lower HDAC4-interaction affinity yet can enhance *COX-2* promoter activity compared with the wild-type LAP1. An *in vivo* DNA-binding assay shows the spatial and temporal binding activity of SUMO1, CEBPD, CEBPB and HDAC4 on the *COX-2* promoter followed EGF treatment. Using the same *in vivo* DNA-binding approach, we further demonstrated that the LAP1K174A and CEBPDK120A attenuated the HDAC4-binding ability to the *COX-2* promoter. These results suggest that the suLAP1 and suCEBPD both act as mediators in modulating HDAC4 recruitment on the *COX-2* promoter.

MATERIALS AND METHODS

Materials

Human EGF was purchased from Peprotech (Rocky Hill, NJ, USA). The COX-2, CEBPB, CEBPD, HDAC1, HDAC4, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and hemagglutinin [HA; for immunoprecipitation (IP)] antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against HA (for western blot) were purchased from BM (Boehringer, Mannheim, Germany) or Babco (Richmond, CA, USA). Antibodies recognizing p300 were purchased from Transduction Laboratories (Lexington, KY, USA). Antibodies against SUMO1 were purchased from Zymed Laboratories (South San Francisco, CA, USA). Antibodies against SUMO2/3 were purchased from Abcam (Cambridge, MA, USA). Lipofectamine 2000, Dulbeco's modified Eagle's medium (DMEM), Opti-MEM medium, the Trizol RNA extraction kit and SuperScriptTM III were obtained from Invitrogen (Carlsbad, CA, USA). The Arrest-In reagent was purchased from Open Biosystems (Huntsville, AL, USA). Monoclonal anti-HDAC1 antibodies, Protein A-agarose and streptavidin–sepharose beads were purchased from Upstate (Charlottesville, VA, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). The Taq DNA polymerases, the *in vitro* transcription/translation kit and luciferase assay system were from Promega (Madison, WI, USA). Expression plasmid pcDNA3/HA was a gift from Dr Hsin-Fang Yang-Yen (IMB, Academia Sinica, Taiwan). All oligonucleotides for DNA affinity precipitation assay (DAPA) and electrophoretic mobility shift assay (EMSA) were synthesized by MDBio Inc. (Taipei, Taiwan). N-ethylmaleimide (NEM), an inhibitor of SUMO proteases, was obtained from Sigma (St Louis, MO, USA).

Plasmid construction

The numbering used to designate residues in CEBPB is based on the human CEBPB sequence (NM_005194). Expression vectors encoding HA-LAP1 (full-length C/EBPB), HA-LAP2 (residues 24–345) and HA-LIP (residues 199–345) were constructed in pcDNA3/HA which was amplified from the A431 cDNA with specific primers as follows: LAP1F/BamHI: 5′-GGATCCCAACGCCTG GTGGCCTGG-3′, LAP2F/BamHI: 5′-GGATCCGAAG TGGCCAACTTCTAC-3′, LIPF/BamHI: 5′-GGATCCG

CGGCGGGCTCCCCGTAC-3′ and hC/EBPBR/EcoRI: 5′-GAATTCCTAGCAGTGGCCGGAGGA-3′. The fragments were verified by sequencing and subcloned into the pcDNA3/HA vector using BamHI and EcoRI. The sumoylation mutants of HA/LAP1, HA/LAP1K174A, and HA/LAP2, HA/LAP2K151A, were generated with a QuikChang site-directed mutagenesis kit (Stratagene, CA, USA). The reporters containing the *COX-2* promoter (−207/+49) and mutants were previously described (14). Expression vectors encoding Flag-HDAC1 and Flag-HDAC4 were generated by Dr Wen-Ming Yang (IMB, National Chung-Hsing University, Taiwan).

Transfection and reporter gene assay

Human epidermoid carcinoma A431 cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. In this series of experiments, cells were cultured in serum-free condition with or without treatment with 25 ng/ml EGF. *COX-2* reporter and indicated expression vectors were cotransfected by lipofection using the Arrest-In reagent or Lipofectamine 2000 according to the manufacturer's instruction. The total DNA amount for each transfection was matched with pcDNA3 or related backbone vectors. After 6-h transfection, cells were changed in fresh serum-free DMEM medium and then were treated with EGF if necessary for 15 h.

Small interfering RNAs assay

Small interfering RNA (siRNA) pools for CEBPB and scrambled control siRNA were purchased from Ambion (Austin, TX, USA). The siRNA of HDAC4 was previously described (37,38) and purchased from Invitrogen were (sense strands): HDAC4-1 (HD4-1), 5′-AAUGUAC GACGCCAAAGAUTT-3′ and HDAC4-2 (HD4-2), 5′-G ACGGGCCAGUGGUCACUG-3′. Cells were separately transfected with CEBPB siRNA and control siRNA by using Lipofectamine 2000. After 24 h of transfection, the lysates of transfectants were harvested after stimulation with or without EGF for 5 h for western blot with indicated antibodies.

In vivo SUMO modification assay and Co-IP assay

A431 cells were transfected with the expression vectors of HA/LAP1, HA/LAP1K174A, HA/LAP2 or HA/LAP2K151A in the presence or absence of SUMO1 expression vectors by using the Arest-In reagent. After 24-h transfection, cells were washed twice with phosphate buffered saline (PBS) containing 20 mM N-ethylmaleimide (NEM) and then harvested by scraping into 200 μl of RIPA buffer containing protease inhibitors as previously described (14). For the Co-IP assay, A431 were transfected with 2 μg of the indicated expression vectors by using Arest-In reagent. After 24 h, cells were harvested with modified RIPA buffer plus protease and phosphatase inhibitors which the details were previously described (14). Five hundred micrograms of cell lysate was immunoprecipitated with 2 μg of anti-HA antibodies in IP buffer in a 1:4 ratio of modified RIPA and IP buffer, 20 mM HEPES (pH 7.9), 2 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM KCl, 10% glycerol and 1 mM dithiothreitol (DTT), under rotation at 4°C for 3 h, and then plus protein A-agarose beads were added for an additional 2 h. Immunoprecipitated beads were washed three times with washing buffer, 1× PBS containing 0.1% IGEPAL CA-630. The immunocomplexes were separated by 10% SDS–PAGE, followed by western blot with anti-HA, anti-SUMO1 anti-SUMO2/3 or anti-HDAC4 antibodies.

DNA affinity precipitation assay

After 6 h serum-free starvation, nuclear extracts of EGF-treated A431 were harvested as indicated time courses. DAPA was performed according to a previously described method (14). Briefly, the 200 μg of lysates extracted from each group were incubated with 2 μg of biotinylated C/EBP or CRE oligonucleotides in the presence of DNA binding buffer. After 1 h of incubation at 4°C, 40 μl of streptavidin–sepharose were added to the reaction mixture and the incubation was continued for 1 h. The complexes were then precipitated by centrifugation and washed three times with DNA binding buffer before they were resolved by SDS–PAGE and subsequently analyzed by immunoblotting with specific antibodies. The sequences of 5′-biotinylated oligonucleotides were followed: CRE: 5′-CACC GGGCTTACGCAATTTTT-3′ and CEBP: 5′ -CAGTCA TTTCGTCACATGGGC-3′.

Electrophoretic mobility shift assay

An EMSA was performed essentially as described by Wang *et al*. (14). Briefly, the ^{32}P-labeled probes (0.2–0.5 ng) containing the individual CEBP or CRE site were incubated with 1 μl of *in vitro*-translated HA/LAP1, HA/LAP2 or HA/LIP in specific binding buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 μg of poly(dI-dC) and 10% glycerol. After 20 min of incubation at room temperature, the reaction mixtures were resolved in 5% native polyacrylamide gels (acrylamide/bisacrylamide ratio, 30:1) at 4°C, and the specific protein–DNA complexes were visualized by autoradiography. The sequences of various oligonucleotides are as described above.

Chromatin IP and re-chromatin IP assay

The chromatin IP (ChIP) assay was performed essentially as described by Wang *et al*. (14). Briefly, A431 cells were treated with 1% formaldehyde for 15 min. The cross-linked chromatin was then prepared and sonicated to an average size of 500 bp. The DNA fragments were immunoprecipitated with antibodies specific to CEBPB, CEBPD, SUMO1, HDAC1, HDAC4 or control rabbit immunoglobulin G (IgG) at 4°C overnight. After reversal of the cross-linking, the immunoprecipitated chromatin was amplified by primers related to specific regions of the *COX-2* genomic locus. For the re-ChIP assay, the first immune complex that had been washed twice with washing buffer of 50 mM Tris–HCl (pH 8.0), 0.1% SDS, 0.5% NP-40, 150 mM NaCl and 2.5 mM EDTA, and three times with low-salt buffer of 10 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA, was then resolved in 10 mM DTT at 37°C, further diluted in ChIP dilution buffer and then

processed according to the same ChIP assay protocol using the indicated antibodies. The primers were as follows: F-186, CTGGGTTTCCGATTTTCTCA and R-49, GAGTTCCTGGACGTGCTCCT. The amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing.

Immunofluoresce assay

A431 cells were seeded onto glass coverslips. Twenty-four hours after reseeding, cells were starved in serum-free medium for 8 h prior to EGF treatment. Fixation was performed in 4% formaldehyde in PBS at room temperature for 20 min. After three times of washing by PBS, cells were permeabilized with 1% Triton X-100 in PBS for 5 min. Next, cells were preincubated with 0.5% Tween 20 in 0.5% BSA in PBS for 15 min, blocked with 1% BSA in PBS for 1 h at room temperature and then incubated for 1 h at room temperature with following primary antibodies diluted in 1% BSA in PBS: anti-HDAC4 (1:100). After washing with PBS for three times, cells were incubated with the relative secondary antibodies (Alexa Fluor 488 anti-rabbit 1:150) in 1% BSA/PBS for 1 h at room temperature followed by washing in PBS. Coversilps were then mounted with ProLong Gold antifade reagent with 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen).

RESULTS

Expression of CEBPBs is induced after long-term EGF treatment in A431 cells

We previously reported that CEBPD can act as a bifunctional regulator and is involved in both basal and EGF-induced *COX-2* expression (14). Since CEBPBs can form a heterodimer with CEBPD and are implied to be involved in the *COX-2* gene regulation, we decided to investigate whether the CEBPBs also participate in the EGF-regulated *COX-2* transcription. To elucidate the relationships between CEBPD and three isoforms of CEBPB in EGF-regulated *COX-2* transcription, their expression patterns were first detected by RT–PCR assay and western blot. Upon EGF treatment, the *COX-2* expression was immediately induced ($\leq$1 h), which is consistent with the increase of CEBPD expression (Figure 1A and B, lane 1–6). This expression started to decrease after 3 h of EGF treatment. Interestingly, the induction of three isoforms of the CEBPB expression occurred in the long-term EGF treatment, along with the decrease of COX-2 expression (Figure 1A and B, lane 4–8), although several studies have suggested that LAP1 and LAP2 act as positive regulators, upregulating the COX-2 expression (12,39). However, the lagging phase induction of LAP1 and LAP2 conflicts with the positive regulation model of *COX-2* transcription. According to this observation, we hypothesized that the slowly induced CEBPBs may play different roles in *COX-2* gene regulation compared with the immediately induced CEBPD.

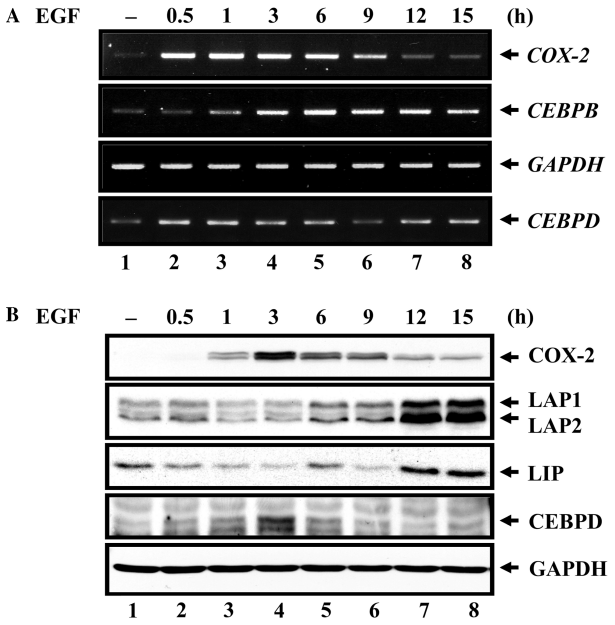

Figure 1. Expression of COX-2 and CEBPs upon long-term EGF treatment in A431 cells. (**A**) A431 cells were starved in serum-free medium for 6 h then treated with 25 ng/ml of EGF. Total RNA was harvested at the indicated times and an RT–PCR assay performed for the following genes: *COX-2*, *CEBPD*, *CEBPB* and *GAPDH*. (**B**) The conditions were the same as those described in (A); the cell lysates were harvested for western blot and protein expressions of COX-2, CEBPD, CEBPB and GAPDH were detected by specific antibodies.

CEBPB participation in repression of EGF-induced COX-2 expression

To verify the repressive effect of CEBPBs on *COX-2* expression, a loss-of-function assay using the knockdown approach was performed. Silencing of the three isoforms of CEBPB reverses the *COX-2* transcripts and protein expression (Figure 2A). This suggests that the CEBPBs indeed do play negative roles on COX-2 expression. As mentioned above, LAP1 and LAP2 consist of transactivation and DNA binding/dimerization domains, whereas LIP lacks the N-terminal transactivation domain. The implication arising from this is that LIP can act as a dominant-negative form protein, repressing transcriptional activation through the formation of a heterodimer with the rest of the C/EBP family members. The three CEBPB variants are translated from one transcript, and the 5′-untranslated region (5′-UTR) of CEBPB mRNA plays a critical role in regulating the selected-translation initiation of isoforms (20,40). To further dissect the effect of individual CEBPB isoforms on the regulation of the *COX-2* transcription, expression vectors bearing coding cDNA of CEBPB isoforms, HA/LAP1, HA/LAP2 or HA/LIP, without the 5′-UTR were constructed for their specific translation (Figure 2B). Compared with the control vectors upon EGF treatment, the well-characterized negative regulator HA/LIP shows an inhibitory effect on the *COX-2* promoter activity. Interestingly, both transfectants of HA/LAP1 and HA/LAP2 show a consistent repressive effect in response to EGF treatment (Figure 2C, right panel). In spite of this, the *COX-2*

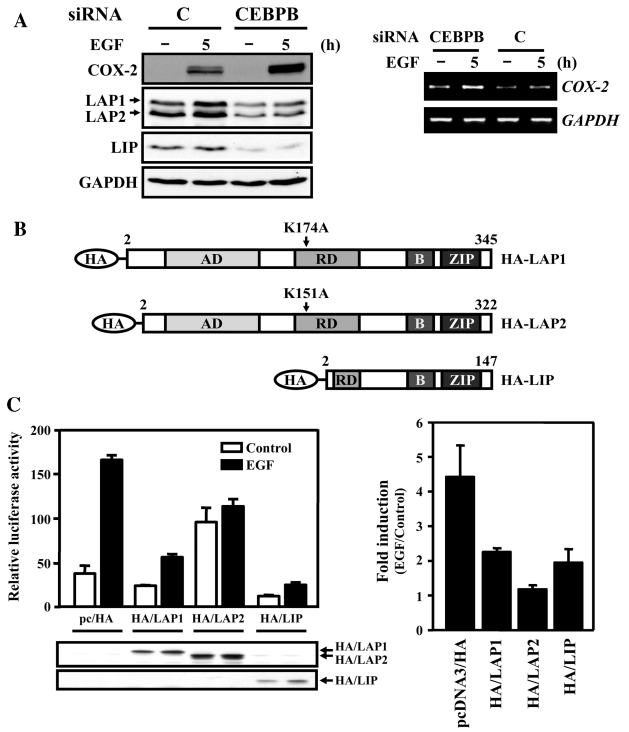

Figure 2. CEBPBs participate in silencing *COX-2* gene expression. (**A**) A431 cells were transiently transfected with siRNA of CEBPB or a scrambled control, C, for 24 h then these transfectants were starved in serum-free medium for 6 h, followed by EGF treatment for 5 h. Western blot (left panel) and RT–PCR (right panel) were performed to detect endogenous CEBPBs and COX-2 expressions. (**B**) Functional domains of the transactivation domain (AD), regulatory (RD), basic (B) and leucine-zipper domains (ZIP) are shown in the diagram. K174 and K151 represent the sumoylation sites of LAP1 and LAP2. (**C**) A431 cells were transiently cotransfected with the *COX-2* reporter and individual expression vectors of CEBPB isoforms with or without EGF treatment for 15 h. Data shown in left panel are representative results from three independent experiments performed in triplicate. Luciferase activities are plotted in arbitrary units. The results shown in right panel are averages from three independent transfection assays. The average fold induction (mean ± SD, $n = 3$) of EGF-treated cells compared to control cells was indicated. Statistical analysis was performed by Student's *t*-test.

promoter activity was inhibited in the HA/LAP1 transfectants showing a repressive effect on the *COX-2* promoter activity but not in the HA/LAP2 transfectants without the EGF treatment (Figure 2C, left panel). These results suggest that LAP1 and LIP, but not LAP2, have consistent repressive roles regardless of EGF existence (Figure 2C).

Sumoylation of LAP1 is enhanced upon long-term EGF treatment, and the sumoylation mutant of LAP1 attenuates its repressive ability on the *COX-2* promoter

A previous study showed that the CEBP regulatory domain motif (RDM) sequences are attachment sites for SUMO1; furthermore, these SUMO1-conjugated sites, LKAEP of LAP1- or LAP2-RDM domain, are necessary and sufficient for the intrinsic inhibitory function of RDMs (26). This report suggests the idea that the sumoylated LAP1 and sumoylated LAP2 (suLAP2) may function as negative regulators in re-inactivation of the COX-2 expression upon long-term EGF treatment, which differs from the LIP-mediated repressive mechanism due to the lack of transactivation domain. To verify whether SUMO1-conjugated lysine residues are involved in the regulation of *COX-2* transcription, the sumoylation mutants of LAP1 and LAP2 expression vectors, HA/LAP1K174A and HA/LAP2K151A, were generated by site-directed mutagenesis. First, an *in vivo* sumoylation assay of LAP1 and LAP2 showed that increases of suHA/LAP1 and suHA/LAP2 were observed when the SUMO1 expression vector was cotransfected with expression vectors of HA/LAP1 or HA/LAP2 in A431 cells (Figure 3A, compare lane 2 with lane 3, and lane 6 with lane 7). This proved that SUMO1-conjugated sumoylation of LAP1 and LAP2 does occur in A431 cells. Moreover, to confirm the SUMO1-conjugated specificity on the lysine 174 of LAP1 and the lysine 151 of LAP2, the HA/sumoylation mutants of LAP1 and HA/LAP2, LAP1K174A and HA/LAP2K151A, were recruited to perform the same *in vivo* sumoylation assay. The SUMO1-conjugated patterns were not visible in the cotransfectants of SUMO1 and HA/LAP1K174A or SUMO1 and HA/LAP2K151A (Figure 3A, compare lane 4 with lane 5, and lane 8 with lane 9). In addition, reciprocal IP assays were performed to re-confirm the *in vivo* SUMO1-cojugated LAP1. The suHA/LAP1 is visible in the SUMO1 antibody-immunoprecipition product of HA/LAP1 transfectant, but not in the transfectants of HA/LAP1K174A or HA/LAP1 with SUMO1 siRNA (Figure 3B and Supplementary Figure S4). These results indicate that the residues of lysine 174 of LAP1 and lysine 151 of LAP2 are the SUMO1-conjugated sites in A431 cells. Meanwhile, no predictable super-shift band was observed in the HA/LIP transfectants, indicating that it is not a SUMO1 substrate (data not shown). Unlike transcriptional activation of the *COX-2* gene, transcriptional repression after long-term EGF treatment has, up till now, been unexplored. Therefore, the issue of whether the amounts of suLAP1 and suLAP2 are coincident with the re-inactivation of *COX-2* transcription needs to be examined. In consideration of whether the long-term EGF treatment changes the amounts of endogenous LAP1 and LAP2 in A431 cells, the *in vivo* sumoylation assay was performed by exogenously expressing the HA/LAP1 and HA/LAP2 to reveal their sumoylated forms. The time frame for EGF-induced suLAP1- and suLAP2-increases (Figure 3C) is consistent with *COX-2* transcription being re-inactivated (Figure 1). This suggests that the suLAP1 and suLAP2 may participate in the re-inactivation of *COX-2* transcription. We further verified whether sumoylation of both LAP1 and LAP2 function in the EGF-regulated *COX-2* promoter activity. Overexpression of HA/LAP1 or HA/LAP2 reduces the *COX-2* promoter activity (Figure 2C), however, the overexpressed HA/LAP1K174A attenuates the repressive activity of the *COX-2* promoter as compared with overexpression of HA/LAP1 (Figure 3D and Supplementary Figure S1). In addition, there was no difference between the cotransfection of the *COX-2* reporter with the HA/LAP2 or the HA/LAP2K151A expression vectors (data not shown). To further confirm whether the sumoylation of LAP1 indeed functions on the *COX-2* promoter, the CEBPB siRNA was cotransfected

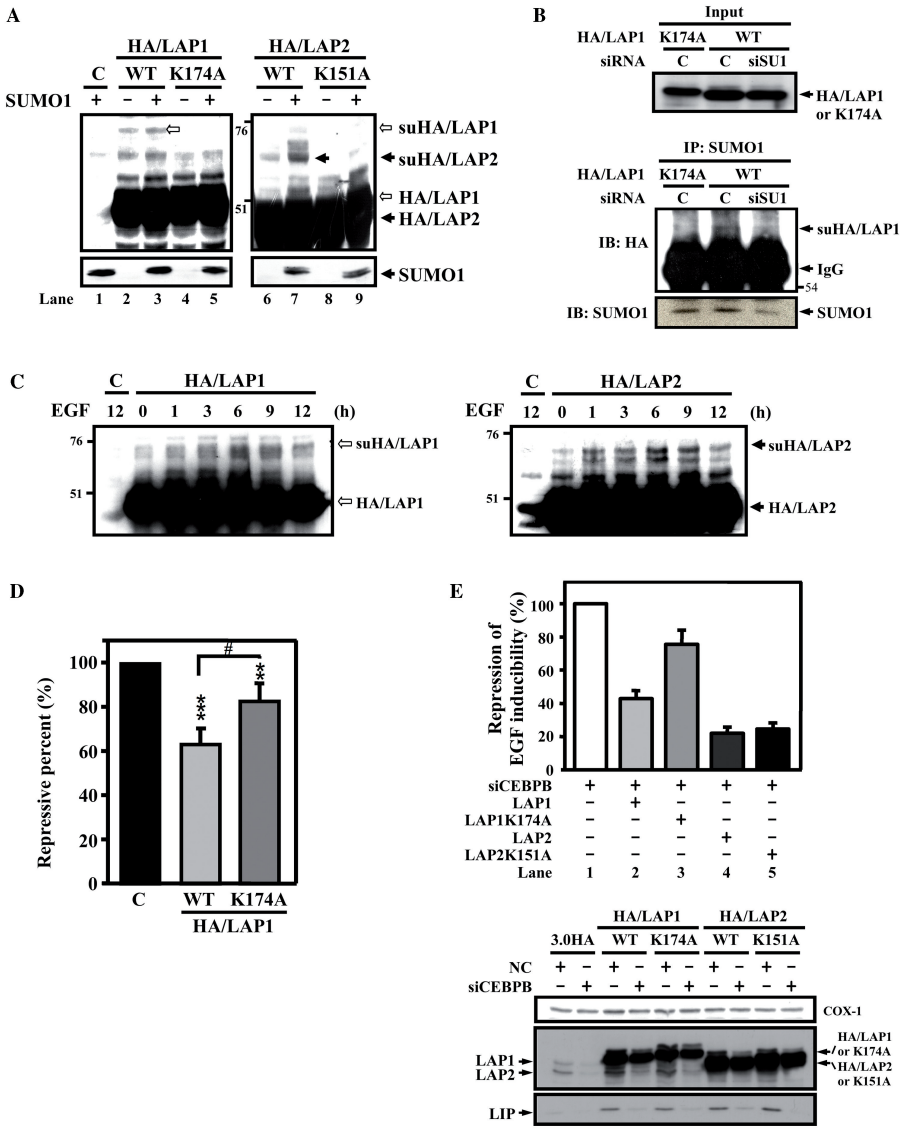

Figure 3. SUMO1-conjugated lysine 174 of LAP1 plays a functional role in the regulation of *COX-2* transcription. (**A**) Western blot was performed with HA antibodies to detect A431 cell lysates from the transfectants of HA/LAP1, HA/LAP1K174, HA/LAP2 and HA/LAP2K151A with or without SUMO1 expression vectors. The bold arrowhead indicates the positions of suHA/LAP1 and suHA/LAP2. (**B**) A431 cells were transfected with expression vectors of HA/LAP1 or HA/LAP1K174A with or without SUMO1 siRNA for 24 h. The lysates of transfectants were harvested, and then for IP by SUMO1 antibody (Zymed). (**C**) The expression vectors of HA/LAP1 and HA/LAP2 were transiently transfected into A431 cells. After 6 h of starvation, these transfectants were stimulated with EGF and harvested at indicated times. The exogenous expressions of HA/LAP1 and HA/LAP2 proteins were detected using HA antibodies. (**D**) A431 cells were transfected with *COX-2* reporter combined with the expression vectors of HA/LAP1 or HA/LAP1K174A as indicated. Cell lysates were then prepared and analyzed for luciferase activity. The percentage of the repression activity of transfectants was normalized to the control transfectants of *COX-2* reporter and pCDNA3/HA vector. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**E**) A431 cells were transfected with *COX-2* reporter and siRNA of CEBPB combined with the expression vectors of HA/LAP1 or HA/LAP1K174A as indicated. Cell lysates were then prepared and analyzed for luciferase activity. Values of EGF-treated cells compared to control cells were indicated. The percentage of the repression activity of transfectants (HA/LAP1, HA/LAP1K174A, HA/LAP2 and HA/LAP2K151A) was normalized to the control transfectants of *COX-2* reporter and siRNA of CEBPB (left panel). Western blot was performed with CEBPB antibodies to confirm the knockdown efficiency of CEBPB siRNA and verify the expression amounts of HA/LAP1, HA/LAP1K174A, HA/LAP2, HA/LAP2K151A and LIP (right panel).

with various indicated expression vectors in A431 cells. Transfected CEBPB siRNA targeted the 3′-UTR of *CEBPBs* mRNA, silencing the endogenous CEBPBs expression. Meanwhile, the CEBPB siRNA has no activity for these cotransfected expression vectors containing only the coding region of CEBPBs (Figure 3E, right panel). Consistent with Figure 3D, LAP1K174A can reverse the LAP1-mediated repression of *COX-2* promoter activity. However, LAP2K151A shows a consistent *COX-2* reporter activity compared with LAP2 upon EGF treatment (Figure 3E and Supplementary Figure S1). The results suggested that an intact lysine 174 of LAP1 is necessary for the repression of *COX-2* promoter activity, and a sumoylation-independent mechanism of LAP2 is involved in the LAP2-mediated inactivation of *COX-2* transcription.

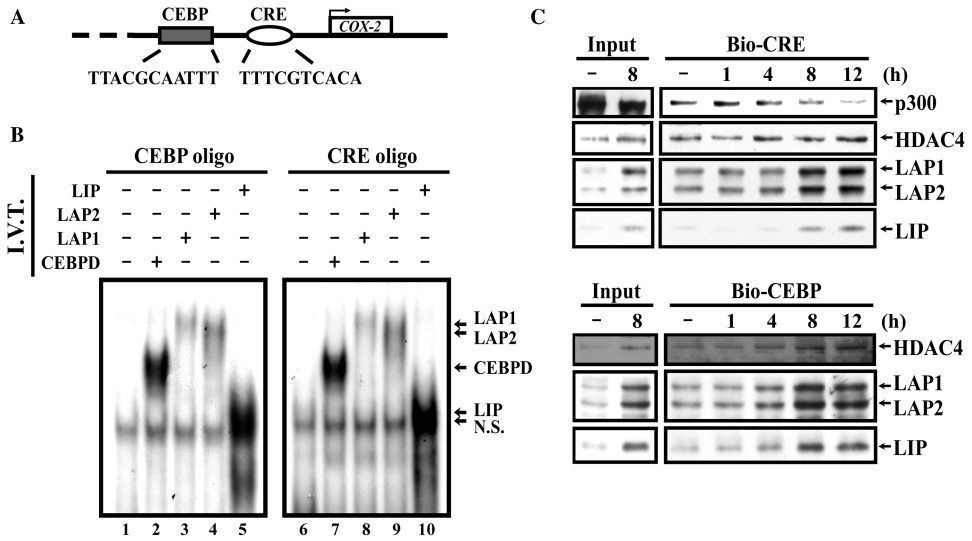

Figure 4. Bindings of CEBPBs parallel with HDAC4 binding upon long-term EGF treatment. (**A**) Schematic representation of the sequences of CEBP and CRE motifs in this study. (**B**) *In vitro* translation of HA/CEBPD, HA/LAP1, HA/LAP2 and HA/LIP was performed, and the mixtures with these products and DNA probes were subjected to a gel-shift (EMSA) assay as described in 'Materials and methods section'. An arrowhead indicates the retarded CEBPs-C/EBP or CEBPs-CRE complex. The *in vitro*-translated lysate without an expression vector is shown for the negative control (lanes 1 and 6). (**C**) Nuclear extracts were harvested from A431 cells at the indicated times and were treated with or without EGF, then incubated with biotin-labeled CRE or C/EBP oligonucleotides. The precipitated products were pulled down by streptavidin–sepharose and analyzed by western blot with antibodies recognizing p300, HDAC4 or CEBPBs.

CEBPB isoforms and HDAC4 can bind to the CEBP and CRE motifs of the *COX-2* promoter

Our previous study showed that the CEBP and CRE motifs (Figure 4A) both played critical roles in the EGF-regulated *COX-2* gene expression in A431 cells (14). To verify whether CEBPBs can participate in *COX-2* transcription through these two motifs, a gel-shift assay was carried out using individual *in vitro*-translated HA/CEBPB isoforms and labeled CEBP or CRE probes. The three CEBPB isoforms bound directly to the CEBP (Figure 4B, lanes 3–5) and CRE (Figure 4B, lanes 8–10) motifs. In addition, HDACs have been proven to be recruited by sumoylated transcription factors, functioning as corepressors to silence gene transcription (41). Our previous study implied that the CEBPD has the ability to modulate the binding of chromatin remodeling enzymes, histone acetyltransferases (HATs) or HDACs, through its posttranslational modification of acetylation or sumoylation. However, the components involved in the suCEBPD- and suLAP1-mediated inactivation of the *COX-2* gene are uncertain. suCEBPD has been identified to contain higher HDAC4-interactive activity by DAPA (Supplementary Figure S2). To further determine the binding activity of chromatin remodeling enzymes, such as p300, HDAC4 and CEBPBs on the CEBP or CRE motifs followed by the EGF treatment, a DAPA was performed to show the change of dynamic transcriptional complex. The pull-down results of CEBP and CRE motifs show similar binding patterns (Figure 4C). Briefly, the transient-increased p300-binding activity is observed from 1 h and returned to the basal form after 4 h of EGF treatment. Similar to the lagging induction of CEBPBs, the apparent binding of CEBPB isoforms was also detected from 4 h and extended to 12 h upon EGF treatment. Interestingly, the binding activity of HDAC4 is consistent with the CEBPBs binding in the lagging stage of EGF treatment. These results suggest that the lagging bindings of CEBPBs and HDAC4 may play a negative role in the regulation of *COX-2* transcription. Additionally, HDAC4 has been implicated in interaction with SUMO1-conjugated substrates (36) and the nucleocytoplasmic shuttling of HDAC4 is reportedly controlled by signaling molecules (42,43). However, which external stimulator can induce HDAC4 redistribution is less studied. In our system, an equal distribution of HDAC4 between nucleus and cytoplasm was observed to pre-exist in nucleus during the EGF-starvation stage. Importantly, the HDAC4 showed a lagging import from 4 h and this extended to 8 h upon EGF treatment (Figure 5A, upper panel). On the other hand, the SUMO1-conjugated proteins are predominant in the nucleus, but transiently exported to the cytoplasm during short-term EGF treatment up until 4 h (Figure 5A, lower panel). The Figure 5B further indicates that HA/LAP1 is coexisting with endogenous HDAC4 and SUMO1 during the long-term EGF treatment. Combining this information with that of Figure 3C and Figure 4C, we suggest that the nuclear importation of HDAC4 and SUMO1 coincides with the LAP1 induction and the amount of sumoylated LAP1 during the inactivated period of *COX-2* gene transcription.

HDAC4, but not HDAC1, plays a functional role in silencing *COX-2*

HDAC4 is a SUMO E3 ligase and displays a transcriptional repressor activity (32,36). However, its interactive transcription factors and the detailed repressive mechanism of transcriptional regulation are still less known. Since the sumoylation of LAP1 plays a negative regulatory role

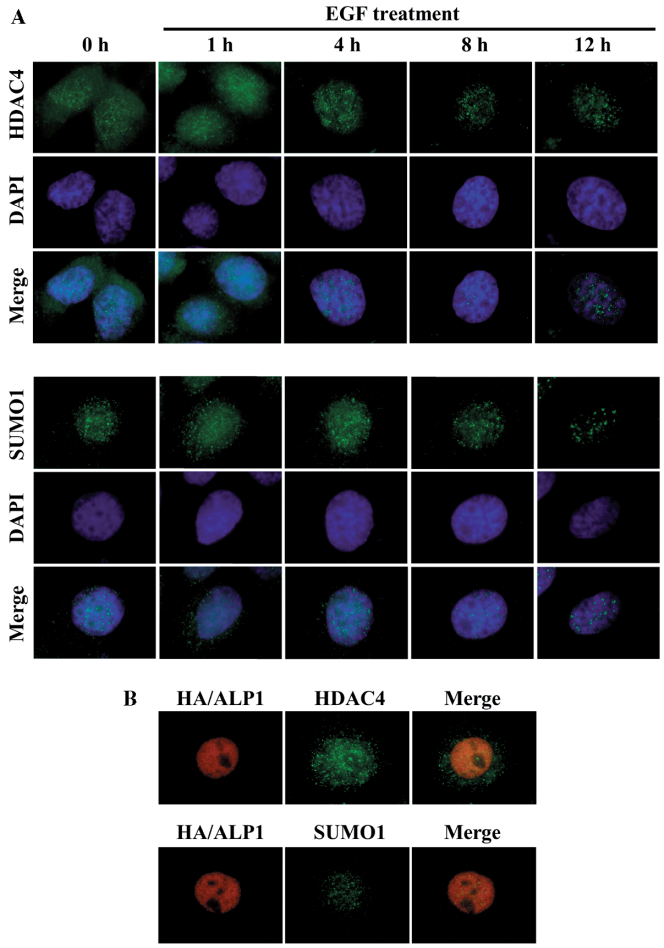

Figure 5. EGF treatment induces HDAC4 and SUMO1 nuclear shuttling. (**A**) A431 cells were starved in serum-free medium for 6 h and then treated with EGF at indicated times. The cells were fixed in formaldehyde and stained with anti-HDAC4 and anti-SUMO1 antibodies. The subcellular localization of HDAC4 (upper panel) and SUMO1 (lower panel) were determined by immunofluorescence microscopy (green). DAPI was used to visualize nuclei distribution (blue). (**B**) A431 cells were transiently transfected with HA/LAP1 expression vector for 24 h and then the transfectants were starved in serum-free medium for 6 h. Cells were fixed and subjected to indirect immunofluorescence staining. HA/LAP1 and endogenous HDAC4 (upper panel) or SUMO1 (lower panel) were stained with monoclonal anti-HA (1:100, red) and polyclonal anti-HDAC4 (1:100, green) or anti-SUMO1 (1:100, green) antibodies, respectively. Coexistence of HA/LAP1 with HDAC4 or SUMO1 were showed in 'Merge'.

in *COX-2* transcription, we further addressed whether HDAC4 plays a functional role in the suLAP1-mediated repression of *COX-2* transcription. Initially, a reporter assay was performed by cotransfection of the *COX-2* reporter with HDAC4 or HDAC1 expression vectors. Interestingly, overexpression of HDAC4 attenuated *COX-2* promoter activity regardless of EGF existence (Figure 6A); whereas, overexpression of HDAC1 showed no effect in A431 cells although it has been implied to be a corepressor in the silencing of *COX-2* transcription (44). To further verify whether HDAC4 plays a functional role through LAP1-binding with CEBP and CRE motifs, the *COX-2* reporters bearing the wild-type or the double mutant of both CEBP and CRE motifs (mCEBP/CRE) were cotransfected with HDAC4 expression vectors. Compared to the HDAC4-mediated repressive effect on the wild-type *COX-2* promoter, HDAC4 lost around 40% of its repressive activity on the *COX-2* reporter bearing the CEBP/CRE mutant (Figure 6B and Supplementary Figure S1). This suggests that the EGF-responsive elements, CEBP and CRE motifs, play a functional role in response to the HDAC4-mediated repression of the *COX-2* promoter. Moreover, a knockdown approach was performed to further confirm the HDAC4-exerted repressive effect on *COX-2* expression. Compared to the scrambled control, the western blot (Figure 6C, left panel) and RT–PCR assay (Figure 6C, right panel) show a consistent result that the silence of HDAC4 enhances *COX-2* expression regardless of EGF treatment. Furthermore, the specific siRNAs of CEBPB or HDAC4 were cotransfected with the *COX-2* reporter to detect whether this repressive effect occurred through the promoter regulation. The silence of either CEBPB or HDAC4 can enhance both the basal and EGF-induced *COX-2* promoter activities (Figure 6D). These results suggest that HDAC4 acts as a repressor on *COX-2* transcription through the CEBPs-binding motifs, and that this repression not only functions during the re-inactivation of the *COX-2* gene in long-term EGF treatment but also in the EGF-starved stage.

suLAP1 cooperates with HDAC4 to repress *COX-2* promoter activity

HDAC4 plays a suppressive role in the regulation of the *COX-2* promoter (Figure 6). suLAP1 is involved in the negative regulation of *COX-2* transcription (Figure 3D and E). Further investigations were performed to determine whether suLAP1 collaborated with HDAC4 to repress *COX-2* promoter activity. To address this issue, a reporter assay was performed by cotransfecting the reporter of *COX-2* promoter with various expression vectors as indicated in Figure 7A and Supplementary Figure S1. The cotransfected LAP1 and HDAC4 expression vectors showed a cooperatively repressive ability against *COX-2* promoter activity compared with transfection with the LAP1 expression vectors only (Figure 7A, compare lane 2 with lane 4). This suggests that an increasing amount of LAP1 can enhance HDAC4-mediated repression of *COX-2* transcription. In addition, the transfection of LAP1K174A not only attenuates the LAP1-mediated repression activity on the *COX-2* reporter (Figure 7A, compare lanes 1 and 2 with lanes 5 and 6), but it also lost the HDAC4-mediated repressive activity on the *COX-2* reporter from 42% to 11% (Figure 7A, compare lanes 2 and 4 with lanes 6 and 8). However, cotransfection with HDAC1 expression vectors shows a consistent *COX-2* promoter activity (Figure 7A, compare lanes 2 and 3 with lanes 6 and 7). This suggests that suLAP1, at least in part, plays a functional role in HDAC4-mediated repression of the *COX-2* promoter. Furthermore, whether LAP1 has a higher capability to interact with HDAC4 than LAP1K174A was addressed by a Co-IP assay with exogenously expressed HA/LAP1 or HA/LAP1K174A. Meanwhile, HA/LAP2, HA/LAP2K151A and HA/LIP were recruited for comparison.

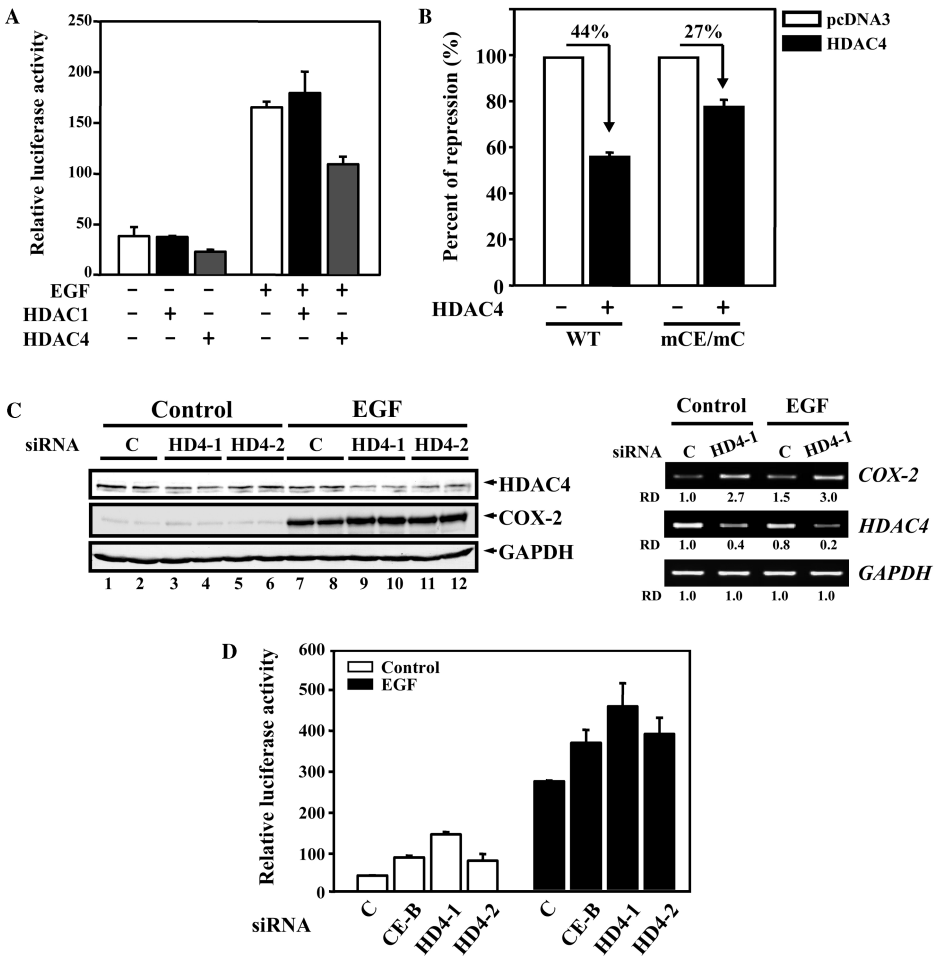

Figure 6. HDAC4, but not HDAC1, plays a negative role in regulating *COX-2* transcription. (**A**) The *COX-2* reporter was cotransfected with HDAC1 or HDAC4 expression vectors for 15 h in complete medium. Cell lysates were prepared for the luciferase assay. Three independent experiments are represented by the statistical analysis. (**B**) Two *COX-2* reporter vectors, pGC207/wt (WT) and pGC207/mCEBP/CRE (mCE/mC), were cotransfected with HDAC4 expression vectors or empty vectors. Cell lysates were harvested for the luciferase assay. The repression ratio was individually normalized with itself. (**C** and **D**) A431 cells were transfected with oligonucleotides-based siRNA of HDAC4 (HD4-1 or HD4-2), CEBPB (CE-B) or the scrambled control (C). After 30 h, these transfectants with or without EGF treatment were harvested to determine the COX-2 expression by western blot analysis (left panel of C) and RT–PCR (right panel of C) or luciferase assay (**D**) (RD: relative density).

The product of HA/LAP1-immunoprecipitated complex showed an obvious HDAC4-interactive activity, but the HA/LAP1K174A-immunoprecipitated complex did not (Figure 7B, right panel). However, the LAP2 and LAP2K151A showed consistent and low HDAC4-interactive activity (Supplementary Figure S3). HDAC4 is not detectable in the HA/LIP precipitated immunocomplex by anti-HA antibodies (data not shown). This implies that the HDAC4-mediated repressive ability on the *COX-2* promoter is suLAP1-dependent but not LIP- and suLAP2-dependent.

suCEBPD and suLAP1 corporately function in the repression of the *COX-2* promoter activity

The transient COX-2 expression responds to external stimuli. Our previous study suggests that suCEBPD can act as a negative regulator and may act through the recruitment of HDACs to repress *COX-2* transcription (14). Herein, we further demonstrated that the suLAP1 negatively regulates *COX-2* transcription. However, the structure/type of *in vivo* binding of these molecules, including CEBPD, CEBPB, SUMO1 and HDAC4, on the *COX-2* promoter under EGF treatment was unclear. The ChIP assay, using extracts of indicated time courses from the EGF-treated A431 cells, was performed to further elucidate the scenario among SUMO1, CEBPD, CEBPB and HDAC4. Consistent with our previous study (14), the increasing binding of CEBPD was observed within a short-term 1 h of EGF treatment, and the binding returned to the basal level upon EGF treatment for 12 h. However, increased CEBPB-binding activity was observed from 3 h and extended to 12 h of EGF treatment. More importantly, the HDAC4 binding pattern is consistent with SUMO1-conjugated proteins; whereas, HDAC1 maintains consistent binding activity throughout the time frame of EGF treatment (Figure 8A). To confirm whether CEBPD and CEBPB conjugated with SUMO1 and form a complex with HDAC4, the re-ChIP assay was performed. In correlation with EGF-regulated

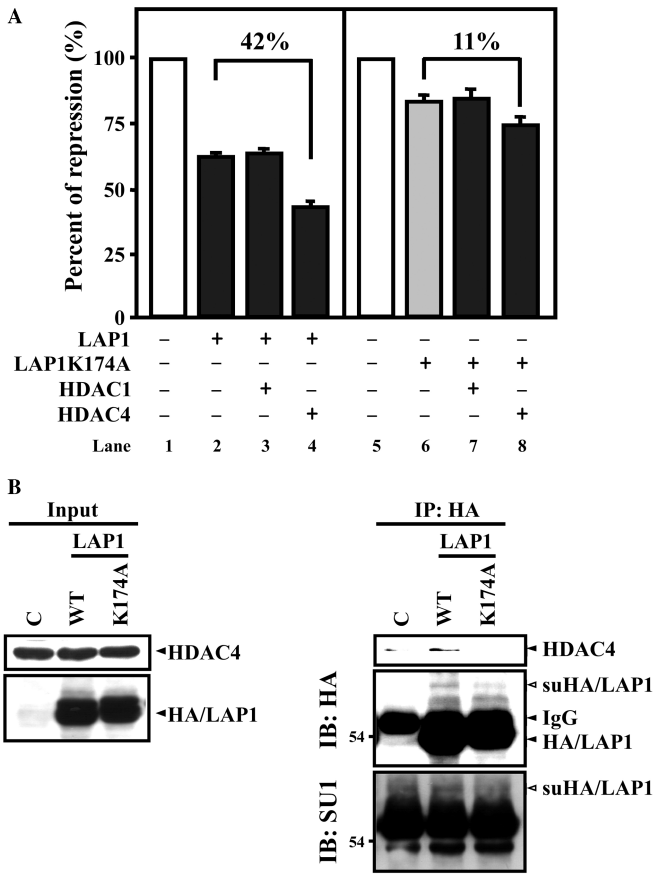

Figure 7. HDAC4 is involved in the suLAP1-mediated repression of *COX-2* promoter activity. (**A**) The *COX-2* reporter was cotransfected with the indicated expression vectors. Cell lysates were harvested for the luciferase activity assay. Reporter activities were normalized to the control transfectant of the reporter and pCDNA3. (**B**) The wild-type (WT) and sumoylation mutant (K174A) of HA/LAP1 were exogenously expressed in A431 cells. HA antibodies were used for the Co-IP assay. HDAC4, SUMO1 and HA antibodies were used for detection in the western blot analysis.

COX-2 transcription, increases in the complex of HDAC4 or SUMO1-conjugated proteins including CEBPD and CEBPB were observed in the long-term 12 h of EGF treatment, which was similar to the results with EGF-untreated cells (Figure 8B). To further confirm if CEBPDK120A and LAP1K174A can attenuate the interaction of HDAC4, the transient transfectants of HA/CEBPD, HA/CEBPDK120A, HA/LAP1 or HA/LAP1K174A were analyzed by the re-ChIP assays. The re-ChIP result demonstrated that the sumoylation mutants of CEBPD and LAP1 lost their HDAC4-binding capabilities (Figure 8C). This *in vivo* DNA binding assay suggests that the suCEBPD and suLAP1 have the potential to interact with HDAC4 and inactivate *COX-2* transcription. Moreover, to clarify whether the suCEBPD and suLAP1 jointly regulated the inactivation of the *COX-2* gene, the reporter assay was carried out by cotransfection with the *COX-2* reporter and various CEBPs expression vectors as indicated. Overexpressed CEBPD reversed HA/LAP1-mediated repression of the *COX-2* promoter activity (Figure 8D, compare lane 4 with lane 6), and further enhanced the HA/LAP1K174A-reversed *COX-2* promoter activity (Figure 8D, compare lane 6 with lane 7). More importantly, an increase of *COX-2* promoter activity was observed when the cells were cotransfected with LAP1K174A and CEBPDK120A expression vectors (Figure 8D, compare lane 8 with lane 7). These results suggest that suLAP1 and suCEBPD cooperate with each other in the regulation of *COX-2* promoter activity.

DISCUSSION

CEBPB is an important transcription factor involved in cellular proliferation (45,46) and differentiation (47,48). It is also involved in *COX-2* gene regulation (12,19,39). Herein, we were interested in the opposite biological phenomenon of EGF-induced lagging CEBPBs expression and the accompanying decrease of *COX-2* transcription (Figure 1). Our goal was to clarify whether CEBPBs potentially play negative roles in *COX-2* transcription. All three individual CEBPBs negatively repressed the *COX-2* promoter via the exogenous expressions of LAP1, LAP2 or LIP (Figure 2C). LIP is a well-studied dominant negative regulator of CEBPs due to an absence of the transactivation domain; however, the mechanism of LAP1- and LAP2-mediated repression in transcriptional regulation is unclear. LAP1 can be sumoylated on the lysine 174 residue which was proven by an *in vivo* sumoylation assay in A431 cells (Figures 3A, B and 7B and Supplementary Figure S4). This is consistent with a previous report stating that LAP1 is a SUMO1 substrate (26). The sumoylated form of LAP1 is coincident with the steady increase of LAP1 upon long-term EGF treatment (Figures 1B and 3C). Combining the LAP1K174A-mediated de-repressive effect (Figure 3D and E), we speculate that the increase of suLAP1 potentially plays a repressive role in *COX-2* transcription upon long-term EGF treatment.

As corepressors, HDACs require specific transcription factors to guide them and target DNA elements for their precise regulatory functions. Several studies have pointed out that HDAC1, HDAC3 and HDAC8 play functional roles in *COX-2* transcription (49–51). suCEBPD shows a high affinity in interaction with HDAC1, 3 and 4 but not HDAC2 (52). We provided evidence to show that the wild-type LAP1, and not the LAP1K174A, has a higher interaction affinity with HDAC4 (Figure 7B). In addition, LIP did not interact with HDAC4 in the Co-IP assay (data not shown). HA/LAP2K151A does not reverse LAP2-repressed *COX-2* promoter activity and shows a consistent HDAC4-binding activity with wild-type LAP2 (Figure 3E and Supplementary Figure S3). The experimental results suggest different roles for each of the three isoforms of CEBPB through interaction with HDAC4, although all of them show a repressive effect on *COX-2* transcription. LAP1 is not only a SUMO1 substrate (26) but also a SUMO2/3 substrate (23). Most importantly, the lysine 174 of LAP1 is the major SUMO-conjugated site. Our results demonstrate that the overexpression of SUMO1 increases the SUMO1-conjugated HA/LAP1 in A431 cells (Figure 3A). We also demonstrate that

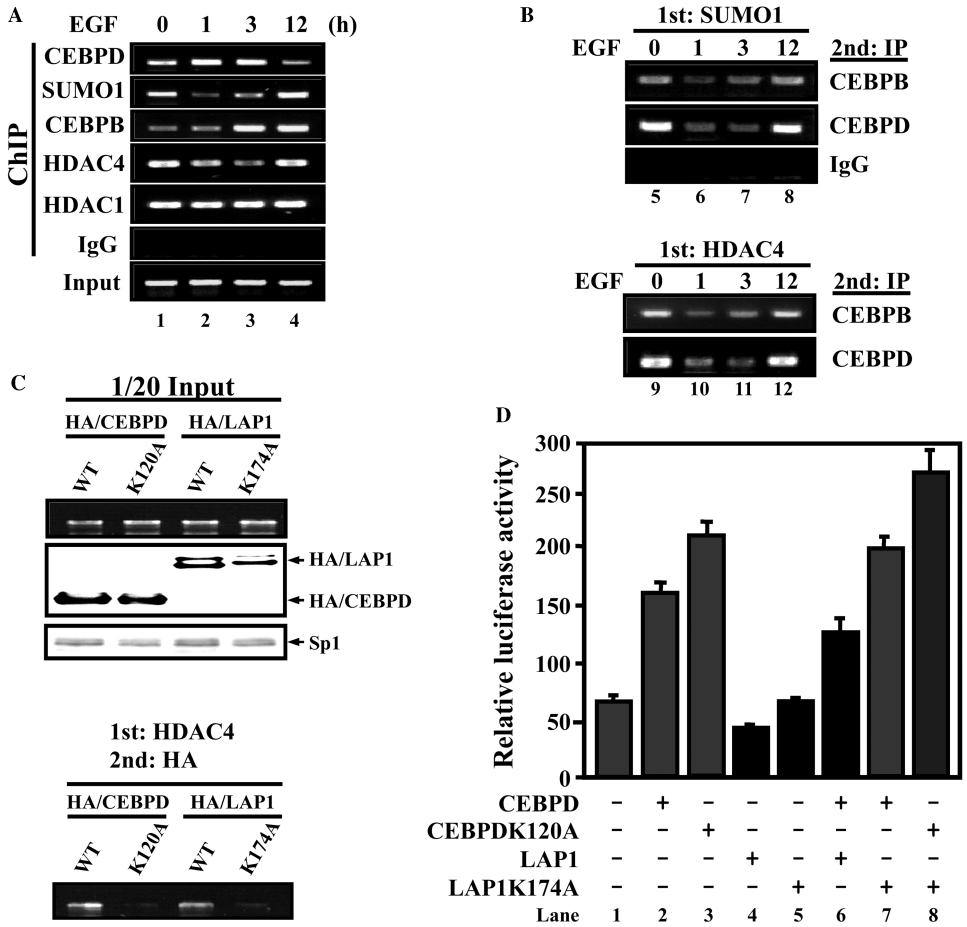

Figure 8. suLAP1 and suCEBPD play a negative coordinating role in the transcriptional regulation of *COX-2* gene, and were consistent with the HDAC4 binding in the long-term EGF treatment. Sheared formaldehyde-cross-linked chromatin with or without EGF treatment from A431 cells was immunoprecipitated with specific antibodies as indicated and the *COX-2* promoter region was processed for PCR amplification with specific primers. As a positive control, PCR amplification was also carried out with input DNA from chromatin before the IP step. The detailed procedures of the ChIP (**A**) and re-ChIP (**B**) assay are described in 'Materials and methods section'. (**C**) A431 cells were transfected with various expression vectors as indicated for 24 h. The re-ChIP assay was performed to determine the chromatin/protein complex of HDAC4 and CEBPD or LAP1 on *COX-2* promoter. The detailed procedures are described in 'Materials and methods section'. (**D**) A431 cells were cotransfected with the *COX-2* reporter and various expression vectors as indicated. Lysates of the transfectants were harvested for the luciferase assay.

SUMO1-LAP1 is detectable in the HA/LAP1-immunoprecipated complex but not in the HA/LAP1K174A-immunoprecipated complex (Figure 3B, 7B and Supplementary Figure S4). Furthermore, the knockdown of SUMO1 attenuates LAP1-mediated repression of the *COX-2* promoter (Supplementary Figure S5). These data suggest that SUMO1-LAP1 can function in the repression of the *COX-2* promoter activity. However, we still could not rule out the possibility that the SUMO2/3-conjugated LAP1 may also participate in the regulation of *COX-2* transcription because the SUMO2/3 is also detectable in the HA/LAP1-immunoprecipated complex but not the HA/LAP1K174A-immunoprecipated complex (Supplementary Figure S6). This issue requires further investigation. In addition, Supplementary Figure S2 shows that the suCEBPD has a higher HDAC4-interactive activity than wild-type CEBPD. This provides explanation that the LAP1K174A cannot fully recover HDAC4-exerted repressive activity (Figure 3D, 3E and 7A), and is also consistent with the result that the co-overexpression of CEBPK120A and LAP1K174A can enhance *COX-2* promoter activity (Figure 8D). These results suggest that the suCEBPD and suLAP1, but not suLAP2 or LIP, respond to HDAC4-mediated repression of *COX-2* transcription. This result also provides evidence implying that the CEBPD and LAP1 are both candidates in responding to the HDAC4-mediated transcriptional regulation including chromatin remodeling.

The CEBP and CRE motifs play critical functional roles in the transcriptional regulation of the *COX-2* gene (4,14,16). CEBPD was shown to directly bind to these two motifs by an *in vitro* DNA binding assay. This current study demonstrates that CEBPBs can also bind to these two sites (Figure 4B), suggesting the possibilities of CEBPBs switching or coordination on the CEBP or CRE motifs of the *COX-2* promoter during the time-dependent EGF treatment (Figure 9). Several papers demonstrate that exogenously overexpressed CEBPD or CEBPB are potential positive regulators on the *COX-2* transcription. However, exogenously expressed CEBPD has greater potential to be an acute responsive protein than CEBPB in the induction of the *COX-2* gene

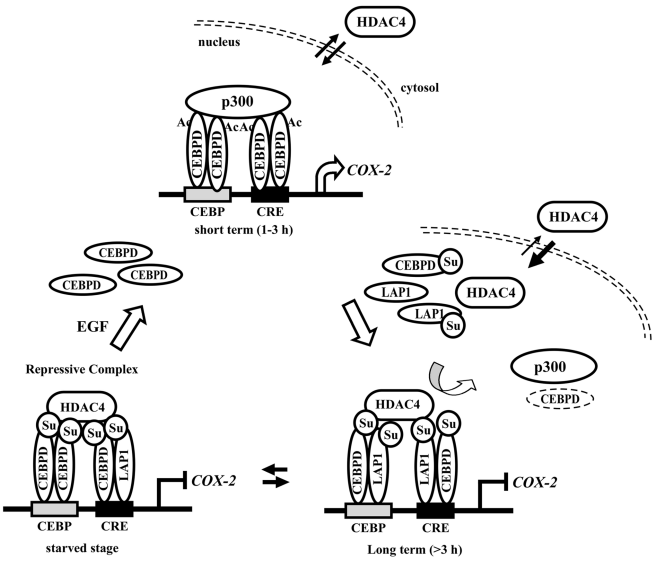

Figure 9. The involvement of CEBPD and LAP1 in the EGF-regulated *COX-2* transcription. In the resting stage or starving stage, suCEBPD and suLAP1 could bind to the CRE and CEBP motifs of *COX-2* promoter and interact with nuclear HDAC4 to form a repressive complex. In the early 1–3 h of EGF treatment, the instantly induced nonsumoylation of CEBPD can be positive regulators that replace the suCEBPD and suLAP1. This replacement will increase the p300 binding and form an intact initiation of the activation complex to stimulate *COX-2* transcription. After 3 h of EGF treatment, combination of decreased binding of nonsuCEBPD and p300, with the increased binding of suLAP1, suCEBPD and increased importation of HDAC4 will re-inactivate *COX-2* transcription through the new formation of the HDAC4/suCEBPD/suLAP1 complex.

(20,53,54). Therefore, the normal physical situation for CEBPD- and CEBPB-mediated *COX-2* transcription needs to be further elucidated. In tumorigenesis, the overexpression of LAP1/LAP2 has been reported in several human tumors with the implication that it is correlated with COX-2 expression, moreover leading to the conclusion that CEBPB might be a crucial positive regulator in *COX-2* transcription. However, LIP draws less attention because it comes from one transcript of the *CEBPB* gene and is a strong repressor of *COX-2* transcription. On the other hand, our unpublished data demonstrates that induction of CEBPD expression is insensitive to external stimulation in cancer cell lines because of hypermethylation of the *CEBPD* promoter. Consequentially, this may result in the inaccurate conclusion that CEBPD may potentially be the most negative regulator just because its expression does not match the increase of COX-2 expression in tumors. Combining previous reports and our studies, we speculated that the CEBPD serves as a bifunctional regulator, at least in part, whereas LAP1/LAP2 may function as repressors acting through spatial and temporal changes to execute the switching of *COX-2* transcription in response to external stimuli (Figure 9). In cancer cells, the overexpressed LAP1/LAP2 or de-sumoylated LAP1/LAP2 can replace the silenced CEBPD and become a potential activator of COX-2 transcription.

The overexpression of HDAC1 represses *COX-2* promoter activity in phorbol 12-myristate 13-acetate (PMA)-treated HFb cells and in lipopolysaccharide (LPS)-treated RAW 264.7 cells (44). CEBPB is suggested to function in the gene repression of PPARβ/δ through a HDAC1-mediated mechanism via a specific C/EBP-responsive element (24). However, this current study demonstrates that the exogenous overexpression of HDAC1 alone has no effect on *COX-2* promoter activity (Figure 6A) even with the cotransfected LAP1 (Figure 7A). A similar experiment was performed in our ongoing study in which the overexpression of HDAC1 can repress the *PPARG2* promoter activity, participating in the suCEBPD-mediated repression in HepG2 cells (52). These results suggest that the HDAC1-mediated transcriptional repression of genes may occur in cell type-dependent or gene-specific manners (49). Class II HDACs show a nucleocytoplasmic shuttling phenomenon through interaction with 14-3-3 protein (55,56). Under long-term EGF treatment, the accumulation of HDAC4 shuttling in the nucleus was observed. Interestingly, the SUMO1 or SUMO1-conjugated proteins can be transiently exported to the cytoplasm during the short-term EGF treatment. Most importantly, the increasing amount of suLAP1 is coincident with the SUMO1 re-importation and HDAC4 accumulation (Figure 5A). These observations are also consistent with the ChIP and re-ChIP results that represent the *in vivo* transcription factor-binding pattern on the *COX-2* promoter in a time-dependent manner (Figure 8A and B). Compared with LAP1, LAP2 is a shorter protein lacking the N-terminal 23 amino acids in human. The integrity of N-terminal LAP1 not only affects the SUMO2/3 conjugation, but is also essential for the specific interaction of SWI/SNF complexes (23,57). Among the CEBPB isoforms, the suLAP1 is the only effecter involved in the HDAC4-mediated repression of *COX-2* transcription. This implies that HDAC4 could be a functional distinguisher between the SUMO1-conjugated LAP1 and LAP2, at least in *COX-2* gene transcription. Additionally, HDAC4 possesses the SUMO E3 ligase activity. This raises an interesting issue of whether LAP1 is a HDAC4 substrate for its activity of SUMO E3 ligase. The overexpressed HDAC4 transfectant shows a consistent sumoylation of LAP1 (Supplementary Figure S7). This implies that the sumoylation of LAP1 is in a HDAC4-independent manner. Our data also demonstrate that the wild-type LAP1 and CEBPD bear higher affinities for HDAC4 interaction than their sumoylation mutants. Moreover, the PCR products of *COX-2* promoter are detectable in the CEBPD- or LAP1-re-ChIP samples from the ChIP sample of HDAC4 (Figure 8C). These results suggest that the suCEBPD and suLAP1 are able to coordinate with HDAC4, and modulate *COX-2* transcription in A431 cells (Figure 9). However, the integration of the EGF-induced signaling pathway and the HDAC4-nucleocytoplasmic shuttling remains unclear.

This precise study not only confirms previous reports suggesting that the CEBPB could be a repressor but also dissects the function of CEBPB isoforms in *COX-2* transcription upon the long-term EGF treatment-induced re-inactivation and the EGF-starved stage. HDAC4 plays a role as a kinetic regulator in a time-dependent manner to coordinate the regulation of the *COX-2* gene. suCEBPD, on the lysine 120 residue, and suLAP1, on the

lysine 174 residue, are involved in the interaction of HDAC4, and function in the HDAC4-mediated repression of *COX-2* transcription.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors would like to thank Christine C. Hsieh and Tony Bourke for critical review of this article.

FUNDING

NSC (96-2320-B-006-044-MY2); NCKU (C007). Funding for open access charge: NSC (96-2320-B-006-044-MY2).

Conflict of interest statement. None declared.

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