CCAAT Enhancer Binding Protein-β Regulates Matrix Metalloproteinase-1 Expression in Interleukin-1β–Stimulated A549 Lung Carcinoma Cells

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Abstract
Matrix metalloproteinase-1 (MMP-1) is an inflammation-inducible neutral protease that mediates extracellular matrix remodeling and promotes tumor invasion. In this study, we examined the activation of MMP-1 gene expression in A549 lung carcinoma cells stimulated with the inflammatory cytokine interleukin-1β (IL-1β). We found that MMP-1 mRNA levels were maximal following 16 hours of IL-1β stimulation and that this correlated with the expression of the transcription factor CCAAT enhancer-binding protein-β (CEBPB). Knockdown of CEBPB expression with short hairpin RNA abrogated the expression of MMP-1, MMP-3, and MMP-10 in IL-1β–stimulated A549 cells. An established CEBP element in the MMP-1 promoter was found to be required for basal and IL-1β–induced transcription. Electrophoresis mobility shift assays showed that CEBPB binds to this promoter element maximally 16 hours after IL-1β stimulation. DNA affinity chromatography studies showed that the LAP1, LAP2, and LIP isoforms of CEBPB bind to the IL-1β–responsive CEBPB site in the MMP-1 promoter. Exogenous expression of the LAP1 and LAP2 isoforms stimulated the MMP-1 promoter, whereas LIP had no effect. Phosphorylation of CEBPB at Thr235 peaked at 16 hours in IL-1β–stimulated cells. The MEK inhibitor U0126 inhibited this phosphorylation and reduced MMP-1 gene induction. These studies establish CEBPB as an important mediator of metalloproteinase gene activation during inflammatory responses in lung cancer cells and highlight the different regulatory roles of CEBPB isoforms.

Introduction
Remodeling of the extracellular matrix is a tightly regulated process during development that is mediated by a family of neutral proteases known as the matrix metalloproteinases (MMP). In disease states such as arthritis and cancer, MMP expression is inappropriately activated, causing pathologic connective tissue destruction and cellular invasion (1). Among the 23 identified human MMP family members (2), MMP-1 (collagenase-1) is broadly expressed by mesenchymal and epithelial cells and degrades interstitial collagens, the most abundant extracellular matrix proteins in the body (1). It has long been appreciated that inflammation-dependent activation of MMP-1 gene expression in synovial cells and chondrocytes contributes to joint destruction in rheumatoid arthritis and osteoarthritis (3). In addition to its classic role in extracellular matrix remodeling, MMP-1 also cleaves protease-activated receptor-1, resulting in the activation of this receptor and tumor invasion (4). This function of MMP-1 as a promoter of tumor invasion may explain why increased MMP-1 expression is associated with increased risk for some forms of breast (5) and lung cancer (6).

Chronic inflammation is a common mediator of disease progression in both arthritis and cancer. Inflammatory cytokines such as IL-1β and tumor necrosis factor α are known to drive MMP-dependent connective tissue destruction in rheumatoid arthritis, and the antagonism of these cytokines is an important line of therapeutic intervention (7). Similarly, these inflammatory cytokines are prevalent in many cancers and are thought to promote tumor progression (8). Genes downstream of inflammatory cytokines, such as cyclooxygenase-2, are known to contribute to the progression of colorectal cancer, but inhibition of this enzyme increases the risk of cardiovascular disease (9). Given that inflammatory cytokines also activate MMP-1, and that this enzyme promotes tumor invasion, IL-1β induction of MMP-1 in cancer cells may have important therapeutic implications.

IL-1β activation of MMP-1 transcription requires the input of multiple signaling pathways with the nuclear factor-κB (NF-κB) transcription factors and mitogen-activated protein kinases playing dominant roles (1). Our studies have shown that RelA is required for IL-1β induction of MMP-1, whereas NF-κB1 and NF-κB2 act as inhibitors of gene expression (10). In parallel with NF-κB activation, IL-1β stimulates the extracellular-regulated kinases (ERK), which in turn activate the transcription factor CCAAT enhancer-binding protein β (CEBPB). Additional studies in our laboratory have established that IL-1β–induced, ERK-dependent phosphorylation of CEBPB contributes to increased MMP-1 transcription in chondrocytes (11).

CEBPB is an intronless gene that is expressed as three distinct isoforms through alternative translational initiation at three in-frame start codons (12). Human CEBPB1 (liver-enriched activator protein; LAP1) and CEBPB2 (LAP2) are 346 and 323

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amino acids, respectively, and function as transcriptional activators (13). CEBPB3 (liver-enriched inhibitory protein; LIP) is 148 amino acids in length, and has been reported to function as a transcriptional repressor due to its lack of a transactivation domain (13). CEBPB becomes transcriptionally active when phosphorylated on Thr235 (Thr217 in the mouse) by the ERK2-stimulated kinase known as p90 ribosomal S kinase (14).

In this study, we have examined the concurrent IL-1β–dependent activation of CEBPB and MMP-1 in the A549 lung carcinoma cell line. We found that CEBPB protein expression and ERK-dependent phosphorylation correlated with the temporal increase of MMP-1 mRNA in these cells. Moreover, gene silencing of CEBPB with short hairpin RNA (shRNA) inhibited IL-1β–induced MMP-1, MMP-3, and MMP-10 gene expression. Interestingly, whereas all three CEBPB isoforms bound to an IL-1β–responsive element in the MMP-1 promoter, only the LAP1 and LAP2 isoforms promoted transcription. These findings highlight the importance of cytokine-induced CEBPB activity with respect to MMP-1 gene expression, and present this pathway as a potential target of therapy in lung cancer.

Results

IL-1β Induces MMP-1 and CEBPB Gene Expression in A549 Cells

We first set out to determine if the inflammatory cytokine IL-1β could promote MMP-1 gene expression in the A549 lung carcinoma cell line. Using quantitative real-time reverse transcription-PCR, we found that MMP-1 mRNA increased following IL-1β treatment during a 24-hour time course, with a peak in fold induction observed at 16 hours (Fig. 1A). Because we previously reported that CEBPB contributes to MMP-1 gene expression in chondrocytes (11), we assayed CEBPB cellular protein levels over a 24-hour period in untreated and IL-1β–treated A549 cells. Western analysis of A549 whole cell extracts revealed that the LAP1 and LAP2 isoforms of CEBPB were expressed at low levels in resting cells and increased to peak levels by 16 hours of IL-1β stimulation (Fig. 1B). In contrast, maximal expression of the LIP isoform occurred later at 24 hours. These data establish a temporal correlation between MMP-1 mRNA and CEBPB protein levels in IL-1β–stimulated A549 cells.

CEBPB Is Required for IL-1β–Dependent MMP Expression in A549 Cells

The concurrent increase in MMP-1 and CEBPB gene expression presented the possibility that CEBPB modulates MMP-1 gene expression in A549 cells. To address this question, we transfected A549 lines with shRNA vectors targeting CEBPB mRNA and then assayed CEBPB and MMP-1 gene expression in untreated and IL-1β–treated A549 cells.
cells. Two separate shRNA plasmids effectively reduced basal and IL-1β-stimulated expression of all three CEBPB isoforms relative to the control shRNA plasmid (Fig. 2A). To assess the effect of CEBPB knockdown for MMP-1 gene regulation, we assayed MMP-1 mRNA from control and CEBPB shRNA-treated A549 cells. CEBPB knockdown with both shRNAs dramatically reduced basal and IL-1β–induced MMP-1 mRNA (Fig. 2B, C, and D). We used the SA Biosciences Extracellular Matrix and Adhesion Molecules PCR array (PAHS-013) to assess the effect of CEBPB silencing on other MMP genes. This analysis indicated that CEBPB knockdown most dramatically affected the expression of MMP-3 and MMP-10 (data not shown). We then used quantitative reverse transcription-PCR to confirm that both CEBPB shRNAs reduced basal and IL-1β–induced MMP-3 and MMP-10 mRNA in A549 cells (Fig. 2B, C, and D). These data show that CEBPB expression is required for maximal MMP-1, MMP-3, and MMP-10 gene expression in IL-1β–stimulated A549 cells.

**FIGURE 2.** Effect of CEBPB knockdown on MMP expression. A549 cells were transfected with a control vector or two separate shRNA vectors specific for CEBPB mRNA. A. Cultures were selected for stable expression with G418, cultured for 24 h both with and without IL-1β, and then assayed for CEBPB and tubulin expression by Western blot. RNA from cells expressing control shRNA (B) or shRNA specific for CEBPB (C and D) were assayed for effects on MMP-1, MMP-3, and MMP-10 gene expression by quantitative reverse transcription-PCR. Gene expression levels are presented relative to untreated cells transfected with the control shRNA. Fold induction by IL-1β is indicated for each transfectant. Columns, mean; bars, SD.

**CEBPB Targets the MMP-1 Promoter in IL-1β-Stimulated A549 Cells**

We next asked if CEBPB regulates MMP-1 gene expression at the transcription level. Our earlier work in chondrocytes mapped an IL-1β–responsive, CEBPB-binding element located at −2921 of the human MMP-1 promoter (11). We therefore tested the responsiveness of this promoter element in A549 cells. Basal expression of a construct containing 2942 bp of the MMP-1 promoter was significantly increased in IL-1β–treated cells, whereas a construct containing 2002 bp of promoter showed dramatically reduced basal activity and no induction by IL-1β (Fig. 3). Deletion of the previously described CEBPB binding site resulted in reduced basal and IL-1β–induced activity (Fig. 3). IL-1β activation of the MMP-1 promoter in A549 cells is consistent with assays of MMP-1 hnRNA levels that showed maximal IL-1β induction after 16 hours (data not shown). Thus, similar to our findings in chondrocytes (11), the CEBPB site at −2921 of the MMP-1 promoter is critical for basal and IL-1β–induced MMP-1 transcription in A549 cells.
To determine if CEBPB directly targets the MMP-1 promoter, we did electrophoresis mobility shift assays of nuclear extracts from A549 cells using the MMP-1 −2921 CEBPB site as a probe. Cells treated with IL-1β for 4 hours contained elevated DNA binding activity that was specific to the putative CEBPB binding site. Moreover, the addition of two separate CEBPB-specific antibodies resulted in supershifts, whereas an antibody to CEBPA did not (Fig. 4A). This specific binding activity increased by 16 hours of IL-1β stimulation (Fig. 4B), which is consistent with maximal CEBPB and MMP-1 expression at 16 hours (Fig. 1A).

CEBPB Isoforms Play Specific Roles with Respect to MMP-1 Promoter Activation

Because LAP1, LAP2, and LIP expression increased in IL-1β–stimulated cells (Fig. 1B), and the antibody used for supershifting does not differentiate between the isoforms, we wanted to determine which CEBPB isoforms participated in the binding activity at the MMP-1 promoter. To address this, we did DNA affinity chromatography using extracts from untreated and IL-1β–treated A549 cells. The wild-type MMP-1 CEBPB site bound detectable levels of LAP1 from extracts of untreated and IL-1β–treated A549 cells. The wild-type MMP-1 CEBPB site bound detectable levels of LAP1 from extracts of untreated cells (Fig. 5A). Treatment with IL-1β for 16 hours resulted in a moderate increase in LAP1 binding and dramatically increased binding of the LAP2 and LIP isoforms (Fig. 5A). Importantly, mutation of the CEBP site in the oligonucleotide substantially reduced its affinity for all three isoforms. Thus, as CEBPB isoforms accumulate in IL-1β–stimulated cells, they can bind the MMP-1 promoter to modulate MMP-1 expression.

To assess the role of the individual CEBPB isoforms in MMP-1 transcription, we cotransfected A549 cells with the 2942 bp MMP-1 promoter construct along with vectors expressing LAP1, LAP2, and LIP isoforms (Fig. 5B). The construct coding for the full-length LAP1 isoform does not express the shorter LAP2 protein in A549 cells (data not shown). Compared with the control expression vector pCMVTAG4a, the LAP1 isoform increased basal MMP-1 promoter activity 10-fold, and IL-1β did not augment basal expression. The LAP2 isoform increased basal MMP-1 promoter activity 13-fold, and the addition of IL-1β resulted in a moderate, albeit a significant, increase in MMP-1 promoter activity. LAP2 was significantly more potent than LAP1 at augmenting MMP-1 transcription in untreated and IL-1β–treated cells. In contrast to LAP1 and LAP2, the LIP isoform did not affect basal or IL-1β–stimulated levels. To show the sequence specificity of LAP2 activation of the MMP-1 promoter, we compared the effects of the LAP2 expression construct in cells transfected with the wild-type MMP-1 promoter and the CEBPB deletion mutant (Fig. 5C). Importantly, deletion of the CEBPB site at −2921 significantly reduced MMP-1 promoter activity in LAP2-expressing cells and fold induction was dramatically reduced. This effect was seen in both untreated and IL-1β–stimulated cells. These data show that although all three CEBPB isoforms bind to the MMP-1 promoter, only LAP1 and LAP2 activate the MMP-1 promoter, with LAP2 being the most active isoform.

ERK-Dependent Phosphorylation of CEBPB Is Required for IL-1β Stimulation of MMP-1 in A549 Cells

It has been reported that phosphorylation of CEBPB on Thr355 by ERK promotes the transcriptional activity of this transcription factor (14). Therefore, we assayed phosphorylation of the ERK-dependent site at Thr355 of CEBPB in IL-1β–stimulated A549 cells. ERK-dependent phosphorylation...
of CEBPB at this site was maximal after 16 hours of IL-1β treatment and then began to decline by 24 hours (Fig. 6A). Interestingly, this correlates directly with the time course for MMP-1 mRNA expression. Inhibition of ERK-dependent phosphorylation with U0126 abrogated IL-1β-induced phosphorylation of CEBPB and significantly reduced IL-1β-induced MMP-1 mRNA levels at 16 hours (Fig. 6B). These data show that IL-1β promotes ERK-dependent phosphorylation of CEBPB in A549 cells, which is concurrent with and required for maximal MMP-1 gene expression. Taken together with our other results, these data suggest that ERK phosphorylation of CEBPB is critical to maintain transcriptional activation of the MMP-1 gene in A549 cells.

**Discussion**

MMP-1 contributes directly to tumor progression and invasion through matrix remodeling and activation of invasion signaling molecules such as protease-activated receptor-1 (4). Inflammatory signals, such as IL-1β, may contribute to tumor invasion by promoting tumor and/or stromal cell expression of MMP-1. In this study, we showed that IL-1β promotes expression and phosphorylation of the transcription factor CEBPB, which can act as an activator of MMP-1 transcription through an established binding site in the MMP-1 promoter. Although it is well established that both transcriptional and posttranscriptional mechanisms contribute to IL-1β-induced MMP-1 gene expression (1), here we present the following model to define CEBPB-dependent MMP-1 transcription in IL-1β-stimulated A549 cells. IL-1β stimulates the expression of CEBPB isoforms, with LAP1 and LAP2 levels maximal at 16 hours and LIP levels maximal at 24 hours. Concurrently, IL-1β promotes transcriptional activity of CEBPB through ERK-dependent phosphorylation at 16 hours, which contributes to maximal MMP-1 gene expression. Our data show that CEBPB is a central mediator of IL-1β-induced MMP-1 gene expression in non–small cell lung carcinoma cells, and suggest that regulating the levels and phosphorylation state of CEBPB isoforms can be important to regulate MMP-1 expression and the invasive potential of lung cancer cells.

It is now widely accepted that inflammatory pathways contribute to the development and progression of cancer in general, and lung cancer, in particular (15-17). Importantly, polymorphisms in the IL-1β and IL-1β receptor antagonist genes have been associated with increased or decreased risk of non–small cell lung cancer (18, 19), suggesting an important role for IL-1β–regulated genes in this disease. Thus, in addition to the established carcinogens such as tobacco smoke, diesel exhaust, and radon, chronic inflammation and aberrant IL-1β expression may lead to effector genes that promote tumor growth and invasion. Cyclooxygenase-2 is another IL-1β–activated gene that is overexpressed in lung cancer that has recently been targeted therapeutically with some success (20). It is important to note that CEBPB has been reported to be an activator of IL-1β–stimulated cyclooxygenase-2 expression (21) and a repressor of epidermal growth factor–stimulated cyclooxygenase-2 expression (22). Our data are consistent with a stimulatory role of CEBPB with respect to transcriptional responses to IL-1β.

Although all three CEBPB isoforms are expressed in IL-1β–stimulated A549 cells, only LAP1 and LAP2 contribute to MMP-1 transcription, with LAP2 being a more effective transactivator than LAP1. Interestingly, Eaton et al. reported a similar dominance of LAP2 with respect to activation of the cyclin D1 promoter in breast cancer cells (13). Elevated LIP expression has been reported in some breast cancers (23) and this may be due to the fact that this isoform antagonizes the CEBPB-dependent cytostatic response to transforming growth factor-β (24). These studies suggest that LIP contributes to transformation due to its blistering effect on CEBPB-dependent MMP-1 transcription in IL-1β–stimulated A549 cells.

**FIGURE 4.** DNA-binding activity at the MMP-1 –2921 CEBP site. Nuclear extracts were prepared from A549 cells cultured in serum-free media for 4 or 16 h, with and without IL-1β stimulation. A, Four-hour extracts were incubated with the –2921 MMP-1 CEBP site depicted, along with unlabelled oligonucleotide competitors or antibodies raised to CEBPB and CEBPA. Sequence-specific, nonspecific, and supershifted bands are indicated. B, Four-hour and 16-h nuclear extracts were assayed by mobility shift assay using the –2921 MMP-1 CEBP site. The resulting shifts were exposed for a shorter period than in A to show intensity differences between 4 and 16 h treatments. These data are representative of two separate experiments.
ability to interfere with the normal functions of CEBPB in the cell cycle. We found that LIP did not dramatically affect transcription from the MMP-1 promoter. Thus, whereas LIP may contribute to cell cycle aberrations in cancer cells, it does not seem to play a role in IL-1β–stimulated MMP-1 expression.

In addition to MMP-1, CEBPB is required for IL-1β activation of MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2). A preliminary analysis of the MMP-3 and MMP-10 promoters using the Transcription Element Search System4 identified multiple putative CEBP elements in both these genes (data not shown). MMP-3 and MMP-10 expand the degradative potential of A549 cells by targeting noncollagen matrix proteins such as fibronectin and laminin (9). MMP-3 also contributes to epithelial-mesenchymal conversion of mammary epithelial cells through cleavage of E-cadherin (2). Recently, Frederick and colleagues (25) reported that MMP-10 contributes to anchorage-independent growth of NSCLC through a PKCι-dependent pathway. Importantly, both MMP-3 and MMP-10 can activate latent MMP-1 (26-28). Consequently, CEBPB promotes collagenolytic activity both at the level of MMP-1 gene activation and enzymatic activity. This concept is consistent with our finding that all the MMP-1 secreted by IL-1β–stimulated A549 cells is in the 42 kDa active form (data not shown). Overall, our findings suggest that targeting CEBPB in lung cancer cells could have a therapeutic benefit by inhibiting three MMPs involved in tumor progression and invasion.

Materials and Methods

Cell Culture and Reagents

A549 cells were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum (HyClone), penicillin/streptomycin, and glutamine (Cellgro, Mediatech). IL-1β was purchased from R&D...

4 http://www.cbil.upenn.edu/cgi-bin/tess/tess
Systems and used at 10 ng/mL. For assays of gene expression, cells were washed with HBSS and placed in DMEM supplemented with 0.2% lactalbumin hydrolysate (Invitrogen). The LAP2 and LIP coding sequences were amplified by PCR from a CEBPB cDNA purchased as an IMAGE clone (cDNA clone MGC:15409 IMAGE:3028673) from Invitrogen. Cloning primers for LAP2 were ATGGGATCCGGAGGA. Cloning primers for LIP were ATGCGGCGGCGCTTCCCGTAC and GCAGTGCCGGAGGAGGAGCA. The resulting PCR products were TOPO cloned into pcDNA 6.2/N-EmGFP-GW/TOPO (Invitrogen). LAP2 and LIP sequences were then digested out of pcDNA 6.2/N-EmGFP-GW/TOPO with EcoRI and subcloned into pcMVTAG4a (Stratagene). The full-length CEBPB sequence coding for LAP1 (pCMV6-Ac-CEBPB-GFP) was purchased from Origene. The coding sequence for LAP1 was then digested out of this plasmid with BamHI/XhoI and subcloned into pCMVTAG4a. All plasmids were confirmed by sequencing at the Dartmouth College Molecular Biology and Proteomics Core Facility.

A

IL-1β + U0126

Phospho-CEBP

Phospho-Erk 1/2

Actin

B

FIGURE 6. IL-1β stimulates ERK-dependent phosphorylation of CEBPB and MMP-1 expression. A, Confluent cultures of A549 cells were placed in serum-free media both with and without the MEK inhibitor U0126 (10 μM/L) for 30 min. Cultures were then stimulated for the indicated times with IL-1β (10 ng/mL) and CEBPB phosphorylated Thr235 was assayed by Western blot. The data presented are representative of three separate experiments. B, A549 cultures were treated with IL-1β, both with and without U0126 pretreatment, for 16 h. Total RNA was isolated and assayed for MMP-1 mRNA by quantitative real-time reverse transcription-PCR.

Western Blot Analysis

A549 cells were cultured in six-well dishes to confluence and then washed and placed in serum-free media. IL-1β was added to 10 ng/mL and incubated for the indicated time points. Cultures were harvested in 200 mL of 2× sample buffer (Sigma Chemical) and heated to 95°C for 5 min. Twenty microliters of each sample were resolved on 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Inc.). Blots were probed with antibodies to total CEBPB (Santa Cruz Biotechnology), phosphorylated Thr235 of CEBPB (Cell Signaling Technology), and Pan Actin (Cell Signaling Technology). Blots were visualized using the Pierce Biotechnology Supersignal West Femto detection kit.

Quantitative Real-time Reverse Transcription-PCR

mRNA levels were assayed by quantitative real-time reverse transcription-PCR as described previously (29, 30). The primers used to detect MMP-1, MMP-3, MMP-10, and glyceraldehyde-3-phosphate dehydrogenase were purchased from SA Biosciences. The SA Biosciences Extracellular Matrix and Adhesion Molecules PCR Array (PAHS-013) was done on a Bio-Rad CFX96 real-time PCR machine according to the instructions of the manufacturer.

Transient Transfection and Reporter Assay

A549 cells were transfected with the human MMP-1 promoter/luciferase reporter plasmids pGL3-2942 and pGL3-2942ΔCEBP (11) using Geneporter Reagent (Genlantis) according to the instructions of the manufacturer. Cells were also transfected with pGL3-2942 along with pcMVTAG4-CEBPB expression plasmids and pCMV6-Ac-GFP (Origene). Twenty-four hours after transfection, cultures were serum-starved and cultured for an additional 18 h both with and without IL-1β (10 ng/mL) and then assayed using Luciferase assay reagent (Promega). Luciferase activity was normalized to GFP fluorescence to control for transfection efficiency.

Stable Transfection and Gene Knockdown in A549 Cells

A549 cells were transfected with the SA Biosystems SureSilencing CEBPB shRNA using LipofectAMINE LTX reagent (Invitrogen) according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were placed in selective media containing 800 μg/mL of geneticin and cultured for 2 to 4 wk. CEBPB knockdown was assessed by Western blot of whole cell extracts.

Electrophoresis Mobility Shift Assay and DNA Affinity Chromatography

Nuclear extracts from A549 cells were prepared as described previously (31). Biotin-labeled double-stranded oligonucleotides, corresponding to −2931 to −2892 of the human MMP-1 promoter, were purchased from Integrated DNA Technologies, Inc. and annealed. Fifty femtomoles of biotin-labeled DNA probes were incubated with 5 μg of nuclear extract in the presence of 100 ng of poly-dI:dC for 30 min at room temperature. DNA-protein complexes were resolved on 5% Tris-borate EDTA gels, transferred to charged nylon membranes (Millipore) and visualized with the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology). For competition assays, molar excess of unlabeled, double-stranded oligonucleotides were included in
the binding reactions. These included the wild-type –2921 to –2892 oligonucleotide (TAAAAAAATATGACAGAAAG-TGAATTTCAAGACTAAGT) or an oligonucleotide with the CEBP binding site mutated (TAAAAAAATATGCCCC-TACCTTTGAATTTCAAGACTAAGT). For supershift as-
says, antibodies (Santa Cruz Biotechnology) raised against CEBPA (SC-9314X) and CEBPB (SC-150X, SC-7962X) were added to the binding reactions. DNA affinity purification was done using the same biotin-labeled oligonucleotide probes and the Miltenyi Biotec FactorFinder kit according to the instructions of the manufacturer. Ten million A549 cells were lysed in the provided buffer, incubated with the biotinylated DNA probe and streptavidin-coated magnetic beads and purified using the μMacs columns and magnetic separator. DNA-binding proteins were eluted from the columns with 2× sample buffer (Sigma-Aldrich Chemical) and assayed by Western blot using the CEBPB and actin antibodies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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