Molecular Biology of the Cell
Vol. 18, 930–942, March 2007

Adipocyte Enhancer-binding Protein-1 Promotes Macrophage Inflammatory Responsiveness by Up-Regulating NF-κB via IκBα Negative Regulation

Amin Majdalawieh, Lei Zhang, and Hyo-Sung Ro

Department of Biochemistry and Molecular Biology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 1X5

Submitted March 21, 2006; Revised December 18, 2006; Accepted December 22, 2006
Monitoring Editor: William Tansey

Nuclear factor κB (NF-κB) subunits comprise a family of eukaryotic transcription factors that are critically involved in cell proliferation, inflammation, and apoptosis. Under basal conditions, NF-κB subunits are kept under inhibitory regulation by physical interaction with NF-κB inhibitors (IκB subunits) in the cytosol. Upon stimulation, IκB subunits become phosphorylated, ubiquitinated, and subsequently degraded, allowing NF-κB subunits to translocate to the nucleus and bind as dimers to κB responsive elements of target genes. Previously, we have shown that AEBP1 enhances macrophage inflammatory responsiveness by inducing the expression of various proinflammatory mediators. Herein, we provide evidence suggesting that AEBP1 manifests its proinflammatory function by up-regulating NF-κB activity via hampering IκBα, but not IκBβ, inhibitory function through protein–protein interaction mediated by the discoidin-like domain (DLD) of AEBP1. Such interaction renders IκBα susceptible to enhanced phosphorylation and degradation, subsequently leading to augmented NF-κB activity. Collectively, we propose a novel molecular mechanism whereby NF-κB activity is modulated by means of protein–protein interaction involving AEBP1 and IκBα. Moreover, our study provides a plausible mechanism explaining the differential regulatory functions exhibited by IκBα and IκBβ in various cell types. We speculate that AEBP1 may serve as a potential therapeutic target for the treatment of various chronic inflammatory diseases and cancer.

INTRODUCTION

Nuclear factor κB (NF-κB) comprises a family of ubiquitously expressed, eukaryotic transcription factors that participate in the regulation of multiple immediate genes that are expressed at the onset of many vital biological processes such as cell growth, immunoregulation, inflammation, and apoptosis (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). NF-κB exists in cells as a heterodimer of members of the Rel family of proteins (e.g., p50, p65 (RelA), RelB, c-Rel, . . . , etc.), which share a high degree of structural similarity (May and Ghosh, 1997). Under basal conditions, most NF-κB subunits are sequestered in the cytosol, where they are constitutively bound by members of the NF-κB inhibitor family of proteins; mainly IκBα and IκBβ (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990). Structurally, IκBα and IκBβ posses a signal-induced kinase domain, six conserved ankyrin (ANK) repeats, and a PEST domain (May and Ghosh, 1997). IκB proteins inhibit NF-κB nuclear translocation by their ability to interact through their ANK repeats with NF-κB subunits. This protein–protein interaction masks the nuclear localization signal (NLS) located within the Rel homology domain (RHD) of NF-κB subunits, which remain trapped in an inactive state in the cytosol (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990).

On stimulation, IκB proteins become phosphorylated by a specific, cytosolic serine-protein-kinase multisubunit complex that includes two IκB kinases (IKKα and IKKβ) along with other nonenzymatic regulatory components (Regnier *et al.*, 1997; DiDonato *et al.*, 1997; Zandi *et al.*, 1997). Subsequently, IκB proteins become ubiquitinated, rendering them susceptible to 26S proteasome-dependent degradation (Alkalay *et al.*, 1995; Chen *et al.*, 1995). Degradation of IκB proteins leads to unmasking of NLS on NF-κB subunits, which translocate to the nucleus and bind selectively as dimers to specific κB DNA-binding elements within the regulatory regions of their target genes leading to transcriptional transactivation (Kunsch *et al.*, 1992). Indeed, NF-κB activation also leads to rapid induction of IκB mRNA synthesis due to the presence of κB DNA-binding element within IκB promoters (Sun *et al.*, 1993; Chiao *et al.*, 1994). This negative feedback regulatory loop promotes NF-κB retention to the cytosol and ceases NF-κB–mediated transactivation of target genes (Sun *et al.*, 1993; Chiao *et al.*, 1994), setting a molecular switch that ensures rapid, transient activation of target genes.

The adipocyte enhancer-binding protein 1 (AEBP1) is a ubiquitously expressed transcriptional repressor with carboxypeptidase and DNA-binding activities (He *et al.*, 1995; Muise and Ro, 1999). AEBP1 is involved in several biological processes including adipogenesis, macrophage cholesterol homeostasis, and inflammation (Park *et al.*, 1999; Kim *et al.*, 2001; Zhang *et al.*, 2005; Majdalawieh *et al.*, 2006). In macrophages, AEBP1 was shown to promote the expression of IL-6, TNF-α, MCP-1, and iNOS (Majdalawieh *et al.*, 2006),

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–03–0217) on January 3, 2007.

Address correspondence to: Hyo-Sung Ro (hsro@dal.ca).

Abbreviations used: AEBP1, adipocyte enhancer-binding protein 1; CHX, cycloheximide; DLD, discoidin-like domain; EMSA, electromobility gel shift assay; IκB, inhibitor of NF-κB; IKK, IκB kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; TLR4, toll-like receptor 4; $t_{1/2}$, half-life.

whose expression is tightly regulated by NF-κB activity (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). In this study, we focus on investigating a potential positive relationship between AEBP1 and NF-κB in an attempt to shed light on the exact molecular mechanism by which AEBP1 enhances macrophage inflammatory responsiveness. Herein, we provide experimental evidence suggesting that AEBP1 up-regulates NF-κB in macrophages by hampering IκBα inhibitory function via protein–protein interaction, which renders IκBα susceptible to increased phosphorylation and proteolytic degradation. Noteworthy, AEBP1-mediated up-regulation of NF-κB activity in macrophages is independent of alterations in the kinetic activity of the IKK complex. Our study provides a novel molecular mechanism by which NF-κB activity can be regulated by AEBP1 through altering the susceptibility of IκBα phosphorylation and proteolytic degradation via protein–protein interaction.

MATERIALS AND METHODS

Mice

Generation of AEBP1^{TG} (Zhang *et al.*, 2005) and AEBP1$^{-/-}$ (Ro *et al.*, 2007) mice was previously described. Phenotype characterization of AEBP1$^{-/-}$ mice was previously described by Ro *et al.* (2007). Age-matched mice were kept on a 12-h light cycle in the Carleton Animal Care Facility at Dalhousie University. Mice were fed chow diet and were killed by cervical dislocation at 8–12 wk of age to isolate thioglycollate-elicited peritoneal macrophages for protein and RNA analyses.

Reagents and Plasmids

Thioglycollate broth medium was purchased from Sigma (St. Louis, MO). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Buckinghamshire, England). Protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DNA oligonucleotides for RT-PCR and gel shift assay were purchased from Sigma. [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from Amersham Biosciences. Poly dI:dC was purchased from Promega (Madison, WI). Lipopolysaccharide (LPS) isolated from *Escherichia coli* 055:B5 and cycloheximide (CHX) were purchased from Sigma. The pcDNA-AEBP1 expression derivative constructs are derived from the pcDNA vector. Detailed plasmid construction is available upon request. The NF-κB-luciferase construct is a kind gift from Dr. Kirill Rosen (Dalhousie University, Halifax, Nova Scotia, Canada). The GST-IκBα (1-54) construct is a kind gift from Dr. Michael Karin (University of California, San Diego, California).

Antisera

Anti-AEBP1 antibody, generated in rabbits against recombinant mouse AEBP1, was affinity-purified from whole serum using recombinant mouse AEBP1 protein immobilized on nitrocellulose, as previously described (Olmsted, 1981). Primary antibodies directed against p65, IκBα, phospho-IκBα, IκBβ, IKKβ, NIK, MEKK1, and c-Myc were purchased from Santa Cruz Biotechnology. Anti-p53 antibody from Santa Cruz Biotechnology was kind gift from Dr. Patrick Lee (Dalhousie University). Anti-actin antibody was purchased from Sigma. Phycoerythrin (PE)-conjugated anti-mouse TLR4 and PE-conjugated mouse IgG1 isotype control antibodies were purchased from *e*Bioscience (San Diego, CA). Purified rat anti-mouse CD14, rat IgG1 isotype control, mouse IgG2a isotype control, FITC-conjugated anti-rat IgG1 antibodies (BD Biosciences PharMingen, San Diego, CA), purified anti-mouse FcγR II/III (CD32/CD16) antibody (Cedarlane Labs, Hornby, Ontario, Canada), and FITC-conjugated anti-mouse IgG isotype control antibody were kindly provided by Dr. Jean Marshall (Dalhousie University). Anti-AKT antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Peritoneal Macrophage Isolation and Culture

Peritoneal macrophages were isolated as previously described (Miles *et al.*, 2000). Briefly, mice were injected intraperitoneally with 3 ml sterile 4% Brewer's thioglycollate broth solution (Sigma). Five days later, mice were killed by cervical dislocation, and peritoneal exudate cells were isolated by peritoneal lavage using sterile, cold RPMI-1640 medium. Peritoneal exudate cells were obtained by centrifugation for 10 min at 4°C at 1100 rpm, resuspended and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin-streptomycin, and 10 mM HEPES under standard culture conditions.

Cell Culture and Transfection

Rat C6 glioma cells (kindly provided by Dr. Kenneth Renton) were cultured in DMEM supplemented with 5% fetal calf serum and 1% penicillin-streptomycin. C6 glioma cells were transiently transfected at 90% confluency using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 3T3-L1 (Neo-1) and anti-sense (AS/Neo-11) stably transfected preadipocytes were cultured in DMEM supplemented with 10% calf serum, 1% penicillin-streptomycin, and 200 μg/ml G418. J774 monocytes/macrophages (kindly provided by Dr. Timothy Lee, Dalhousie University) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin-streptomycin, and 10 mM HEPES. All cell lines were cultured under standard culture conditions. Where applicable, cells were treated with 1 μg/ml LPS (Sigma), or phosphate-buffered saline (PBS) as a control, for 30 min to induce NF-κB activation. Where applicable, J774 cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 48 h to induce their differentiation into macrophages. For protein stability studies, cells were treated with 50 μg/ml CHX (Sigma) or DMSO as a control, for the indicated time points.

Flow Cytometry

Flow cytometry was performed as previously described (Jawdat *et al.*, 2004). Briefly, 0.5×10^6 macrophages were washed twice with immunofluorescence buffer (2% BSA, 0.02% NaN_3 in PBS). Macrophages were resuspended in immunofluorescence buffer and incubated for 20 min on ice with 5 μg/ml anti-mouse FcγR II/III (CD32/CD16) antibody (Cedarlane) to block free antibody-binding sites. Subsequently, macrophages were incubated for 20 min on ice with 1 μg/ml PE-conjugated anti-mouse TLR4 antibody, purified anti-mouse CD14 antibody, or isotype control antibodies (mouse IgG1 and rat IgG1, respectively). Macrophages were then washed three times with immunofluorescence buffer. For CD14 detection, macrophages were incubated with 1 μg/ml FITC-conjugated anti-rat IgG1 antibody for 20 min on ice. Subsequently, stained macrophages were washed three times with immunofluorescence buffer and finally were fixed in 1% paraformaldehyde in PBS for 30 min on ice. Fixed macrophages were washed twice with immunofluorescence buffer and resuspended in 300 μl immunofluorescence buffer. For flow cytometric analysis, macrophages were acquired with an FACSCalibur (BD Biosciences) and analyzed with CellQuest (BD Biosciences). Positive staining using specific antibodies was evaluated relative to negative staining using isotype control antibodies.

RNA Isolation and RT-PCR

Total RNA was isolated and purified from peritoneal macrophages using RNA STAT-60 isolation reagent (TEL-TEST, Friendswood, TX) following the manufacturer's recommendations. RNA, 1 μg, was subjected to reverse transcription using the Omniscript reverse transcriptase kit (Qiagen, Chatsworth, CA) along with oligo $(dT)_{12–18}$ primers (Invitrogen) according to the manufacturer's directions. Specific murine PCR primers were used to amplify PCR products corresponding to the genes of interest using Hotstar Taq DNA polymerase kit (Qiagen). The following primer sets were used to amplify the genes of interest using the MJ Research PTC-100 Thermal Cycler (Scientific Support, Waltham, MA): for *p65*, 5′-CTCTGGGGCGGCACGTTTTAC-3′ and 5′-CATCCACCTGTTCCCCTTGG-3′; *IκBα*, 5′-CAGGACTGGGCCATGGAGGG-3′ and 5′-TGGCCATTGTAGTTGGTGGC-3′; *IκBβ*, 5′-GGACACAGCCCTGCACTTGG-3′ and 5′-GTAGCCTCCAGTCTTCATCA-3′; *AEBP1/ACLP*, 5′-GTAGTAGCCCCAAGGAGGAC-3′ (PCR4) and 5′-CGATCCACTGGGTCTGCGAC-3′ (PCR7); and *β-actin*, 5′-CTGGCACCACACCTTCTACA-3′ and 5′-TCGTACTCCTGCTTGCTGATC-3′. The PCR conditions and number of thermal cycles were optimized for each gene examined.

Whole-Cell and Nuclear Protein Extraction and Western Blot Analysis

For whole-cell protein extraction, cells were washed with cold PBS and subsequently lysed in cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol [DTT], 0.25% sodium deoxycholate, 0.1% NP-40) containing 1 mM phenylmethysulfonyl fluoride (PMSF), 50 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, 5 mM EGTA, and protease inhibitors cocktail (Roche, Indianapolis, IN). Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4°C. For nuclear protein extraction, the Nuclear Protein Extraction Kit (Active Motif) was used according to the manufacturer's recommendations. Protein extraction from white adipose tissue (WAT) was performed as previously described (Zhang *et al.*, 2005). Protein concentration was determined by a Bradford protein assay using BSA for standardization. Proteins were resolved on 8.5% polyacrylamide gels and subsequently transferred onto nitrocellulose membranes (Amersham). For immunoblotting, nitrocellulose membranes were incubated with specific antibodies recognizing target proteins overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody (1:3000) for 1 h at room temperature and subsequently analyzed by ECL detection system (Amersham).

Coimmunoprecipitation Analyses

For immunoprecipitation analysis, whole-cell protein extracts were obtained by shearing cells in cold RIPA lysis buffer as described above. Protein extracts (0.5 mg) were first incubated with protein A/G plus agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. The precleared protein extracts were

subsequently incubated with 2 μg/ml normal IgG or the indicated specific antibodies for 16 h at 4°C and then with protein A/G plus agarose beads for 4 h at 4°C to allow precipitation of target proteins. Then, the protein A/G plus agarose beads were collected and washed twice with cold RIPA lysis buffer, once with cold RIPA lysis buffer containing 500 mM NaCl, and finally once with cold RIPA lysis buffer. Subsequently, 20 μl sample buffer was added to the immunoprecipitate samples, which were then boiled for 5 min and spun at 14,000 rpm for 10 min. Finally, the immunoprecipitate samples were resolved on 8.5% polyacrylamide mini-gels, transferred onto nitrocellulose membranes, and subjected to immunoblotting as described above.

Luciferase Reporter and β-Galactosidase Assays

Luciferase reporter activity was assessed using the luciferase assay system (Promega) according to the manufacturer's directions. In brief, C6 glioma cells were transiently cotransfected with NF-κB-luciferase construct (kindly provided by Dr. Kirill Rosen), pCMV-β-galactosidase expression vector (pHermes-lacZ), and pcDNA-AEBP1 expression plasmids in 12-well plates. Forty-eight hours later, transfectants were washed twice in cold PBS and subsequently lysed in 120 μl passive lysis buffer (Promega). Protein extract, 30 μl, was used to measure reporter activity using the BMG FLUOstar Galaxy microplate reader (BMG Lab Technologies, Offenburg, Germany). Protein extract, 70 ml, was used to measure β-galactosidase activity as previously described (He *et al.*, 1995). Luciferase activity was adjusted based on β-galactosidase activity to normalize for transfection efficiency.

Electrophoretic Mobility Gel Shift Assay

The probe containing the κB DNA-binding consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was radiolabeled with [α-^{32}P]ATP (Amersham) by Klenow fill-in reaction. Nuclear protein extracts (2 μg) were incubated with the κB-radiolabeled probe and 1 μg poly dI:dC (Promega) for 20 min at room temperature in binding reaction buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2.5% glycerol). Specific (unlabeled oligonucleotide) or nonspecific (unrelated oligonucleotide) competitors were used at 50× excess. The DNA–protein complexes were resolved on 5% 0.25× TBE polyacrylamide mini-gels, which were then dried and subjected to autoradiography.

In Vitro Kinase Assay

Endogenously expressed IKKα was immunoprecipitated from 0.5 mg precleared protein extracts by incubation with 2 μg/ml anti-IKKα antibody (Santa Cruz Biotechnology) for 16 h at 4°C and then with protein A/G plus agarose beads for 4 h at 4°C. The immunoprecipitate samples were washed twice with cold RIPA lysis buffer, once with cold RIPA lysis buffer containing 500 mM NaCl, and finally once with cold kinase reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, and 0.3 mM cold ATP). Kinase assays were performed by adding 1 μg of bacterially expressed GST-IκBα (1-54), as a substrate, and 5 μCi of [γ-^{32}P]ATP (Amersham) to the IKKα immunoprecipitate samples in a total of 20 μl kinase reaction buffer. The kinase reaction was allowed to take place for 30 min at 30°C. The reaction was stopped by the addition of 2× SDS sample loading buffer followed by boiling for 5 min and centrifugation for 10 min at 14,000 rpm. The samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, analyzed by autoradiography, and finally subjected to immunoblotting using specific antibodies against the indicated proteins.

Statistical Analysis

Data are expressed as the mean ± SEM of the indicated number of experiments. Statistical significance was determined using Student's *t* test for unpaired observations. $p < 0.05$ was considered statistically significant.

RESULTS

AEBP1 Up-Regulates NF-κB Activity in Macrophages

We have previously demonstrated that AEBP1 expression in macrophages positively correlates with IL-6, TNF-α, MCP-1, and iNOS (Majdalawieh *et al.*, 2006). Hence, we suspected that AEBP1 overexpression and deficiency in macrophages would lead to enhanced and inhibited NF-κB activity, respectively. To examine this likely possibility, we assessed NF-κB activity upon AEBP1 overexpression in unstimulated and LPS-stimulated C6 glioma cells, which express LPS-responsive toll-like receptor 4 (TLR4; Jack *et al.*, 2005) and its coreceptor CD14 (Deininger *et al.*, 2003), by luciferase reporter assay, due to repeatedly unsuccessful attempts to transfect THP-1, U937, and J774 monocyte/macrophage cell lines using various transfection reagents. C6 glioma cells were cotransfected with NF-κB-luciferase construct along with increasing amounts of the pcDNA-AEBP1 plasmid expressing AEBP1. As shown in Figure 1A, AEBP1 enhances NF-κB activity in a dose-responsive manner.

To examine whether this regulation takes place in macrophages, we evaluated p65 protein levels in the nuclei of unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. Western blot analysis clearly demonstrated that the nuclear p65 protein level is significantly lower in AEBP1$^{-/-}$ macrophages under basal and LPS-stimulatory conditions (Figure 1, B and C). Indeed, AEBP1-mediated NF-κB hyperactivation seems to be independent of LPS treatment because nuclear p65 protein level is lower in AEBP1$^{-/-}$ macrophages even under basal conditions (Figure 1, B and C). To further confirm AEBP1's ability to modulate NF-κB activity in macrophages, nuclear proteins extracted from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages were subjected to electrophoretic mobility gel shift assay using ^{32}P-labeled κB DNA-binding consensus sequence. Consistent with the Western blotting findings (Figure 1, B and C), electrophoretic mobility gel shift assay (EMSA) analysis suggests that NF-κB activity is not completely diminished, but dramatically decreased in AEBP1$^{-/-}$ macrophages (Figure 1D). Because changes in cytoplasmic-nuclear localization are important in NF-κB signaling, it was important to examine the subcellular distribution of AEBP1 in macrophages before and upon LPS stimulation. As shown in Figure 1E, AEBP1 displays almost equal distribution in the cytoplasm and nucleus, and this subcellular distribution of AEBP1 is not altered by LPS stimulation. AKT and c-Myc were used as cytoplasmic and nuclear markers, respectively.

TLR4 has been shown to function as the functional receptor responsive to signaling initiated by bacterial LPS (Poltorak *et al.*, 1998) after specific binding to CD14 and LPS-binding protein (LBP; Wright *et al.*, 1990) that are expressed on various cell types including macrophages. Thus, it was important to compare the expression of TLR4 and CD14 on the cell surface of AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages to eliminate the possibility that the differential NF-κB activity displayed by those macrophages is not due to altered expression of TLR4 or CD14. Flow cytometric analysis revealed that AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages express equivalent cell surface TLR4 and CD14 levels (Figure 1F), suggesting indiscriminative LPS-induced signaling cascade leading to NF-κB activation. Taken together, these results suggest that AEBP1 is capable of enhancing NF-κB activity in vitro and in vivo under basal and LPS-stimulatory conditions.

Noteworthy, *AEBP1* gene extends over 10 kb and gives rise to two transcripts by an alternative splicing mechanism (Ro *et al.*, 2001). One transcript is translated into an 82-kDa protein, AEBP1, whereas the other transcript is translated into a 175-kDa protein, ACLP (Aortic Carboxypeptidase-Like Protein), the latter having an additional 380-amino acid coding sequence at its N-terminus (Ro *et al.*, 2001). ACLP was initially identified in human aortic smooth muscle cells, and its expression was shown to be up-regulated during smooth muscle cell differentiation (Layne *et al.*, 1998). Although its transcript can be detected in several tissues including the kidney, colon, aorta, brain, stomach, spleen, white adipose tissue, and thymus, ACLP protein can only be detected in the aorta (Layne *et al.*, 1998) and adipose tissue (Layne *et al.*, 2001). Unlike AEBP1, ACLP is an extracellular matrix (ECM)-associated secretory protein that is excluded from the nuclear and cytoplasmic compartments of mouse aortic smooth muscle cells (Layne *et al.*, 2001). Thus, it is inconceivable that ACLP can have any effect on NF-κB activity in macrophages or C6 glioma cells, despite the fact that ACLP possesses the exact same amino acid sequence of AEBP1 at its C-terminus (Ro *et al.*, 2001). Most importantly, experimen-

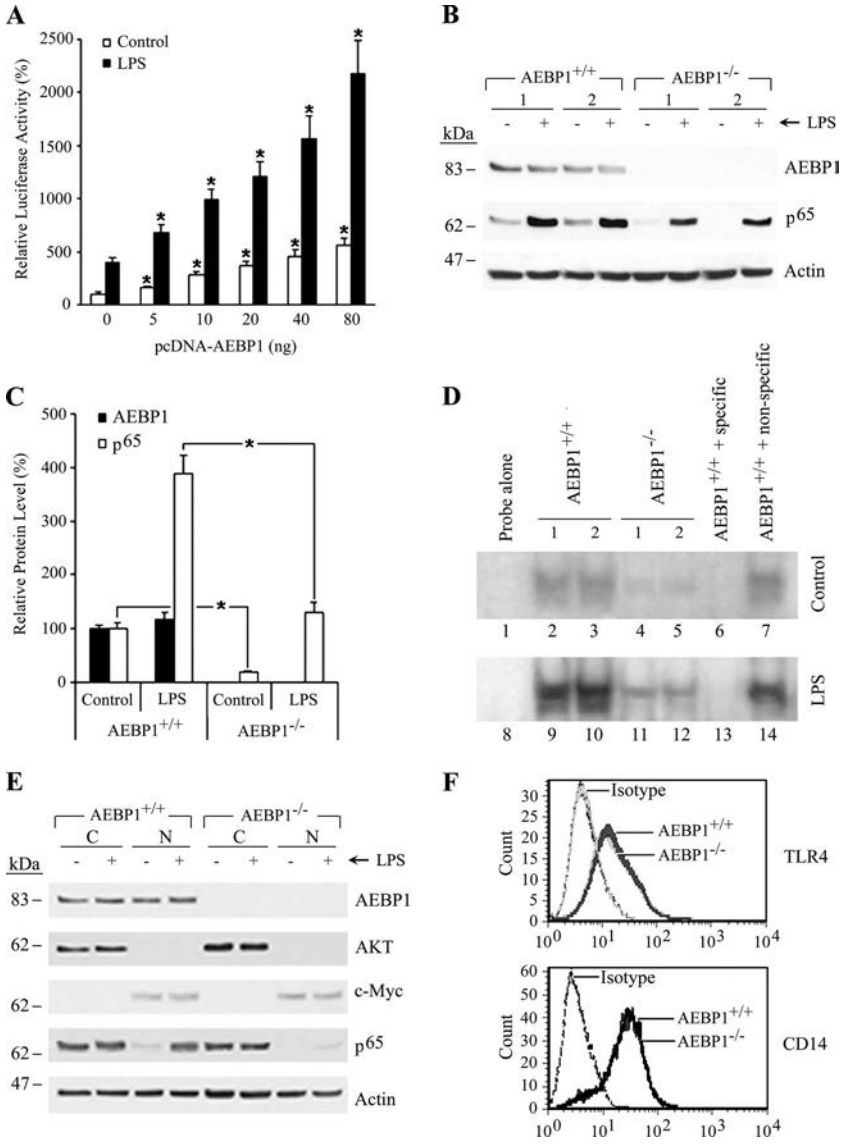

Figure 1. AEBP1 up-regulates NF-κB activity in vitro and in vivo. (A) Exogenously expressed AEBP1 enhances NF-κB activity in a dose-responsive manner in C6 glioma cells cotransfected with NF-κB-luciferase reporter and increasing amounts of a pcDNA-AEBP1 plasmid. Relative luciferase activity was set to 100% for the 0-ng control sample. Statistical significance was determined relative to 0-ng transfection under control and LPS-stimulatory conditions (n = 6). (B–D) AEBP1$^{-/-}$ macrophages exhibit significantly lower NF-κB activity. (B) Nuclear protein extracts isolated from unstimulated and LPS-stimulated macrophages were subjected to SDS-PAGE and immunoblotting for the indicated proteins. (C) A histogram illustrates densitometric analysis of protein levels shown in B. Nuclear protein levels were normalized based on actin expression (n = 6). (D) Nuclear protein extracts isolated from unstimulated and LPS-stimulated macrophages were subjected to EMSA in presence of specific (unlabeled NF-κB probe) or nonspecific (unlabeled, unrelated probe) competitors. This data are representative of three independent experiments. (E) Equal amounts of cytoplasmic and nuclear protein extracts isolated from unstimulated and LPS-stimulated macrophages were subjected to SDS-PAGE and immunoblotting for the indicated proteins. This data are representative of three independent experiments. (F) Flow cytometry shows that AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages express equivalent levels of TLR4 and CD14 on their cell surface.

tal evidence clearly indicates that neither ACLP mRNA nor ACLP protein is expressed in primary macrophages, J774 macrophages, or C6 glioma cells (Figure 2, A and B, respectively), despite their detection in white adipose tissue (Figure 2). Equally important, experimental evidence indicates that targeted overexpression of AEBP1 in macrophages isolated from AEBP1^{TG} mice leads to significant induction of NF-κB activity (data not shown) and macrophage inflammatory responsiveness (Majdalawieh *et al.*, 2006), indicating that NF-κB activity can only be regulated by AEBP1, not ACLP, because overexpression of AEBP1 in AEBP1^{TG} macrophage is driven by a transgene carrying the cDNA of AEBP1 (Zhang *et al.*, 2005). Therefore, unlike AEBP1, ACLP is incapable of modulating NF-κB activity in macrophages.

AEBP1 Modulates NF-κB Activity Independent of Its Transcriptional Repression Function

Because AEBP1 is known to function as a potent transcriptional repressor of several target genes (He *et al.*, 1995; Majdalawieh *et al.*, 2006), it is conceivable to suspect that AEBP1 transcriptionally represses known or unknown genes whose products function as negative regulators of NF-κB gene expression. Similarly, it is possible that AEBP1 directly represses the expression of *IκBα* and/or *IκBβ*, whose products are known to antagonize NF-κB activity. If any of these valid possibilities is true, then p65 mRNA level should positively correlate with AEBP1 expression in macrophages. To assess these possibilities, total RNA was isolated from unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages and subsequently subjected to RT-PCR in order to assess p65, IκBα, and IκBβ mRNA levels. RT-PCR and densitometric analysis based on β-actin expression clearly demonstrated that differential expression of AEBP1 has no correlation with p65 mRNA level (Figure 3, A and B). Moreover, and consistent with a role of AEBP1 in NF-κB enhanced activation, the mRNA levels of IκBα and IκBβ are higher in AEBP1$^{+/+}$ macrophages, compared with AEBP1$^{-/-}$ macrophages, under basal and LPS-stimulatory conditions (Figure 3, A and B). These findings suggest that AEBP1 up-regulates NF-κB activity independent of its transcriptional repression function.

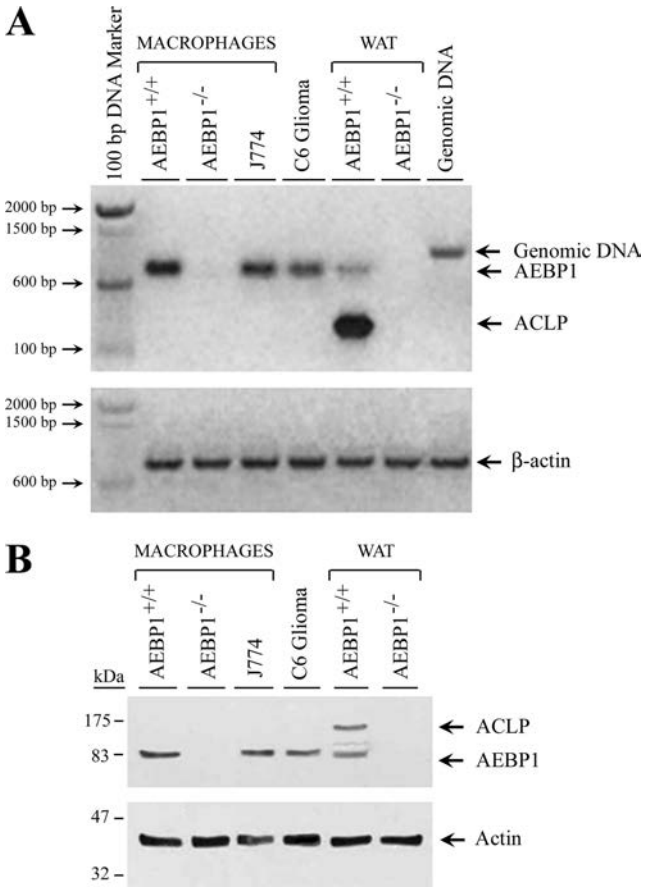

Figure 2. ACLP, unlike AEBP1, is not expressed in macrophages and C6 glioma cells. (A) Total RNA obtained from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, PMA-treated J774 macrophages, C6 glioma cells, as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ white adipose tissue (WAT) was subjected to RT-PCR using PCR4 and PCR7 primer set (refer to Ro *et al.*, 2001) to assess AEBP1 and ACLP mRNA levels (top panel). Ear genomic DNA from AEBP1$^{+/+}$ mouse was used as a control. Using PCR4 and PCR7 primer set, the expected amplicon size corresponding to genomic DNA, AEBP1 transcript, and ACLP transcript is 955, 787, and 300 base pairs, respectively. β-actin expression served as a control (bottom panel). (B) Whole-cell protein extracts obtained from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, PMA-treated J774 macrophages, and C6 glioma cells, as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ white adipose tissue (WAT) were subjected to SDS-PAGE and immunoblotting using anti-AEBP1 antibody (top panel). Actin protein levels are shown as an input control (bottom panel). This data are representative of three independent experiments.

AEBP1 Negatively Influences IκBα Protein Stability in Macrophages

In an attempt to determine the mechanism by which AEBP1 up-regulates NF-κB activity in macrophages, we first assessed the expression levels of IκBα and IκBβ in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. To this end, whole-cell protein extracts were obtained from unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages and were subsequently subjected to SDS-PAGE. Interestingly, Western blot analysis clearly revealed that IκBα protein level is threefold higher in AEBP1$^{-/-}$ macrophages, in comparison to AEBP1$^{+/+}$ macrophages, under basal and LPS-stimulatory conditions (Figure 3, C and D). Again, the significant difference in IκBα protein level between AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages is observed under basal and LPS-stimulatory conditions (Figure 3, C and D). Noteworthy, IκBβ protein level in AEBP1$^{-/-}$ macrophages is similar to that in AEBP1$^{+/+}$ macrophages, both under basal and LPS-stimulatory conditions (Figure 3, C and D). Interestingly, the level of phosphorylated IκBα is significantly lower in AEBP1$^{-/-}$ macrophages under basal and LPS-stimulatory conditions (Figure 3, C and D). Thus, this data suggests that AEBP1 modulates IκBα, but not IκBβ, protein levels in macrophages by enhancing its phosphorylation on Ser^{32}/Ser^{36}.

Because IκBα phosphorylation renders it susceptible to proteolytic degradation (Alkalay *et al.*, 1995; Chen *et al.*, 1995), we wanted to evaluate IκBα and IκBβ protein stability in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. To this end, macrophages were treated with 50 μg/ml CHX for various time points, and whole-cell protein extracts were subjected to SDS-PAGE. As shown in Figure 3, E and F, IκBα protein half-life is significantly higher in AEBP1$^{-/-}$ macrophages compared with AEBP1$^{+/+}$ macrophages ($t_{1/2}$ = ~250 min vs. $t_{1/2}$ = ~50 min, respectively; Figure 3F). Noteworthy, IκBβ protein levels in AEBP1$^{-/-}$ macrophages upon CHX treatment were comparable to those in AEBP1$^{+/+}$ macrophages (Figure 3, E and F). As a control, tumor suppressor p53 protein level was assessed in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages upon CHX treatment. As shown in Figure 3E, p53 protein stability trend is very similar in both types of macrophages (i.e., $t_{1/2}$ = ~50 min). This IκBα and IκBβ protein stability study was also performed in control 3T3-L1 preadipocytes (Neo-1) and stably transfected AEBP1 antisense (AS/Neo-11) preadipocytes, and very similar results were obtained (data not shown). Taken together, these findings strongly suggest that lack of AEBP1 promotes IκBα protein stability, significantly inhibiting its turnover.

Because modulation of IKKβ activity is associated with modulation of IκBα phosphorylation status, and subsequently IκBα proteolytic degradation (Li *et al.*, 1999), it is possible that AEBP1 may serve as a direct or indirect up-regulator of IKKβ activity, leading to increased phosphorylation of IκBα in macrophages. To examine this possibility, we assessed IKKβ kinetic activity in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages by in vitro kinase assay. To this end, endogenously expressed IKK complex was immunoprecipitated from whole-cell protein extracts obtained from unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages using anti-IKKα antibody, and IKKβ kinetic activity was assessed using a bacterially expressed GST-IκBα (1-54) fusion protein as a substrate in an in vitro kinase assay. Clearly, IKKβ kinetic activity is indistinguishable in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages (Figure 4A), indicating that AEBP1 does not alter the enzymatic activity of IKKβ in macrophages. Equivalent GST-IκBα (1-54) and IKKα and IKKβ immunoprecipitate levels was confirmed by blotting the membrane with anti-GST, anti-IKKα, and anti-IKKβ antibodies, respectively (Figure 4A).

The 700-9000 kDa IKK complex is composed of the three IKK proteins (IKKα, IKKβ, IKKγ), NF-κB essential modulator (NEMO), and other adaptor proteins complex (Mercurio *et al.*, 1997, 1999; Woronicz *et al.*, 1997Zandi *et al.*, 1997; Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998). Many studies have also shown that NF-κB inducing kinase (NIK) and MEK kinase 1 (MEKK1), upstream up-regulators of the IκB kinases, are normally associated with the IKK complex (Regnier *et al.*, 1997; Woronicz *et al.*, 1997; Nakano *et al.*, 1998; Nemoto *et al.*, 1998). Figure 4A clearly demonstrates that the physical association of NIK and MEKK1 with the IKK complex is not altered by the loss of AEBP1. Figure 4B represents immunoblotting of whole-cell protein extracts for the indicated proteins as an input control. Taken together, these findings

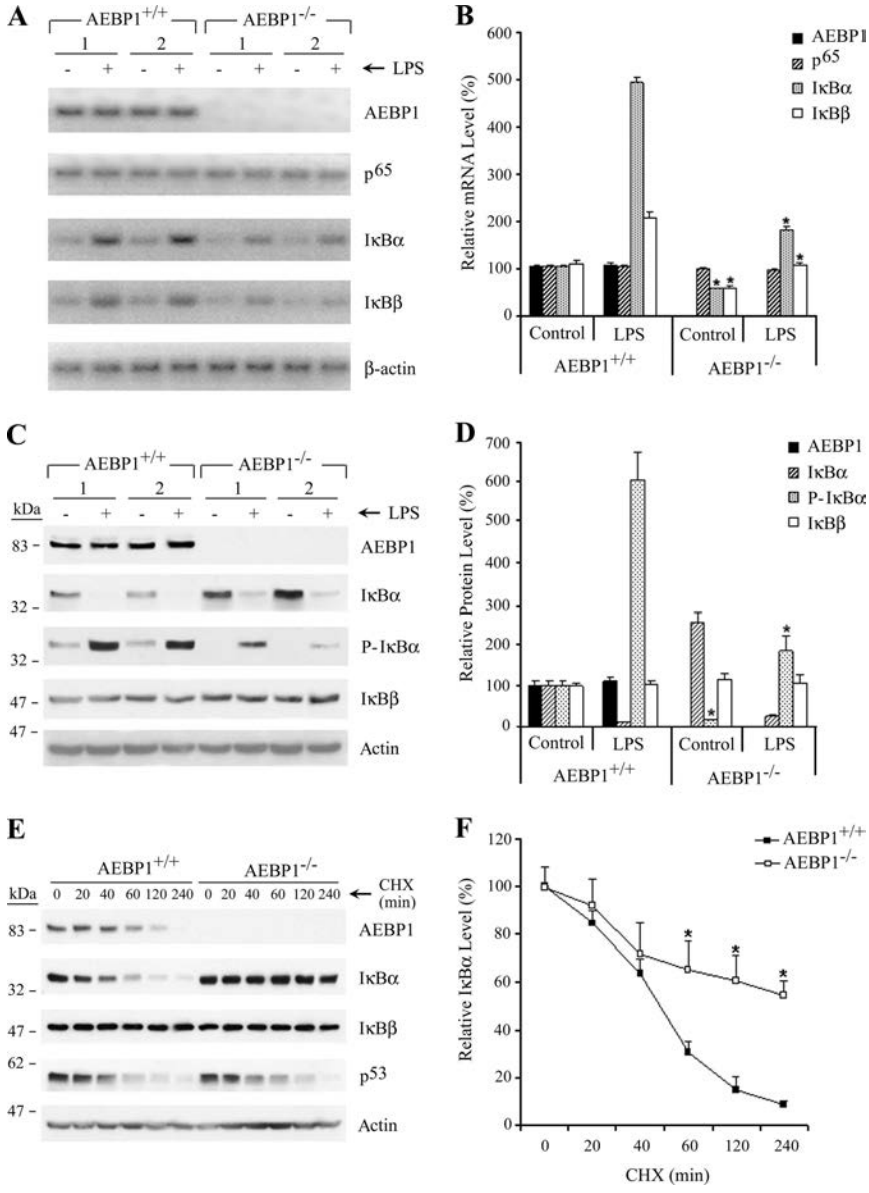

Figure 3. AEBP1 promotes IκBα phosphorylation and proteolytic degradation in macrophages. (A and B) AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages express equivalent p65, but higher IκBα and IκBβ, mRNA levels. (A) Total RNA obtained from macrophages treated with 1 μg/ml LPS or PBS (control) for 1 h was subjected to RT-PCR to assess p65, IκBα, and IκBβ mRNA levels. (B) A histogram demonstrates densitometric analysis of RT-PCR results shown in A based on β-actin expression. (C and D) AEBP1$^{-/-}$ macrophages display reduced IκBα phosphorylation and elevated IκBα, but not IκBβ, protein levels. (C) Whole-cell protein extracts isolated from macrophages were subjected to SDS-PAGE and immunoblotting for the indicated proteins. (D) A histogram illustrates densitometric analysis of the immunoblotting analysis shown in C. Statistical significance was determined relative to protein levels in unstimulated or LPS-stimulated AEBP1$^{+/+}$ macrophages. (E and F) AEBP1$^{-/-}$ macrophages display prolonged IκBα, but not IκBβ, protein stability. (E) Whole-cell protein extracts isolated from macrophages treated with vehicle (ethanol) or 50 μg/ml CHX were subjected to SDS-PAGE and immunoblotting for the indicated proteins. (F) A line-curve illustrates densitometric analysis of protein levels shown in E. Protein levels at 0 min was taken to be 100% for every group, and protein levels at other time points are expressed relative to that at 0 min within the same group.

suggest that AEBP1 is capable of modulating IκBα protein stability and NF-κB activity in macrophages without altering the kinetic activity of the IKK complex.

AEBP1 Is Not a Component of the IKK Complex

It is possible that AEBP1 may be part of the IKK complex by means of physical interaction with one or more of its known or unknown components, and thus, it may be directly involved in regulating IκBα phosphorylation and proteolytic degradation. To examine this possibility, AEBP1 was immunoprecipitated from unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, and Western blot analysis was performed on the immunoprecipitate samples to check for IKKα and IKKβ coimmunoprecipitation. Protein extracts obtained from LPS-stimulated AEBP1$^{+/+}$ macrophages were incubated with normal IgG to serve as a negative control for AEBP1 coimmunoprecipitation analysis. Figure 4C suggests that neither IKKα nor IKKβ coimmunoprecipitates along with AEBP1, despite successful AEBP1 immunoprecipitation. Figure 4D represents immunoblotting of whole-cell protein extracts for the indicated proteins as an input control. Consistently, AEBP1 does not coimmunoprecipitate along with IKKα, IKKβ, NIK, and MEKK1, major components of the IKK complex (Figure 4A). These findings strongly suggest that AEBP1 is not a component of the IKK complex in macrophages.

AEBP1 Physically Interacts with Endogenous IκBα, but not IκBβ, in Macrophages

In an attempt to shed light on the molecular mechanism by which AEBP1 contributes to IκBα protein instability, we hypothesized the AEBP1 may be capable of physically interacting with IκBα in a way that renders IκBα more prone to phosphorylation, and subsequently, proteolytic degradation. To examine this possibility, we performed coimmunoprecipitation studies in which endogenously expressed IκBα was immunoprecipitated from whole-cell protein extracts isolated from unstimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages using anti-IκBα antibody. Protein extracts obtained from LPS-stimulated AEBP1$^{+/+}$ mac-

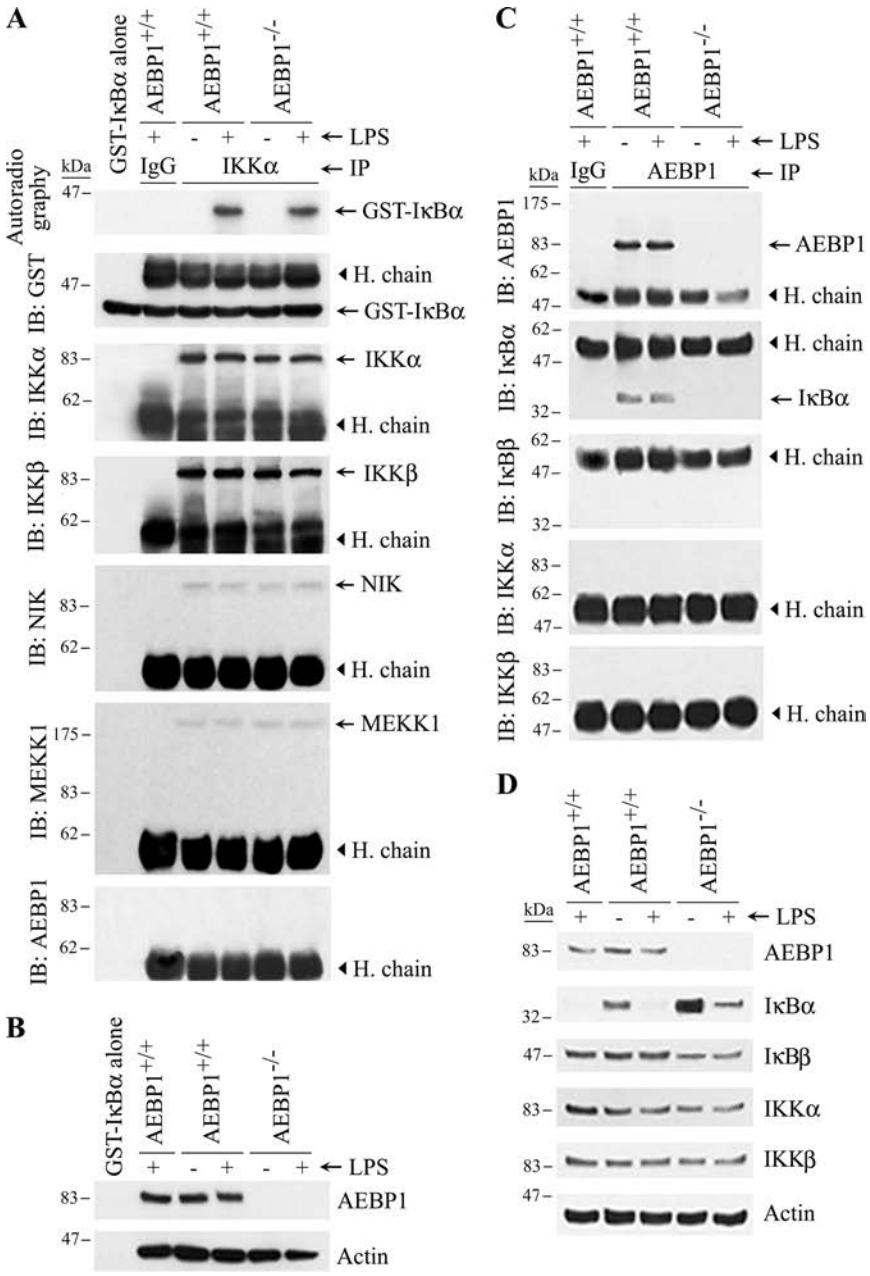

Figure 4. AEBP1 does not influence the kinetic activity of the IKK complex and is not part of it. (A and B) AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages exhibit similar IKKβ kinetic activity in vitro. (A) In vitro kinase assay using immunoprecipitated, endogenously expressed IKK complex and GST-IκBα (1-54) fusion protein as a substrate was performed followed by autoradiography. The IKK complex was immunoprecipitated using anti-IKKα antibody, and normal IgG was used as a negative control. Anti-GST antibody was used to detect GST-IκBα (1-54) as a loading control, and antisera against the indicated proteins were used to confirm successful immunoprecipitation of the indicated IKK complex components. (B) AEBP1 and actin protein levels are shown as an input control. (C and D) AEBP1 is not a component of the IKK complex. (C) Whole-cell protein extracts isolated from unstimulated and LPS-stimulated macrophages were subjected to coimmunoprecipitation analysis using anti-AEBP1 antibody or normal IgG as a control. SDS-PAGE was performed and followed by immunoblotting with specific antisera against the indicated proteins. (D) Whole-cell protein extracts were immunoblotted for the indicated proteins as an input control. This data are representative of two independent experiments.

rophages were incubated with normal IgG to serve as a negative control for IκBα coimmunoprecipitation analysis. Figure 5A, top left panel, clearly demonstrates that endogenous AEBP1 physically interacts with endogenous IκBα in macrophages. Importantly, no AEBP1 band was detectable in the normal IgG control sample or IκBα immunoprecipitate samples from AEBP1$^{-/-}$ macrophages. Notably, coimmunoprecipitation analysis suggests that AEBP1 physically interacts with IκBα to the same extent under basal and LPS-stimulatory conditions Figure 5A, top left panel. Reciprocal coimmunoprecipitation analysis revealed detection of 37-kDa bands corresponding to IκBα when endogenously expressed AEBP1 was immunoprecipitated from LPS-stimulated AEBP1$^{+/+}$ macrophages (Figure 4C), confirming AEBP1-IκBα physical interaction.

Because of the relatively high structural similarity between IκBα and IκBβ (May and Ghosh, 1997), we wondered whether AEBP1 is capable of interacting with IκBβ, as it does with IκBα. Surprisingly, coimmunoprecipitation studies failed to reveal any interaction between endogenously expressed AEBP1 and IκBβ in macrophages (Figure 5A, top right panel), despite successful IκBβ immunoprecipitation (Figure 5A, middle right panel). In agreement, AEBP1 coimmunoprecipitation analysis in macrophages revealed no detection of IκBβ (Figure 4C).

To determine the region or domain of AEBP1 that is required for protein–protein interaction with IκBα, we performed coimmunoprecipitation analysis using C6 glioma cells that are transfected with various mutant derivatives of AEBP1 (Figure 5B). On transfection, whole-cell protein extracts were obtained from C6 transfectants and endogenously expressed IκBα was immunoprecipitated. Subsequently, whole-cell protein extracts and immunoprecipitate samples were subjected to SDS-PAGE followed by immuno-

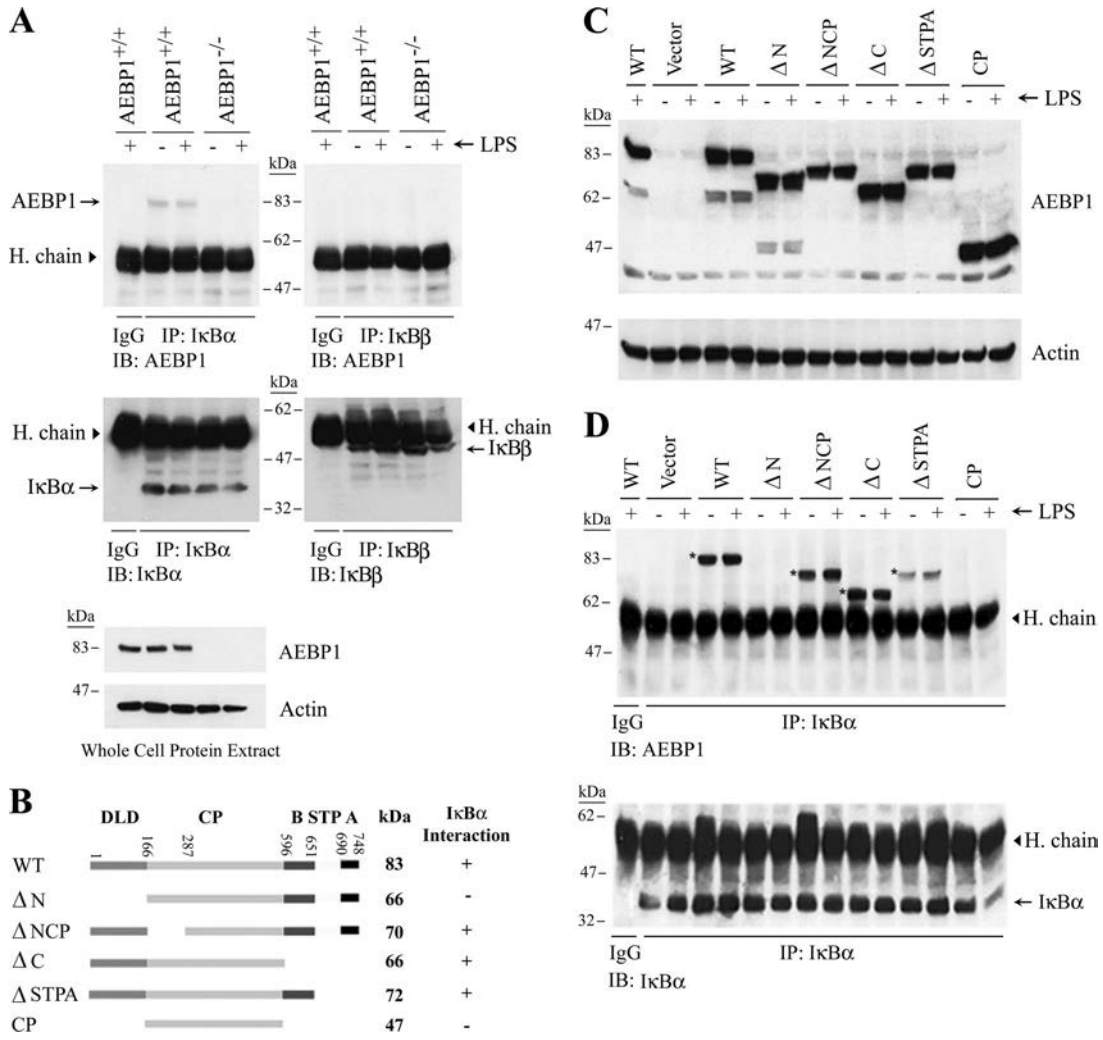

Figure 5. AEBP1 physically interacts with endogenous IκBα in macrophages via its DLD. (A) Whole-cell protein extracts isolated from unstimulated and LPS-stimulated macrophages were subjected to coimmunoprecipitation analysis using anti-IκBα antibody (top left panel), anti-IκBβ antibody (top right panel), or normal IgG followed by immunoblotting with anti-AEBP1 antibody. IκBα and IκBβ immunoprecipitation was confirmed by immunoblotting using anti-IκBα and anti-IκBβ antibodies (middle panels). AEBP1 and actin protein levels are shown as an input control (bottom panel). (B) A schematic representation illustrates AEBP1 derivatives with expected protein size. The ability of AEBP1 derivatives to physically interact with endogenous IκBα is indicated (− or +). (C) Whole-cell protein extracts isolated from unstimulated and LPS-stimulated C6 glioma transfectants were subjected to SDS-PAGE and immunoblotting using anti-AEBP1 antibody to detect exogenously expressed AEBP1 derivatives. (D) IκBα immunoprecipitate samples obtained from unstimulated and LPS-stimulated C6 glioma transfectants were subjected to SDS-PAGE and immunoblotting using anti-AEBP1 (top panel) anti-IκBα (bottom panel) antibodies. An asterisk represents AEBP1 derivatives that coimmunoprecipitated with endogenous IκBα. Data are representative of three independent experiments.

blotting using anti-AEBP1 antibody. As shown in Figure 5C, almost similar levels of AEBP1 derivatives were exogenously expressed in transfectants. Interestingly, the WT form of AEBP1 specifically coimmunoprecipitated with IκBα, in which AEBP1 band was absent in the normal IgG control sample (Figure 5D, top panel). It is evident that all AEBP1 mutant forms, except ΔN and CP, are capable of binding IκBα (Figure 5D, top panel), despite equivalent IκBα immunoprecipitation (Figure 5D, bottom panel). Because ΔN and CP are the only mutant derivatives of AEBP1 that lack DLD, these findings suggest that AEBP1 physically interacts with IκBα via its DLD. Consistently, coimmunoprecipitation analysis using whole-cell protein extracts obtained from unstimulated and LPS-stimulated C6 glioma transfectants confirmed that LPS has no detectable effect on AEBP1-IκBα protein–protein interaction in vitro (Figure 5D, top panel). In agreement with a lack of protein–protein interaction between endogenously expressed AEBP1 and IκBβ in macrophages, coimmunoprecipitation studies using C6 glioma transfectants failed to reveal any physical interaction between AEBP1 and IκBβ (data not shown). Taken together, these findings suggest that AEBP1 physically interacts with IκBα, but not IκBβ, in macrophages via its DLD in an LPS-independent manner.

DLD Is Essential for AEBP1-mediated IκBα Proteolytic Degradation and NF-κB Up-Regulation.

Because AEBP1 mutant derivatives that lack DLD were unable to physically interact with IκBα, we next examined the effect of deleting DLD of AEBP1 on IκBα protein stability, as well as NF-κB transcriptional activity. Whole-cell protein

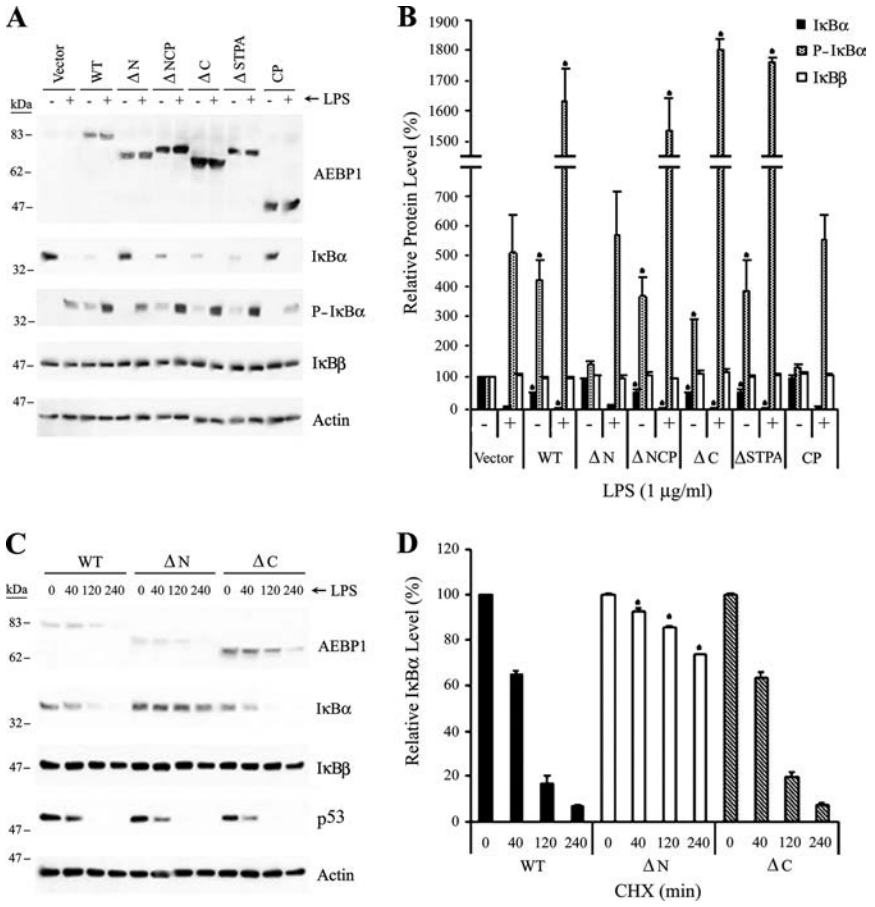

Figure 6. DLD is required for AEBP1-mediated enhanced IκBα phosphorylation and proteolytic degradation. (A) Whole-cell protein extracts obtained from unstimulated or LPS-stimulated C6 glioma transfectants were subjected to SDS-PAGE and immunoblotting for the indicated proteins. (B) A histogram illustrates densitometric analysis of protein levels shown in A. Statistical significance was determined relative to individual protein levels in unstimulated or LPS-stimulated, empty vector transfectants. (C) Whole-cell protein extracts isolated from control and CHX-treated C6 glioma transfectants expressing WT, ΔN, and ΔC forms of AEBP1 were subjected to SDS-PAGE and immunoblotting for the indicated proteins. (D) A histogram illustrates densitometric analysis of protein levels shown in C. Relative protein levels were determined based on actin expression, values at 0 min were taken to be 100% for every transfectant group, and statistical significance was determined relative to IκBα protein level in WT-transfected cells at each time point (n = 3).

extracts obtained from unstimulated and LPS-stimulated C6 glioma cells that are transiently transfected with plasmids expressing various AEBP1 derivatives were subjected to SDS-PAGE and immunoblotting to assess IκBα and IκBβ protein levels. As shown in Figure 6, A and B, overexpression of WT, ΔNCP, ΔC, and ΔSTPA, but not ΔN and CP, forms of AEBP1 is accompanied by decreased protein levels of IκBα, but not IκBβ, under basal and LPS-stimulatory conditions, despite equal loading. Moreover, C6 glioma transfectants that express AEBP1 derivatives retaining DLD (i.e., WT, ΔNCP, ΔC, and ΔSTPA) display about threefold higher levels of phosphorylated IκBα, under basal and LPS-stimulatory conditions (Figure 6, A and B).

To evaluate the physiological importance of DLD in AEBP1-promoted IκBα protein degradation, C6 glioma cells were transiently transfected with plasmids expressing the WT, ΔN, and ΔC derivatives of AEBP1 and subsequently treated with CHX to assess IκBα protein stability. Immunoblotting and densitometric analyses revealed that DLD deletion eliminates AEBP1's ability to promote IκBα proteolytic degradation, indicating that DLD is indispensable for AEBP1-promoted IκBα protein instability (Figure 6, C and D). Importantly, the ΔC mutant form of AEBP1, which was used as a positive control, behaved similar to the WT form (Figure 6, C and D). In those cells expressing WT, ΔN, or ΔC derivatives, the half-life of IκBα protein turnover is estimated to be 60, >240, and 60 min, respectively. Notably, transfectants expressing various derivatives of AEBP1 displayed no significant differential patterns with respect to IκBβ or p53 protein turnover (Figure 6C), substantiating AEBP1's function as an IκBα proteolytic degradation mediator.

Next, we compared the ability of AEBP1 derivative proteins to modulate NF-κB transcriptional activity in vitro. First, immunoblotting and densitometric analyses revealed that C6 glioma cells that express WT, ΔNCP, ΔC, and ΔSTPA possess higher nuclear p65 protein levels, whereas cells that express ΔN and CP have nuclear p65 protein levels that are very similar to those in cells transfected with empty vector, under basal and LPS-stimulatory conditions (Figure 7, A and B). Second, NF-κB-luciferase reporter assay was performed using C6 glioma cells expressing various AEBP1 derivatives, and NF-κB activity was evaluated under basal and LPS-stimulatory conditions. Compared with cells that were transfected with empty vectors, cells that transiently expressed the WT, ΔNCP, ΔC, or ΔSTPA forms of AEBP1 displayed significantly enhanced (5-fold) NF-κB activity under basal and LPS-stimulatory conditions (Figure 7C). The WT, ΔNCP, ΔC, and ΔSTPA derivatives of AEBP1 exhibited no significant differential ability among themselves to promote NF-κB activity. On the other hand, the ΔN and CP derivatives caused no significant increase in NF-κB activity under basal and LPS-stimulatory conditions (Figure 7C). Third, to further confirm these findings, we performed EMSA using κB DNA-binding consensus sequence and nuclear protein extracts obtained from unstimulated and LPS-stimulated C6 transfectants expressing various derivative forms of AEBP1. As shown in Figure 7D, the WT, ΔNCP, ΔC, and ΔSTPA, but not ΔN and CP, forms of AEBP1 enhance the activity of NF-κB. Importantly, TLR4 and CD14 are expressed at similar levels in those C6 glioma transfectants (data not shown). Taken together, these findings strongly suggest that lack of DLD eliminates AEBP1-mediated NF-κB

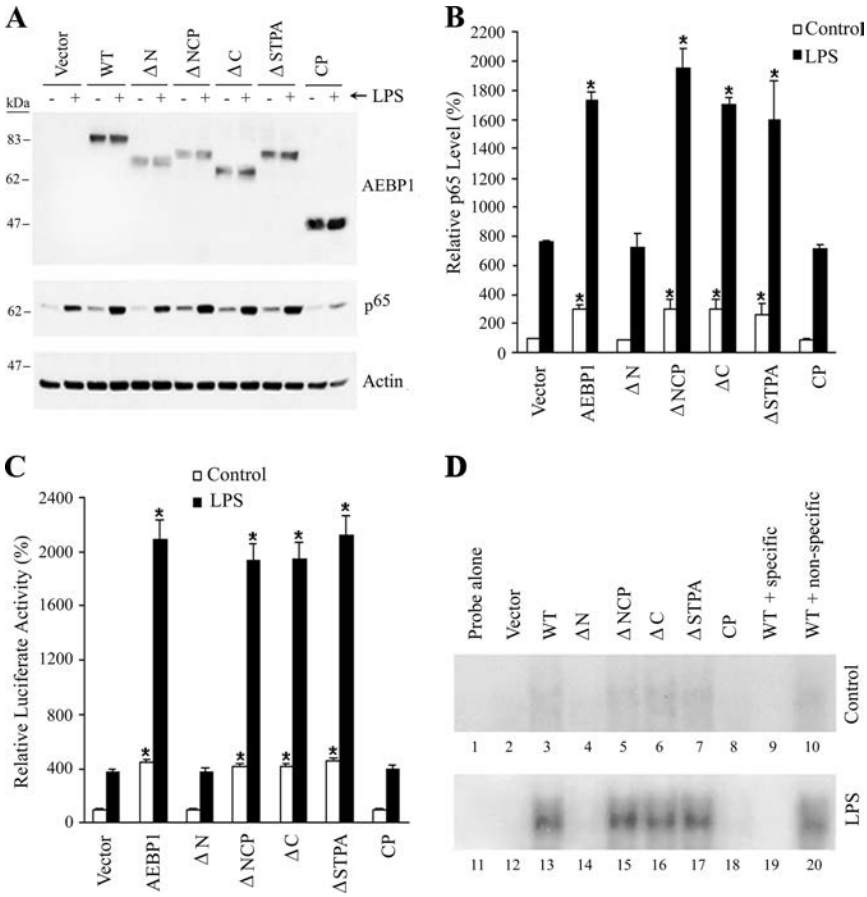

Figure 7. AEBP1-mediated up-regulation of NF-κB activity is DLD-dependent. (A) Nuclear protein extracts obtained from unstimulated or LPS-stimulated C6 glioma transfectants were subjected to SDS-PAGE and immunoblotting for p65. (B) A histogram illustrates densitometric analysis of nuclear protein levels shown in A, after normalization based on actin expression. Statistical significance was determined relative to protein level in unstimulated or LPS-stimulated, empty vector-transfectants. (C) Whole-cell protein extracts isolated from unstimulated or LPS-stimulated C6 glioma transfectants that are cotransfected with NF-κB-luciferase reporter were subjected to luciferase reporter assay. Statistical significance was determined relative to luciferase activity in unstimulated or LPS-stimulated, empty vector-transfectants (n = 3–8). (D) Nuclear protein extracts isolated from unstimulated and LPS-stimulated C6 glioma transfectants were subjected to EMSA in presence of specific (unlabeled NF-κB probe) or nonspecific (unlabeled, unrelated probe) competitors. This data are representative of three independent experiments.

hyperactivation, consistent with the effect of DLD deletion on AEBP1-mediated IκBα proteolytic degradation.

DISCUSSION

Because its initial identification by Sen and Baltimore (1986) about two decades ago, NF-κB has been the focus of many researchers in an attempt to understand the various molecular mechanisms involved in inflammatory diseases and cancer. Modulation of NF-κB activity can result in many abnormal cellular processes and diseases including asthma, arthritis, atherosclerosis, obesity, and various types of cancers (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Shoelson *et al.*, 2003; Monaco and Paleolog, 2004). In this study, we focus on examining the potential interplay between AEBP1 and NF-κB in an attempt to shed light on the molecular mechanism by which AEBP1 manifests its proinflammatory function in macrophages.

Herein, we provide experimental evidence establishing a positive relationship between AEBP1 expression and NF-κB activity in macrophages (Figure 1). Indeed, nuclear p65 protein level is significantly reduced in AEBP1$^{-/-}$ macrophages, compared with AEBP1$^{+/+}$ counterparts (Figure 1, B and C). Consistently, EMSA clearly illustrates that ablation of AEBP1 expression in macrophages correlates with markedly inhibited NF-κB DNA-binding activity (Figure 1D). Interestingly, AEBP1 expression in macrophages negatively correlates with IκBα, but not IκBβ, protein levels (Figure 3, C and D). Our data suggest that the negative regulation imposed by AEBP1 on IκBα function is independent of alterations in the IKK complex kinetic activity (Figure 4A) and is mediated by protein–protein interaction that requires DLD of AEBP1 (Figure 5D). It is clear that the half-life of IκBα is significantly prolonged in macrophages that lack AEBP1, compared with macrophages that express normal levels of AEBP1 (~250 vs. ~50 min, respectively; Figure 3, E and F), and that DLD of AEBP1 is crucial for such a biological regulation (Figure 6, C and D). Although modulation of IκBα expression has been previously shown to be a mechanism explaining altered NF-κB activity (Azuma *et al.*, 1999; Delerive *et al.*, 2000; Saile *et al.*, 2001), our study is the first of its kind to propose a molecular mechanism behind modulated NF-κB activity by which IκBα protein stability is altered via protein–protein interaction and is independent of alterations in IKK complex kinetic activity.

Our findings suggest that AEBP1 physically interacts with IκBα via its DLD, which is known be a protein–protein interaction mediator (Johnson *et al.*, 1993; Prag *et al.*, 2004). Indeed, DLD of AEBP1 was found to be required for protein–protein interaction between AEBP1 and MAPK (Kim *et al.*, 2001). Similarly, this study suggests that AEBP1 is capable of physically interacting with IκBα by means of its DLD, whose deletion eliminated AEBP1-IκBα interaction (Figure 5D). It is worth mentioning that despite the structural similarities between IκBα and IκBβ, our data suggests that AEBP1 is capable of interacting with IκBα, but not IκBβ, in macrophages (Figure 5A). Analysis of IκBα-IκBβ amino acid sequence alignment reveals that there are three main structural differences between IκBα and IκBβ. First, the first 12 amino acid residues in IκBα are absent in IκBβ. Second, there is a 41-amino acid stretch located between the third and forth ANK repeat of IκBβ that is not present in IκBα.

Third, there is an 18-amino acid stretch at the C-terminus of IκBβ that is absent in IκBα. Based on sequence analysis, it is conceivable that either the presence of the first 12 amino acid residues in IκBα is required for interaction with AEBP1 or that the presence of the extra amino acid stretches in IκBβ allows the formation of a tertiary structure that does not permit protein–protein interaction with AEBP1. It is also conceivable that the extra amino acid stretches in IκBβ somehow mask the region, or domain, that is necessary for protein–protein interaction with AEBP1. Identification of the region of IκBα that mediates protein–protein interaction with AEBP1, which will shed more light on the differential ability of AEBP1 to regulate IκBα and IκBβ functions in vivo, is currently underway. Further investigation of the AEBP1-interacting region of IκBα will shed more light on how AEBP1 is capable of differentially regulating IκBα and IκBβ functions in vivo.

Consistent with our proposal that AEBP1-IκBα protein–protein interaction provokes destabilization of IκBα shortening its half-life, ΔN and CP mutant forms of AEBP1 had no effect on IκBα protein stability (Figure 6). Importantly, in contrast to WT form of AEBP1, ΔN, and CP mutant forms possess minimal up-regulatory function toward NF-κB activity (Figure 7), confirming that AEBP1-IκBα interaction is a key biological event that is crucial for AEBP1-mediated IκBα-induced degradation and subsequent NF-κB up-regulation.

Despite their structural similarities, IκBα and IκBβ are known to be differentially regulated in various cell types and under several stimulatory conditions (Thompson *et al.*, 1995; Weil *et al.*, 1997). For instance, PMA or TNF-α treatment was shown to induce IκBα degradation, while IκBβ was resistant to degradation (Thompson *et al.*, 1995). In addition, although both IκBα and IκBβ become ubiquitinated upon phosphorylation, the two lysine residues (Lys^{21} and Lys^{22}) at the N-terminus of IκBα are required for ubiquitination, whereas the lysine residue (Lys^{9}) at the N-terminus of IκBβ is not required for ubiquitination (Weil *et al.*, 1997). Nonetheless, the exact mechanism(s) responsible for the differential regulation and function of IκBα and IκBβ remains unrevealed. Our study shed some light on a possible mechanism that can satisfactorily explain the differential regulatory functions exhibited by these two molecules. This mechanism implicates the differential capability of AEBP1 to physically interact with IκBα, but not IκBβ.

Up to date, two pathways have been suggested as molecular mechanisms responsible for IκBα proteolytic degradation. First, upon stimulation, IκBα is thought to be degraded via a classical, signal-induced proteosome-dependent pathway that involves the 26S proteosome (Alkalay *et al.*, 1995). Second, in vitro studies using immature B cells have shown that IκBα can be subject to constitutive proteosome-independent, Ca^{2+}-dependent degradation under basal conditions (Miyamoto *et al.*, 1998). It was also shown that constitutive phosphorylation of serine/threonine residues within the C-terminal PEST domain of IκBα by casein kinase II (CKII) is required for IκBα turnover (Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996). Also, accumulation of free IκBα in the cytosol triggers its rapid degradation through a phosphorylation, ubiquitination-independent proteosome-dependent pathway (Krappmann *et al.*, 1996). So, how does AEBP1-IκBα interaction lead to IκBα phosphorylation and subsequently proteolytic degradation? Although the exact molecular mechanism by which this regulatory process takes place is yet to be identified, we can provide a few speculative points that may enlighten the role of AEBP1-IκBα interaction in IκBα phosphorylation and proteolytic degradation. First, it is conceivable that AEBP1-IκBα interaction causes a conformational change in the latter, rendering it more susceptible to Ser^{32}/Ser^{36} phosphorylation and degradation via the ubiquitination-dependent proteosome-dependent pathway. Second, it is possible that IκBα-bound AEBP1 serves as a "recruiting" scaffold protein that facilitates recruitment of the constitutive proteosome-independent Ca^{2+}-dependent proteolytic or ubiquitination-independent proteosome-dependent machineries. Third, it is possible that AEBP1-bound IκBα is more prone to constitutive phosphorylation on serine/threonine residues within the PEST domain, inducing its proteosome-dependent proteolytic degradation.

Because AEBP1 represses PPARγ1 (Majdalawieh *et al.*, 2006), and because PPARγ interferes with NF-κB activity (Chinetti *et al.*, 1998; Ricote *et al.*, 1998; Chung *et al.*, 2000), it is possible that PPARγ1 repression by AEBP1 may contribute to AEBP1-mediated NF-κB up-regulation in macrophages. However, this effect may be marginal because of the following reasons. First, the ΔN mutant derivative, which mimics the WT AEBP1 with regard to PPARγ1 repression (data not shown), is incapable of up-regulating NF-κB activity (Figure 7). In agreement, ΔC mutant derivative, which is incapable of down-regulating PPARγ1 because of its lack of the DNA-binding domain (Majdalawieh *et al.*, 2006), is capable of mediating NF-κB up-regulation (Figure 7). Second, Glass and colleagues have shown that neither treatment of RAW 264.7 macrophages with PPARγ ligand nor PPARγ overexpression in absence of its ligand had any anti-inflammatory effects (Ricote *et al.*, 1998). The PPARγ-mediated anti-inflammatory effects are only observed when PPARγ is overexpressed and ligand activated (Weil *et al.*, 1997). In our study, AEBP1 enhances NF-κB activity in macrophages expressing endogenous PPARγ in absence of PPARγ ligand. Collectively, coordinate AEBP1-mediated NF-κB up-regulation seems to be independent of AEBP1-mediated PPARγ1 repression in macrophages.

It is important to mention that abrogation of NF-κB activity has been shown to cause embryonic lethality due to liver

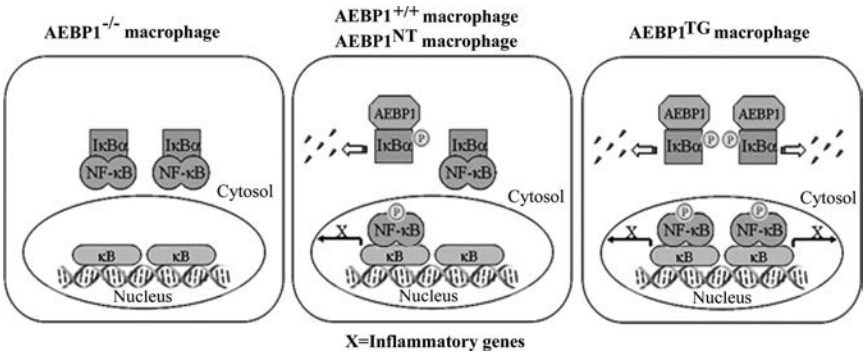

Figure 8. A model implicating AEBP1 in IκBα phosphorylation and proteolytic degradation, leading to enhanced NF-κB activation in macrophages. AEBP1 is proposed to promote IκBα phosphorylation and proteolytic degradation via protein–protein interaction that is DLD-dependent. Hence, AEBP1 overexpression and deficiency in macrophages is postulated to ultimately result in enhanced and reduced NF-κB transcriptional and DNA-binding activities, respectively, explaining the proinflammatory function of AEBP1 in macrophages.

apoptosis (Beg *et al.*, 1995; Doi *et al.*, 1997; Li *et al.*, 1999). This is very interesting given that NF-κB activity is severely inhibited in AEBP1$^{-/-}$ macrophages because of prolonged IκBα protein half-life. If coordinate regulation of NF-κB by AEBP1 in macrophages occurs in other cell types, such as hepatocytes, one would expect AEBP1$^{-/-}$ mice to suffer from liver apoptosis and possibly embryonic lethality. Interesting enough, experimental evidence suggests that AEBP1 loss leads to significant elevation of hepatic levels of apoptotic markers such as cleaved caspase-3 and p-STAT-3 (data not shown), suggesting that AEBP1 plays a critical role in apoptosis and cell survival. Remarkably, AEBP1$^{-/-}$ mice exhibit ~50% embryonic lethality (Ro *et al.*, 2007). Hence, it is plausible that the diminished NF-κB activity caused by AEBP1 deficiency can serve as a molecular mechanism underlying embryonic lethality in AEBP1$^{-/-}$ mice. Elucidation of AEBP1 role in hepatic regulation of NF-κB signaling and liver apoptosis is currently underway.

Figure 8 depicts a model implicating AEBP1 as an IκBα physical interacting partner that enhances macrophage inflammatory responsiveness by promoting IκBα phosphorylation and proteolytic degradation, subsequently leading to NF-κB activation. Experimental evidence indicates that AEBP1 physically interacts with endogenous IκBα via its DLD, and this protein–protein interaction renders IκBα prone to phosphorylation and proteolytic degradation. This AEBP1-mediated IκBα down-regulatory process is physiologically significant because it is accompanied by enhanced NF-κB activity leading to augmented macrophage inflammatory responsiveness. Because NF-κB is involved in biological processes implicated in many diseases, we anticipate that AEBP1 may potentially serve as a molecular candidate toward the development of therapeutic strategies for the treatment of various chronic inflammatory diseases and cancer.

ACKNOWLEDGMENTS

We thank Dr. Kirill Rosen, Dr. Patrick Lee, Dr. Kenneth Renton, Dr. Timothy Lee, Dr. Rongtuan Lin (Lady Davis Institute, Quebec, Canada), and Dr. Michael Karin for kindly providing us with the NF-κB-luciferase reporter construct, anti-p53 antibody, C6 glioma cells, J774 monocytes/macrophages, anti-IKKα antibody, and GST-IκBα (1-54) construct, respectively. We are grateful to Dr. Jean Marshall for kindly providing us with antibodies used in flow cytometric analysis. We thank Dr. Wojciech Dawicki and Mary Ann Trevors for their technical support. This work was supported by grants from Canadian Institute of Health Research (CIHR) and Natural Science and Engineering Research Council (NSERC).

REFERENCES

Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A., and Ben-Neriah, Y. (1995). Stimulation-dependent IκB-α phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA *92*, 10599–10603.

Azuma, M., Motegi, K., Aota, K., Yamashita, T., Yoshida, H., and Sato, M. (1999). TGF-β1 inhibits NF-κB activity through induction of IκB-α expression in human salivary gland cells: a possible mechanism of growth suppression by TGF-β1. Exp. Cell Res. *250*, 213–222.

Baeuerle, P. A., and Baltimore, D. (1989). A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. *3*, 1689–1698.

Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995). Embyonic lethality and liver degeneration in mice lacking the RelA component of NF-κβ. Nature *376*, 167–170.

Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκB-α to the ubiquitin-proteasome pathway. Genes Dev. *9*, 1586–1597.

Chiao, P. J., Miyamoto, S., and Verma, I. M. (1994). Autoregulation of IκB-α activity. Proc. Natl. Acad. Sci. USA *91*, 28–32.

Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., and Staels, B. (1998). Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. J. Biol. Chem. *273*, 25573–25580.

Chung, S. W., Kang, B. Y., Kim, S. H., Pak, Y. K., Cho, D., Trinchieri, G., and Kim, T. S. (2000). Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB. J. Biol. Chem. *275*, 32681–32687.

Deininger, M. H., Meyermann, R., and Schluesener, H. J. (2003). Expression and release of CD14 in astrocytic brain tumors. Acta Neuropathol. *106*, 271–277.

Delerive, P., Gervois, P., Fruchart, J. C., and Staels, B. (2000). Induction of IκBα expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-α activators. J. Biol. Chem. *275*, 36703–36707.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997). A cytokine responsive IkB kinase that activates transcription factor NF-kB. Nature *388*, 548–554.

Doi, T. S., Takahashi, T., Taguchi, O., Azuma, T., and Obata, Y. (1997). NF-κ B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. J. Exp. Med. *185*, 953–961.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-kB by phosphorylation of its inhibitor IkB. Nature *344*, 678–682.

He, G. P., Muise, A., Li, A. W., and Ro, H.-S. (1995). A eukaryotic transcriptional represser with carboxypeptidase act. Nature *378*, 92–96.

Jack, C. S., Arbour, N., Manusow, J., Montgrain, V., Blain, M., McCrea, E., Shapiro, A., and Antel, J. P. (2005). TLR signaling tailors innate immune responses in human microglia and astrocytes. J. Immunol. *175*, 4320–4330.

Jawdat, D. M., Albert, E. J., Rowden, G., Haidl, I. D., and Marshall, J. S. (2004). IgE-mediated mast cell activation induces Langerhans cell migration in vivo. J. Immunol. *173*, 5275–5282.

Johnson, J. D., Edman, J. C., and Rutter, W. J. (1993). A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain. Proc. Natl. Acad. Sci. USA *90*, 5677–5681.

Kim, S. W., Muise, A. M., Lyons, P. J., and Ro, H.-S. (2001). Regulation of adipogenesis by a transcriptional repressor that modulates MAPK activation. J. Biol. Chem. *276*, 10199–10206.

Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1996). Different mechanisms control signal-induced degradation and basal turnover of the NF-κB inhibitor IκB α in vivo. EMBO J. *15*, 6716–6726.

Kunsch, C., Ruben, S. M., and Rosen, C. A. (1992). Selection of optimal κ B/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. Mol. Cell. Biol. *12*, 4412–4421.

Layne, M. D., Endege, W. O., Jain, M. K., Yet, S. F., Hsieh, C. M., Chin, M. T., Perrella, M. A., Blanar, M. A., Haber, E., and Lee, M. E. (1998). Aortic carboxypeptidase-like protein, a novel protein with discoidin and carboxypeptidase-like domains, is up-regulated during vascular smooth muscle cell differentiation. J. Biol. Chem. *273*, 15654–15660.

Layne, M. D., Yet, S. F., Maemura, K., Hsieh, C. M., Bernfield, M., Perrella, M. A., and Lee, M. E. (2001). Impaired abdominal wall development and deficient wound healing in mice lacking aortic carboxypeptidase-like protein. Mol. Cell. Biol. *21*, 5256–5261.

Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). The IKKβ subunit of IκB kinase (IKK) is essential for nuclear factor κB activation and prevention of apoptosis. J. Exp. Med. *189*, 1839–1845.

Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996). Phosphorylation of IκBα in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol. Cell. Biol. *16*, 1401–1409.

Majdalawieh, A., Zhang, L., Fuki, I. V., Rader, D. J., and Ro, H.-S. (2006). Adipocyte enhancer-binding protein 1 is a potential novel atherogenic factor involved in macrophage cholesterol homeostasis and inflammation. Proc. Natl. Acad. Sci. USA *103*, 2346–2351.

May, M. J., and Ghosh, S. (1997). Rel/NF-κ B and I κ B proteins: an overview. Semin. Cancer Biol. *8*, 63–73.

McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996). Casein kinase II phosphorylates IκB-α at S-283, S-289, S-293, and T-291 and is required for its degradation. Mol. Cell. Biol. *16*, 899–906.

Mercurio, F. *et al.* (1999). IκB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol. Cell. Biol. *19*, 1526–1538.

Mercurio, F. *et al.* (1997). IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science *278*, 860–866.

Miles, E. A., Wallace, F. A., and Calder, P. C. (2000). Dietary fish oil reduces intercellular adhesion molecule 1 and scavenger receptor expression on murine macrophages. Atherosclerosis *152*, 43–50.

Miyamoto, S., Seufzer, B. J., and Shumway, S. D. (1998). Novel IκB-α proteolytic pathway in WEHI231 immature B cells. Mol. Cell. Biol. *18*, 19–29.

Monaco, C., and Paleolog, E. (2004). Nuclear factor κB: a potential therapeutic target in atherosclerosis and thrombosis. Cardiovasc. Res. *61*, 671–682.

Muise, A. M., and Ro, H.-S. (1999). Enzymic characterization of a novel member of the regulatory B-like carboxypeptidase with transcriptional repression function: stimulation of enzymic activity by its target DNA. Biochem. J. *343*, 341–345.

Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998). Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc. Natl. Acad. Sci. USA *95*, 3537–3542.

Nemoto, S., DiDonato, J. A., and Lin, A. (1998). Coordinate regulation of IκB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-κB-inducing kinase. Mol. Cell. Biol. *18*, 7336–7343.

Olmsted, J. B. (1981). Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J. Biol. Chem. *256*, 11955–11957.

Park, J.-G., Muise, A., He, G. P., Kim, S.-W., and Ro, H.-S. (1999). Transcriptional regulation by the γ5 subunit of a heterotrimeric G protein during adipogenesis. EMBO J *18*, 4004–4012.

Poltorak, A. *et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Prag, S., Collett, G. D., and Adams, J. C. (2004). Molecular analysis of muskelin identifies a conserved discoidin-like domain that contributes to protein self-association. Biochem. J. *381*, 547–559.

Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997). Identification and characterization of an IκB kinase. Cell *90*, 373–383.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998). The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. Nature *391*, 79–82.

Ro, H.-S., Kim, S. W., Wu, D., Webber, C., and Nicholson, T. E. (2001). Gene structure and expression of the mouse adipocyte enhancer-binding protein. Gene *280*, 123–133.

Ro, H.-S. *et al.* (2007). Obesity (*in press*).

Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998). IKK-γ is an essential regulatory subunit of the IκB kinase complex. Nature *395*, 297–300.

Saile, B., Matthes, N., El Armouche, H., Neubauer, K., and Ramadori, G. (2001). The bcl, NFκB and p53/p21WAF1 systems are involved in spontaneous apoptosis and in the anti-apoptotic effect of TGF-β or TNF-α on activated hepatic stellate cells. Eur. J. Cell Biol. *80*, 554–561.

Schwarz, E. M., Van Antwerp, D., and Verma, I. M. (1996). Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. Mol. Cell. Biol. *16*, 3554–3559.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705–716.

Shoelson, S. E., Lee, J., and Yuan, M. (2003). Inflammation and the IKK β/IκB/NF-κB axis in obesity- and diet-induced insulin resistance. Int. J. Obes. Relat. Metab. Disord. *27*, S49–S52.

Sun, S.-C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. (1993). NF-κB controls expression of inhibitor IκB-α: evidence for an inducible autoregulatory pathway. Science *259*, 1912–1915.

Tak, P. P., and Firestein, G. S. (2001). NF-κB: a key role in inflammatory diseases. J. Clin. Invest. *107*, 7–11.

Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995). IκB-β regulates the persistent response in a biphasic activation of NF-κB. Cell *80*, 573–582.

Weil, R., Laurent-Winter, C., and Israel, A. (1997). Regulation of IκBβ degradation. Similarities to and differences from IκBα. J. Biol. Chem. *272*, 9942–9949.

Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997). IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science *278*, 866–869.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science *249*, 1431–1433.

Yamamoto, Y., and Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J. Clin. Invest. *107*, 135–142.

Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998). Complementation cloning of NEMO, a component of the IκB kinase complex essential for NF-κB activation. Cell *93*, 1231–1240.

Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. Cell *91*, 243–252.

Zhang, L., Reidy, S. P., Nicholson, T. E., Lee, H.-J., Majdalawieh, A., Webber, C., Stewart, B. R., Dolphin, P., and Ro, H.-S. (2005). The role of AEBP1 in sex-specific diet-induced obesity. Mol. Med. *11*, 39–47.