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C/EBP α Expression is Partially Regulated by C/EBP β in Response to DNA Damage and C/EBP α Deficient Fibroblasts Display an Impaired G₁ Checkpoint

Rakesh Ranjan¹, Elizabeth A. Thompson¹, Kyungsil Yoon², and Robert C. Smart¹

¹Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, North Carolina 27695-7633

²National Cancer Center, Division of Common Cancers, Lung Cancer Branch, South Korea

Abstract

We observed that C/EBP α is highly inducible in primary fibroblasts by DNA damaging agents that induce strand breaks, alkylate and crosslink DNA as well as those that produce bulky DNA lesions. Fibroblasts deficient in C/EBP α (C/EBP α ^{-/-}) display an impaired G₁ checkpoint as evidenced by inappropriate entry into S-phase in response to DNA damage and these cells also display an enhanced G₁ to S transition in response to mitogens. The induction of C/EBP α by DNA damage in fibroblasts does not require p53. EMSA analysis of nuclear extracts prepared from UVB- and MNNG-treated fibroblasts revealed increased binding of C/EBP β to a C/EBP consensus sequence and ChIP analysis revealed increased C/EBP β binding to the C/EBP α promoter. To determine whether C/EBP β has a role in the regulation of C/EBP α we treated C/EBP β ^{-/-} fibroblasts with UVB or MNNG. We observed C/EBP α induction was impaired in both UVB- and MNNG- treated C/EBP β ^{-/-} fibroblasts. Our study reveals a novel role for C/EBP β in the regulation of C/EBP α in response to DNA damage and provides definitive genetic evidence that C/EBP α has a critical role in the DNA damage G₁ checkpoint.

Introduction

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors that contain a C-terminal basic DNA binding domain and a leucine zipper domain involved in homo- or hetero-dimerization (Ramji & Foka, 2002). The N-terminal region contains transcription activation and regulatory domains that interact with basal transcription apparatus and transcription co-activators. There are six members of the C/EBP family and C/EBPs play important roles in fundamental cellular processes including proliferation, apoptosis, differentiation, inflammation, senescence and energy metabolism (Ramji & Foka, 2002; Johnson, 2005).

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Address Correspondence to: Robert C. Smart, Ph.D., Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC 27695-7633, USA. Phone: 919-515-7245, Fax: 919-515-7169, Email: rcsmart@ncsu.edu.

C/EBP α mediates cell cycle arrest associated with terminal differentiation in adipocytes (Umek et al., 1991; Lin & Lane, 1994), hepatocytes (Diehl et al., 1996) and myeloid cells (Radomska et al., 1998; Wang et al., 1999) and regulates the expression of genes associated with the differentiated phenotype in these cell types. Consistent with its role in the regulation of differentiation, C/EBP α has been shown to be a tumor suppressor gene in acute myeloid leukemia where it is inactivated in ~9% of AML cases through specific somatic mutations (Pabst et al., 2001; Gombart et al., 2002). The inactivating mutations in C/EBP α result in a block in granulocytic differentiation and contribute to the uncontrolled proliferation of undifferentiated immature granulocytic blasts. Ectopic or forced expression of C/EBP α inhibits cell cycle progression in nearly all cell types examined (Hendricks-Taylor & Darlington, 1995; Watkins et al., 1996; Halmos et al., 2002; Johnson, 2005; Shim et al., 2005); however, the intrinsic cellular signals and pathways that regulate C/EBP α expression and its growth arrest properties are not fully understood. In some cases these pathways are linked to cellular differentiation.

C/EBP α expression is ablated or greatly diminished in a number of epithelial cancers including lung (Halmos et al., 2002), skin (Oh & Smart, 1998; Shim et al., 2005), liver (Xu et al., 2001), head and neck (Bennett et al., 2007), endometrial (Takai et al., 2005) and breast (Gery et al., 2005) suggesting a tumor suppressor function. In many cases, the C/EBP α gene is silenced through promoter hypermethylation (Gery et al., 2005; Tada et al., 2006; Bennett et al., 2007). Causal or genetic evidence for a suppressor role of C/EBP α in epithelial tumorigenesis is lacking due to the absence of C/EBP α mutations in epithelial tumors. Moreover, genetically engineered mouse models to document the suppressor function of C/EBP α in tumorigenesis have been problematic as germline or lung specific deletion of C/EBP α is perinatally lethal (Wang et al., 1995; Basseres et al., 2006). Recently, a genetically engineered mouse model in which C/EBP α was ablated in the epidermis was successfully developed (Loomis et al., 2007). These mice survived and were highly susceptible to carcinogen-induced skin tumorigenesis, thus providing the first genetic evidence for C/EBP α as a suppressor of epithelial tumorigenesis. Surprisingly, the epidermal specific C/EBP α knockout mice did not show alterations in stratified squamous differentiation or proliferation of epidermal keratinocytes, suggesting their enhanced susceptibility to tumorigenesis is not related to alterations in keratinocyte differentiation (Loomis et al., 2007).

To prevent or reduce stress-induced injury and cellular damage cells have evolved intricate pathways that permit them to respond to both intrinsic and extrinsic stressors. In terms of DNA damage, cells respond by engaging cell cycle checkpoints and repairing damaged DNA in order to maintain genome integrity and to prevent heritable mutations which can lead to genomic instability, aging and cancer (Kastan & Bartek, 2004; Sancar et al., 2004; Ishikawa et al., 2006). In UVB-treated skin keratinocytes, C/EBP α expression is highly induced through a p53-dependent pathway and the partial siRNA knockdown of C/EBP α in the BALB/MK2 keratinocyte cell line resulted in a diminished G₁ checkpoint after UVB-induced DNA damage (Yoon & Smart, 2004). While C/EBP α was highly induced by UVB in keratinocytes, UVB treatment of HepG2, NRK and NIH3T3 cells failed to induce C/EBP α . Thus, C/EBP α , at least in keratinocytes, is regulated by stress involving DNA damage through a p53 pathway and C/EBP α appears to have a role in the maintenance of

genomic stability via its role in the G₁ checkpoint. However, genetic evidence supporting a role for C/EBP α in the G₁ checkpoint is lacking and as mentioned above no other cell types other than keratinocytes have been reported to respond to DNA damage with the induction of C/EBP α .

In light of C/EBP α 's role in tumorigenesis and DNA damage response, it is important to understand the stress pathways or mechanisms through which C/EBP α is regulated in response to DNA damage and the functional importance of C/EBP α 's induction. In the current study, we show that fibroblasts respond to multiple types of DNA damage with the induction of C/EBP α and we present genetic evidence utilizing C/EBP α ^{-/-} cells to demonstrate a role for C/EBP α in the G₁ checkpoint. Importantly, we have identified a novel stress pathway in which C/EBP β has a role in the induction of C/EBP α in response to DNA damage.

Results

UVB Induces C/EBP α In Fibroblasts and C/EBP α ^{-/-} Fibroblasts Display Alterations in the G₁/S Transition and G₁ Checkpoint

Initially, we attempted cell cycle studies in wild type and C/EBP α ^{-/-} primary keratinocytes; however, these studies were not informative due to the inherent complexity of using primary keratinocytes for cell cycle regulation studies owing to the presence of mixed populations of differentiating and proliferating primary keratinocytes. To provide direct genetic evidence for C/EBP α in the G₁ checkpoint and to extend the findings on UVB-induced expression of C/EBP α in keratinocytes to another cell type, mouse dermal wild type and C/EBP α ^{-/-} primary fibroblasts were utilized. Wild type primary and C/EBP α ^{-/-} fibroblasts were exposed to a single dose of UVB radiation (5 mJ/cm²). UVB produced a significant increase in C/EBP α protein levels in wild type fibroblasts (Fig 1A) and C/EBP α was not expressed in C/EBP α ^{-/-} fibroblasts (Fig 1B). In wild type fibroblasts, C/EBP α was maximally induced at 6 h post-UVB treatment and returned to control levels by 24 h post-UVB treatment. Elevated levels of C/EBP α were detected as early as 1 h post-UVB treatment (data not shown). UVB also induced a modest transient increase in C/EBP β at 6 h post-UVB (Fig. 1A).

To examine the effect of the genetic ablation of C/EBP α on cell cycle regulation, wild type and C/EBP α ^{-/-} and primary fibroblasts were synchronized by serum deprivation and then released into the cell cycle by the addition of serum containing media. Fibroblasts were either left untreated or treated with a single dose of UVB (5 mJ/cm²) 4 h after release. Cells were pulsed with BrdU 1 h before each collection time point (4, 15, 18, 21 and 24 h post release) and FACS analysis was conducted to monitor entry into S-phase. Serum released C/EBP α ^{-/-} and wild type fibroblasts not treated with UVB displayed a synchronized entry into S-phase. However, C/EBP α ^{-/-} fibroblasts consistently displayed 10-20% more cells in S-phase than wild type fibroblasts indicating that C/EBP α ^{-/-} cells have an enhanced mitogen-induced entry into S-phase (Fig. 1C, E). Serum released wild type and C/EBP α ^{-/-} fibroblasts treated with UVB exhibited a significant decrease in the number of S-phase cells at 15 and 18 h compared to untreated control cells and this decrease was followed by a recovery at 21 and 24 h, indicating that cells from both genotypes engaged a G₁ checkpoint response.

However, UVB-treated *C/EBP α* ^{-/-} fibroblasts exhibited a significantly attenuated G₁ checkpoint as there were ~ 70% more *C/EBP α* ^{-/-} cells in S-phase at 15 and 18 h than similarly treated wild type cells indicating inappropriate entry into S-phase (Fig. 1D, F). Collectively, these results demonstrate that *C/EBP α* is highly induced by UVB in fibroblasts and the genetic ablation of *C/EBP α* results into an enhanced mitogen induced G₁/S transition in untreated cells and a significantly diminished G₁ checkpoint in response to DNA damage.

DNA damage and the regulation of *C/EBP α*

As shown in Fig 2A, UVB treatment of fibroblasts with 5, 10 and 20 mJ/cm² resulted in the induction of *C/EBP α* with the higher doses displaying a more prolonged induction of *C/EBP α* . To determine whether *C/EBP α* can be induced by DNA damaging agents other than UVB, we treated fibroblasts with MNNG, a direct acting mutagen that methylates DNA; cisplatin, a cancer therapeutic that cross links DNA; camptothecin, an alkaloid with anti-tumor activity that induces single stranded DNA breaks by inhibiting topoisomerase I enzyme; and bleomycin, an antineoplastic drug that induces both single and double stranded DNA breaks. As shown in Fig. 2B-E, MNNG, cisplatin, camptothecin and bleomycin were inducers of *C/EBP α* . To determine whether the increases in UVB-induced *C/EBP α* protein levels involve altered stability of *C/EBP α* protein, untreated and UVB-treated fibroblasts were incubated with cycloheximide, an inhibitor of protein synthesis, and the stability of the *C/EBP α* protein was examined over time. The degradation of *C/EBP α* protein was similar in both untreated (Fig. 2F, left panel) and UVB-treated fibroblasts (Fig. 2F, right panel). Similar results were observed for *C/EBP β* (Fig. 2F). To determine whether *C/EBP α* mRNA levels are increased by UVB treatment, we utilized quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR). UVB treatment (5 mJ/cm²) of primary fibroblast resulted in significant increases in *C/EBP α* mRNA at 6 and 12 h post UVB (Fig. 2G) and this increase was blocked by actinomycin D, an inhibitor of transcription (data not shown). These results indicate that the increased levels of *C/EBP α* in UVB-treated fibroblasts are due to increased transcription of *C/EBP α* .

In keratinocytes, the transcription factor p53 is essential for the UVB induction of *C/EBP α* (Yoon & Smart, 2004). As shown in Figure 3A, UVB treatment of wild type primary keratinocytes resulted in significant increases in the protein levels of *C/EBP α* and p53, as well as the p53 target gene p21, while UVB treatment of p53^{-/-} primary keratinocytes failed to significantly induce the expression of both *C/EBP α* and p21 protein (Fig. 3A). These results verify that in keratinocytes, p53 is required for *C/EBP α* induction by UVB. In contrast to keratinocytes, p53 was dispensable for the UVB-induction of *C/EBP α* in fibroblasts. As shown in Figure 3B, UVB treatment of p53^{-/-} fibroblasts efficiently induced *C/EBP α* and as anticipated failed to induce the p53 target, p21, thus verifying the ablation of p53 activity (Fig. 3B). These results demonstrate p53 is dispensable for the UVB induction of *C/EBP α* in fibroblasts indicating a p53 independent pathway contributes to the induction of *C/EBP α* in fibroblasts.

UVB Increases C/EBP β Binding To C/EBP Consensus Sequence And Is Bound To The C/EBP α Promoter

During the process of L1 preadipocyte differentiation, both C/EBP β protein levels and DNA binding activity are increased and C/EBP β has been shown to regulate C/EBP α levels during process (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004). Since we observed that UVB produces an increase in C/EBP β protein levels, we initiated studies to examine whether C/EBP β has role in the regulation of C/EBP α expression in response to DNA damage. First, we used EMSA analysis to examine whether nuclear extracts isolated from UVB and MNNG treated fibroblasts display increases in the binding of C/EBP β to a canonical C/EBP consensus sequence (TGCAGATTGCGCAATCTGCA) (Osada et al., 1996). As shown in Fig. 4A, nuclear extracts from cells treated with UVB and MNNG displayed increased C/EBP binding to the C/EBP consensus sequence and this increase appeared somewhat greater than the increase in C/EBP α and C/EBP β protein levels (Fig. 4 A, lower panel). As shown in Figure 4B, no binding was detected with these nuclear extracts when a mutant C/EBP consensus sequence (TGCAGAGACTAGTCTCTGCA) was utilized and in competition studies, only the cold wild type C/EBP consensus sequence could compete for binding. Supershift assays with antibodies to C/EBP α and C/EBP β , but not IgG control antibodies revealed that the increase in C/EBP DNA binding post-UVB was due to both C/EBP α and C/EBP β binding with C/EBP β being present in all complexes (Fig. 4C). Due to alternative translation start sites, C/EBP β protein can be present in three isoforms, termed LAP*(liver activating protein*), LAP *aka* C/EBP β and LIP (liver inhibitory protein) which functions as a dominant negative inhibitor of LAP* and LAP. To begin to understand which C/EBP β isoforms are responsible for the increase binding in the EMSA analysis, we first conducted immunoblot analysis of protein extracts from fibroblasts to examine the levels of the three C/EBP β isoforms and then overexpressed LAP and LIP and examined their DNA binding location using EMSA. As shown in Fig 4D, LAP is the predominate C/EBP β isoform in fibroblasts and is also the predominate C/EBP β binding isoform in UVB-treated fibroblasts (Fig 4E). As shown in Fig. 4F, we also conducted EMSA analysis using the C/EBP binding sequence ((-188) GCGTTGCGCCACGATCTCTC (-169)) that was previously identified as a bona-fide C/EBP site in the C/EBP α promoter (Tang et al., 1999; Tang et al., 2004). We observed increases in C/EBP binding after UVB or MNNG to the C/EBP α promoter consensus oligomer (Fig 4F) similar to those observed using the canonical C/EBP consensus sequence (Fig 4A). This binding could be competed away with cold consensus sequence (data not shown). To determine whether the C/EBP β binds to this C/EBP site in the C/EBP α promoter in vivo, we conducted ChIP analysis utilizing a C/EBP β antibody to immunoprecipitate C/EBP β -DNA complexes. PCR was conducted on the input DNA and C/EBP β immunoprecipitated DNA with primers that flank the C/EBP site in C/EBP α promoter. We observed that C/EBP β was bound to the C/EBP α promoter in the basal untreated state and that UVB treatment consistently produced a modest increase in C/EBP β binding (N=3) at the early time point (Fig. 4G). To further confirm the specificity of immunoprecipitation of C/EBP β , we performed ChIP analysis on the C/EBP α promoter in C/EBP β ^{-/-} fibroblasts. We observed an absence of a PCR product in the C/EBP β immunoprecipitated samples further supporting our results that C/EBP β directly binds to the C/EBP α promoter in wild type fibroblasts. We also conducted ChIP analysis on the C/EBP α promoter with a C/EBP α antibody to determine whether C/EBP α is bound to its own

promoter in vivo. We observed that C/EBP α is bound to its own promoter at basal state and UVB treatment resulted in increased C/EBP α binding (Fig. 4H). Taken together, these results suggest C/EBP α expression is regulated by C/EBP β in response to DNA damage and that C/EBP α has an autoregulatory role.

C/EBP β has a Role in the UVB- and MNNG-Induction of C/EBP α

To determine whether C/EBP β has a functional role in the regulation of C/EBP α in response to DNA damage, we isolated dermal fibroblasts from wild type and C/EBP β ^{-/-} mice and treated these cells with UVB (5 mJ/cm² or 10 mJ/cm²) or MNNG (35 μ M). As shown in Fig. 5A, UVB (10 mJ/cm²) induction of C/EBP α protein in C/EBP β ^{-/-} fibroblasts was impaired as both the level of protein induction was reduced and the time course for its induction was altered. While the induction of C/EBP α was impaired the increase in p53 protein was comparable in wild type and C/EBP β ^{-/-} fibroblasts (Fig. 5A). The induction of C/EBP α was also impaired in C/EBP β ^{-/-} fibroblasts treated with a lower dose of UVB (5 mJ/cm²) (Fig 5B) or with the alkylating mutagen MNNG (Fig 5C). To determine whether C/EBP β deficiency has an effect on UVB-induced C/EBP α mRNA levels, we isolated RNA from wild type and C/EBP β ^{-/-} fibroblasts before and after UVB treatment and examined C/EBP α mRNA levels using quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR), (Fig. 5D). The UVB induction of C/EBP α mRNA was significantly decreased in C/EBP β ^{-/-} fibroblasts compared to wild type fibroblasts (Fig 5D). Collectively, these results indicate that in fibroblasts C/EBP β functions downstream of DNA damage to partially regulate C/EBP α mRNA and protein expression.

Discussion

Previously we have shown that C/EBP α is a UVB/DNA damage-inducible gene in mouse and human primary keratinocytes, however, UVB treatment did not induce C/EBP α in three other cell lines examined (HepG2, NRK, or NIH3T3 cells) (Yoon & Smart, 2004) suggesting the induction of C/EBP α by UVB may be a keratinocytes-specific effect. In keratinocytes, UVB-induction of C/EBP α is solely dependent upon p53 and this is mediated through p53 binding to a p53 response element in the distal promoter of C/EBP α (Yoon & Smart, 2004). The results presented in this study demonstrate that C/EBP α is a DNA damage responsive gene in mouse primary fibroblasts and that p53 is dispensable for the UVB-induction of C/EBP α in fibroblasts. Instead, we observed C/EBP α is regulated by C/EBP β in response to DNA damage and C/EBP α likely has an autoregulatory role in response to DNA damage. C/EBP α induction by DNA damage was impaired in C/EBP β ^{-/-} fibroblasts, both at the protein and mRNA level and the time course for their induction was altered, however, there was not a complete ablation of C/EBP α induction indicating that other pathways are also involved. While C/EBP β has been shown to regulate C/EBP α expression during pre-adipocyte differentiation (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004), our study is the first to demonstrate that C/EBP β functions downstream of DNA damage to regulate the induction of C/EBP α . Therefore, C/EBP β is a protein that can be activated by numerous cues including those involving differentiation (Yeh et al., 1995; Sterneck et al., 1997; Oh & Smart, 1998; Zhu et al., 1999) and DNA damage (Ewing et al., 2008) as well as oncogenes (Sundfeldt et al., 1999; Rask et al., 2000;

Zhu et al., 2002; Shuman et al., 2004) and inflammatory cytokines (Akira et al., 1990; Mukaida et al., 1990; Drouet et al., 1991). Recently, Ewing et al (Ewing et al., 2008) reported C/EBP β represses p53 levels and function to promote cell survival downstream of DNA damage. Therefore, emerging evidence indicating that both C/EBP α and C/EBP β participate in DNA damage response pathways.

UVB treatment produced a modest increase in C/EBP β levels and ChIP analyses also revealed modest increases in C/EBP β binding to the C/EBP α promoter and yet C/EBP β ^{-/-} fibroblasts displayed a significantly impaired induction of C/EBP α mRNA and protein in response to UVB or MNNG treatment. Our ChIP results also revealed that C/EBP β is constitutively bound to the C/EBP α promoter in untreated fibroblasts. Taken together these results suggest that post-translational modifications of C/EBP β may contribute to the regulation of C/EBP α in response to UVB. It is generally accepted that C/EBP β exists in a repressed state and post-translational modifications de-repress C/EBP β and increase its transcriptional activity. For example, phosphorylation or deletion of the repressor domain unfolds and induces conformational change in C/EBP β , which results in de-repression and increased transactivation activity of C/EBP β (Kowenz-Leutz et al., 1994; Williams et al., 1995). In addition to phosphorylation, C/EBP β is modified by acetylation and a mutant C/EBP β that can no longer be acetylated on Lys-39 has an impaired ability to transactivate a C/EBP α promoter reporter construct (Cesena et al., 2007; Cesena et al., 2008). Post-translational modifications involving methylation (Pless et al., 2008) and sumoylation (Kim et al., 2002; Eaton & Sealy, 2003; Berberich-Siebelt et al., 2006) also have regulatory roles and changes in mediator complex also result in C/EBP β activation (Mo et al., 2004). Recently, it was reported that C/EBP β is involved in the opening of chromatin allowing other transcription factors to bind to the gene promoter and increase gene expression (Plachetka et al., 2008). Further studies are required to understand how C/EBP β is activated in response to DNA damage and how these events contribute to the regulation of C/EBP α .

Previous studies in keratinocytes utilizing siRNA knockdown of C/EBP α indicated that C/EBP α has a role in the G₁ checkpoint in response to DNA damage (Yoon & Smart, 2004). One goal of the current study was to use a genetic approach to confirm and verify the role of C/EBP α in the G₁ checkpoint as siRNA knockdown studies can be complicated by the unintentional interaction of the siRNA with other unidentified targets. We observed a diminished G₁ checkpoint response in C/EBP α ^{-/-} fibroblasts compared to wild type fibroblasts in response to UVB treatment, and our results provides important genetic evidence that C/EBP α is involved in DNA damage induced G₁ checkpoint. Moreover, we observed that serum deprived C/EBP α ^{-/-} fibroblasts display an enhanced mitogen-induced entry into S-phase compared to wild type fibroblasts suggesting that C/EBP α has a direct role in the regulation of the G₁ to S transition in response to mitogens. To our knowledge there are no previous studies that have utilized synchronous cultures of primary cells genetically deficient in C/EBP α to define a functional role for C/EBP α in the regulation of G₁/S transition in response to mitogens. Proposed mechanisms for C/EBP α -induced cell cycle arrest involve interactions with cell cycle proteins including; Rb family members (Chen et al., 1996; Timchenko et al., 1999), CDK4 and CDK2 (Wang et al., 2001), E2F (Slomiany et al., 2000; Porse et al., 2001), p21 (Timchenko et al., 1996) and the SWI/SNF chromatin remodeling complex (Muller et al., 2004). Further studies are required to

understand the molecular mechanisms of C/EBP α 's involvement in the G₁/S transition and how this impinges on the DNA-damage induced G₁ checkpoint and neoplasia.

Materials and Methods

Cell lines and cell culture

Mouse primary keratinocytes were isolated from epidermis of newborn mice by overnight floatation of skin in trypsin at 4°C (Hennings et al., 1980; Dlugosz et al., 1995) and dermal fibroblasts were isolated from dermis after the removal of epidermis from skin. Primary keratinocytes were cultured as described by Yoon et al (Yoon & Smart, 2004). For fibroblasts isolation, dermis was subjected to digestion in collagenase (0.35%) for 25 mins, followed by DNAase (250 units/skin) treatment for 5 mins at 37°C while shaking. Cells were filtered, and collected after centrifugation, and isolated fibroblasts were plated in Eagle's minimal essential medium (EMEM; BioWhittaker) (2 mM CaCl₂) supplemented with 10% Fetal Bovine Serum (FBS)(Sigma), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B (GIBCO) in 100 mm tissue culture dish. Upon confluence, fibroblasts were passaged and plated in 60 mm tissue culture dish. When cells reached 70% confluence they were treated with UVB or other DNA damaging agents.

Animals

Germline C/EBP α ^{-/-} mice do not survive; pups die prenatally or survive only few hours after birth (Wang et al., 1995). A keratin 5, K5Cre transgenic line mouse can be used for generalized Cre-mediated recombination or tissue specific gene ablation. When floxed females carrying transgene K5Cre are mated to floxed male animals, recombination occurs in all the tissues in all mice produced from the above mating pair (Ramirez et al., 2004). Germline C/EBP α ^{-/-} pups were produced by mating epidermal conditional C/EBP α ^{-/-} female (K5Cre;C/EBP α ^{fl/fl}) (C57BL/6;DBA;129SV) (Loomis et al., 2007) and C/EBP α floxed (C/EBP α ^{fl/fl}) male mice (C57BL/6;DBA;129SV) (Lee et al., 1997). Primers and PCR conditions used to genotype mice were published previously (Lee et al., 1997; Ramirez et al., 2004). p53^{+/-} male mice were mated with p53^{+/-} female mice to generate p53^{-/-} as well as wild type newborn pups (C57BL/6). Primers and PCR conditions were published previously (Hulla et al., 2001). The C/EBP β ^{-/-} mice used in this study have been described previously (Sterneck et al., 1997). The C/EBP β ^{-/-} and wild-type new born pups were generated by mating C/EBP β ^{+/-} females to C/EBP β ^{+/-} males (C57BL/6;129/SV). For all other studies not utilizing genetically modified mice, fibroblasts were isolated from the dermis of wild type 129SV mice.

Treatment of cells

The UV lamp (model EB 280C; Spectronics) used for treating cells emits wavelengths between 280 and 320 nm with a spectrum peak at 312 nm. The intensity of light emitted was measured by NIST Traceable Radiometer Photometer (Model IL1400A, International Light). Cells were treated with UVB as described by Yoon et al (Yoon & Smart, 2004). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), camptothecin and cycloheximide were dissolved in dimethyl sulfoxide (DMSO). Cisplatin {cis-Diammineplatinum (II)}, and bleomycin were dissolved in water. Cells were either treated with MNNG, cycloheximide,

camptothecin, bleomycin, dimethyl sulfoxide or water. Cells were treated with cisplatin for 2 h, and then media was replaced with fresh media without cisplatin. Actinomycin D was dissolved in ethanol and cells were treated with Actinomycin D or ethanol.

Preparation of cell lysates

Nuclear extracts were prepared as previously described by Schreiber et al (Schreiber et al., 1989). For preparation of whole cell lysates, cells were washed with cold PBS and harvested by scraping. Cells were collected by centrifugation and protein was isolated in radio-immunoprecipitation assay buffer as previously described (Ewing et al., 2008).

Western blot analysis

Protein from cell lysates were loaded onto a 12% polyacrylamide Tris-glycine gel (Invitrogen), then separated by electrophoresis and transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (Oh & Smart, 1998), the membranes were probed with rabbit polyclonal immunoglobulin G (IgG) raised against C/EBP α (sc-61), p53 (sc-6243), C/EBP β (sc-150), p21 (sc-757), or mouse monoclonal raised against α tubulin (sc-8035) (1:2000) (Santa Cruz Biotechnology) and then probed with a horseradish peroxidase-linked secondary antibody (Amersham). Detection was made with an enhanced chemiluminescence reagent (Perkin Elmer life Science) followed by exposure of membrane to the film.

Quantitative real time PCR

Total RNA was isolated from either control or UVB treated fibroblasts using TRI reagent (Sigma) and then purified by RNeasy Mini Kit (Qiagen). cDNA was prepared from 50 ng RNA by ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol. cDNA was used to perform Quantitative PCR using mouse C/EBP α TaqMan Gene Expression Assays, 18S TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universal PCR mix (Applied Biosystems). Data were analyzed using comparative C_T method.

Chromatin immunoprecipitation (ChIP) assay

Primary fibroblasts were plated in 100 mm culture plates and were left untreated or treated with UVB dose 5 mJ/cm². After 1 and 6 h of UVB treatment cells were treated with 1% formaldehyde, thereafter ChIP assay was performed as per manufacturer's instruction (Upstate Biotechnology). The formaldehyde treated cells were lysed with SDS lysis buffer and sonicated to produce 200-500 bp long DNA fragments. Samples were pre-cleared with salmon sperm DNA/ protein A, then immunoprecipitated with polyclonal antibody against C/EBP β , C/EBP α or rabbit IgG at 4°C overnight. Immunoprecipitated DNA was decrosslinked with 5 M NaCl and extracted by ethanol/ chloroform precipitation and amplified by PCR. Primer set for PCR was designed to flank C/EBP regulatory sequence in C/EBP α promoter (-188) GCGTTGCGCCACGATCTCTC (-169) (Tang et al., 1999; Tang et al., 2004). Primer set flanking the corresponding site were 5'-(324) GGCTGGAAGTGGGTGACTTA (-305)-3' and 5'-(115) CGCCTTCTCCTGTGACTTTC (-134)-3' to produce a 210 bp PCR product.

Electrophoretic mobility shift assay (EMSA) and supershift

Nuclear extracts, 2 µg in 10 µl buffer C, were incubated with 10 µl of master binding mix buffer with ³²P-labeled C/EBP probe (Santa Cruz) or ³²P labeled probe corresponding to C/EBP responsive element in C/EBPα promoter for 30 minutes at room temperature. For the supershift assays, samples were treated as above but incubated with either C/EBPα (sc-61), C/EBPβ (sc-150) or IgG (sc-2027) (Santa Cruz Biotechnology) antibody. For competition assays, samples were incubated for 20 minutes with cold wild type and cold mutant C/EBP consensus oligonucleotide probe (50 fold in excess) in 10 µl master mix binding buffer and then with wild type ³²P-labeled C/EBP probe for 20 minutes at room temperature. Samples were loaded onto 6% polyacrylamide gel, and subjected to electrophoresis in 0.025X TBE buffer at 200V at 4-8° C for 5-7 h.

5-Bromo-2'-deoxyuridine (BrdU) labeling and fluorescence-activated cell sorting (FACS) Analysis

When fibroblasts reached 30-40% confluence, cells were synchronized by serum deprivation in 0.5% FBS for 28 h. Fibroblasts were released by adding 10% FBS containing fibroblast medium. After 4 h of release, fibroblasts were either left untreated or treated with UVB (5 mJ/cm²). One hour before the cells were harvested at each time point, the cells were incubated with 10 µM BrdU. Cells were then fixed in 70% alcohol, treated with 2 N HCl–Triton X-100 to denature DNA, followed by neutralization with Na₂B₄O₇. Cells were pelleted, resuspended in 0.5% Tween 20–1% bovine serum albumin–PBS with anti-BrdU–fluorescein isothiocyanate antibody (1:50; Becton Dickinson) and 0.5 mg of RNase/ml, and incubated at 4°C overnight. Cells were pelleted and resuspended in PBS containing 5 µg/ml propidium iodide (PI) and subjected to FACS analysis.

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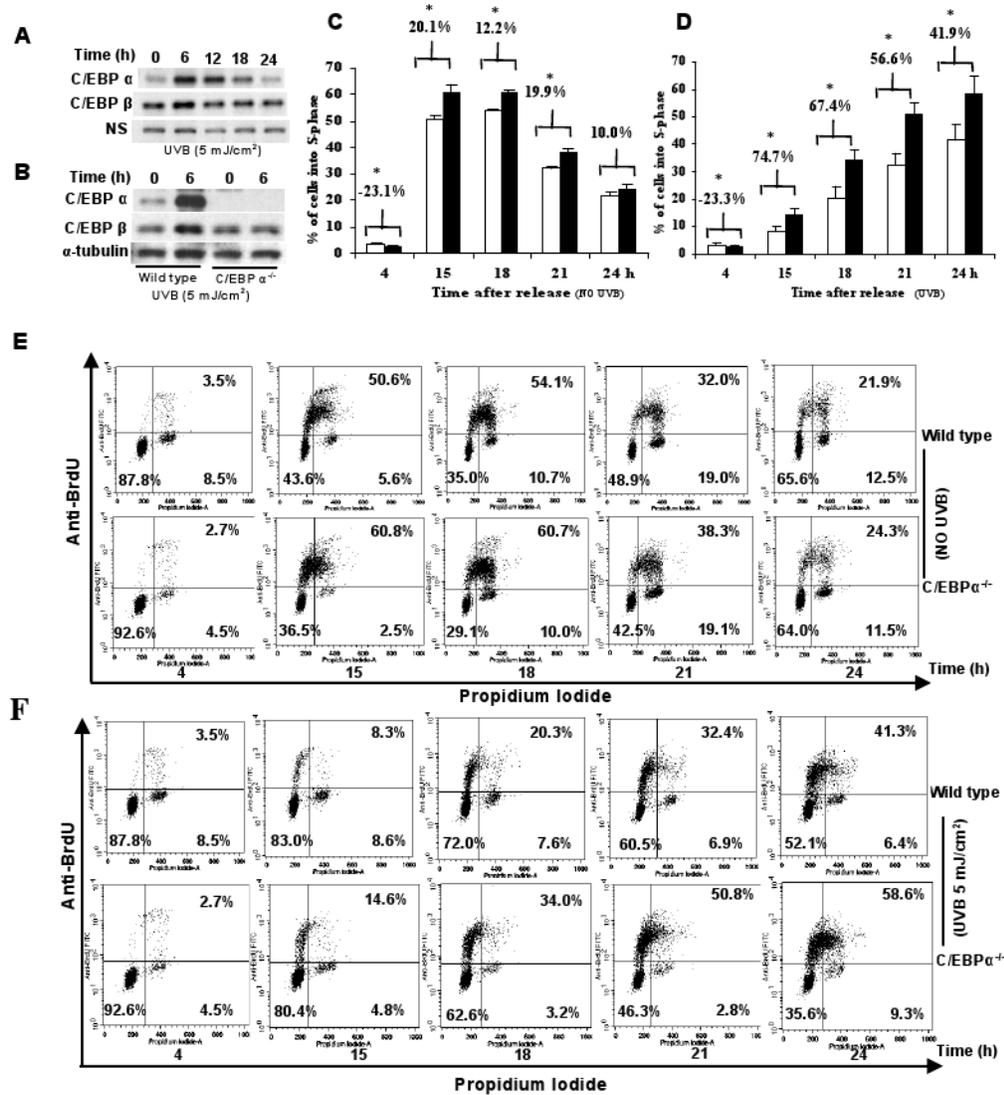


FIG. 1. UVB induces C/EBP α in primary fibroblasts and C/EBP α is involved in the G₁/S transition as well as in UVB-induced G₁ checkpoint
 (A) Primary fibroblasts were exposed to UVB (5 mJ/cm²) and cell lysates were prepared at various time points and immunoblot analysis conducted. Non-specific (NS) band is shown to confirm equal loading. (B) Primary fibroblasts from newborn wild type or C/EBP α ^{-/-} were treated with UVB (5 mJ/cm²) and cell lysates were prepared at various time points and immunoblot analysis conducted. (C,D) Wild type (open column) and C/EBP α ^{-/-} (black column) fibroblasts were synchronized by serum deprivation for 28 h in 0.5% serum and then released into the cell cycle by the addition of serum containing medium. Fibroblasts were either not treated (C) or treated with UVB (5 mJ/cm²) (D) at 4 h after the addition of serum containing medium. Cells were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) 1 h before collection and then the cells were fixed, incubated with anti-BrdU antibody, stained with PI, and subjected to FACS analysis. The number above each column pair represents the percent increase in S-phase cells in C/EBP α ^{-/-} fibroblasts compared to wild type. Data represents mean \pm S.D, N=3/time point/genotype. Two-factor ANOVA demonstrated

significant interaction between genotype and time for both untreated and treated cells ($P < 0.05$). *Significantly different from wild type fibroblasts ($p < 0.05$) at the indicated time point as determined by Student's t-test. (E) Representative scatter plots for untreated fibroblasts (for C) after release, showing the mean percentage of cells ($N=3/\text{time point}/\text{genotype}$) in G_1 (lower left), S (top), and G_2M (lower right) phase of the cell cycle. (F) Representative scatter plots for UVB-treated fibroblasts (for D) showing the mean percentage of cells ($N=3/\text{time point}/\text{genotype}$) in G_1 , S and G_2M . The data presented represents one of four independent experiments all showing similar results.

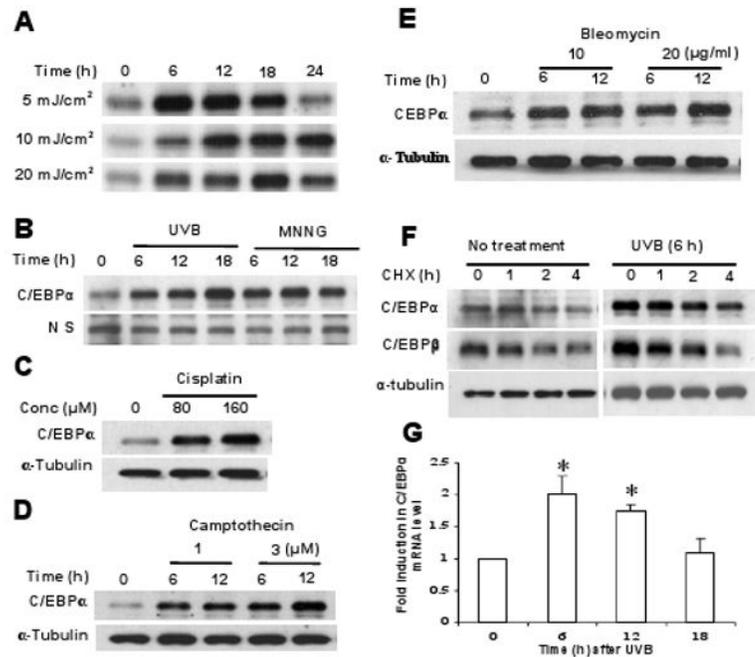


FIG. 2. DNA damaging agents induce C/EBPα in primary fibroblasts

(A) Primary fibroblasts were exposed to different doses of UVB and cell lysates were prepared at indicated time points and immunoblot analysis conducted. (B-E) Primary fibroblasts were treated with UVB (10 mJ/cm²), MNNG (35 μM), cisplatin (80 and 160 μM), camptothecin (1 and 3 μM), bleomycin (10 and 20 μg/ml), DMSO or water alone, and immunoblot analysis conducted. Non-specific (NS) and α-tubulin band is shown to confirm equal loading. (F) Fibroblasts were either not treated (left panel) or treated (right panel) with UVB and 6 h later were incubated with cycloheximide (50 μg/ml). Cells were harvested at indicated time points after the start of cycloheximide treatment and immunoblot analysis was conducted. (G) Total RNA was isolated from fibroblasts at different time points after UVB (5 mJ/cm²) treatment. Quantitative RT-PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized to 18 S and analyzed using the comparative C_T method. Data is expressed as mean ± standard error (N=3) and each experiment was run in triplicate. *Significantly different from time zero (p < 0.05) as determined by Student's t-test

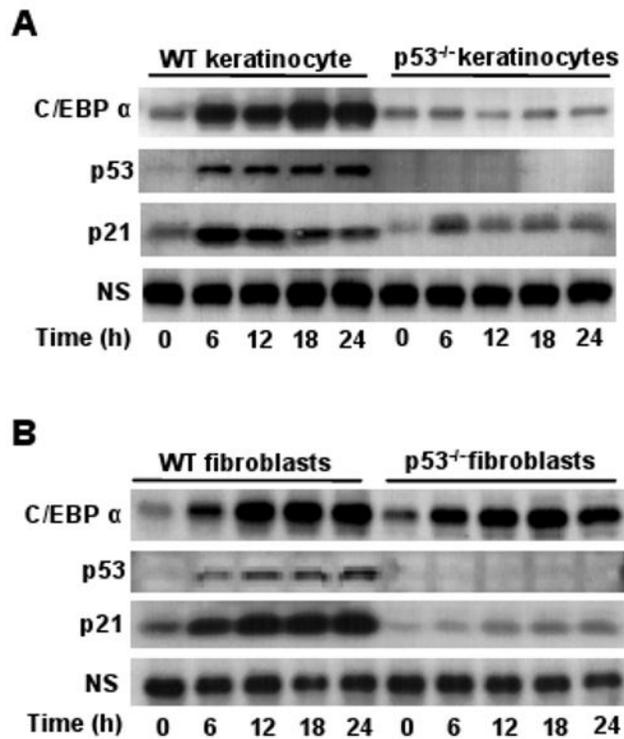


FIG. 3. UVB induction of C/EBPα does not require p53 in fibroblasts

(A) Primary keratinocytes were isolated from wild type and p53^{-/-} newborn mice and were treated with UVB (10 mJ/cm²). Keratinocytes were harvested at the indicated time points and immunoblot analysis conducted. (B) Primary fibroblasts isolated from wild type and p53^{-/-} newborn mice were treated with UVB (10 mJ/cm²). Fibroblasts were harvested at indicated time points and immunoblot analysis conducted.

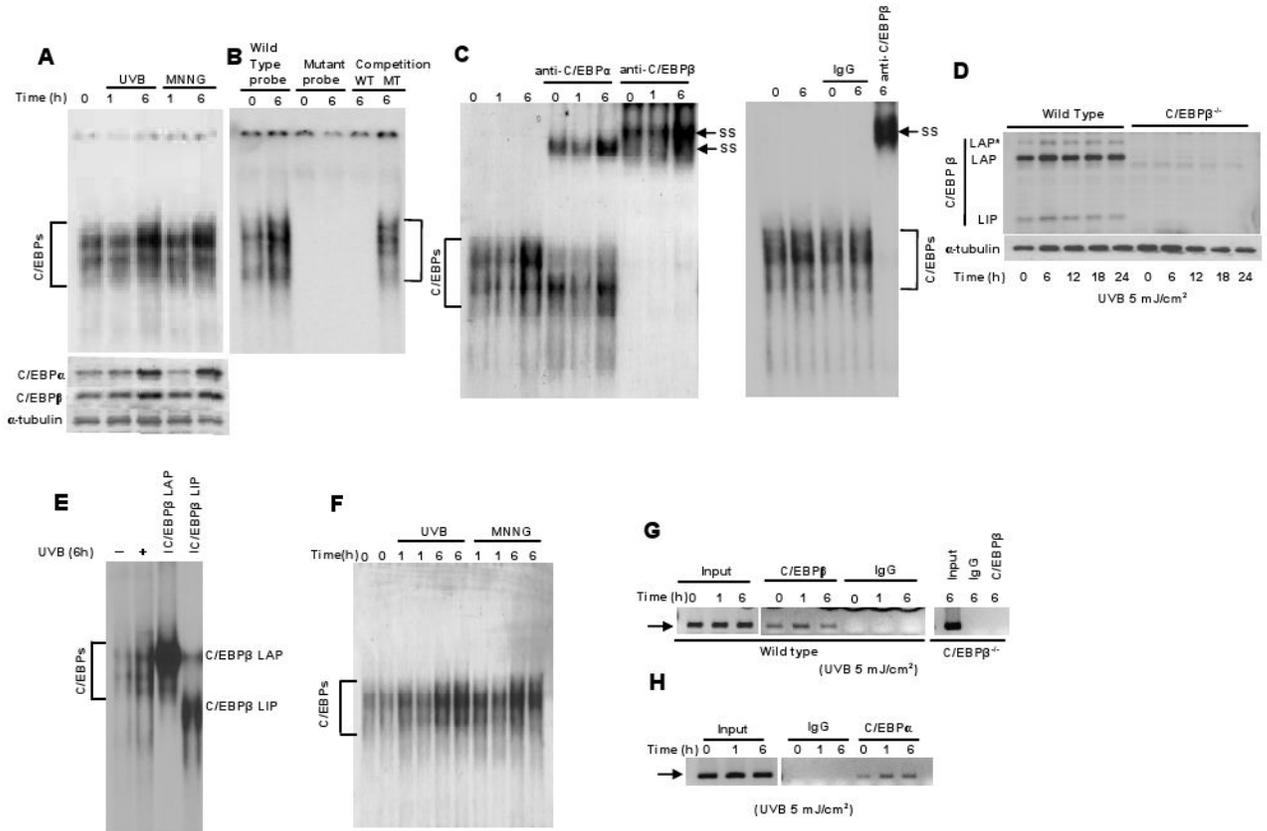


FIG. 4. UVB and MNNG increase binding of C/EBP α and C/EBP β to C/EBP consensus sequence and to the C/EBP α promoter in vivo

(A) Wild type fibroblasts were treated with UVB (5 mJ/cm²) or MNNG (35 μ M) and nuclear extracts were prepared. EMSA was conducted with 2 μ g of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. (Lower panel) Nuclear extract from above experiment was used to conduct immunoblot analysis. (B) Wild type fibroblasts were treated with UVB (5 mJ/cm²) and nuclear extracts were prepared. EMSA was conducted with 2 μ g of nuclear extract and a labeled wild type or mutant C/EBP consensus oligonucleotide probe. Competition assays were performed with cold wild type (WT) or cold mutant (MT) C/EBP probe (50 fold in excess). (C) Wild type fibroblasts were treated with UVB (5 mJ/cm²) and nuclear extracts were prepared. EMSA was conducted with 2 μ g of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. Supershift assays were performed with anti-C/EBP α , anti-C/EBP β antibody or IgG (SS-Supershift). (D) Primary fibroblasts from wild type and C/EBP β ^{-/-} mice were treated with UVB (5 mJ/cm²). Cells were harvested at the indicated time points and immunoblot analysis conducted. (E) Nuclear extracts were prepared from non-treated and UVB (5 mJ/cm²) treated fibroblasts (Lane 1 and 2) and from HEK 293 cells transfected with pcDNA3.1 C/EBP β -LAP or pcDNA3.1-C/EBP β LIP (Lane 3 and 4). EMSA was conducted with 2 μ g of nuclear extract and a labeled C/EBP consensus oligonucleotide probe. (F) Wild type fibroblasts were treated with UVB (5 mJ/cm²) or MNNG (35 μ M) and nuclear extracts were prepared at indicated time points. EMSA was conducted with 2 μ g of nuclear extract and labeled C/EBP consensus sequence corresponding to the C/EBP responsive element in C/

EBP α promoter. (G) Wild type or C/EBP $\beta^{-/-}$ fibroblasts were treated with UVB (5 mJ/cm 2) and ChIP assay using a C/EBP β antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBP β samples. (H) Wild type fibroblasts were treated with UVB (5 mJ/cm 2) and ChIP assay using a C/EBP α antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBP α samples.

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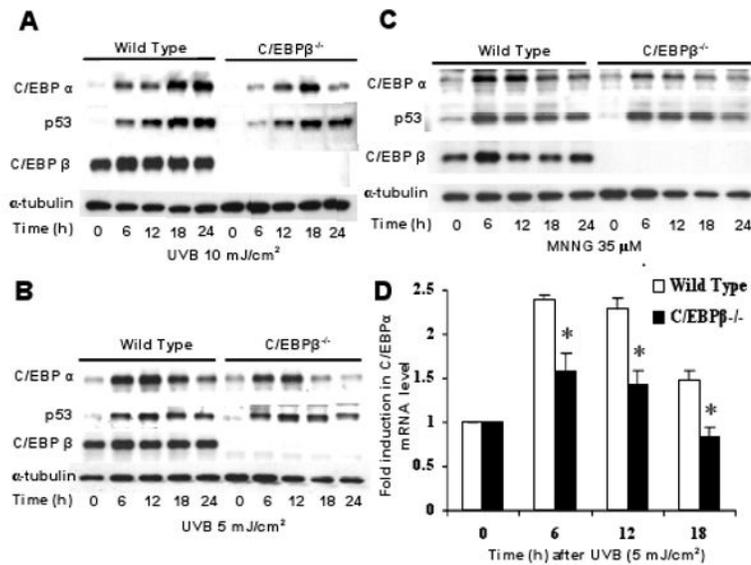


FIG. 5. C/EBP α is Regulated by C/EBP β in Response to DNA Damage

(A-C) Primary fibroblasts from newborn wild type or C/EBP β ^{-/-} mice were treated with UVB (10 mJ/cm²), UVB (5 mJ/cm²) or MNNG (35 μ M). Cells were harvested at the indicated time points and immunoblot analysis conducted. (D) Primary fibroblasts from newborn wild type or C/EBP β ^{-/-} mice were treated with UVB (5 mJ/cm²) and RNA isolated at the indicated time points. Quantitative RT-PCR was conducted for C/EBP α and 18 S mRNA levels. Data was normalized using 18 S and was analyzed using comparative C_T method. Data is expressed as mean \pm standard error (N = 4) and each experiment was run in triplicate. Two-factor ANOVA demonstrated significant interaction between genotype and time (P<0.05). *Significantly different from wild type fibroblasts (p< 0.05) at the indicated time point as determined by Student's t-test.