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Research paper

P53 regulates CCAAT/Enhancer binding protein β gene expression

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ABSTRACT

Background: The transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) is implicated in diverse processes and diseases. Its two isoforms, namely liver-enriched activator protein (LAP) and liver-enriched inhibitor protein (LIP) are translated from the same mRNA. They share the same C-terminal DNA binding domain except LAP has an extra N-terminal activation domain. Probably due to its higher affinity for its DNA cognate sequences, LIP can inhibit LAP transcriptional activity even at substoichiometric levels. However, the regulatory mechanism of C/EBPβ gene expression and the LAP: LIP ratio is unclear.

Methods: In this study, the C/EBPβ promoter sequence was scanned for conserved P53 response element (P53RE), and binding of P53 to the C/EBPβ promoter was tested by Electrophoretic Mobility Shift Assay (EMSA) and chromatin immunoprecipitation assay. P53 over-expression and dominant negative P53 expression plasmids were transfected into rat lung fibroblasts and tested for C/EBPβ gene transcription and expression. Western blot analysis was used to test the regulation of C/EBPβ LAP and LIP isoforms. Constructs containing the LAP 5′untranslated region (5′UTR) or the LIP 5′UTR region were used to test the importance of 5′UTR in the control of C/EBPβ LAP and LIP translation.

Results: The C/EBPβ promoter sequence was found to contain a conserved P53 response element (P53RE), which binds P53 as demonstrated by Electrophoresis Mobility Shift Assay and chromatin immunoprecipitation assays. P53 over-expression suppressed while dominant negative P53 stimulated C/EBPβ gene transcription and expression. Western blot analysis showed that P53 differentially regulated the translation of the C/EBPβ LAP and LIP isoforms through the regulation of eIF4E and eIF4E-BP1. Further studies with constructs containing the LAP 5′untranslated region (5′UTR) or the LIP 5′UTR region showed that the 5′UTR is important in differential control of C/EBPβ LAP and LIP translation.

Conclusion: Analysis of the effects of P53 on C/EBPβ expression revealed a novel mechanism by which P53 could antagonize the effects of C/EBPβ on its target gene expression. For the first time, P53 is shown to be a repressor of C/EBPβ gene expression at both transcriptional and translational levels, with a differential effect in the magnitude of the effect on LAP vs. LIP isoforms.

1. Introduction

CCAAT/enhancer-binding protein β (C/EBPβ) is highly expressed in the liver, adipose tissue, blood cells, lung, and the endocrine pancreas in development (Birkenmeier et al., 1989; Thomassin et al., 1992; Williams et al., 1991). It plays significant roles in diverse processes and diseases (Matherne et al., 2023). C/EBPβ deficient mice exhibit defective mammary gland development (Zahnow et al., 2001; Robinson et al., 1998), failure to ovulate (Sterneck et al., 1997), and reduced lung fibrotic response to bleomycin-induced injury (Hu et al., 2012; Hu et al., 2007; Hu et al., 2004). CEBPβ expression is increased in many tumors and implicated in promoting tumor cell proliferation and/or differentiation (Matherne et al., 2023; Barakat et al., 2015; Ewing et al., 2008; Ferrini et al., 2001). Thus, an improved understanding of the regulation of its expression will advance the field both for its clinical and scientific significance.

Abbreviations: LAP, liver-enriched activator protein; LIP, liver-enriched inhibitor protein; C/EBPβ, CCAAT/enhancer-binding protein β; ChIP assay, Chromatin immunoprecipitation assay; eIF4E, eukaryotic translation initiation factor 4E; eIF4E-BP, eukaryotic translation initiation factor 4E binding protein; P53RE, P53 response element; EMSA, Electrophoresis Mobility Shift Assay; RT-PCR, reverse transcription polymerase chain reaction.

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Several different isoforms of C/EBPβ have been described (Zahnow et al., 2001; Hu et al., 2004; Descombes and Schibler, 1991; Dearth et al., 2001; Campion et al., 2014; Ossipow et al., 1993; Saint-Auret et al., 2011; Calkhoven et al., 2000). The ~ 38 KD and 35 KD isoforms are referred to as liver-enriched activator protein (LAP) while the ~ 21 KD isoform is referred to as liver-enriched inhibitor protein (LIP) (Hu et al., 2004; Descombes and Schibler, 1991; Ossipow et al., 1993). Both LAP and LIP are translated from the same mRNA (Descombes and Schibler, 1991; Ossipow et al., 1993) and share the same C-terminal DNA binding domain except LAP has an extra N-terminal activation domain (Descombes and Schibler, 1991; Ossipow et al., 1993). LAP functions mostly as a transcriptional activator, whereas LIP is regarded as a functional LAP antagonist (Hu et al., 2004; Descombes and Schibler, 1991; Dearth et al., 2001; Ossipow et al., 1993; Saint-Auret et al., 2011; Bae and Kim, 2005).

Probably due to its higher affinity for its DNA cognate binding sequences, LIP can counteract the functions of LAP isoforms even at sub-stoichiometric levels (Hu et al., 2004; Descombes and Schibler, 1991; Dearth et al., 2001; Saint-Auret et al., 2011; Raught et al., 1995). Thus, the ratio of C/EBPβ isoforms (i.e. LAP/LIP ratio) may be decisive in determining the ultimate effect of C/EBPβ on multiple biological processes (Hu et al., 2004; Descombes and Schibler, 1991; Dearth et al., 2001; Saint-Auret et al., 2011; Calkhoven et al., 2000; Raught et al., 1995). For example, interleukin-1β (IL-1β) induces C/EBPβ transcription but reduces the LAP/LIP ratio by preferentially increasing the expression of the LIP isoform resulting in net suppression of myofibroblast differentiation (Hu et al., 2004). The mechanism for controlling the LAP/LIP ratio is not fully understood. Initially, a proteolytic mechanism is proposed to account for the presence of LIP in tissue extracts (Baer et al., 1998; Welm et al., 1999). However, subsequent studies indicated the presence of alternate translation initiation sites at codons Met1 or Met22 in the rat C/EBPβ mRNA, which generated LAP proteins of 38 and 35 KD, respectively, as well as downstream codon Met153 yielding the 20 KD LIP (Calkhoven et al., 2000). Furthermore, eukaryotic translation initiation factor 4E (eIF4E) is known to reduce the LIP: LAP ratio due to increased LAP expression (Calkhoven et al., 2000). Binding to methyl guanosine-containing cap of mRNAs by eIF4E (Sonenberg, 1981; Trachsel et al., 1980; Sonenberg et al., 1979) enhances eukaryotic translation initiation factor 4G and eukaryotic translation initiation factor 4A complexation (Lachance et al., 2002; Mader et al., 1995). By their RNA helicase activity (Feoktistova et al., 2013), the 5′-untranslated regions of mRNA are unwound for binding of the 40S ribosome subunit to start protein synthesis (Mader et al., 1995; Feoktistova et al., 2013; Walsh and Mohr, 2014; Svitkin et al., 2005; Jia et al., 2012). The presence of eIF4E determines whether the translation is cap-dependent or from an internal ribosomal entry site (Svitkin et al., 2005), which is inhibited by eukaryotic translation initiation factor 4E-binding protein 1 (eIF4E-BP1) by binding to eIF4E to inhibit translation initiation complex formation (Haghighat et al., 1995). However (Duncan and Song, 1999; Sukarieh et al., 2009), phosphorylation of eIF4E-BP1 by diverse signals causes release from eIF4e with consequent cap-dependent translation (Sukarieh et al., 2009; El-Chaar et al., 2004; Hay and Sonenberg, 2004). Interestingly p53 induction enhances the formation of a shortened form of non-phosphorylated eIF4E-BP1 that is less susceptible to degradation and has a greater affinity for eIF4E relative to full-length eIF4E-BP1 (Constantinou et al., 2008).

P53 has a complex structure with domains for multimer formation, and activation of transcription necessitating binding to DNA (Miyashita and Reed, 1995; Gupta et al., 2001). The tetrameric form comprises two dimers that are known to interact with the palindromic DNA sequence 5′-PuPuPuC(A/T)(T/A)GPy-PyPy-3′ (McLure and Lee, 1998; el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B, 1992; Wang et al., 1995), although individual DNA parts of the palindrome retain the ability to interact effectively with P53 (Wang et al., 1995). This binding of P53 results in stimulation or inhibition of target gene expression (Miyashita and Reed, 1995) depending on the presence of other factors (Miyashita and Reed, 1995; Ho and Benchimol, 2003; Lee et al., 1999). The myriad roles of P53 in cancer and cell senescence argue for the importance of advancing the current understanding of its target genes and how they are regulated to discover novel mechanisms of both scientific and potential clinical relevance.

Although P53 has the potential to regulate indirectly C/EBPβ expression and the LAP/LIP ratio via its effect on eIF4E-BP1, the possibility that P53 could also regulate C/EBPβ gene expression directly is unknown. In this study, the ability of P53 to directly regulate C/EBPβ gene expression was analyzed. Initial analysis revealed a putative P53 binding site in the C/EBPβ gene promoter suggesting the potential role of P53 in the direct regulation of C/EBPβ gene expression. First, using a combination of P53 over and under-expression strategies the results revealed that deficiency of P53 enhanced, while its overexpression diminished C/EBPβ expression. This P53 effect appeared to be mediated by its direct binding to the identified binding consensus in the C/EBPβ promoter, and mutation of this binding sequence resulted in enhanced C/EBPβ expression along with loss of regulation/repression by P53. Thus, P53 directly repressed C/EBPβ gene expression by binding to its cognate DNA consensus sequence.

2. Materials and Methods

Animals and cells. Fischer 344 rats, P53 deficient mice (Stock No: 002101) and wild type control mice (Stock No.: 000664) were purchased from Charles River Breeding Laboratories, Wilmington, MA. and Jackson Laboratory, Bar Harbor, ME respectively. The C/EBPβ deficient mice were bred in the animal facility of the University of Michigan which are originally received from Dr. Peter F. Johnson (Center for Cancer Research, National Cancer Institute, Frederick, MD)(Sterneck et al., 1997; Hu et al., 2004). The rat lung and mouse lung fibroblasts were isolated from adult animals and cultured as described previously (Hu et al., 2003). For IL-1β treatment, the fibroblasts were washed with PBS and cultured in DMEM containing 0.5 % plasma-derived serum (PDS) and indicated dose of IL-1β (R&D systems, Inc. Minneapolis, MN) for 12 h or 48 h before harvesting for RNA or protein analysis, respectively.

Plasmids. The P53 expression plasmid PCG-P53 and dominant negative P53 (DN53) expression plasmid were gifts from Dr. Gilbert F. Morris (Tulane University)(Morris et al., 1998). The rat C/EBPβ promoter from −1886 to + 117 was amplified by PCR from the rat genome with primers GGGGTACCAGTCTGCCAGAGACC and TGGGTCTAAAGGCGGCGGGCGGC. It was inserted into a promoterless pGL3-Basic vector (Promega Corporation, Madison, WI) to form plasmid pGL3-rC/EBPBp where the luciferase reporter gene expression was driven by the C/EBPβ promoter. The pGL3-rC/EBPBp was then used as templates in site-directed mutagenesis with primer pair A (GGGGGGCTTCCTGGAGTAAAGCTCAGCCGAGACCCCAGCAG) and B (CTGCTGGGGTCTCGGCTGAGCTTTACTCCAGGAAGCCCCCC) or primer pair C (GGTGGCCTAGAGGCAGAAAGCTTAGTCACCAGTGT TGGATG) and D (CATCCAACACTGGTGACTAAGCTTTCTGCCTCTAG GCCACC) to generate the P53RE mutated C/EBPβ promoter mutants (pGL3-rC/EBPBp-P53REm) and control mutant with a site other than the P53RE mutated (pGL3-rC/EBPBp-Controlm). A SnoB1 and HindIII restriction endonuclease sites were introduced respectively for screening. All the C/EBPβ promoter mutants are confirmed by DNA sequencing.

Rat C/EBPβ cDNA (35-kDa LAP) expression plasmid pCMV-LAP was a gift from Dr. J. Schwartz (University of Michigan), which was originally obtained from Dr. U. Schibler (University of Geneva, Geneva, Switzerland) (Descombes and Schibler, 1991).

The pCMV-GLO vector was generated by replacing the pGK promoter in the pmir-GLO vector (Promega Corporation, Madison, WI) with PCR amplified CMV promoter from the pCMV-sport6 vector (Life Technologies, Carlsbad, CA) to introduce the restriction endonuclease site for cloning. The 5′ untranslated region (5′UTR) of LAP (+1 to + 116 from

transcriptional start site) and LIP (+120 to + 509 from transcriptional start site) were amplified from rat genomic DNA and inserted into pCMV-GLO vector to form pCMV-GLO-LAP5′UTR and pCMV-GLO-LIP5′UTR respectively. The Renilla luciferase control vector pRL-CMV used in luciferase assays was purchased from Promega Corporation, Madison, WI.

Electrophoresis Mobility Shift Assay (EMSA). The EMSA was performed as before(Hu et al., 2007). The double-stranded oligonucleotide probe with sequence 5′ GGA GGC CTT CCT GGA GGC AAG CTC AGC CGA GAC CCC AGC AGG GGA ACT CC 3′ spanning the P53RE at −1525 to −1477 from the transcriptional start site according to the rat C/EBPβ promoter sequence was labeled with ^{32}P and then incubated with nuclear extract from lung fibroblast or purified P53 (Active Motif, Carlsbad, CA) at 25℃ for 20 min. They were then electrophoresed through a 4% non-denaturing polyacrylamide gel in 1x TBE. For indicated samples, the nuclear extracts were preincubated with anti-P53 antibody, rabbit IgG, or unlabeled probes on ice for 30 min before adding the ^{32}P labeled DNA probe. The dried gels were then exposed to X-ray film at different times to visualize radioactive bands.

Chromatin immunoprecipitation (ChIP) assay. Chromatin immunoprecipitation assay was performed using a kit from Millipore Co. Billerica, MA following the manufacturer's protocol as previously described (Hu et al., 2011). The oligonucleotide primers G (5′-ACAGACAGACAGACCCCTCC-3′) and H (5′-AGTGGGACATTGGGGCTTC-3) were used to amplify the rat C/EBPβ promoter region spanning the P53RE area.

Transient transfection and reporter gene assay. All transient transfections of cells were performed using the FuGENE® 6 Transfection Reagent (Promega Corporation, Madison, WI.) according to the manufacturer's instructions as previously described (Hu et al., 2003). 2 μg DNA of the C/EBPβ promoter-luciferase constructs of interest and 100 ng plasmid pRL-SV40 control vector (used for normalization) were co-transfected per culture into lung fibroblasts in serum-free DMEM medium. Four hours after the transfection, the media were replaced with DMEM containing 0.5% plasma-derived serum. In experiments to examine the effects of P53 on C/EBPβ gene expression, 2 μg of the P53 expression plasmid, dominant negative P53 expression plasmid, or the corresponding expression vectors were transfected alone or co-transfected with the indicated C/EBPβ promoter-luciferase construct respectively. The pCMV-GLO, pCMV-GLO-LAP5′UTR, and pCMV-GLO-LIP5′UTR containing both firefly luciferase and Renilla luciferase in the same plasmid were transfected individually. The activity of firefly or Renilla luciferase was measured using the dual luciferase assay kit (Promega Corporation, Madison, WI) 48 h after transfection. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase. Experiments with each construct were repeated 2–4 times and the resulting relative light units were expressed as mean ± SE.

Western blot. Western blotting was conducted as previously described (Hu et al., 2003) using antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), and LI-COR Biosciences, (Lincoln, NE, USA). Selected blots were scanned and digitized, and band intensities were quantified using Carestream Molecular Imaging software version 5.0.2.30 (Carestream Health, Rochester, NY).

Real-time RT-PCR. Semi-quantitative real-time RT-PCR was conducted as previously described (Hu et al., 2012; Hu et al., 2011). 100 ng of total RNA extract with by Trizol reagent (Invitrogen life technologies, Carlsbad, CA) was input into each well of 96 well plates as the template and GAPDH was used as an internal control. All the primer and probe sets were purchased from Applied Biosystems, Foster City, CA and used with the TaqMan™ RNA-to-CT™ 1-Step Kit (Catalog: 4392938) from Thermo Fisher Scientific, Waltham, MA.

Statistical analysis. ANOVA with post hoc Scheffe's test was undertaken as before (Hu et al., 2012; Hu et al., 2011).

3. Results

Identification of P53RE in the C/EBPβ gene promoter. A variety of factors such as IL-1, IL-6, and lipopolysaccharide can activate and modulate C/EBPβ gene expression in many cell types and tissues (Hu et al., 2004; Chano and Descoteaux, 2002; Buck et al., 1994). For example, increased C/EBPβ expression was observed during myofibroblast differentiation and bleomycin-induced pulmonary fibrosis (Hu et al., 2012; Hu et al., 2007; Hu et al., 2004). However, the regulatory mechanism for this increased expression is unknown. In an attempt to search for potential regulators of C/EBPβ gene expression, the rat C/EBPβ gene promoter was scanned for potential *cis*-acting elements. This analysis revealed a highly conserved P53 response element (P53RE) at −1511 to −1485 from the transcriptional start site, which is conserved in mouse and human C/EBPβ promoters (Figure S1). To evaluate if this P53RE is functionally important in the P53-mediated regulation of C/EBPβ gene expression, a series of experiments were undertaken.

Effects of IL-1β on P53 and C/EBPβ expression in fibroblasts. Previously IL-1β is shown to stimulate C/EBPβ expression (Hu et al., 2004) but the mechanism is unknown. Given the presence of the P53RE in the C/EBPβ promoter, the possibility that P53 might mediate this IL-1β effect on C/EBPβ expression was examined. In this experiment, rat lung fibroblasts were treated with or without interleukin-1β for 12 h and then analyzed for both P53 and C/EBPβ mRNA levels. The results showed that both P53 and C/EBPβ were expressed in fibroblasts, but IL-1β treatment caused divergent responses in their expression (Fig. 1a). Thus IL-1β caused a significant decrease (35% inhibition) in P53 mRNA levels while causing a > 2-fold increase in C/EBPβ mRNA. Furthermore, analysis of the P53 protein by western blotting revealed that IL-1β inhibited P53 protein expression in a dose-dependent manner (Fig. 1b), which is also opposite to the dose-dependent stimulation of C/EBPβ expression as shown previously (Hu et al., 2004). These results indicated that the response of P53 and C/EBPβ expression to IL-1β treatment were negatively correlated, suggesting that P53 might be a repressor of C/EBPβ expression.

P53 binding to the P53RE in the C/EBPβ promoter. To evaluate this potential direct repressor role of P53 on the C/EBPβ gene promoter, gel shift assays were undertaken to initially analyze whether P53 could bind the identified P53RE in the C/EBPβ promoter. A double-stranded oligonucleotide DNA probe corresponding to the sequence of the rat C/EBPβ gene promoter at −1525 to −1477 from the transcriptional start site spanning the P53RE was used in these assays. The results showed that a complex was formed between the nuclear extract and the oligo DNA probe containing the P53RE in the rat C/EBPβ promoter (Fig. 1c). The complex indicative of protein binding to the radioactive probe was abolished in the presence of a 100-fold excess of the unlabeled probe, indicating specific binding to the probe. A super-shifted band was noted when the nuclear extract was preincubated with an anti-P53 antibody but not control IgG. Complex formation was absent when nuclear extracts from P53-deficient fibroblasts were incubated with the labeled probe, confirming that P53 was responsible for the formation of the DNA-protein complex detected using wild-type nuclear extracts. Moreover, when recombinant P53 was used instead of nuclear extracts in the gel shift assay, two shifted bands were noted which were abolished by incubation with 100-fold excess of unlabeled probe (Fig. 1d). Notably, no shifted band was noted when P53 was incubated with the probe in which the P53RE was mutated. To confirm that P53 could directly bind the P53RE in the C/EBPβ promoter in intact cells, rat lung fibroblasts were fixed with formaldehyde and the cell lysates were used in a ChIP assay with anti-P53 antibody. A 323 bp DNA fragment was amplified by PCR when anti-P53 antibody precipitated DNA or the input control DNA were used as a template together with primers spanning the P53RE in the C/EBPβ gene promoter region (Fig. 1e). No band was detected when control IgG was used indicating specific precipitation by the anti-P53 antibody. These results taken together indicated that P53 could bind to the P53RE in the C/EBPβ gene promoter in fibroblasts.

P53 regulates C/EBPβ transcription. To test if the P53-P53RE

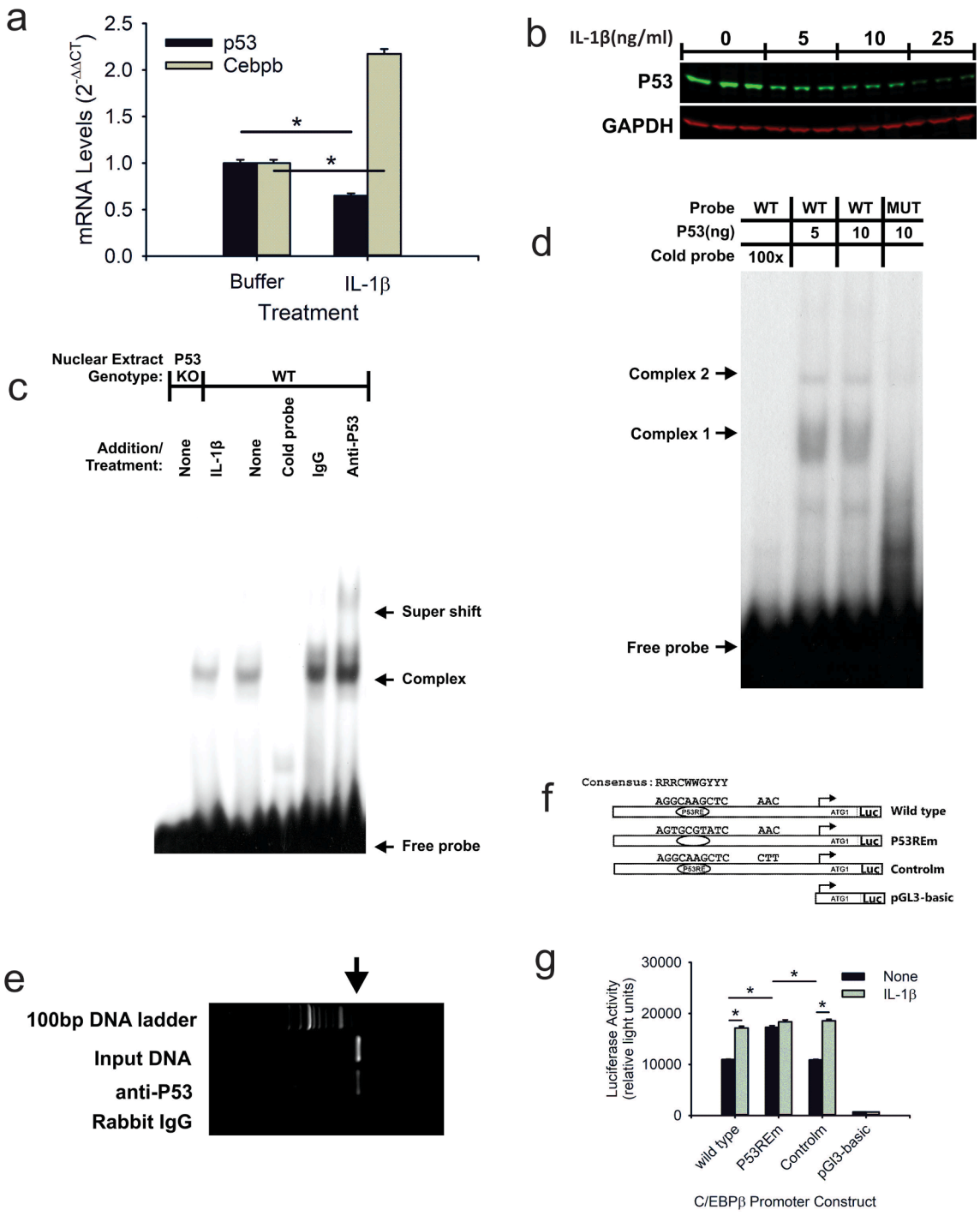

Fig. 1. Expression of P53 in rat lung fibroblasts. (a) Rat lung fibroblasts were treated with IL-1β for 12 h and then P53 and C/EBPβ gene expression was analyzed by real-time PCR. * indicates a significant difference ($p < 0.05$) from the respective ('Buffer') control group. (b) Total proteins from the lung fibroblasts treated with the indicated concentration of IL-1β treated for 48 h were analyzed for P53 protein levels by western blot analysis. GAPDH was used as a loading control. (c)Binding of P53 to P53RE in C/EBPβ gene promoter. ^{32}P labeled double-stranded oligonucleotide probe containing P53RE was incubated with nuclear extracts from wild type ('WT') or P53 deficient ('P53 KO') rat lung fibroblasts (WT). Some of the WT cells were pre-treated with IL-1β. Where indicated WT cell extracts were preincubated with 100-fold excess of the cold probe, anti-P53 antibodies, or the relevant IgG control. Arrows indicated the bands corresponding to the free probe and retarded bands corresponding to the complex formation and a super-shifted band upon incubation with anti-P53 antibodies. (d). The wild-type or P53RE mutated probes were incubated with recombinant P53 protein (P53) in a gel shift assay. 'Cold probe' indicates samples that were incubated in the presence of a 100-fold excess cold probe. The specific DNA-protein complexes were indicated by solid arrows. (e) Binding of P53 to the C/EBPβ gene promoter was analyzed by ChIP assay using anti-P53 antibodies. After incubation with the antibodies ("anti-P53") or control IgG ("Rabbit IgG"), the precipitated DNA, as well as unfractionated DNA ("input DNA"), were analyzed by PCR using primers spanning the P53RE region in C/EBPβ promoter region. The PCR products were then separated in a 1.3% agarose gel. Arrow indicated a band corresponding to the amplified P53RE region. (f) C/EBPβ promoter mutant activity. Wild type, P53RE mutated (P53REm), and control mutant (Controlm) C/EBPβ promoter luciferase reporter constructs were used for this experiment. The relative locations of mutations in the various constructs are diagrammed in (f). In (g) The luciferase activity was normalized to its respective Renilla luciferase control activity and the results were expressed as relative light units and shown as means ± SE of triplicates. * indicates statistical significance ($p < 0.05$) in comparisons between the indicated two groups.

interaction is functionally important for the expression of C/EBPβ gene expression, a P53RE mutated rat C/EBPβ gene promoter and a control C/EBPβ gene promoter mutant with mutation outside the P53RE were generated by site-directed mutagenesis. These promoter mutants and the wild-type rat C/EBPβ promoter were inserted into a promoterless pGL3-basic vector to drive the expression of luciferase reporter gene (Fig. 1f). The impact of P53RE mutation on C/EBPβ gene expression was evaluated by luciferase activity after transient transfection of these plasmids into lung fibroblasts. The results showed that the P53RE mutated C/EBPβ promoter activity was significantly (>70%) higher than that of the wild-type promoter (Fig. 1g). The activity of the control promoter mutant was essentially the same as the wild-type promoter activity. IL-1β treatment caused the expected stimulation (>70%) of wild type C/EBPβ and control mutant promoter activity but not that of the P53RE mutated promoter construct, which appeared to be already maximally stimulated. These data indicated that the P53RE is functionally important in the repression of C/EBPβ gene expression presumably by P53.

To further confirm the role of P53 as a repressor of C/EBPβ gene expression the effects of P53 over-expression or functional deficiency on C/EBPβ gene expression were evaluated. When fibroblasts were transfected with a P53 expression plasmid to induce over-expression, the co-transfected C/EBPβ promoter exhibited significantly reduced activity, even when promoter activity was enhanced by IL-1β treatment (Fig. 2a). In contrast transfection with a dominant negative P53 construct to reduce P53 function resulted in a significant enhancement of C/EBPβ promoter activity, albeit the stimulation was smaller in magnitude in IL-1β treated cells (Fig. 2b). Similar results were obtained when C/EBPβ mRNA was measured instead of promoter activity, namely inhibition by P53 over-expression and stimulation by the dominant negative P53 (Fig. 2c and 2d, respectively). Furthermore, when the C/EBPβ promoter construct was transfected into P53 deficient lung fibroblasts isolated from P53 knockout mice, C/EBPβ promoter activity was > 2-fold higher than the activity in wild-type lung fibroblasts (Fig. 2e). However, in IL-1β treated cells, the promoter activity appeared to be maximally stimulated and could not be further increased by P53 deficiency. Similar results were obtained when C/EBPβ mRNA was measured, namely significantly higher C/EBPβ mRNA levels in the absence of P53 (Fig. 2f). Further analysis of the effects of P53 over-expression or functional deficiency on C/EBPβ isoform proteins by western blotting confirmed the repressive effects of P53 on protein expression as well (Fig. 2g). Thus, P53 over-expression caused diminished levels of both LAP and LIP (Fig. 2g), while transfection with the dominant negative P53 was associated with higher levels of these proteins (Fig. 2h). Thus, the role of P53 as a repressor of C/EBPβ gene expression was confirmed.

P53 regulation of C/EBPβ translation. The preceding experiments consistently showed that P53 deficiency or functional impairment significantly increased C/EBPβ expression. IL-1β treatment stimulated the C/EBPβ protein levels in wild-type fibroblasts but not in P53-deficient fibroblasts, which already exhibited heightened levels of both LAP and LIP isoforms (Fig. 3a). In addition, in wild-type cells, the IL-1β-induced increase in the LIP isoform (fold change) was much higher than the increase in LAP isoforms. As a result, the LAP/LIP ratio decreased from 3.52 in wild-type fibroblasts to 2.57 in IL-1β treated cells (Fig. 3a). The LAP/LIP ratio was lower in P53 deficient fibroblasts compared to that in wild-type fibroblasts, however, unlike in wild-type fibroblasts, this ratio was not altered by IL-1β treatment (Fig. 3a). Since the C/EBPβ isoforms are translated from the same RNA (Descombes and Schibler, 1991), the different C/EBPβ isoform expression patterns in wild type vs. P53 deficient fibroblasts suggested that P53 may play additional roles in the regulation of C/EBPβ expression at the translational level. As previous studies have suggested that the LAP/LIP ratio may be regulated at the translational level via the translation initiate factor eIF4E (Calkhoven et al., 2000), the effects of P53 on the expression of eIF4E and its associate regulator eIF4E-BP1 were investigated. The results showed that in P53 deficient fibroblasts, the expression of eIF4E was much higher than that in wild-type cells (Fig. 3b). However, expression of eIF4E-BP1 was lower in P53 deficient fibroblasts relative to that in wild-type fibroblasts. IL-1β treatment stimulated eIF4E expression while inhibiting eIF4E-BP1 expression in wild-type fibroblasts but these effects were not apparent in P53-deficient cells. In addition, relative to wild-type cells P53 deficient fibroblasts exhibited increased levels of phosphorylated eIF4E-BP1 but decreased levels of the truncated form of eIF4E-BP1. IL-1β treatment had little effect on the phosphorylation of eIF4E-BP1 in both wild-type and P53-deficient cells. However, IL-1β reduced the truncated form of eIF4E-BP1 in the wild type but not as much in P53 deficient cells.

To further evaluate the potential regulation of C/EBPβ translation by P53, a plasmid containing the C/EBPβ LAP coding region (35 KD) and driven by the constitutive CMV promoter (pCMV-LAP) was co-transfected with P53 expression plasmid (pCG-P53) or empty expression vector control into C/EBPβ deficient fibroblasts. When the cell lysates were analyzed by western blotting, two bands corresponding to the LAP (35 kD) and LIP (21 kD) isoforms of C/EBPβ were detected. The levels of LIP isoforms were dramatically decreased while the level of LAP was slightly decreased in fibroblasts co-transfected with the P53 expression plasmid compared to those in fibroblasts co-transfected with the control vector (Fig. 4a). This reduction in isoform expression was neither due to differences in transfection efficiency nor the transcriptional regulation of the CMV promoter as the luciferase expression driven by the same CMV promoter in the control plasmid in the same co-transfections was essentially unaffected. Thus, in addition to the effect on transcription (Fig. 1 and Fig. 2), this reduction of LAP and LIP expression by the P53 expression plasmid was likely due to regulation at the translational level, perhaps via eIF4E and/or eIF4E-BP1.

Plasmid pMIR-GlO contains both firefly luciferase and renilla luciferase controlled by PGK promoter and SV40 promoter respectively (Promega Corporation, Madison, WI). To further confirm the translational regulation of C/EBPβ gene expression by P53, the pGK promoter in the pMIR-GLO vector was replaced by the CMV promoter to produce the plasmid, pCMV-GLO. The 5′ untranslated region (5′UTR) of the firefly luciferase reporter gene in the pCMV-GLO vector was then further replaced by the C/EBPβ LAP 5′UTR or C/EBPβ LIP 5′UTR to form plasmids pCMV-GLO-LAP-5UTR and pCMV-GLO-LAP-5UTR, respectively (Figure S2). Examination of the effects of transfecting these plasmids into wild-type and P53 deficient fibroblasts would allow evaluation of the effect of the respective 5′UTR on the translation of the firefly luciferase. There was no detectable difference in luciferase activity in samples from wild-type vs P53 deficient cells when transfected with pCMV-GLO, which had the original firefly 5′UTR (data not shown). In contrast, the firefly luciferase activity was significantly higher in P53 deficient cells relative to wild-type cells when the original 5′UTR was replaced with either the C/EBPβ LAP 5′UTR or C/EBPβ LIP 5′UTR (Fig. 4B). In addition, the increased luciferase activities in P53 deficient cells were much more pronounced (>6-fold higher than in wild-type cells) for the construct containing the LIP 5′UTR vs. < 2-fold higher for the construct containing the LAP 5′UTR. As the expression of firefly luciferase was controlled by the constitutive CMV promoter, the observed difference in luciferase activities in wild type vs. P53 deficient cells should be primarily due to P53-dependent regulation of translation of the LAP or LIP 5′UTR containing plasmids. Thus the 5′ untranslated region might mediate the translational regulation by P53 in addition to effects on eIF4E and eIF4E-BP1.

4. Discussion

Transcription factors of the CCAAT/enhancer binding protein (C/EBP) family have decisive roles in the differentiation of various cell types, including adipocytes (Bae and Kim, 2005), hepatocytes (Ferrini et al., 2001; Kurash et al., 2004; Lilja et al., 1999; Hungness et al., 2002), enterocytes (Zhu et al., 1999), keratinocytes (Sterneck et al., 2006; Atwood and Sealy, 2011), myofibroblasts (Hu et al., 2012; Hu et al.,

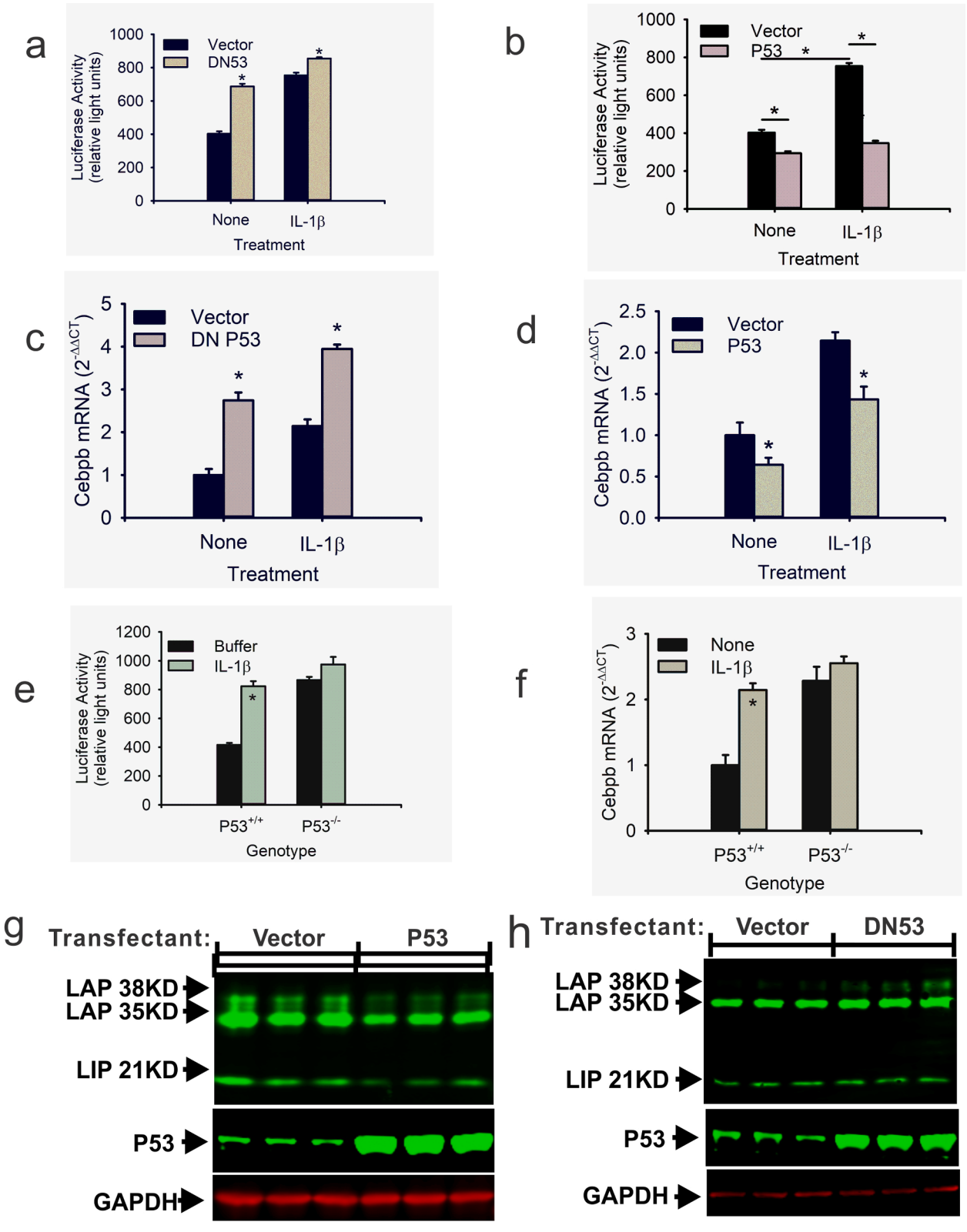

Fig. 2. Effect of P53 on C/EBPβ gene transcription. The effect of ectopically expressed P53 ('P53') (a and c) or dominant negative P53 expression ('DN53') (b and d) on C/EBPβ promoter activity (a and b) and C/EBPβ mRNA (c and d) are shown. In (a) and (b) promoter activity was evaluated by luciferase assay and results were expressed as in the legend in Fig. 1G. In (c) and (d) C/EBPβ mRNA was analyzed by real-time PCR. * indicates statistical significance ($p < 0.05$) in comparisons between the indicated two groups. (e) The effect of P53 deficient on C/EBPβ gene transcription was evaluated by comparing C/EBPβ promoter activity and mRNA levels in wild type vs P53 deficient mouse lung fibroblasts (P53KO) by luciferase assay. (f)Effect of P53 deficiency on C/EBPβ gene transcription evaluated by real-time PCR analysis. * indicates statistical significance ($p < 0.05$) in comparisons between the indicated two groups. (g) Rat lung fibroblasts were transfected with P53 ('P53') or the expression vector only ('Vector'). The cell extracts were evaluated for C/EBPβ isoform proteins and P53 by western blot analysis. (h) Rat lung fibroblasts were transfected with dominant negative P53 ('DN53') or the expression vector only ('Vector'). The cell extracts were evaluated for C/EBPβ isoform proteins and P53 by western blot analysis using GAPDH as the loading control.

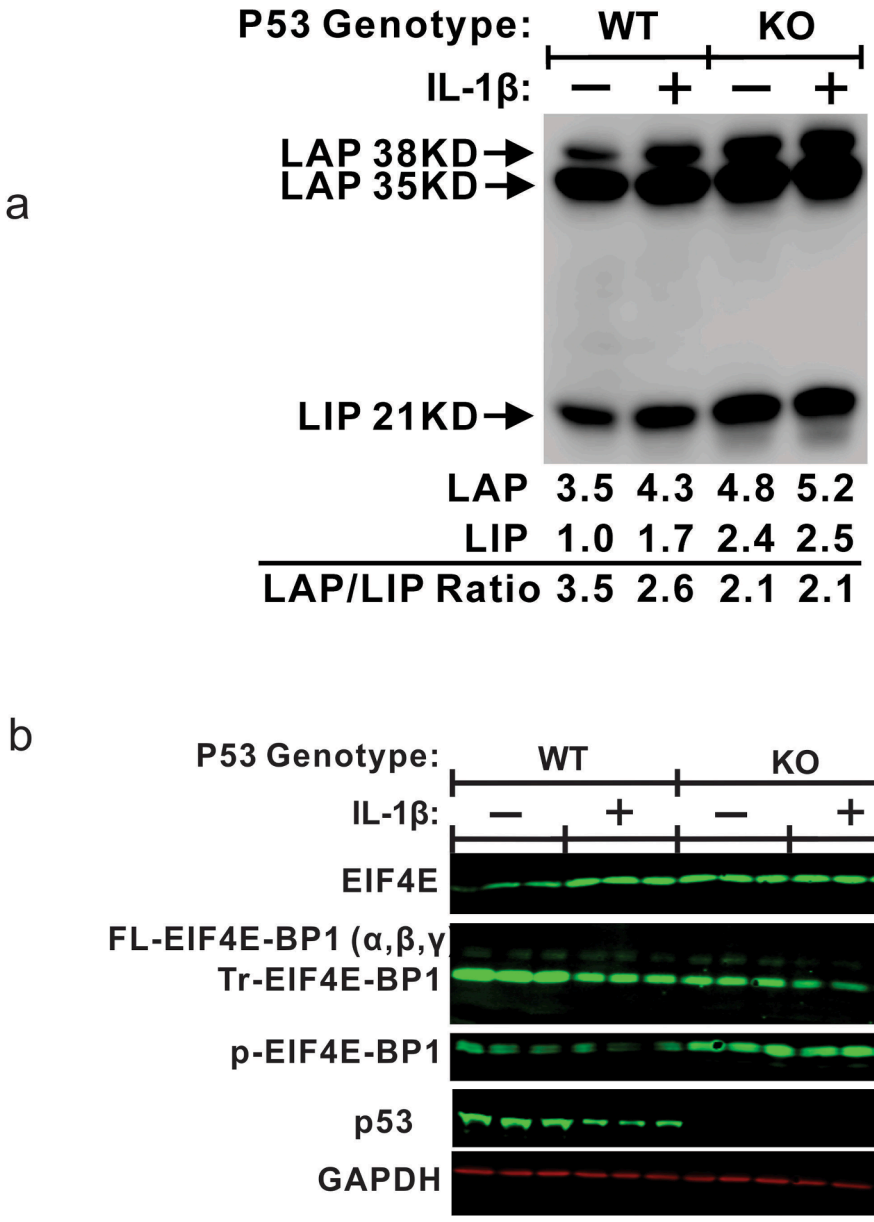

Fig. 3. (a) Effects of P53 deficiency on C/EBPβ. Wild type and P53 deficient ('P53 KO') fibroblasts were treated with IL-1β or the vehicle buffer only as indicated for 48 h. The cell protein extracts were evaluated by western blot analysis for the C/EBPβ isoforms in (a) and eIF4E plus the various forms of eIF4E-BP1. The bands corresponding to the various C/EBPβ isoforms were quantified and the relative band intensities of LAP and LIP as well as the LAP/LIP ratios were calculated with the wild-type buffer treated LIP isoform set to 1.0 as indicated in the lower panel of (a). (b) the same samples as mentioned in (a) were analyzed for eIF4E and eIF4E-BP1. the FL-eIF4E-BP1, Tr-eIF4E-BP1, and P-eIF4E-BP1 represented full-length eIF4E-BP1, truncated eIF4EBP1, phosphorylated eIF4E-BP1, and P53 respectively. GAPDH was used as a loading control.

2007; Hu et al., 2004), mammary gland, the hematopoietic system, as well as in ovulation. It also plays important roles in the processes that are controlled by the tumor repressor gene P53, such as cell senescence (Barakat et al., 2015; Rufini et al., 2013; Yoon et al., 2007), cell survival (Ewing et al., 2008; Jin et al., 2013), and tumorigenesis (Atwood and Sealy, 2011; Stiewe, 2007; Moll and Schramm, 1998; Begay et al., 2015; Kubicka et al., 1999). Notably reciprocal inhibition between C/EBPβ and P53 has been reported in a variety of studies (Ewing et al., 2008; Jin et al., 2013; Stiewe, 2007; Margulies and Sehgal, 1993; Gingras et al., 1999). C/EBPβ inhibits p53 gene transcription (Stiewe, 2007) and suppresses P53 regulation of cell survival (Jin et al., 2013) and the development of cancer (Stiewe, 2007). On the other hand, P53 reduces the binding of C/EBPβ to the interleukin-6 and albumin gene promoters resulting in the inhibition of their activation (Margulies and Sehgal, 1993; Gingras et al., 1999). However, the antagonistic inhibition mechanism between C/EBPβ and P53 is complex and far from clear although *trans*-repression of P53 and C/EBPβ through direct interaction has been previously reported (Margulies and Sehgal, 1993).

In this study, an alternative or additional novel mechanism by which P53 could inhibit C/EBPβ function is described. Following the identification of a conserved P53 binding consensus (P53RE) in the C/EBPβ gene promoter by promoter sequence analysis, the possibility of P53 regulation of C/EBPβ gene expression was examined. Firstly, direct binding of P53 to P53RE in the C/EBPβ promoter was demonstrated by gel shift mobility and ChIP assays. Secondly, the functional significance of the P53RE was revealed by the finding that mutation of the P53RE enhanced C/EBPβ gene promoter activity suggesting P53 as a potential repressor of C/EBPβ transcription. Consistent with this role is the observation that ectopically expressed P53 reduced C/EBPβ promoter activity and gene expression, while P53 deficiency or impairment of its function enhanced C/EBPβ gene expression. Thirdly, IL-1β stimulation of C/EBPβ expression was associated with a reduction in P53 expression, and the IL-1β stimulatory effect was not observed in the absence of P53 or when its function is inhibited. Thus the inhibitory effects of P53 on C/EBPβ function noted previously could be mediated by its repression of C/EBPβ gene expression. Furthermore, enhanced expression or function of C/EBPβ, such as by IL-1β, could be mediated by suppression of P53 expression and/or function.

Further analysis of the effects of P53 on C/EBPβ protein isoforms suggested additional potential regulation at the translational level. Thus

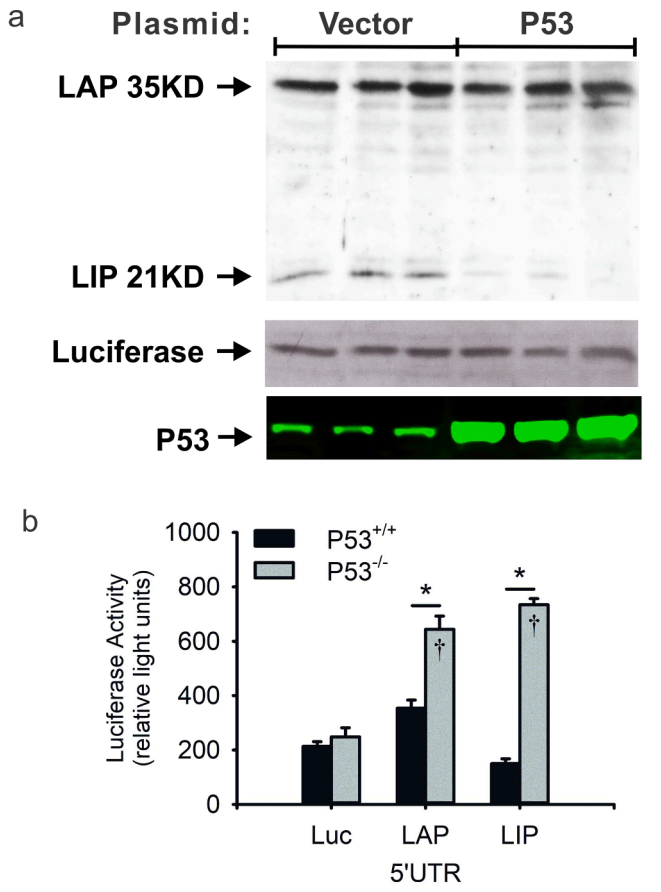

Fig. 4. P53 inhibits C/EBPβ translation. (a)The C/EBPβ LAP expression plasmid pCMV-LAP and the CMV promoter-driven luciferase vector pRL-CMV were co-transfected into C/EBPβ deficient fibroblasts, together with either P53 expression plasmid pCG-P53 or the vector only. Two days after transfection, the total protein extracts from the transfected cells were analyzed for C/EBPβ proteins and P53 by western blotting. The membrane was reblotted for luciferase protein for confirmation of uniform transfection efficiency between samples. (b) Effects of C/EBPβ isoform 5′UTR on translational regulation by P53. The pCMV-GLO vectors containing either the LAP 5′UTR (LAP) or the LIP 5′UTR (LIP) were transfected into wild type ('WT') or P53 deficient ('P53 KO') fibroblasts. The activity of these constructs measured as luciferase activity was expressed as relative light units after normalization to the respective renilla luciferase activity expressed by the same plasmids. * indicates statistical significance ($p < 0.05$) in comparisons of the indicated two groups; † indicates significance vs. the p53 knockout Luc control group.

P53 deficiency or functional inhibition caused a greater increase in the C/EBPβ LIP isoform relative to the increase of the LAP isoform. Further studies revealed that P53 deficiency caused enhanced expression of eIF4E, a key component for translation. Binding to eIF4E-BP1 inhibits eIF4E function (Gingras et al., 2001; Rau et al., 1996; Raught and Gingras, 1999; Sachdeva et al., 2009) and interestingly P53 deficiency suppressed eIF4E-BP1 expression. Thus P53 could also regulate at the translational level via effects on eIF4E expression and function. It is noteworthy that the C/EBPβ isoforms are translated from the same mRNA (Descombes and Schibler, 1991) and eIF4E is implicated in the differential translation of C/EBPβ isoforms. Moreover, P53 inhibits eIF4E gene expression by suppressing its activation by C-MYC (Zhu et al., 2005; Nathan et al., 2002). Interestingly overexpression of eIF4E and mutations of p53 are correlated in certain cancers (Nathan et al., 2002; Nathan et al., 2000). Functionally eIF4E is involved in directing ribosomes to the 7-methyl-guanosine five-prime cap structure of mRNAs (Sonenberg, 1981; Trachsel et al., 1980; Sonenberg et al., 1979) and is the rate-limiting component of the eukaryotic translation apparatus (Raught and Gingras, 1999). eIF4E-BP1 strongly binds to eIF4E and prevents eIF4E assembly into the eIF4F complex for translation initiation (Gingras et al., 2001; Rau et al., 1996; Raught and Gingras, 1999; Sachdeva et al., 2009). Phosphorylation of eIF4E-BP1 dissociates the eIF4E-eIF4E-BP1 complex and thus enhances translation initiation (Gingras et al., 2001; Rau et al., 1996; Sachdeva et al., 2009). Truncation of eIF4E-BP1 results in a more stable molecule, which is not phosphorylated (Constantinou et al., 2008). Thus the truncated form of eIF4E-BP1 forms a more stable complex with eIF4E than the full-length eIF4E-BP1 (Constantinou et al., 2008). In this study, P53 deficiency resulted in diminished eIF4E-BP1 truncation but increased eIF4E-BP1 phosphorylation, further supporting a role for P53 in translational regulation of C/EBPβ gene expression. Additionally, when the CMV promoter-driven C/EBPβ LAP expression plasmid pCMV-LAP was transfected into C/EBPβ deficient fibroblasts, expression of both the 35 kD LAP and 21 kD LIP isoforms was suppressed in cells co-transfected with the P53 expression plasmid. As the C/EBPβ expression in these cells was solely derived from the transfected pCMV-LAP under the control of the constitutive CMV promoter, the translational regulation of C/EBPβ gene expression by P53 was demonstrated.

Finally, the effect of P53 on the LAP vs. LIP isoforms was not equal in magnitude, resulting in alterations in the LAP/LIP ratio with potential consequences on the regulation of C/EBPβ target genes. Investigation of the basis for this differential effect on these isoforms was initially focused on the potential role of differing 5′UTRs of the respective isoforms. The C/EBPβ LAP or LIP 5′ UTR were engineered in the same location before the firefly luciferase reporter gene and under the control of the constitutive CMV promoter. While the construct containing the LAP 5′UTR exhibited greater activity than the one with the LIP 5′UTR, the stimulatory effect of P53 deficiency was greater in the latter than in the former. This differential response between the two constructs could account for the altered LAP/LIP ratio in P53 deficient cells wherein a greater stimulation of the LIP expression resulted in a reduced LAP/LIP ratio. As the 5′UTRs of LAP and LIP contain different Kozak sequences and micro ORFs that are important for efficient translation initiation (Calkhoven et al., 2000), one possible mechanism is that eIF4E may differentially interact with these elements. Since P53 could regulate eIF4E expression and function, the level of P53 expression could impact on this differential translational effect on LAP vs. LIP isoform expression. Further studies are needed to delineate and confirm such a mechanism.

5. Conclusion

In summary, analysis of the effects of P53 over-expression and deficiency indicated a novel mechanism by which P53 could antagonize the effects of C/EBPβ on its target gene expression. For the first time, P53 is shown to be a repressor of C/EBPβ gene expression at both transcriptional and translational levels, with a differential effect in the magnitude of the response between LAP and LIP isoforms. These effects are expected to have consequences on the expression of C/EBPβ target genes, and thus the functional role of C/EBPβ in diverse cellular processes regulated by these genes. Hence the regulation of P53 expression and function can be contemplated as a means of controlling C/EBPβ dependent processes, and any attempt to manipulate P53 expression should consider potential effects on C/EBPβ and its role in diverse biological and pathophysiological processes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

Biao Hu, Zhe Wu: Conceptualization, Data curation, Formal analysis, Validation, Investigation, Review & editing. **Tianju Liu:** Formal analysis, Validation, Investigation, Review & editing. **Sem H. Phan:** Conceptualization, Methodology, Formal analysis, Validation, Investigation, Fund acquisition, Resources, Supervision, Review & editing.

Availability of data and material

Not applicable.

Ethics approval and consent to participate

All animal experiments were carried out by following the Basel Declaration, the National Research Council's Guide for the Care, the NIH guidelines in the USA, and the ARRIVE guidelines. The study was reviewed and approved by the University of Michigan Institutional Biosafety Committee and the University Committee on the Use and Care of Animals (No. PRO00007605 and No. PRO00006691).

Consent for publication

Not applicable.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2023.147675.

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