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Activated p53 induces NF-κB DNA binding but suppresses its transcriptional activation

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ABSTRACT

NF-κB plays an important role in oncogenesis. Recently, we have demonstrated that loss of p53 function enhances DNA binding and transcriptional activities of NF-κB via IKKα and IKKβ, and that glycolysis, activated by NF-κB, has an integral role in oncogene-induced cell transformation. Here, we show that ectopically expressed p53 induces acetylation and phosphorylation at Ser 536 of p65, an NF-κB component, and enhances DNA-binding activity of NF-κB. However, activated p53 suppresses transcriptional activity of NF-κB. Under non-stimulating conditions, p65 formed a complex with IKKα and IKKβ. Activated p53 bound to p65 on DNA and disrupted binding of p65 to IKKβ. Moreover, histone H3 kinase activity, which requires transcriptional activation of NF-κB, was diminished by p53. Thus, activated p53 may suppress transcriptional activity of NF-κB through inhibition of IKK and histone H3 kinase on DNA, suggesting a novel p53-mediated suppression system for tumorigenesis.

Nuclear factor-κB (NF-κB) is a critical regulator of innate and adaptive immune responses, and the inflammatory response [1,2]. Upon stimulation of pro-inflammatory cytokines or conditions of stress, the inhibitor of κB (IκB), which sequesters NF-κB into the cytoplasm, is phosphorylated by the IκB kinase (IKK) complex, consisting of IKKα and IKKβ, and degraded by the ubiquitin–proteasome system [3]. Subsequently, NF-κB translocates into the nucleus and binds to target DNA sequence (κB-site) to induce gene transcription. Numerous studies have demonstrated that constitutive activation of NF-κB is frequently observed in many types of cancer cells [4,5]. It is also known that NF-κB activates expression of genes involved in apoptosis inhibition and cell adhesion [6], suggesting its role in oncogenesis.

The *p53* tumor suppressor gene is the most frequent target for genetic alterations in human cancer [7]. It was reported that p53 inhibits the transcriptional activity of NF-κB [8–11]. On the other hand, it has also been shown that p53 activates NF-κB [12]. Recently, we have shown that p53 regulates glucose metabolism through the IKK-NF-κB pathway and that, in the absence of p53, enhanced glycolysis plays an integral role in oncogene-induced cell transformation [13]. We found that kinase activities of IKKα and IKKβ and subsequent NF-κB activity are enhanced in p53-deficient mouse embryonic fibroblasts (MEFs) and also in wild-type MEFs expressing mutant p53. Moreover, we found, in response to DNA damage, acceleration of NF-κB/p65 binding and the recruitment of p53 to the promoter region of *IκBα*, a well-known NF-κB-inducible gene. The amount of transcriptionally activated phosphorylation of histone H3 was markedly decreased when accompanied by p53 binding, suggesting that activated p53 inhibits NF-κB on the chromatin.

Here, we show that ectopically expressed p53 enhances the DNA-binding activity of NF-κB by acetylation and phosphorylation at Ser 536 of p65, but suppresses its transcriptional activity. Our results provide a possible mechanism underlying p53-mediated suppression of the transcriptional activity of NF-κB.

Materials and methods

Plasmids. pNF-κB Luc was obtained from Clontech Laboratories, Inc. The control plasmid phRL-TK (*Renilla* luciferase reporter) was obtained from Toyobo. pEF-p53 L22Q/W23S is a p300/CBP-binding mutant, in which residues 22 and 23 were replaced by glutamine and serine residues, respectively [14]. pEF-p53 S15A and S46A are mutants, in which the serine residues15 and 46 were replaced by alanine.

Antibodies and materials. Anti-IKKα (sc-7182; Santa Cruz Biotechnology), anti-IKKβ (Upstate Biotechnology), anti-HA (Covance), and anti-p65 (sc-372; Santa Cruz Biotechnology) antibodies were used for immunoprecipitation. Anti-p53 (FL393; Santa Cruz Biotechnology and Ab-5; Neo Marker, Inc.), anti-phospho-p53 (Ser15 and Ser46; Cell Signaling Technology), anti-IKKα (Oncogene), anti-IKKβ (Cell Signaling), anti-phospho-IKK (IKKα [Ser180]/IKKβ [Ser181]; Cell Signaling Technology), anti-p65 (sc-372 and sc-8008; Santa Cruz Biotechnology), anti-phospho-p65 (Ser536; Cell Signaling Technology), anti-acetylated-Lysine (Cell Signaling Technology),

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and anti-TFIIB (sc-225; Santa Cruz Biotechnology) antibodies were used for immunoblot analysis. Adriamycin, U0126, and U0124 were obtained from Calbiochem.

Luciferase assay and electrophoretic mobility shift assay (EMSA). Luciferase assay, preparation of nuclear extracts, and EMSA were performed as described previously [13].

DNA pull-down assay and in vitro histone H3 phosphorylation assay. A DNA pull-down assay was performed as described previously with slight modifications [15]. Nuclear extract (100 μg) pre-cleared with streptavidin agarose (Invitrogen) was mixed with 3 μg of poly (dI-dC) and 5 μg of biotinylated double-strand oligonucleotide (NF-κB-binding sequence [κB-sites]; primer pair 5′-biotin-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ and 5′-TCGACCTCTCGGAAAGTCCCCTCTGA-3′, p53-binding sites; 5′-biotin-GTCGGGAGCGTGTCCGGGCAGGTCGCGCT-3′ and 5′-GCTGAGCGCGACCTGCCCGGACACGCTCC-3′) in a buffer (20 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 10% glycerol, 0.01% NP-40, and 1 mM DTT). After the mixture was incubated at room temperature for 30 min, the complex was "pulled down" with streptavidin agarose beads and then washed with three times in the same buffer with 0.03% sarkosyl. The bound proteins were subjected to SDS–PAGE. For the *in vitro* histone H3 phosphorylation assay, the precipitated complexes were washed three times with buffer (20 mM Tris, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 1 mM DTT, protease inhibitor cocktail, and 1% Triton X-100) and incubated in 10 μl of reaction mixture (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.05 μCi [γ-^{32}P]ATP, and 5 μg of purified histone H3 [Upstate Biotechnology] as a substrate) at 30 °C for 20 min. After SDS–PAGE, the degree of phosphorylation of histone H3 was measured by autoradiography.

Results and discussion

We have shown that in p53 deficient or dominant negative p53-expressing MEFs, IKKα, and IKKβ activity was increased and subsequently, DNA-binding activity of NF-κB was enhanced [13]. On the other hand, in wild-type MEFs, adriamycin-induced activated p53 also enhanced DNA binding of NF-κB in spite of down-regulation of IKK activity. As shown in Fig. 1A, ectopically expressed p53, from an expression vector, in p53-null Saos-2 cells enhances DNA-binding activity of NF-κB, similar to a previous report using adenovirus-expressed p53 [12]. Although it was reported that ectopically expressed p53 enhanced DNA binding of NF-κB through

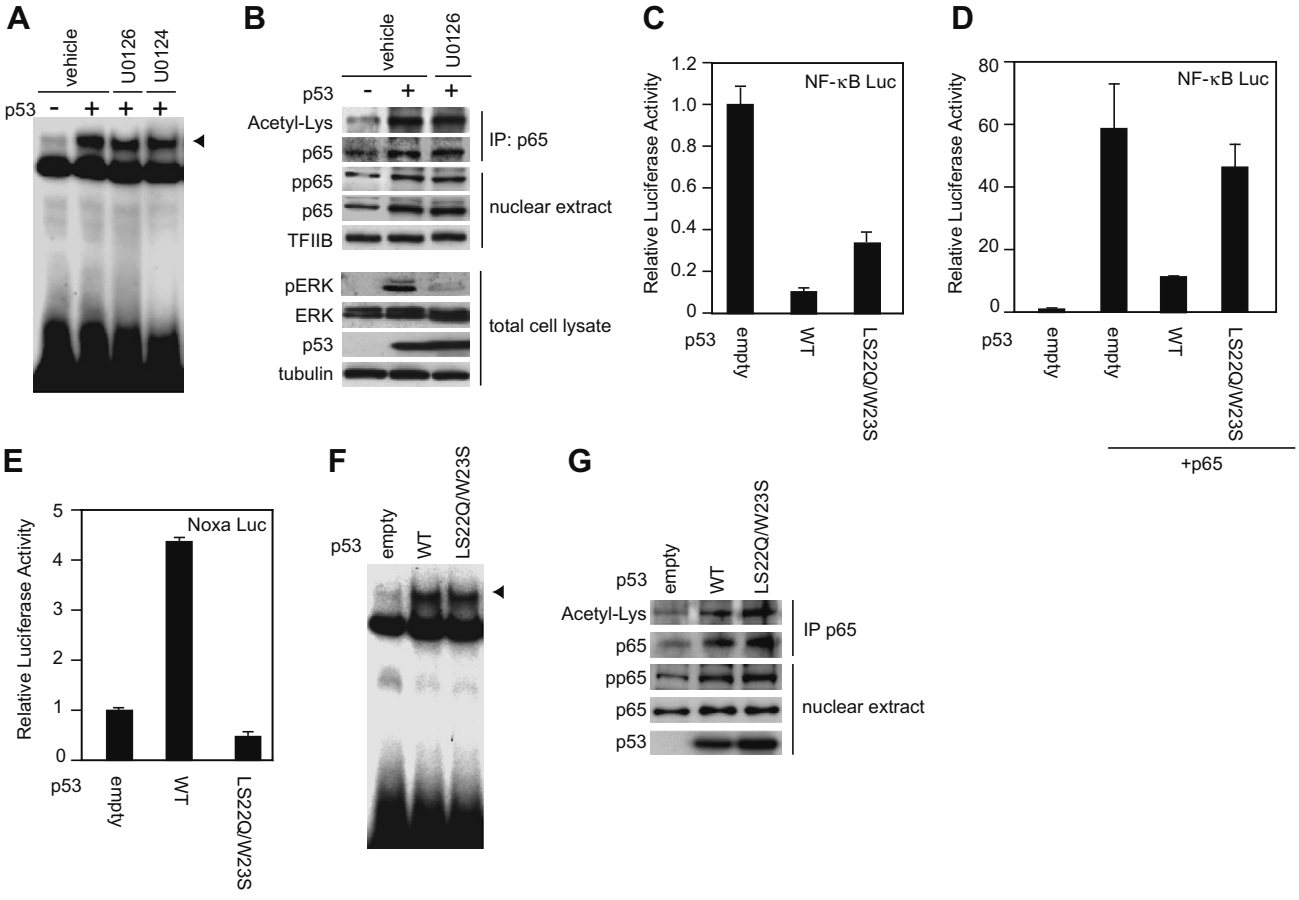

Fig. 1. Ectopically expressed p53 enhances DNA-binding activity of NF-κB. (A,B) The p53 expression vector was transfected into Saos-2 cells pretreated with MEK1 inhibitor U0126 or negative control U0124 (25 mM) for 1 h. After 24 h from transfection, the nuclear protein and total cell protein were extracted. NF-κB DNA-binding activities were determined by EMSA (A). Arrowhead indicates the position of NF-κB. The expression levels or modification levels of p65 were determined by immunoblot analysis with indicated antibodies (B). Acetylated p65 was determined by immunoblot analysis with an anti-acetyl lysine antibody against immunoprecipitated p65 from the nuclear extract. pp65 indicates the band of phosphorylated p65 at Ser 536. TFIIB and tubulin were used as loading controls. The phosphorylated ERK1/2 level was determined by immunoblot analysis with phospho-ERK1/2 antibody. (C–E) Saos-2 cells were cotransfected with the NF-κB Luc (C,D) or Noxa Luc (E) reporter plasmid, phRL-TK (internal control), and expression vectors as indicated. After 24 h from transfection, relative luciferase activity was evaluated. Data represent means ± SD of three independent assays. (F,G) The expression vector of wild-type or mutant p53 (L22Q/W23S) was transfected into Saos-2 cells. After 24 h from transfection, the nuclear protein and total cell protein were extracted. NF-κB DNA-binding activities were determined by EMSA (F). The expression levels or modification levels of p65 were determined by immunoblot analysis with indicated antibodies as described in B (G).

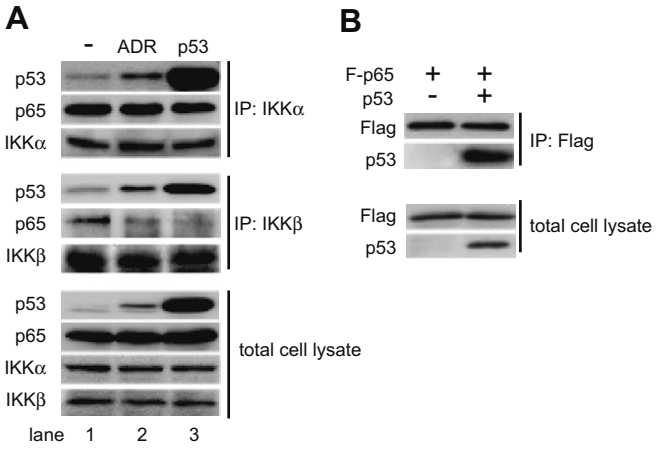

Fig. 2. p53 binds to IKKα, IKKβ, and p65. (A) MCF-7 cells were treated with adriamycin (ADR) for 16 h or transfected with the p53 expression vector. Cell lysates were subjected to immunoprecipitation (IP) with antibodies against IKKα and IKKβ. Immunoprecipitates were analyzed by immunoblotting with the anti-p53, anti-p65, anti-IKKα, and anti-IKKβ antibodies. (B) The expression vector of Flag-tagged p65 was transfected into MCF-7 cells with (+) or without (−) the p53 expression vector, and cell extracts were prepared 24 h after transfection. Cell extracts were subjected to immunoprecipitation with anti-Flag antibodies, and immunoprecipitates were analyzed by immunoblotting with anti-p53 antibodies.

MEK1-dependent pp90^{rsk} activation [12], enhanced DNA-binding activity of NF-κB was scarcely altered in the presence of MEK inhibitor U0126 (Fig. 1A). Because p65 acetylation and phosphorylation at Ser 536 were reported to induce DNA-binding activity of NF-κB [16,17], we next examined whether p53 enhanced these modifications of p65. As shown in Fig. 1B, p65 modifications of both acetylation and phosphorylation at Ser 536 were enhanced by p53 expression in the absence and presence of U0126. As shown in Fig. 1C, the ectopically expressed p53 clearly suppressed the transcriptional activity of NF-κB in spite of enhanced DNA-binding activity of NF-κB. Previously, p300/CBP-dependent transcriptional interference between NF-κB and p53 has been reported [8–11]. Although the p300/CBP-binding-deficient p53 mutant (p53 L22Q/W23S), which does not have transcriptional activity (Fig. 1E), hardly suppressed transcriptional activity of ectopically expressed p65 (Fig. 1D), it suppressed the transcriptional activity of endogenous NF-κB (Fig. 1C). In addition, using p53 L22Q/W23S, DNA-binding activity of NF-κB (Fig. 1F) and acetylation and phosphorylation at Ser536 of p65 (Fig. 1G) were also enhanced. These results suggested that ectopically expressed p53 enhanced DNA binding of NF-κB by induction of modifications of p65, but suppressed transcriptional activity of NF-κB in a p300-independent manner.

To examine how activated p53 suppresses NF-κB transcriptional activity, we performed a co-immunoprecipitation assay. p65 bound to both IKKα and IKKβ (lane 1 in Fig. 2A) and adriamycin-activated p53 also bound to IKKα and IKKβ (lane 2 in Fig. 2A). Interestingly, following adriamycin treatment, IKKβ was excluded from the IKKα/p65 complex; however the binding of IKKα to p65 was not affected. Exogenous p53 excluded IKKβ more strongly from the complex, but did not affect IKKα binding (lane 3 in Fig. 2A). Considering the fact that ectopically expressed p53 also bound to p65 (Fig. 2B) [8], it is possible that activated p53 binds to IKKβ and the complex including p65 and IKKα, which may lead to elimination of IKKβ from the complex.

To determine the mechanism of p53-mediated exclusion of IKKβ from the IKKα/p65 complex in detail, we performed a GST

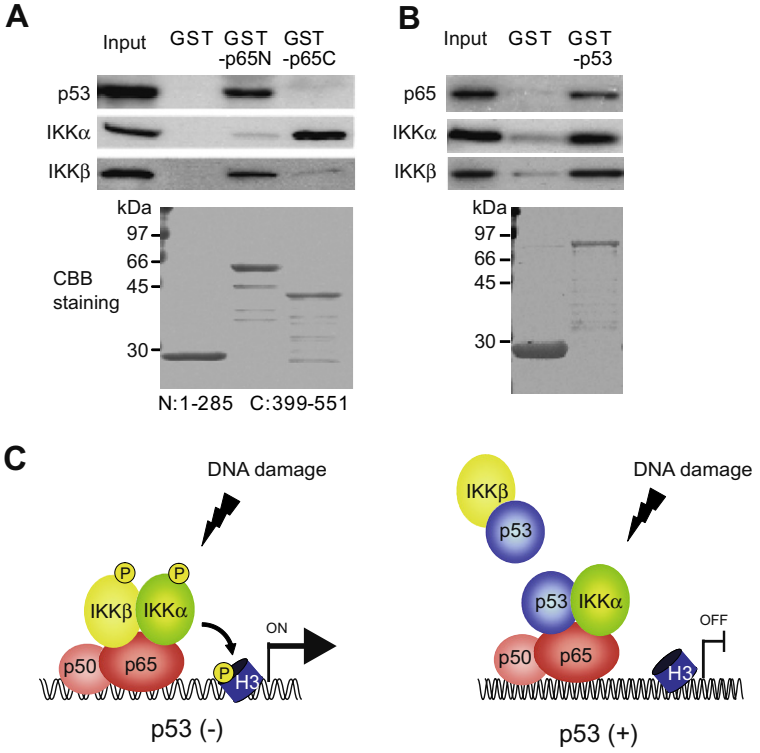

Fig. 3. p53 and IKKβ binds to N-terminal portion of p65. Glutathione–Sepharose beads preloaded with GST, GST-p65N (1-285), or GST-p65C (399-551) were incubated with the extracts from MCF-7 cells expressing Flag-tagged p53, HA-tagged IKKα, or HA-tagged IKKβ. The *in vitro* bindings of p53, IKKα, and IKKβ to p65 were examined by immunoblot analysis with the anti-Flag and anti-HA antibodies (top). (B) Glutathione–Sepharose beads preloaded with GST or GST-p53 were incubated with the extracts of MCF-7 cells expressing Flag-tagged p65, HA-tagged IKKα, or HA-tagged IKKβ. The *in vitro* bindings of p65, IKKα, and IKKβ to p53 were examined by immunoblot analysis with the anti-Flag and anti-HA antibodies (top). A Coomassie brilliant blue (CBB)-stained gel shows the expression of GST fusion proteins used in the top panel (bottom). (C) The schema represents a model of the activated p53-mediated suppression mechanism of NF-κB activation.

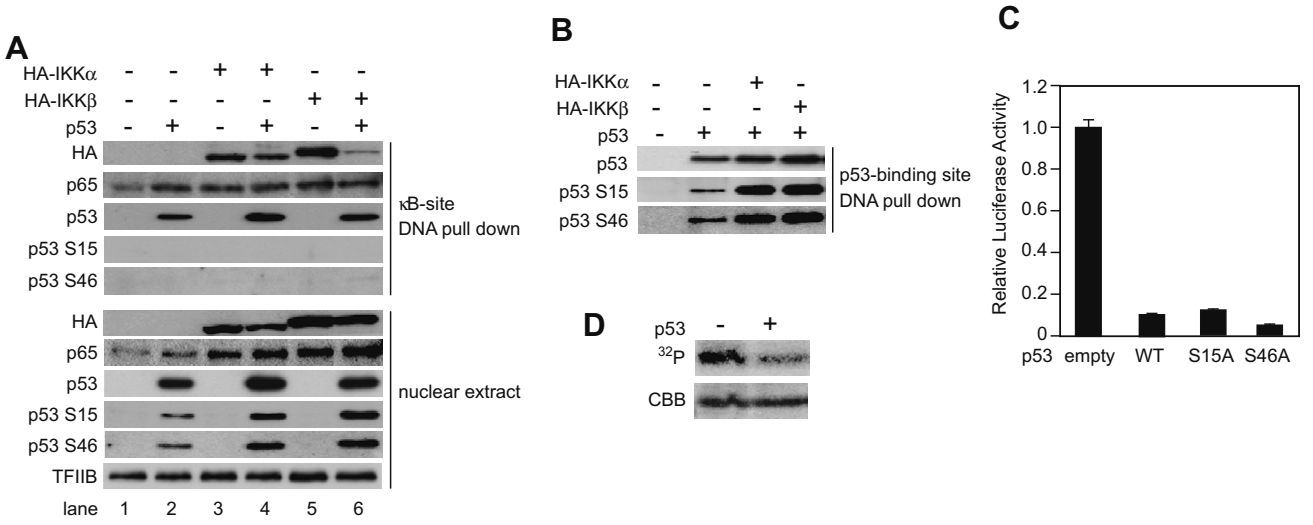

Fig. 4. p53 disrupts the recruitment of IKKβ to κB-site. (A,B) The HA-tagged IKKα or HA-tagged IKKβ expression vector was transfected into Saos-2 cells with or without the p53 expression vector, and nuclear extracts were prepared 24 h after transfection. The bindings of p53, p65, IKKα, or IKKβ to the κB-site (A) or the p53-binding site (B) were analyzed by DNA pull-down assay using a biotin-labeled κB-site or p53-binding site oligonucleotide, followed by immunoblotting with the indicated antibodies. TFIIB was used as a loading control. (C) Saos-2 cells were cotransfected with the NF-κB Luc reporter plasmid, phRL-TK (internal control), and expression vectors as indicated. After 24 h from transfection, relative luciferase activity was evaluated. Data represent means ± SD of three independent assays. (D) The nuclear extracts from Saos-2 cells transfected with or without the p53 expression vector were subjected to a DNA pull-down assay with biotin-labeled κB-site oligonucleotides, and the kinase activity of proteins recruited to the NF-κB-binding sequences was estimated by *in vitro* kinase assay using purified histone H3 as a substrate. Radioactive protein was visualized by autoradiography (top panels). The CBB stained gels in the bottom panel showed an equal loading of histone H3.

pull-down assay. As shown in Fig. 3A, p53 bound to the N-terminal portion (amino acids 1–285) of p65 that has a Rel homology domain, as previously reported [8]. IKKβ also bound to the same region of p65. In contrast, IKKα mainly bound to the C-terminal portion corresponding to the transcriptional activation domain (amino acids 399–551). We also performed a GST-p53 pull-down assay and confirmed the bindings of p65, IKKα and IKKβ to p53 (Fig. 3B). These results suggest that, following p53 activation, IKKα, p65, and p53 form a ternary complex, whereas p53 and IKKβ bind to each other and this binding competes with IKKβ binding to p65 (Fig. 3C).

Next, we analyzed the constitution of the complexes formed on oligonucleotides containing a κB-site by DNA pull-down assays. p65 weakly bound to the κB-site DNA (lane 1 in Fig. 4A). The ectopically expressed p53 exhibited a significant complex formation with p65 on the κB-site (lane 2 in Fig. 4A). Moreover, IKKα or IKKβ also formed a complex with p65 on the κB-site (lanes 3 and 5 in Fig. 4A). Importantly, p53, p65 and IKKα formed a complex on the κB-site DNA (lane 4 in Fig. 4A); however p53 excluded IKKβ from the complex containing p65 and p53 (lane 6 in Fig. 4A). It was shown that recruited p53 was exclusively in the unphosphorylated form at Ser 15 and Ser 46, in contrast to exogenous p53, which exhibited phosphorylation at these serines. In contrast, exogenous p53 on the p53-binding sequence element of Noxa promoter [18] was phosphorylated at these serines (Fig. 4B). Although it is unclear how recruitment of p53 to the NF-κB-binding sequence and the p53-binding sequence were regulated by phosphorylation, these results suggest that p53, phosphorylated at Ser 15 and Ser 46, predominantly binds to the p53-binding site and unphosphorylated p53 binds to p65 at the κB-site and inhibits recruitment of IKKβ. It was also shown that p53 S15A and S46A mutants, as well as the wild-type, suppressed NF-κB transcriptional activation (Fig. 4C), indicating that the suppression by p53 was not related to phosphorylation at Ser 15 and Ser 46. We had shown that in $p53^{-/-}$ MEFs, histone H3 phosphorylation and recruitment of IKKβ to the *IκBα* promoter, which includes a κB-site, were enhanced by adriamycin [13]. As it is known that IKKβ phosphorylates IKKα, regulating the kinase activity of IKKα [19,20], recruitment of IKKβ as well as IKKα to the promoter may be closely related to histone H3 phosphorylation. Furthermore, adriamycin-induced p53 in wild-type MEFs clearly diminished the IKKβ recruitment to the promoter and histone H3 phosphorylation [13]. An *in vitro* histone H3 phosphorylation assay revealed that the phosphorylation of histone H3 by a κB-site DNA-bound complex was markedly suppressed by ectopically expressed p53, suggesting that activity or recruitment of certain histone H3 kinase(s) to the κB-site is inhibited by p53 (Fig. 4D). Taken together, although activated p53 enhances DNA-binding activity of NF-κB through modification of p65, p53 may suppress transcriptional activity of NF-κB via inhibition of histone H3 phosphorylation by histone H3 kinase(s) including IKKα.

Accumulating evidence also shows that the IKK/NF-κB signaling pathway plays a key role in inflammation-associated tumor development [4,5]. In this context, it is considered that NF-κB is a major factor controlling the ability of both pre-neoplastic and malignant cells to resist apoptosis-based tumor-surveillance mechanisms. Recently, it has been shown that aberrant DNA replication evokes a DNA damage response, resulting in p53 activation and elimination of oncogene-expressing cells by induction of apoptosis or senescence [21,22]. Therefore our results suggest a possible role in p53-mediated tumor-surveillance through inhibition of NF-κB.

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