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**Vitamin D₃ Upregulated Protein 1 Suppresses TNF-α-Induced NF-κB Activation in Hepatocarcinogenesis**

Hyo-Jung Kwon,*†,1 Young-Suk Won,*1 Hyun-Woo Suh,‡,1 Jun-Ho Jeon,§,*1 Yan Shao,‡ Suk-Ran Yoon,‡ Jin-Woong Chung,‡ Tae-Don Kim,‡ Hwan-Mook Kim,* Ki-Hoan Nam,* Won-Kee Yoon,* Dae-Ghon Kim,* Jeong-Hwan Kim,# Young-Sung Kim,# Dae-Yong Kim,‡ Hyoung-Chin Kim,* and Inpyo Choi‡

Vitamin D₃ upregulated protein 1 (VDUP1) is a candidate tumor suppressor, the expression of which is dramatically reduced in various tumor tissues. In this study, we found that VDUP1 expression is suppressed during human hepatic carcinogenesis, and mice lacking VDUP1 are much more susceptible to diethylnitrosamine-induced hepatocarcinogenesis compared with wild type mice. VDUP1-deficient tumors proliferated significantly more than wild type tumors and had corresponding changes in the expression of key cell cycle regulatory proteins. In addition, the hepatomitogen-induced response was associated with a considerable increase in the release of TNF-α and subsequent enhancement of NF-κB activation in VDUP1-deficient mice. When cells were treated with TNF-α, the VDUP1 level was markedly reduced, concomitant with elevated NF-κB activation. Furthermore, the overexpression of VDUP1 resulted in the robust suppression of TNF-α-activated NF-κB activity via association with HDAC1 and HDAC3. These results indicate that VDUP1 negatively regulates hepatocarcinogenesis by suppressing TNF-α-induced NF-κB activation. *The Journal of Immunology,* 2010, 185: 000–000.

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Abbreviations used in this paper: ALT, alanine transaminase; C, control nontreated; CBP, CREB-binding protein; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; KO, knockout; NT, nontumor; 8-OHdG, 8-hydroxy-2′-deoxy-guanosine; PCNA, proliferating cell nuclear Ag; pRL–CMV, cytomegalovirus–Renilla luciferase construct; T, tumor; Trx, thioredoxin; VDUP1, vitamin D₃ upregulated protein 1; WT, wild type.

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Hepatocellular carcinoma (HCC), the most common type of liver cancer, is the fifth most common malignant tumor worldwide and the third leading cause of cancer death (9, 10). The prognosis of HCC is poor, and worldwide the overall 5-y survival rate is estimated at only 3%, mainly because HCC is frequently diagnosed at an advanced stage. The highest incidences of HCC are in sub-Saharan Africa and southeastern Asia, where hepatitis B virus infection is endemic (11). In Europe and the United States, the incidence of HCC is low but slowly increasing, probably as a result of the increase in hepatitis C virus infection. In addition to hepatotropic viruses like hepatitis B virus and hepatitis C virus, the other major risk factors for HCC include conditions that lead to chronic liver injury and inflammation, such as alcohol abuse and metabolic liver disease, and mutagens, such as aflatoxin (10, 12). However, the development of HCC is a multifactorial process, and the causal and mechanistic relationships between HCC risk factors and carcinogenic mechanisms have not yet been clarified. In human HCC, the constant activation of NF-κB is one of the key early events in neoplastic progression. Chan et al. (13) reported that 20 of 32 (62.5%) patients with HCC showed increased NF-κB activity compared with the corresponding controls. They also demonstrated that NF-κB activation was closely associated with urokinase plasminogen activator expression and led to more aggressive tumor behavior in terms of venous invasion, liver invasion, and absence of tumor encapsulation. In addition, NF-κB also functionally interacts with inducible NO synthase. The overexpression of inducible NO synthase contributes to growth deregulation in preneoplastic and neoplastic liver cells through the activation of the IKK/NF-κB signal axis (14). Thus, inhibition of the activated NF-κB signaling pathway may provide potential therapeutic targets for human HCC.

Vitamin D₃ upregulated protein 1 (VDUP1) is a multifunctional 46-kDa protein that was originally identified as a differentially expressed gene in 1a,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-treated HL-60 leukemia cells (15) and B16 melanoma cells (16). VDUP1 interacts with the antioxidant thioredoxin (Trx) to inhibit the reducing activity of Trx and blocks the interactions of Trx with...
other factors, such as ASK-1 and PAG, thereby increasing the vulnerability of cells to oxidative stress (17, 18). In addition, the ectopic expression of VDUP1 inhibits tumor cell growth and cell cycle progression, demonstrating its role as a novel tumor suppressor (19). Clinically, VDUP1 has also been related to tumorigenesis, because its expression is dramatically reduced in various tumors including breast, renal, and gastrointestinal cancers (20–22). Moreover, it has been suggested that VDUP1 downregulation may play a role in liver carcinogenesis, because VDUP1 deficiency is sufficient to induce HCC in a mouse model (23). To our knowledge, this study provides the first in vivo evidence that the loss of VDUP1 contributes to the progression of HCC, but its role in human hepatocarcinogenesis and the precise mechanisms underlying tumor development remain unclear.

In this study, we mechanistically address the role of VDUP1 in liver carcinogenesis using a well-characterized model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis. We found that the loss of VDUP1 greatly increased the susceptibility to DEN-induced hepatocarcinogenesis and NF-κB activity. We investigated the role of VDUP1 in the regulation of NF-κB activity, and we demonstrate that VDUP1 downregulated NF-κB activity via interactions with HDAC1 and HDAC3.

Materials and Methods

Animal experiments
VDUP1-knockout (KO) mice were generated as described previously (24) and were bred onto C57BL/6 mice for 10 generations. All mice were housed in a pathogen-free animal facility under a 12-h light-dark cycle and maintained on standard rodent chow and water provided ad libitum. VDUP1-KO and wild type (WT) mice were injected i.p. with 25 or 100 mg/kg DEN (Sigma-Aldrich, St. Louis, MO) at 13 d of age. For experiments involving TNF-α, 5-kd-old mice were injected i.v. with 25 μg/kg recombinant murine TNF-α (R&D Systems, Minneapolis, MN) dissolved in saline. The mice were euthanized and analyzed at the time points indicated in the text. All experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals, from the Institute for Laboratory Animal Research.

Liver histology, immunohistochemistry, and TUNEL assays
The liver tissues were fixed in 10% buffered formalin. Fixed samples were embedded in paraffin under a 12-h light-dark cycle and maintained on standard rodent chow and water provided ad libitum. VDUP1-KO and wild type (WT) mice were injected i.p. with 25 or 100 mg/kg DEN (Sigma-Aldrich, St. Louis, MO) at 13 d of age. For experiments involving TNF-α, 5-kd-old mice were injected i.v. with 25 μg/kg recombinant murine TNF-α (R&D Systems, Minneapolis, MN) dissolved in saline. The mice were euthanized and analyzed at the time points indicated in the text. All experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals, from the Institute for Laboratory Animal Research.

Liver histology, immunohistochemistry, and TUNEL assays
The liver tissues were fixed in 10% buffered formalin. Fixed samples were embedded in paraffin, sectioned, and stained with H&E for histological examination. Immunohistochemical analysis was performed using the Avidin-Biotin Complex Staining Kit (Vector Laboratories, Burlingame, CA). The Abs used were: anti-proliferating cell nuclear Ag (PCNA; Santa Cruz Diagnostics, Santa Cruz, CA), anti-VDUP1 (Medical and Biological Laboratories, Nagoya, Japan), and anti–8-hydroxy-2’-deoxy-guanosine (8-OHdG; Japan Institute for the Control of Aging, Shizuoka, Japan). Apoptosis was assessed using the TUNEL method and the ApopTag Plus Peroxidase In situ Apoptosis Detection Kit (Chemicon International, Temecula, CA), according to the manufacturer’s protocol.

Human tumor tissues and patient information
To investigate the relative mRNA expression level of VDUP1, a total of 19 primary HCC samples, with surrounding nontumor liver tissues, were obtained from the surgical resections of primary HCC. Written informed consent was obtained from each patient. This protocol conformed to the ethical guidelines of the institutional review board. The HCC and nontumor tissues were histologically confirmed by pathologists at Chonbuk National Medical School and Hospital.

Lipid peroxidation assay
The malondialdehyde level in the liver tissue was measured using the thiobarbituric acid reduction method with a commercially available kit (Cell Biolabs, San Diego, CA).

RNA isolation and real-time quantitative PCR
Total RNA was extracted from liver samples using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed and analyzed by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with specific primers. The reactions were amplified and quantified using the ABI 7700 sequence detection system and the manufacturer’s software (Applied Biosystems). The mRNA expression level was calculated using β-actin as the control.

EMSA
Nuclear extracts were prepared from mouse lung fibroblasts or transiently transfected HEK 293T cells treated with or without TNF-α (10 ng/ml). An EMSA was performed using an NF-κB consensus oligonucleotide (5’-AGT TGA GGC GAC TTT CCC AGG C-3’) that specifically binds NF-κB. Double-stranded oligonucleotides were end-laabeled with 2.5 μM [γ-32P]ATP using T4 kinase (Takara Shuzo, Otsu, Japan) and purified using PROBER columns (iNtRON Bio, Seongnam, Korea). The radioactive oligonucleotides were incubated with nuclear extracts (10 μg) for 20 min in the binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg of poly (dl-dC), and 1 μg of BSA at room temperature. The samples were loaded on a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA) and subjected to electrophoresis at 10 V/cm for 3–5 h at 4°C. The gels were then vacuum-dried and autoradiographed.

NF-κB–dependent luciferase reporter assay
To assay NF-κB activity, the indicated expression vectors were transiently cotransfected into HEK 293T cells with pNF-κB–Luc plasmid (Stratagene, La Jolla, CA) and cytomegalovirus-Renilla luciferase construct (pRL–CMV) (Promega, Madison, WI), a Renilla-derived luciferase reporter plasmid for transfection efficiency control, using lipofectamine (Invitrogen, Carlsbad, CA) reagents. Following the indicated treatments, the cells were lysed and subjected to luciferase assay. The values obtained from luciferin were normalized by the values obtained from coelenterate-luciferin (Promega).

Precipitation of GST-fusion proteins and coimmunoprecipitation assay
For the pull-down assay, HEK 293T cells were transfected with various combinations of expression vectors, as indicated in the text. Twenty-four hours after transfection, the cells were harvested and lysed in lysis buffer containing 50 mM NaCl, 10% glycerol, 20 mM HEPES (pH 7.2), and 1× protease inhibitor mixture (Calbiochem, San Diego, CA). After lysis, aliquots of the cell lysates were incubated with glutathione-Sepharose 4B (GE Healthcare, Uppsala, Sweden) for 12 h at 4°C. These beads were then washed five times with lysis buffer. The bound proteins were resuspended in SDS sample buffer, subjected to SDS-PAGE, and detected by autoradiography. Coimmunoprecipitation of endogenous VDUP1 with endogenous HDAC1 or p65 was analyzed in nuclear lysates of HeLa cells. Protein G-Agarose beads (Roche, Basel Switzerland) were preincubated with mouse monoclonal anti-VDUP1 antiserum or control preimmune serum at 4°C for 30 min. The mixtures were then incubated with nuclear lysates of HeLa cells for an additional 5 h. These precipitations were washed three times with lysis buffer. The bound proteins were visualized by Western blot with anti-HDAC1 Ab or anti-p65 Ab.

Immunofluorescence microscopy
HeLa cells were plated on coverslips in 12-well plates 24 h prior to transfection. Cells transfected with FLAG-VDUP1 and HA-tagged HDAC1 or HDAC3 expression plasmids were washed with PBS and fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, followed by permeabilization with 0.3% Triton X-100. The cells were incubated for 1 h at room temperature with the appropriate primary Abs (anti-HA, anti-FLAG, anti-HDAC3, or anti-p65). After three washes with PBS, the cells were incubated with Texas Red–conjugated goat anti-mouse IgG (BD Pharmingen, San Diego, CA) and/or FITC-conjugated anti-rabbit IgG for 1 h at room temperature, and washed again with PBS. The samples were examined with an LSM510 confocal microscope (Carl Zeiss, Gottingen, Germany).

Statistical analysis
For the statistical analysis of the data, p values were analyzed using an unpaired two-tailed Student t test. A p value <0.05 was considered statistically significant.

Results
Loss of VDUP1 promotes hepatocarcinogenesis
A single injection of DEN into 2-wk-old male mice results in the formation of HCC (25). To determine the role of VDUP1 in HCC, male VDUP1-KO and WT mice were injected with DEN on postnatal day 13. The number and maximal diameter of detectable tumor
nODULES WERE 5- AND 8-FOLD GREATER IN VDUP1-KO MICE AT 36 WK, RESPECTIVELY (FIG. 1A). A HISTOPATHOLOGIC EXAMINATION REVEALED THAT 100 AND 70% OF THE VDUP1-DEFICIENT LIVERS EXHIBITED HEPATOCELLULAR ADENOMA AND HCC, RESPECTIVELY, COMPARED WITH ONLY 75 AND 6% IN WT MICE AT 36 WK (FIG. 1B, TABLE I). IN ADDITION, VDUP1 EXPRESSION LEVELS WERE SIGNIFICANTLY DECREASED IN TUMOR TISSUES (FIG. 1C, D).
C). Next, we assessed the levels of VDUP1 in human HCC samples. When compared with the expression in nontumor tissue, 63.2% (12 of 19) of the HCC samples showed a more than 2-fold decrease in the mRNA expression of \textit{VDUP1} (Fig. 1D). Overall, these data from HCC indicate that VDUP1 is suppressed during hepatic carcinogenesis.

Increased cell proliferation in VDUP1-deficient tumors

Cell proliferation and apoptosis were analyzed in tumor and nontumor tissues to determine the role of VDUP1 on tumor growth. There was no significant difference in the apoptotic index between WT and VDUP1-KO tumors 36 wk after DEN treatment (Fig. 1E). In contrast, the proliferating index, as assessed by PCNA immunohistochemistry, was ∼2-fold higher in VDUP1-deficient tumors 36 wk after DEN treatment (Fig. 1E). In addition, the expression of NF-κB p50 and p65, PCNA, cyclin D1, and cyclin A was substantially higher in VDUP1-deficient tumors compared with WT tumors, whereas the absence of VDUP1 attenuated p21 and p27 expression (Fig. 1F).

Next, we examined in detail the immediate effects of DEN on signal transduction and cell behavior in precancerous tissues. Measurement of circulating liver enzymes and TUNEL assay revealed no differences in hepatocyte death in livers of DEN-treated WT compared with VDUP1-KO mice (Fig. 2A). A histologic analysis confirmed these results, revealing similar apoptotic and necrotic cell numbers in both groups of mice after DEN treatment (Fig. 2A). In addition, an analysis of the apoptotic signaling pathway revealed that DEN injection led to a comparable induction of p-ASK1, p-p38 MAPK, Bax, and caspase cleavage in WT and VDUP1-KO mice (Fig. 2B). Furthermore, the level of phosphorylated-ASK1 was almost same in VDUP1<sup>2/2</sup> lung fibroblasts compared with WT after H<sub>2</sub>O<sub>2</sub> exposure in vitro (Supplemental Fig. 1A). In this situation, the percentage of apoptotic cells was no significant difference in both TNF-α/cycloheximide and H<sub>2</sub>O<sub>2</sub> treatment between WT and TXNIP-KO lung fibroblasts (Supplemental Fig. 1B). These results suggest

### Table 1. Incidence of hepatic tumors in DEN-treated WT and TXNIP-KO mice

| Weeks after DEN | VDUP1 Genotype | Total Incidence (%) | Hepatocellular Adenoma (%) | HCC (%) |
|----------------|---------------|---------------------|---------------------------|--------|
| 16             | WT            | 0/9 (0)             | 0/9 (0)                   | 0/9 (0) |
|                | KO            | 1/10 (10)           | 1/10 (10)                 | 0/10 (0) |
| 26             | WT            | 5/10 (50)           | 5/10 (50)                 | 0/10 (0) |
|                | KO            | 9/9 (100)*          | 9/9 (100)*                | 1/9 (11) |
| 36             | WT            | 12/16 (75)          | 12/16 (75)                | 1/16 (6) |
|                | KO            | 10/10 (100)*        | 10/10 (100)*              | 7/10 (70)* |

Data are presented as mean ± SEM.

*p < 0.05 versus respective WT mice.
that the earlier development of HCC in the absence of VDUP1 was not due to deregulated apoptotic pathways in our model.

Increased production of reactive oxygen species is a major contributor to the initiation and progression of DEN-induced liver cancer (26). The content of malondialdehyde, the major aldehyde end product of membrane lipid peroxidation, was higher in WT livers compared with VDUP1-KO livers at 5 h after DEN treatment. However, no significant differences were observed at 24 and 48 h (Fig. 2, top). The livers of VDUP1-KO mice exhibited levels of anti-8-OHdG reactivity similar to those observed in WT livers 48 h after DEN administration (Fig. 2, bottom), indicating that VDUP1 does not affect oxidative damage after DEN administration.

Meanwhile, the number of PCNA-positive hepatocytes increased in both groups of mice after DEN exposure (Fig. 2D). Moreover, this response was significantly augmented in VDUP1-KO mice, suggesting that the loss of VDUP1 may accelerate DEN-induced hepatocarcinogenesis by increasing cell proliferation rather than by deregulating apoptotic pathways or oxidative stress.

**Loss of VDUP1 promotes TNF-α–induced NF-κB activity**

Growth factors and cytokines, such as TNF-α, IL-6, and HGF, play a significant role in compensatory hepatocyte proliferation. As shown in Fig. 3A, the absence of VDUP1 increased the mRNA expression of TNF-α and IL-6 following DEN treatment, but had no significant effect on the mRNA expression of HGF. In addition, the mRNA expression of IL-1β was slightly elevated 5 h after DEN administration in VDUP1-KO mice compared with WT mice (Fig. 3A).

TNF-α and IL-6 mediate hepatocyte proliferation by activating several transcription factor complexes, including NF-κB, STAT, and AP-1 (27). In VDUP1-KO mice, NF-κB activation was significantly increased in VDUP1-KO mice compared with WT counterparts (Fig. 3B). In addition, the activation of JNK, as assessed by the phosphorylation of c-Jun, was detected earlier in DEN-exposed VDUP1-deficient livers than in WT livers, whereas there was no significant difference in the activation of STAT-3 between WT and VDUP1-KO mice (Fig. 3B).

Based on elevated TNF-α expression and NF-κB activation in VDUP1-KO mice, we next examined whether VDUP1 affects TNF-α–mediated NF-κB activation. TNF-α treatment led to rapid activation of NF-κB in VDUP1-deficient and WT livers (Fig. 3C). These responses, however, were significantly increased in VDUP1-KO livers compared with WT livers, suggesting that VDUP1 regulates NF-κB activation via TNF-α. Similarly, NF-κB DNA binding activity was increased in TNF-α–treated VDUP1-KO fibroblasts compared with WT fibroblasts (Fig. 3D). Overall, these results suggest that VDUP1 is involved in the NF-κB activation pathway.

**VDUP1 inhibits TNF-α– or p65-induced NF-κB activation**

TNF-α–mediated activation of the NF-κB pathway has been relatively well characterized (28). To understand the molecular mechanisms associated with VDUP1-mediated NF-κB regulation, we examined whether ectopically overexpressed VDUP1 can modulate TNF-α–induced NF-κB activation. TNF-α–induced NF-κB activation was markedly inhibited by VDUP1 overexpression in a dose-dependent manner (Fig. 4A). In addition, p65 subunit strongly induced NF-κB–dependent luciferase activity, which was also downregulated by VDUP1 (Supplemental Fig. 2A). The VDUP1 C247S mutant, which is unable to interact with Trx (29), had...
relatively reverse effects compared with WT-VDUP1 (Fig. 4A). Meanwhile, VDUP1 C63S/C120S mutant, which is not mutated at the cysteine 247 residue, showed a somewhat similar effect that observed for WT VDUP1 (Supplemental Fig. 2B). Again, VDUP1 C247S moderately suppressed p65 subunit-induced NF-κB activity than WT VDUP1 (Supplemental Fig. 2A). Thus, we assessed whether Trx is involved in the VDUP1-mediated regulation of NF-κB activity. The inhibitory patterns of VDUP1 and the VDUP1 mutant were not significantly changed in response to Trx overexpression (Fig. 4B), implying that residue 247 of VDUP1 is responsible for regulating NF-κB activity via an interaction with an unidentified factor, but not with Trx. Furthermore, an EMSA was performed to evaluate the DNA binding activity of NF-κB. TNF-α treatment increased NF-κB binding to the labeled probe containing the NF-κB binding sites (Fig. 4C). VDUP1 decreased TNF-α-induced NF-κB DNA binding activity in a dose-dependent manner, reversibly of which activities were recovered by increasing amounts of VDUP1 C247S mutant (Fig. 4C).

We next investigated whether VDUP1 could exert its negative effect on diverse NF-κB activation signals. Ectopically overexpressed VDUP1 also inhibited IL-1β-, TNF-α-, and LPS-mediated NF-κB activation (Fig. 4D). To determine in detail how VDUP1 modulates NF-κB activity, we examined whether VDUP1 modulates the activity of various signal transducers known to be NF-κB activators downstream of the TNF-α and IL-1βRs (Fig. 4E). VDUP1 inhibited TRAF2-, IKKβ-, and MEKK1-mediated NF-κB activation, suggesting that VDUP1 might function at a location in the NF-κB pathway where divergent signals merge (Fig. 4E). Indeed, VDUP1 inhibited NF-κB activation induced by NF-κB p65, one of the end point molecules in the NF-κB pathway (Supplemental Figs. 2A, 4E). In the same line with these observations, VDUP1 overexpression did not inhibit TNF-α–induced IκB degradation in HEK 293T cells (Fig. 4F). These results suggest that VDUP1 might target the NF-κB p65 subunit to regulate NF-κB activity.

**VDUP1 synergistically inhibits p65-induced NF-κB activation with HDAC1 and HDAC3**

HDACs are known to inhibit NF-κB activity by binding to NF-κB p50 or p65 (30–32). Based on the observation that VDUP1 inhibits NF-κB p65 activity, we investigated the potential relationship between VDUP1 and HDACs in NF-κB activation. Overexpressed VDUP1, HDAC1, and HDAC3 suppressed p65-induced NF-κB reporter activity (Fig. 5A). The synergistic suppressive effects on p65-induced NF-κB activity were shown for the combination of both VDUP1 and HDAC1 or HDAC3, and these effects were recovered by treatment with the HDAC inhibitor trichostatin A (Fig. 5A). NF-κB p65 binds to CREB-binding protein (CBP) and its homolog p300, as well as PCAF (p300/CBP-associated factor), whereas p50 fails to recruit transcriptional coactivators (33–37). Therefore, a transient transfection system was used to evaluate whether VDUP1 modulates CBP-induced p65 acetylation and NF-κB coactivation. Expression of CBP increased p65-induced NF-κB activity; however, the overexpression of VDUP1 suppressed this activity in a dose-dependent manner (Fig. 5B). Furthermore, the acetylation of p65 by CBP was synergistically decreased by HDAC1, HDAC3, and VDUP1 (Fig. 5C). We next analyzed the ability of VDUP1 or the VDUP1 C247S mutant to coprecipitate with HDAC1 and HDAC3. VDUP1 bound strongly to HDAC1 or HDAC3, but the VDUP1 C247S mutant bound relatively weakly to HDAC1 or HDAC3 (Fig. 5D). VDUP1 was colocalized with HDAC1 or HDAC3 in the nucleus (Fig. 5E; yellow). In addition, endogenous HDAC1 also bound to endogenous VDUP1, and more strongly when treated by TNF-α (Fig. 5F, left). Interestingly, when treated by TNF-α, although nuclear VDUP1 decreased significantly, nuclear p65 was found to bind VDUP1 substantially (Fig. 5F, right). Overall, these results indicate that VDUP1 interacts with HDAC, and residue 247 is crucial for the HDAC interaction to modulate p65 activity.

**TNF-α downregulates VDUP1 expression**

To further elucidate the molecular mechanism underlying the inhibitory effect of VDUP1 on TNF-α–induced NF-κB activation, we analyzed VDUP1 expression during TNF-α signaling. TNF-α treatment downregulated VDUP1 at early time points after stimulation (Fig. 6A). VDUP1 expression was decreased early after TNF-α treatment, and it remained low for at least 6 h in HeLa cells (Fig. 6B). As expected, the levels of phospho–NF-κB p65 and total NF-κB p65 significantly increased in the nucleus 5 min after TNF-α treatment. Moreover, the level of NF-κB p65 remained high for at least 6 h (Fig. 6B). Meanwhile, the level of VDUP1 markedly decreased in both the cytosol and the nucleus shortly after treatment (Fig. 6A, 6B). These results indicate that TNF-α induces downregulation of VDUP1 in parallel with NF-κB activation.

We analyzed in detail TNF-α–mediated downregulation of VDUP1. The VDUP1 mRNA level was also decreased early after TNF-α treatment (Fig. 6C). VDUP1 protein levels were decreased in a dose- and time-dependent manner (Fig. 6D). Overall, it seems that TNF-α induces the suppression of VDUP1 both at the transcriptional level and posttranscriptional level. Thus, it facilitates TNF-α signaling and NF-κB activation, which is attenuated by VDUP1 and HDACs, as shown in Fig. 6E.

**Discussion**

In this study, we focused on the molecular mechanisms that account for the elevated susceptibility of VDUP1-KO mice to chemically induced hepatocarcinogenesis. We found that the tumor-promoting effect of VDUP1 deficiency was associated with an increased compensatory proliferation rate. Importantly, the loss of VDUP1 results in an increased TNF-α level and leads to NF-κB activation, processes that are critical for compensatory proliferation and hepatocarcinogenesis.

A number of studies have suggested that VDUP1 is involved in the regulation of cell proliferation. These results indicate that VDUP1 inhibits cell proliferation by associating with diverse cell cycle regulators (38). In the current study, we initially showed that the loss of VDUP1 confers a growth advantage to DEN-induced tumor cells. In accordance with previous data, this finding was correlated with the altered expression of cell cycle regulators, including cyclin D1, cyclin A, p27, and p21. However, during the immediate response to DEN exposure, VDUP1 deficiency promoted the proliferation of surviving hepatocytes via increased TNF-α release and subsequent enhancement of NF-κB activation. Notably, cyclin D1 is the most important transcriptional target of NF-κB (39, 40). These data suggest that the increased activity of NF-κB is one of the reasons for increased cell proliferation and the altered expression of cell cycle regulators detected in VDUP1-deficient tumors. This finding correlates well with previous studies reporting that VDUP1 is a transcriptional repressor that suppresses the cyclin A2 promoter activity in association with other corepressors (19). In the current study, we provide further evidence that VDUP1 deficiency acts as a transcriptional activator of NF-κB–mediated gene expression and function, and that this effect is a major driving force of the accelerated development and progression of hepatocyte proliferation and HCC.

Based on the altered patterns of NF-κB, however, dysregulated apoptosis gains advantages during HCC formation in mice lacking VDUP1. This is due to the ability of NF-κB to promote cell survival over TNF-α–driven apoptosis by inducing several antiapoptotic proteins or by blocking sustained JNK activation (3). Moreover, pre-
vious in vitro studies had reported that overexpression of VDUP1 caused dissociation of Trx from ASK1 and subsequent ASK1-dependent apoptosis (41). Thus, it would be expected that loss of VDUP1 promotes reduction of DEN-induced apoptosis in VDUP1 KO mice. We also speculated that, in VDUP1-deficient liver tissue, TNF-α triggers apoptotic cell death in response to DEN through activation of JNK. However, the elevated NF-κB owing to VDUP1 deficiency compensates for the JNK-induced apoptosis, resulting in no difference in apoptosis. Nevertheless, no apparent differences in the cell death rates were observed between VDUP1-KO and control livers, indicating that the acceleration of DEN-induced compensatory hepatocyte proliferation as a consequence of VDUP1 deficiency may be due to its strong mitogenic activity rather than an increase in cell survival.

While NF-κB activation has been previously associated with liver tumor development, emerging evidence indicates that the loss

FIGURE 4. VDUP1 inhibits TNF-α–induced NF-κB activation. A and B, HEK293 cells were transiently cotransfected with the indicated plasmids together with NF-κB reporter plasmid and pRL–CMV. Twenty-four hours posttransfection, the cells were treated with or without TNF-α (10 ng/ml) for 6 h, and NF-κB–derived luciferase activities were measured after normalizing for transfection efficiency against Renilla luciferase activity. The data are expressed as the mean ± S.D. of three independent experiments. C, HEK 293T cells were transiently transfected with increasing amounts of pFLAG-VDUP1 or pFLAG-VDUP1 C247S. Nuclear extracts of cells stimulated with or without TNF-α (10 ng/ml, 6 h) were analyzed by EMSA, and VDUP1 levels were visualized by Western blot (bottom). D, VDUP1 inhibits NF-κB activation induced by TNF-α, IL-1β, and LPS. HEK 293T cells were transiently transfected either with pEGFP (1 μg) or with pEGFP-VDUP1 (1 μg) vector together with 0.1 μg NF-κB reporter plasmid and 0.1 μg pRL–CMV. Twenty-four hours after transfection, the cells were treated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), or LPS (1 μg/ml) for an additional 8 h. The luciferase activities were measured and normalized. E, VDUP1 inhibits NF-κB activation through various signal transducers. HEK 293T cells were transiently transfected either with empty vector (1 μg) or with VDUP1 expression vector (1 μg) together with 1 μg of expression plasmid encoding TRAF2, MEKK1, IKKβ, or NF-κB p65 along with 0.1 μg NF-κB reporter plasmid and 0.1 μg pRL–CMV. Twenty-four hours after transfection, lysates were analyzed for luciferase activity as in A and B. F, HEK 293T cells were transiently transfected with pFLAG Alone or pFLAG-VDUP1. After 24 h, the cells were incubated with TNF-α (10 ng/ml) for the indicated periods of time. The levels of IκBα and VDUP1 were quantified by Western blot and normalized to β-actin.
of activators of the NF-κB cascade may also paradoxically induce liver tumor development. Pikarsky et al. (42) demonstrated that NF-κB promotes hepatocarcinogenesis in MDR2-deficient mice. On the contrary, a study from Maeda et al. (26, 43) showed that the hepatocyte-specific deletion of IKKβ promoted DEN-induced hepatocarcinogenesis in IKKβ−/− mice. This finding was also supported by a recent report demonstrating that hepatocyte-specific IKKβ/NEMO-deficient mice, in which the NF-κB pathway was inhibited in hepatocytes, were prone to develop HCC (44).

In this study, we showed that activation of NF-κB owing to VDUP1 deficiency promoted chemically induced hepatocarcinogenesis. Although the role of NF-κB in hepatocarcinogenesis is dependent on cell type, these observations reveal that VDUP1 deficiency-mediated NF-κB activation has an essential physiologic function in the development of HCC, identifying that NF-κB functions as a tumor promoter in the liver.

Furthermore, we checked VDUP1-mediated regulation of NF-κB activity in lung cells in addition to liver cells. We performed NF-κB reporter assay using H1299 lung carcinoma cells stably overexpressing VDUP1. TNF-α–induced NF-κB activation was decreased ∼30% in VDUP1-tranfected H1299 cells compared with control H1299 cells (Supplemental Fig. 3A; 3.4- versus 2.4-fold).
The phosphorylation of p65 after TNF-α treatment was less increased in VDUP1-transfected H1299 cells compared with control H1299 cells (Supplemental Fig. 3B). Next, we tested the effects of VDUP1 on lung carcinoma cell growth in an in vivo tumor xenograft model. VDUP1 overexpression completely suppressed tumorigenesis in vivo, indicating that VDUP1 also functions as a tumor suppressor in lung cancer model (Supplemental Fig. 3C,3D).

In addition, we examined the functions of VDUP1 in TNF-α–mediated NF-κB activation. We identified VDUP1 as a new component of the corepressors by demonstrating its ability to repress NF-κB activation, during which HDAC1 and HDAC3 interact with NF-κB p65 to suppress the NF-κB activity (30–32). In addition, our pull-down experimental results suggest that the formation of the complex of VDUP1, HDAC1, and NF-κB p65 may play a crucial role in the suppression function of VDUP1 to inhibit TNF-α–induced NF-κB activity. However, the detailed mechanisms remain to be addressed further.

VDUP1 was initially isolated as a protein of which expression is upregulated by 1,25(OH)2D3 (15). Based on the previous study showing that active vitamin D prevents cancer progression by reducing cell proliferation (45, 46), it was investigated whether 1,25(OH)2D3 has an anticancer effect in WT, but not in VDUP1-KO mice. The results of our experiment showed that 1,25(OH)2D3 treatment prior to DEN resulted in a significant decrease in hepatocyte proliferation and NF-κB p65 activation in both WT and VDUP1-KO mice during tumor initiation step (Supplemental Fig. 4), indicating that vitamin D also showed an antitumor effect in VDUP1-KO mice. The hepatocarcinogenesis is a complex process in which many mediators participate. Indeed, antiproliferative activity of 1,25(OH)2D3 has been associated with altered induction of TGF-β signaling, amphiregulin, cyclin-dependent kinase inhibitors p21WAF1/CIP1 and p27KIP1 and several mitogenic factors, including VEGF-related protein, Cyr61, and midkine (47, 48). Therefore, despite VDUP1 deficiency, induction of other factors by 1,25(OH)2D3 may contribute to decrease of hepatocyte proliferation, resulting in suppression of DEN-induced tumorigenesis.

In summary, this study demonstrates a crucial role for VDUP1 in tumor formation. Using a chemically induced liver carcinogenesis model, we demonstrated that increased cell proliferation caused by TNF-α–induced NF-κB activation is a primary mechanism by
which these findings provide new insights into the role of VDUP1 in tumorigenesis and demonstrate that VDUP1 may represent an attractive target for antiproliferative cancer therapy.

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The authors have no financial conflicts of interest.

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