

DDX3, a DEAD Box RNA Helicase with Tumor Growth–Suppressive Property and Transcriptional Regulation Activity of the $p21^{waf1/cip1}$ Promoter, Is a Candidate Tumor Suppressor

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Abstract

DDX3 is a DEAD box RNA helicase with diverse biological functions. Using colony formation assay, our results revealed that DDX3 inhibited the colony formation ability of various tumor cells, and this inhibition might be due to a reduced growth rate caused by DDX3. Additionally, we identified $p21^{waf1/cip1}$, a cyclin-dependent kinase inhibitor, as a target gene of DDX3, and the up-regulation of $p21^{waf1/cip1}$ expression accounted for the colony-suppressing activity of DDX3. Moreover, DDX3 exerted its transactivation function on $p21^{waf1/cip1}$ promoter through an ATPase-dependent but helicase-independent mechanism, and the four Sp1 sites located within the -123 to -63 region, relative to the transcription start site of $p21^{waf1/cip1}$ promoter, were essential for the response to DDX3. Furthermore, DDX3 interacted and cooperated with Sp1 to up-regulate the promoter activity of $p21^{waf1/cip1}$. To determine the relevance of DDX3 in clinical cancers, the expression profile of DDX3 in various tumors was also examined. A declined expression of DDX3 mRNA and protein was found in $\sim 58\%$ to 73% of hepatoma specimens, which led to the reduction of $p21^{waf1/cip1}$ expression in a manner independent of $p53$ status. Additionally, an alteration of subcellular localization from nuclei to cytoplasm was also observed in $>70\%$ of cutaneous squamous cell carcinoma samples. Because DDX3 exhibits tumor suppressor functions, such as a growth-suppressive property and transcriptional activation of the $p21^{waf1/cip1}$ promoter, and is inactivated through down-regulation of gene expression or alteration of subcellular localization in tumor cells, all these features together suggest that DDX3 might be a candidate tumor suppressor. (Cancer Res 2006; 66(13): 6579-88)

Introduction

DDX3, also named as CAP-Rf (1) or DBX (2), is a hepatitis C virus core protein-associated cellular factor that belongs to the DEAD box RNA helicase family (1, 3, 4). DDX3 harbors nucleotide triphosphatase-deoxynucleotide triphosphatase activity (1), ATPase-dependent RNA helicase activity, and a nuclear-cytoplasmic shuttling localization function (5). However, the precise biological

functions of DDX3 are still not fully understood. It has been reported that the yeast homologue of DDX3, Ded1, is essential for translation initiation (6), and DDX3 is able to rescue Ded1-deficient yeast mutants (4). Additionally, DDX3 has also been suggested to be involved in pre-mRNA splicing because of its association with functional spliceosome (7) and its possession of an RS-like domain (1). Recently, DDX3 has been reported to play a role in mRNA migration (8) and is required for HIV-1 Rev-RRE export function (5). Moreover, DDX3 is identified as a subunit of mammalian Mediator complex (9) and has a transcriptional activation function (1). These findings indicate a regulatory role for DDX3 in gene expression.

The cell cycle progression of eukaryotic cells is well controlled by cyclin, cyclin-dependent kinases (cdk), and cdk inhibitors. $p21^{waf1/cip1}$ is one of the critical cdk inhibitors. Through interaction with the cdk/cyclin complexes, $p21^{waf1/cip1}$ modulates their kinase activity, resulting in cell growth arrest (10). Additionally, $p21^{waf1/cip1}$ also prevents DNA synthesis and regulates DNA replication through interaction with proliferating cell nuclear antigen (11). Therefore, $p21^{waf1/cip1}$ may serve as a growth inhibitor and cell cycle checkpoint. In addition to the modulation of cell proliferation, $p21^{waf1/cip1}$ is also involved in the regulation of apoptosis, differentiation, and stress response (11). Furthermore, although mutation of $p21^{waf1/cip1}$ is uncommon in cancers (12), $p21^{waf1/cip1}$ -deficient mice show susceptibility to spontaneously or chemically induced tumor formation (13–15). Hence, $p21^{waf1/cip1}$ acts as a tumor suppressor.

Several factors have been reported to induce the expression of $p21^{waf1/cip1}$ and the most important one is the tumor suppressor $p53$ (16). Additionally, $p53$ -independent stimulation including certain extracellular signals, chemical drugs (17), and viral products (18, 19) also induce $p21^{waf1/cip1}$ expression. The mechanism of $p21^{waf1/cip1}$ induction caused by these factors often involves the action of corresponding transcription factors on the $p21^{waf1/cip1}$ promoter (17). These transcription factors include signal transducers and activators of transcription, nuclear hormone receptors, Smad3, and the ubiquitous transcription factor Sp1. Sp1 binds to the GC-rich sequence on the proximal region of $p21^{waf1/cip1}$ promoter and acts as a mediator for the induction of $p21^{waf1/cip1}$ gene caused by transforming growth factor β (TGF- β ; ref. 20) and several chemical drugs including mitogen-activated protein kinase inhibitor and histone deacetylase inhibitor (21, 22). Furthermore, Sp1 also interacts with other regulatory transcription factors of $p21^{waf1/cip1}$ promoter physically and functionally (23–25). Therefore, Sp1 plays a critical role in the regulation of $p21^{waf1/cip1}$ promoter activity.

In this study, we have identified a tumor growth–suppressive property of DDX3. Additionally, we also identified $p21^{waf1/cip1}$ as a downstream gene of DDX3. DDX3 enhances the expression

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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of $p21^{waf1/cip1}$ gene and up-regulates the promoter activity of $p21^{waf1/cip1}$ through an ATPase-dependent but helicase-independent mechanism. The modulation of $p21^{waf1/cip1}$ gene expression accounts for the growth-suppressive effect of DDX3. Moreover, our data also revealed that DDX3 transactivates the $p21^{waf1/cip1}$ promoter through its multiple Sp1 sites, and DDX3 collaborates with Sp1 on $p21^{waf1/cip1}$ promoter activation. Finally, we also showed that DDX3 expression was decreased in hepatocellular carcinoma (HCC) specimens, and the reduced expression of DDX3 might lead to a p53-independent decrease of $p21^{waf1/cip1}$ expression. Additionally, an extensive loss of its nuclear localization was observed in cutaneous squamous cell carcinoma (SCC) compared with normal squamous cells. Together, our results show that DDX3 exerts tumor suppressor functions, including growth inhibition and transcriptional modulation of the promoter activity of $p21^{waf1/cip1}$, and is inactivated through deregulated expression or nuclear exclusion in tumor cells. All these features strongly suggest that DDX3 might act as a tumor suppressor.

Materials and Methods

Cell lines and cultures. HuH-7 (human HCC), HepG2 (human hepatocellular blastoma), 293T (human embryonic kidney fibroblast), HeLa (human cervical cancer cells), NIH 3T3 (murine fibroblast), and HCT116 (human colon cancer) and its derived cell line HCT116 $p21^{-/-}$ (kindly provided by Dr. B. Vogelstein, Howard Hughes Medical Institute, Chevy Chase and the Sydney Kimmel Comprehensive Cancer Center of the Johns Hopkins University School of Medicine, Baltimore, MD; ref. 26) were grown in DMEM supplemented with 10% fetal bovine serum or bovine calf serum (NIH 3T3 cells; Invitrogen-Life Technologies Corp., Carlsbad, CA).

Plasmid construction. Plasmid p21-Luc was constructed by insertion of a 2.3-kb *HindIII* fragment (spanning the promoter region -2,325 to +8 related to transcription start site of human $p21^{waf1/cip1}$ gene) from pWWP-Luc (16) into *HindIII*-treated GL2 vector (Promega Corp., Madison, WI). To construct the serial 5'-deleted $p21^{waf1/cip1}$ promoter-driven luciferase reporters (-1,002/+8)p21-Luc, (-463/+8)p21-Luc, (-223/+8)p21-Luc, (-210/+8)p21-Luc, (-159/+8)p21-Luc, (-106/+8)p21-Luc, (-84/+8)p21-Luc, (-76/+8)p21-Luc, (-63/+8)p21-Luc, and (-56/+8)p21-Luc, *HindIII*/*BglII*-treated, PCR-amplified $p21^{waf1/cip1}$ promoter fragments were ligated with *HindIII*/*BglII*-treated pGL2 vector. To construct the $p21^{waf1/cip1}$ internal promoter region (nucleotide -127 to -63) deleted reporter plasmid (-2,325/+8) Δ (-127/-63)p21-Luc, the p21-Luc construct was digested with *ApaI*, blunted, and then self-ligated. The $p21^{waf1/cip1}$ promoter-driven luciferase reporters (pGL3 derivatives) containing point mutations in six Sp1 elements, (Mut1)p21-Luc, (Mut2)p21-Luc, (Mut3)p21-Luc, (Mut4)p21-Luc, and (Mut5/6)p21-Luc and the parental wild-type reporter, (Wt)p21-Luc, were kindly provided by Dr. D. Kardassis (Department of Basic Sciences, University of Crete Medical School, Heraklion, Crete, Greece; ref. 27). Other $p21^{waf1/cip1}$ promoter-driven luciferase reporters containing multiple point mutations of the Sp1 sites or point mutation of the AP-2 and E2F responsive elements (Mut1/2)p21-Luc, (Mut3/4)p21-Luc, (Mut1/2/3)p21-Luc, (Mut2/3/4)p21-Luc, (Mut1/2/3/4)p21-Luc, (mAP-2-1)p21-Luc, (mAP-2-2)p21-Luc, (mE2F(b)-1)p21-Luc, and (mE2F(b)-2)p21-Luc were generated using the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA). To obtain the pcDNA-SR α /FLAG vector, pcDNA3-FLAG vector (Invitrogen Corp., Carlsbad, CA) was treated with *HindIII* and *NruI* to eliminate the cytomegalovirus promoter fragment, blunted, and then ligated with 0.8 kb of *ClaiI*-treated and Klenow filled-in SR α promoter fragment from pSR α vector (28). The mammalian expression vector pcDNA-SR α /FLAG-DDX3 was constructed by insertion of a 2-kb *EcoRI*/*ApaI*-treated fragment of the *DDX3* gene derived by reverse transcription-PCR (RT-PCR) into *EcoRI*/*ApaI*-treated pcDNA-SR α /FLAG vector. Plasmids pcDNA-SR α /FLAG-DDX3(AAA) and pcDNA-SR α /FLAG-DDX3(DQAD), which direct the expression of the *DDX3* mutants harboring mutation of the SAT or DEAD motif, were constructed by changing the

Ser³⁸² and Thr³⁸⁴ residues to Ala and the Glu³⁴³ residue to Gln by the QuickChange site-directed mutagenesis system (Stratagene). To obtain pGEX-5 \times -1/DDX3(1-226), which expresses the glutathione *S*-transferase/DDX3(1-226) fusion protein in *E. coli*, an *EcoRI*/*XhoI*-treated PCR-amplified cDNA fragment encoding NH₂-terminal 1-226 amino acid region of DDX3 was ligated with *EcoRI*/*XhoI*-treated pGEX-5 \times -1 vector (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Plasmids pMC2P/FLAG-Sp1 and pMC2P/FLAG-Sp3, which direct the expression of FLAG-Sp1 or FLAG-Sp3, were kindly provided by Dr. G. Suske (Philipps University, Marburg, Germany). The plasmid pcDNA-SR α /FLAG-Sp1 was generated by direct insertion of an *EcoRI*/*ApaI*-digested, PCR-amplified Sp1 cDNA fragment into *EcoRI*/*ApaI*-treated pcDNA-SR α -FLAG. The bacterial expression vector pGEX-5 \times -1/Sp1 was constructed by introducing an *EcoRI*/*XhoI*-treated Sp1 gene fragment into *EcoRI*/*XhoI*-treated pGEX-5 \times -1 vector. All of these constructs were verified by DNA sequencing.

Antibody preparation. To generate anti-DDX3 antibody, glutathione *S*-transferase fusion protein containing the NH₂-terminal 1-226 amino acid fragment of DDX3 [GST/DDX3(1-226)] was used as antigen for immunization. Immunization and collection of antisera were done by the Custom Antibody Services of Protech Technology Enterprise Co., Ltd. (Taipei, Taiwan). Anti-DDX3 sera were depleted with purified GST protein before application to the immunohistochemistry analysis.

Colony formation assay. For the colony formation assay, HuH-7, HeLa, HCT116, and NIH 3T3 cells were transfected with pcDNA-SR α /FLAG-DDX3 or its parental empty vector by the calcium phosphate method or by Effectene (Qiagen, Hilden, Germany). Transfected cells were under G418 (Calbiochem; Merck Ltd., Darmstadt, Germany) selection (400 μ g/mL for HuH-7 and HeLa cells, 600 μ g/mL for HCT116 cells, and 900 μ g/mL for NIH 3T3 cells) for 2 to 3 weeks. Colonies were fixed with methanol/acetone (1:1) and stained with crystal violet (1 mg/mL). To address the $p21^{waf1/cip1}$ -dependent inhibition on colony formation, HCT116 and HCT116 $p21^{-/-}$ cells were cotransfected with pSilencer 3.1-H1 puro (Ambion, Austin, TX) and pcDNA-SR α /FLAG-DDX3 or its parental vector by Effectene (Qiagen) and selected with puromycin (0.4 μ g/mL) for 2 weeks.

Reporter assay. For the luciferase reporter assay, correct amounts of cells (HuH-7, HeLa, NIH 3T3, 293T, HCT116, and HepG2 cells) were transfected with an appropriate amount of reporter plasmid, p21-Luc, or its derivatives and either the empty parental vector or the DDX3 expression construct. The TK-Relina reporter (Promega) was also cotransfected for normalization of transfection efficiency. Transfected cells were harvested at 48 hours posttransfection and a dual luciferase assay was done according to the instruction of the manufacturer. The luciferase activity was measured with an AutoLumat LB953 luminometer (Berthold) and normalized with the cotransfected Relina activity.

Determination of growth rate in DDX3-producing stable clones. HuH-7 and HeLa cells were transfected with pcDNA-SR α /FLAG-DDX3 or parental vector pcDNA-SR α /FLAG and selected with G418 (400 μ g/mL) for 3 to 4 weeks. Independent clones with exogenous DDX3 expression were isolated and maintained in G418 (400 μ g/mL)-containing medium. To compare the growth rate between DDX3-producing cells and control cell lines, cells were seeded at a density of 1.0×10^5 onto 6-cm plates. At each 24-hour interval, the cells were collected, stained with trypan blue, and counted with a hemocytometer. At least two independent experiments were done.

In vitro pull-down assay. GST, GST/Sp1 fusion proteins were purified as previously described (29). For the *in vitro* GST pull-down assay, 20 μ L of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) with immobilized GST or GST/Sp1 fusion proteins (4 μ g each) were incubated with HuH-7 whole-cell extracts (500 μ g) at 4°C for 6 hours. The beads were extensively washed. Proteins bound to the beads were then analyzed by Western blotting using anti-DDX3 polyclonal antibody.

In vivo coimmunoprecipitation assay. For the coimmunoprecipitation experiments, HuH-7 cells transfected with pcDNA-SR α /FLAG-Sp1 were harvested and cell lysates were prepared using immunoprecipitation lysis buffer [20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, and 1% Triton X-100]. Cell extracts (1.5 mg) were incubated with 15 μ L of anti-FLAG M2-agarose affinity gel (Sigma-Aldrich, St. Louis, MO). After

extensive washing with immunoprecipitation lysis buffer, the immunoprecipitated proteins were analyzed by immunoblotting using specific antibodies against DDX3 and FLAG epitope (ab1257, Abcam, Cambridge, United Kingdom).

Cancer profiling array. The cancer-profiling array II (BD Biosciences Clontech, Palo Alto, CA) includes normalized cDNA from 154 tumor specimens and corresponding normal tissues from individual patient. To address the mRNA expression profile of *DDX3* in these tissues, a 0.7-kb ³²P-labeled cDNA probe spanning the 3'-coding region (400 bp) and 3'-untranslated region of *DDX3* (300 bp) was generated by the Rediprime II probe labeling kit (Amersham Pharmacia Biotech) and used to probe the cancer profiling array according to the user instruction.

Quantitative real-time PCR. cDNAs prepared from 45 adjacent normal-tumor paired hepatoma samples were provided by different sources (30, 31) and used in quantitative RT-PCR assay. mRNA expression of *DDX3* and *p21^{waf1/cip1}* in adjacent normal liver tissues and HCC samples was examined using quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Applied Science, Mannheim, Germany) according to the instruction manual using the primer set DDX3-4987F, TTCTCAGATGT-TTGTGTGTGGATT; DDX3-5142R, AAACCTTGCTCAAATGCTATTGCTG; p21-F, AATCCCAGCTACTTGAAGGC; and p21-R, GCTGACTGCAACCTC-TGCC. Ribosomal protein S24 (RPS24) gene served as an internal control for quantitation using the primer set TGGCTTTGGCATGATTATGAT and CTTTTTGCCAGCACCAACATT. Real-time detection of the emission intensity of SYBR Green was done with a LightCycler 2.0 instrument (Roche Applied Science). Each experiment was repeated in triplicate, including a no-template negative control.

Identification of p53 mutations in HCC specimens. To determine the *p53* status in individual HCC samples, cDNA templates (1 µg) from 45 HCC samples were mixed with the primer set p53-1-F, ATGGAGGAGCCGAGT-CAG, and p53-1181-R, TCAGTCTGAGTCAGGCCCTTC, and high-fidelity Easy-A Hi-Fi PCR cloning enzyme (Stratagene) for PCR reactions. Full-length *p53* cDNA fragments were subsequently cloned with pGEM-T easy vector system (Promega) to generate *p53* constructs. At least 10 constructs of each individual HCC sample were sequenced and mutations appearing in more than five constructs were considered as somatic mutations of the *p53* gene, not PCR-induced errors.

Tumor specimens. Tissue specimens were obtained commercially from different sources. Human hepatoma tissues were collected from Cybrdi (Frederick, MD; liver carcinoma tissue array, CC03-02-001), AccuMax (New York, NY; liver cancer tissues-hepatocellular carcinoma array, A204), and SuperBioChips Laboratories (Seoul, South Korea; human liver cancer array, CSA2). Human normal epidermis and cutaneous SCC samples were collected from SuperBioChips Laboratories (skin cancer array CX-1 and HA1 array). All of the tissue arrays are formalin-fixed, paraffin-embedded, and 4-µm-thick sections and are pathologically confirmed.

Immunohistochemistry. Tissue sections were placed in 10 mmol/L citric acid buffer (pH 6.0) and subjected to antigen retrieval by microwaving for 20 minutes after deparaffinization and rehydration. After incubation with 3% hydrogen peroxide for 10 minutes and 0.2% horse serum for 20 minutes, a 1:200 diluted rabbit anti-DDX3 polyclonal antibody was applied for 1 hour at room temperature and followed by incubation with biotinylated linker and streptavidin-horseradish peroxidase (LSAB2 system-HRP, DAKO Cytomation, Carpinteria, CA) for 30 and 10 minutes, respectively. The signals were visualized using AEC⁺ (DAKO Cytomation) as the substrate-chromagen at room temperature for 13 minutes. Sections were counterstained with hematoxylin and mounted. A semiquantitative evaluation was used to score the immunoreactivity. For analysis of the normal-tumor paired HCC samples, the staining intensity was compared directly between the tumor part and the normal part. Assessment of the DDX3 staining in the normal skin and cutaneous SCC specimens was done in two ways. Specifically, the percentage of nuclear immunoreactive cells was graded into five categories: 0% to 10% cells stained, 11% to 25% cells stained, 26% to 50% cells stained, 51% to 75% cells stained, and 76% to 100% cells stained. The intensity of nuclear staining was divided into four levels: negative, slight, moderate, and strong. In addition, a χ^2 test was used to analyze the loss of nuclear

localization in SCC specimens. Differences with $P < 0.05$ were accepted as statistically significant.

Results

DDX3 exerts an inhibitory effect on cell growth. To explore the biological function of DDX3 on cell growth control, a colony formation assay was done in several tumor cell lines (HuH-7, HCT116, and HeLa) and normal murine fibroblast cells (NIH 3T3) under the selection of G418. As shown in Fig. 1A, forced expression of DDX3 inhibited colony formation activity of HuH-7 and NIH 3T3 cells in a dose-dependent manner and a strong suppression of colony formation was also observed in HeLa and HCT116 cells. This inhibition of colony formation was due to the growth-suppressive ability of DDX3 on tumor cells but was not a result of the inhibitory effect of DDX3 on the expression of G418 resistance gene because DDX3 up-regulated, but not down-regulated, the SV40 early promoter that directs G418 resistance gene expression (data not shown). Moreover, we also established DDX3-overproducing clones in HuH-7 and HeLa cells (Fig. 1B, top) and determined their growth rates by cell counting. As shown in Fig. 1B (bottom), the growth rates of DDX3-overproducing clones, including HuH-7/DDX3#6, HuH-7/DDX3#7, HuH-7/DDX3#8, HeLa/DDX3#9, and HeLa/DDX3#14, were much slower than the control cell line harboring parental vector without DDX3. This may account for the inhibition of colony formation by DDX3. Taken together, these results indicate that DDX3 is a negative regulator of cell growth control.

DDX3 up-regulates the *p21^{waf1/cip1}* gene expression and inhibits the colony formation activity of tumor cells in a *p21^{waf1/cip1}*-dependent manner. Recently, several RNA helicases, such as RNA helicase A and CHAMP, have been shown to modulate the expression of cell cycle regulator *p16^{INK4a}* or *p21^{waf1/cip1}* (32, 33). To address the mechanism of growth inhibition by DDX3, we examined whether the promoter activity and the expression level of the cell cycle regulator *p21^{waf1/cip1}* are regulated by DDX3. As shown in Fig. 2A, exogenous expression of *DDX3* in various cell lines led to a 2- to 4-fold up-regulation of *p21^{waf1/cip1}* promoter-driven luciferase activity. Additionally, Western blot analysis indicated that the expression level of *p21^{waf1/cip1}* gene was also enhanced by the ectopic expression of *DDX3* in HuH-7, HeLa, and 293T cells (Fig. 2B). Because DDX3 up-regulates the promoter activity and the expression level of *p21^{waf1/cip1}* gene without cell type specificity, this suggests that DDX3 acts as a general regulator of *p21^{waf1/cip1}* gene expression.

To address the essential role of *p21^{waf1/cip1}* in DDX3-mediated colony-suppressing activity, a colony formation assay was also carried out in HCT116 and its derived cell line HCT116 *p21^{-/-}* that is deficient in *p21^{waf1/cip1}* expression. As shown in Fig. 2C, forced expression of DDX3 in HCT116 cells led to an 85% reduction of colony formation, whereas in HCT116 *p21^{-/-}* cells this DDX3-mediated suppression of colony formation was dramatically decreased to 20%. This result strongly suggests that DDX3 exerts its suppressing activity on colony formation in a *p21^{waf1/cip1}*-dependent manner.

The ATPase activity, but not the helicase activity, of DDX3 contributes to DDX3-mediated transcriptional modulation on the *p21^{waf1/cip1}* promoter. Because DDX3 harbors ATPase and RNA unwinding activities, we are interested to know whether these two enzymatic activities are required for the transcriptional regulatory effect of DDX3 on *p21^{waf1/cip1}* promoter. To address this issue, we generated mutations within the DEAD box or SAT motif

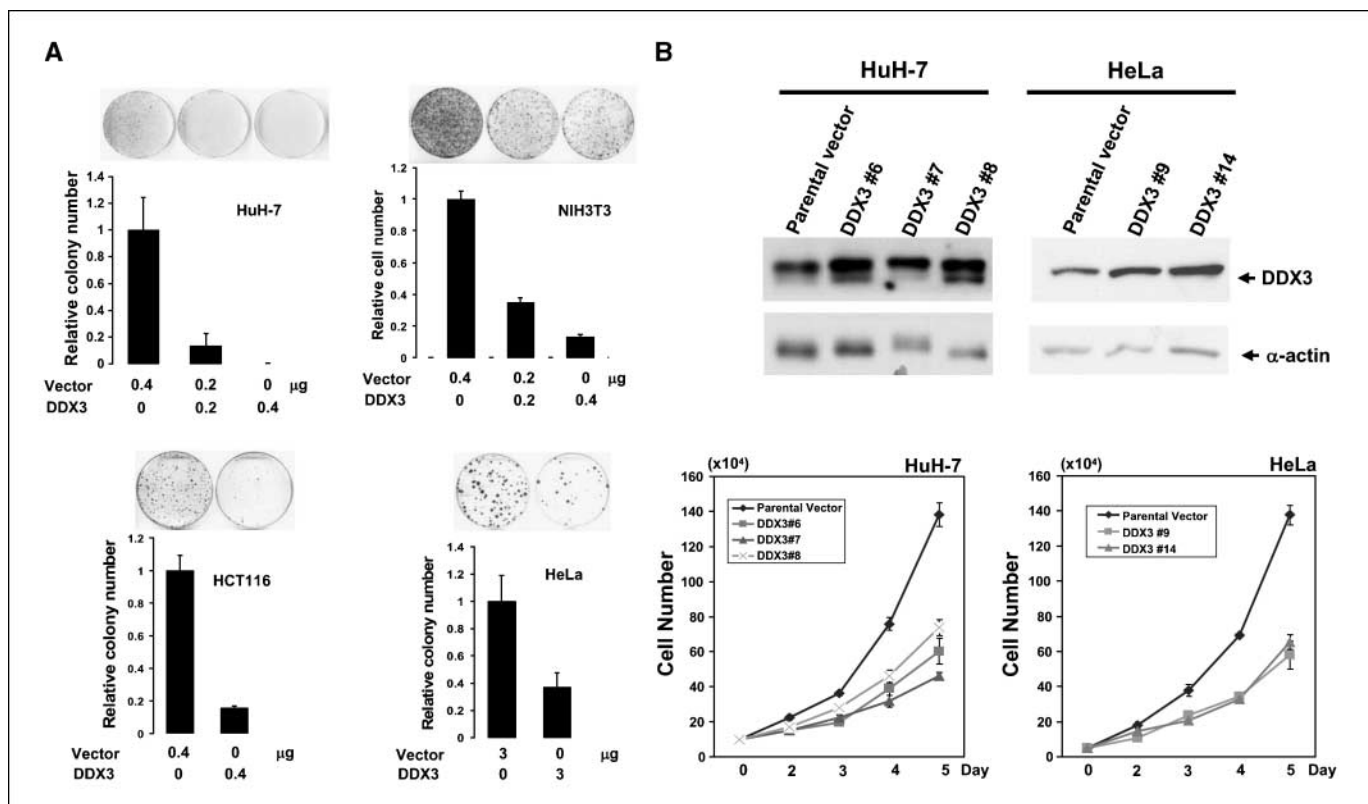


Figure 1. Ectopic expression of *DDX3* inhibits cell growth. **A**, *DDX3* inhibited the colony formation ability of several tumor and normal murine fibroblast cells. HuH-7, HeLa, HCT116, or NIH 3T3 cells were transfected with parental vector or FLAG-*DDX3* expression plasmid at the indicated amount by the Effectene or calcium phosphate method (see Materials and Methods). The total amount of transfected DNA was kept constant by adding control vector. Transfected cells were selected with G418 for 2 to 4 weeks. Colonies were then fixed, stained with crystal violet, and counted. In all cases, the relative colony number in the absence of FLAG-*DDX3* transfection was arbitrarily assigned as one. Results were derived from at least three independent experiments done in triplicate. **B**, *top*, expression levels of *DDX3* in various *DDX3*-overexpressing HuH-7 and HeLa cell lines and parental cells were examined by immunoblotting with anti-*DDX3* specific antibody. *Bottom*, different clones of *DDX3*-overproducing cells were seeded and the numbers of viable cells were counted at various time intervals (see Materials and Methods). Results were derived from at least two independent experiments done in triplicate.

The conversion of DEAD motif to DQAD sequence impairs both ATPase activity and ATPase-dependent helicase activity (34). On the other hand, a point mutation of the SAT motif to AAA sequence leads to a loss of RNA unwinding activity but retains the ATPase activity of the helicase (34). Using these two mutants, we are able to distinguish which activity is required for the transcriptional activation of *DDX3* on *p21^{waf1/cip1}* promoter. To this end, increasing amounts of expression constructs of *DDX3*/DQAD, *DDX3*/AAA and wild-type *DDX3* were cotransfected with *p21^{waf1/cip1}* promoter-driven luciferase reporter. Interestingly, our data revealed that at a higher dosage (2 μg/well), the wild-type *DDX3* and mutant *DDX3*/AAA up-regulated the activity of *p21^{waf1/cip1}* promoter ~12-fold whereas the *DDX3*/DQAD mutant only activated the *p21^{waf1/cip1}* promoter activity ~5-fold (Supplementary Fig. S1). Because a similar expression level of these three *DDX3* expression constructs was detected (data not shown), their differential induction of *p21^{waf1/cip1}* promoter activity suggests that the ATPase activity, but not the RNA helicase activity, contributes to the transcriptional activation of *DDX3* on the *p21^{waf1/cip1}* promoter.

***DDX3* transactivates the *p21^{waf1/cip1}* promoter through multiple Sp1 sites.** Next, to examine the underlying mechanism by which *DDX3* activates the *p21^{waf1/cip1}* promoter, we mapped the *DDX3* responsive region within the *p21^{waf1/cip1}* promoter by serial 5'-deleted promoter-driven luciferase reporters (Fig. 3A and Supplementary Fig. S2A). The results suggested that the levels of

activation induced by *DDX3* of the luciferase activity of serial reporter constructs from (–1,002/+8)*p21*-Luc to (–123/+8)*p21*-Luc were comparable with the level of activation elicited by the full-length promoter-driven reporter *p21*-Luc (5- to 10-fold; Fig. 3B and Supplementary Fig. S2B). However, this *DDX3*-induced activation was dramatically decreased in reporter (–84/+8)*p21*-Luc and (–76/+8)*p21*-Luc by ~2- to 3-fold and was completely lost in (–63/+8)*p21*-Luc and (–56/+8)*p21*-Luc reporters (Fig. 3B). This observation suggested that the *DDX3* responsive region within the *p21^{waf1/cip1}* promoter was located between –123 to –63 upstream of the transcription start site. This conclusion is further supported by another reporter assay, which was directed by a *p21^{waf1/cip1}* promoter lacking the –127 to –63 nucleotide region (Fig. 3C).

As noted, several regulatory elements including six Sp1 sites, two E2F sites, and one AP-2 site reside within the *DDX3* responsive region of the *p21^{waf1/cip1}* promoter (17). To delineate which element is essential for the transactivation of *DDX3* on *p21^{waf1/cip1}* promoter, *p21^{waf1/cip1}* promoter-directed reporter plasmids harboring single mutation within Sp1-1, Sp1-2, Sp1-3, Sp1-4, Sp1-5/6, E2F(b)-1, E2F(b)-2, and AP-2 sites (see Supplementary Fig. S3A) were assayed for luciferase activity in the presence or absence of *DDX3*. Interestingly, our data indicated that the induction of reporters destroying AP-2, E2F, and each individual Sp1 site was comparable with that of wild-type reporter (Supplementary

Fig. S3B and C). However, for those reporters harboring more than two Sp1 sites mutations, the DDX3-mediated induction of reporter activity was decreased compared with the wild-type reporter, and this decreased level was proportional to the numbers of Sp1 sites destroyed (Fig. 3D). All together, our results indicate that DDX3 transactivates $p21^{waf1/cip1}$ promoter through multiple Sp1 sites.

Functional cooperation and physical interaction between DDX3 and Sp1. It is well known that Sp1 and Sp3 can transactivate the $p21^{waf1/cip1}$ promoter through Sp1 sites (35). To examine whether DDX3 transactivates the $p21^{waf1/cip1}$ promoter activity through Sp1 site-interacting proteins, the Sp1 or Sp3 expression plasmid together with the DDX3 expression construct and the $p21^{waf1/cip1}$ promoter-driven luciferase reporter were introduced into HuH-7 cells. As shown in Fig. 4A, transient expression of DDX3 alone resulted in a

4-fold activation of $p21^{waf1/cip1}$ promoter activity whereas transiently expressed Sp1 or Sp3 of increasing amount led to a 1.6- to 1.8-fold activation of the $p21^{waf1/cip1}$ promoter. Interestingly, the induction effect of DDX3 on the $p21^{waf1/cip1}$ promoter was increased to 10-fold when coexpressing with Sp1; however, this coactivation effect was not found when coexpressing with Sp3 (Fig. 4A). This result strongly suggests that Sp1, but not Sp3, is involved in the transcriptional regulation of DDX3 at the $p21^{waf1/cip1}$ promoter.

The functional cooperation between DDX3 and Sp1 proteins strongly implies the physical interactions of these two factors. To test this possibility, an *in vitro* GST fusion protein pull-down assay was done. As shown in Fig. 4B, endogenous DDX3 protein was pulled down by GST-Sp1 but not by GST control beads, suggesting a specific interaction between DDX3 and Sp1 *in vitro*. The *in vivo*

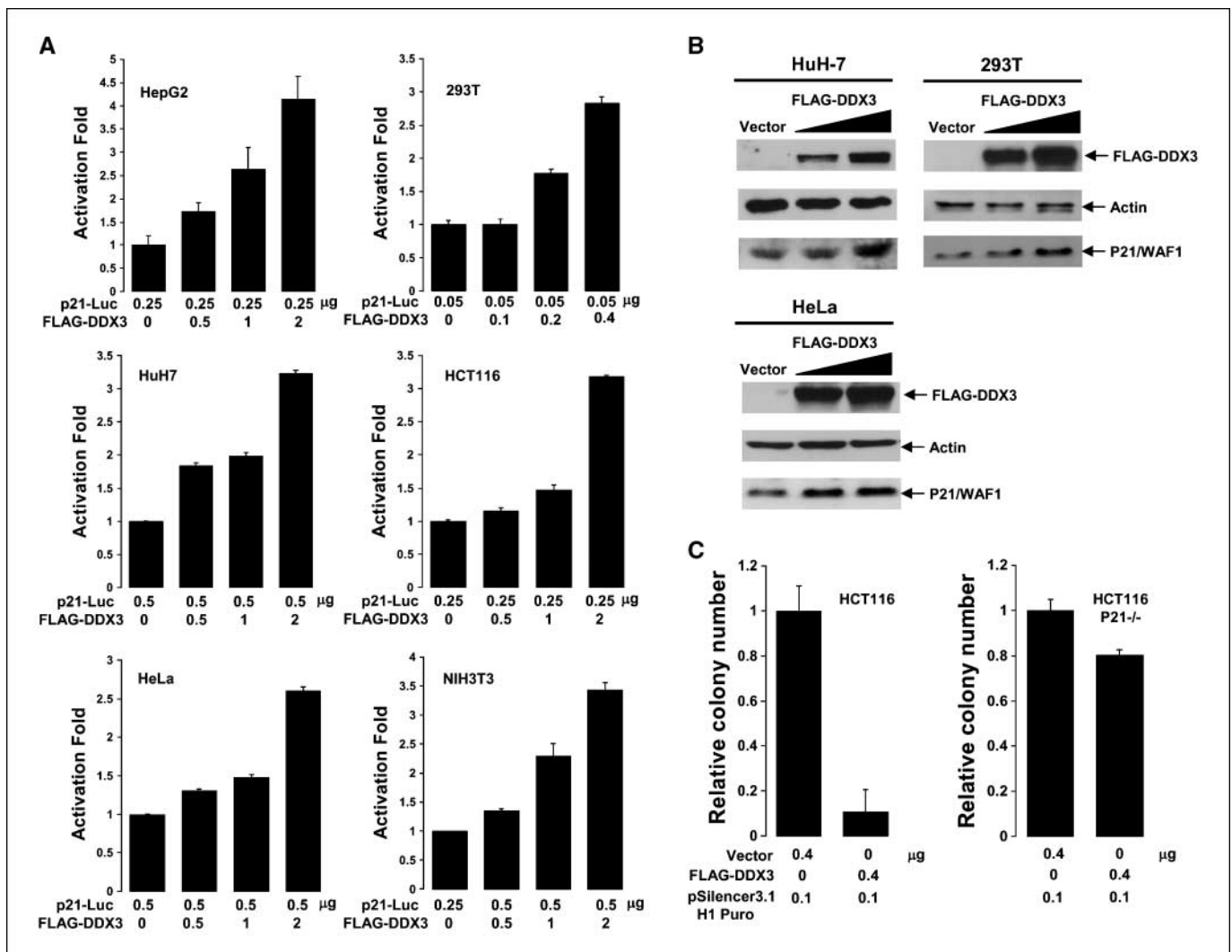


Figure 2. $p21^{waf1/cip1}$ is a target gene of DDX3. A, DDX3 up-regulates the $p21^{waf1/cip1}$ promoter activity. $p21^{waf1/cip1}$ promoter-driven luciferase reporter (p21-Luc; 0.05-0.5 μg) and increasing amount (0.1-2 μg) of FLAG-DDX3 expression construct were cotransfected into various cell lines as indicated. The total amount of transfected DNA was kept constant by adding control vector (pcDNA3-SRα/FLAG). Luciferase activity was measured at 48 hours posttransfection. In all cases, the relative luciferase activity shown is presented as fold activation relative to the control transfection and is derived from at least three independent experiments done in triplicate. B, the expression of endogenous $p21^{waf1/cip1}$ is enhanced by forced expression of DDX3. HuH-7, HeLa, and 293T cells were transfected with increasing amount (10 or 20 μg) of FLAG-DDX3 expression plasmid. After 48 hours of transfection, cell extracts were prepared and the expression level of FLAG-DDX3, $p21^{waf1/cip1}$, and α -actin was detected by immunoblotting with anti-FLAG, anti- $p21^{waf1/cip1}$, and anti- α -actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The expression level of α -actin was served as a loading control. C, DDX3 exerts the colony suppressive effect through a $p21^{waf1/cip1}$ -dependent mechanism. HCT116 and HCT116 $p21^{-/-}$ cells were cotransfected with pSilencer 3.1-H1 puro and pcDNA-SRα/FLAG-DDX3 (FLAG-DDX3) or its parental vector at indicated amount by Effectene and selected with puromycin for 14 days. Colonies were fixed, stained with crystal violet, and the colony number was counted. Relative colony number in the absence of FLAG-DDX3 transfection was arbitrarily assigned as one. Results were derived from at least two independent experiments done in triplicate.

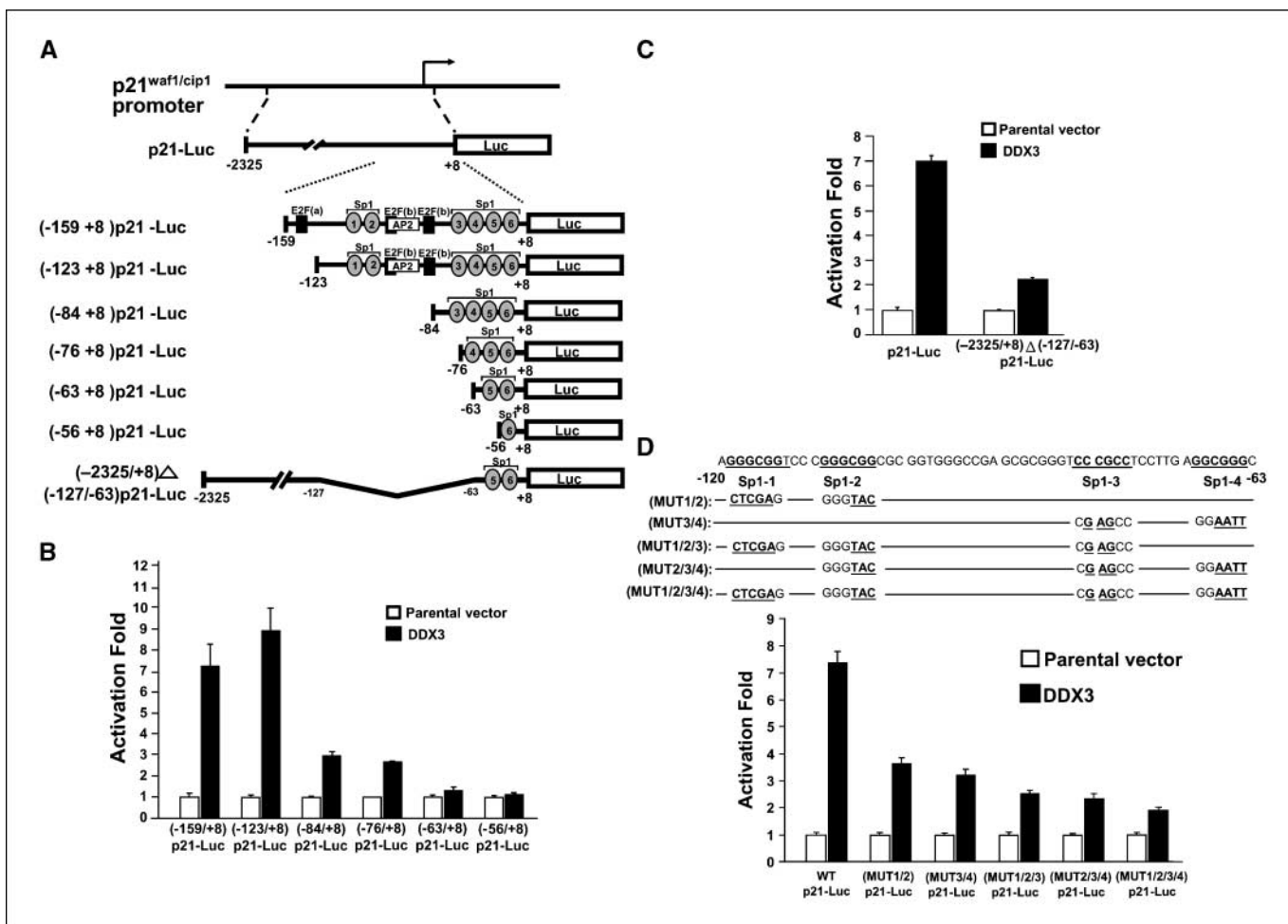


Figure 3. Multiple Sp1 sites (Sp1-1, Sp1-2, Sp1-3, and Sp1-4) located at -123 to -63 related to transcription start site are essential for the transactivation of DDX3 on *p21^{waf1/cip1}* promoter. **A**, schematic representation of serial *p21^{waf1/cip1}* promoter-driven luciferase reporters used in transactivation assay: p21-Luc contains the 2.3-kb full-length of *p21^{waf1/cip1}* promoter (from -2,326 to +10 nucleotide related transcription start site); its derivatives (-159/+8)p21-Luc, (-123/+8)p21-Luc, (-84/+8)p21-Luc, (-76/+8)p21-Luc, (-63/+8)p21-Luc, and (-56/+8)p21-Luc contain a series of deleted promoters of *p21^{waf1/cip1}*. (-2,326/+8) Δ (-127/-63)p21-Luc is also a derivative of p21-Luc reporter containing the full-length *p21^{waf1/cip1}* promoter lacking -127 to -63 region. **B** and **C**, HuH-7 cells were cotransfected with a *p21^{waf1/cip1}* promoter-driven reporter (p21-Luc) or its derivatives represented in (A) (0.25 μ g of each) together with DDX3 expression construct (pcDNA3-SR α /FLAG-DDX3) or control vector (pcDNA3-SR α /FLAG; 2 μ g). Luciferase assay was done at 48 hours posttransfection. The fold transactivation of the various *p21^{waf1/cip1}* promoter-directed reporter constructs by DDX3 is shown as fold activation relative to the control transfection and is derived from at least three independent experiments done in triplicate. **D**, analysis of the transactivation effect of DDX3 on *p21^{waf1/cip1}* promoters mutated at multiple Sp1 elements. **Top**, schematic representation of generated mutations within DDX3 responsive region of the human *p21^{waf1/cip1}* promoter. Nucleotide substitutions introduced into the regulatory elements of *p21^{waf1/cip1}* promoter region in mutant constructs are shown in boldface and underlined. The DDX3-mediated transactivation of *p21^{waf1/cip1}* promoter driven by mutant constructs defective in two Sp1 sites (MUT1/2 and MUT3/4), three Sp1 sites (MUT1/2/3 and MUT2/3/4), or four Sp1 sites (MUT1/2/3/4) was measured as described in (B and C).

interaction between DDX3 and Sp1 was further examined by a coimmunoprecipitation assay. As shown in Fig. 4C, transiently expressed FLAG-Sp1 and endogenous DDX3 were coimmunoprecipitated by anti-FLAG antibody, suggesting that DDX3 could interact with Sp1 *in vivo*. Thus, the *in vitro* and *in vivo* binding analyses clearly show that there is an interaction between DDX3 and the transcription factor Sp1.

The expression or subcellular localization of DDX3 is altered in tumor cells. Because DDX3 exhibits tumor suppressor functions, it is interesting to know whether DDX3 is inactivated in tumor cells by alteration of gene expression. To address this issue, a DDX3-specific DNA probe was hybridized against the cancer-profiling array II (see Materials and Methods). Interestingly, the mRNA expression of DDX3 was declined in the liver tumor specimens (three of three samples) and SCC of the vulva (three of five samples; Supplementary Fig. S4). To further confirm the

alteration of DDX3 expression in cancer specimens, the expression profiles of DDX3 were examined in normal-tumor paired HCC samples by immunohistochemical staining analysis using specific anti-DDX3 antibody. As shown in Fig. 5A, decreased expression of DDX3 at the protein level was found in 73% of HCC from 26 patients. Additionally, the mRNA expression of DDX3 was also examined by quantitative RT-PCR, and our data revealed that the DDX3 mRNA level was reduced in 57.8% of 45 HCC specimens (Fig. 5A and B). These results clearly indicate that the expression of DDX3 is down-regulated in liver tumor cells. Furthermore, we also delineated the relationship between DDX3 and *p21^{waf1/cip1}* mRNA expression in these HCC cases by quantitative RT-PCR (Fig. 5B). Our result showed that, among 26 cases with decreased DDX3 expression status, 20 cases (77%) also showed simultaneous *p21^{waf1/cip1}* down-regulation, and the correlation between the expression status of DDX3 and *p21^{waf1/cip1}* was statistically

significant ($P = 0.039$; Fig. 5C). Notably, a positive correlation between the mRNA expression levels of *DDX3* and *p21^{waf1/cip1}* in 45 HCC specimens was also found by linear regression analysis ($r = 0.501$, $P < 0.001$; Fig. 5D). Most interestingly, among those 20

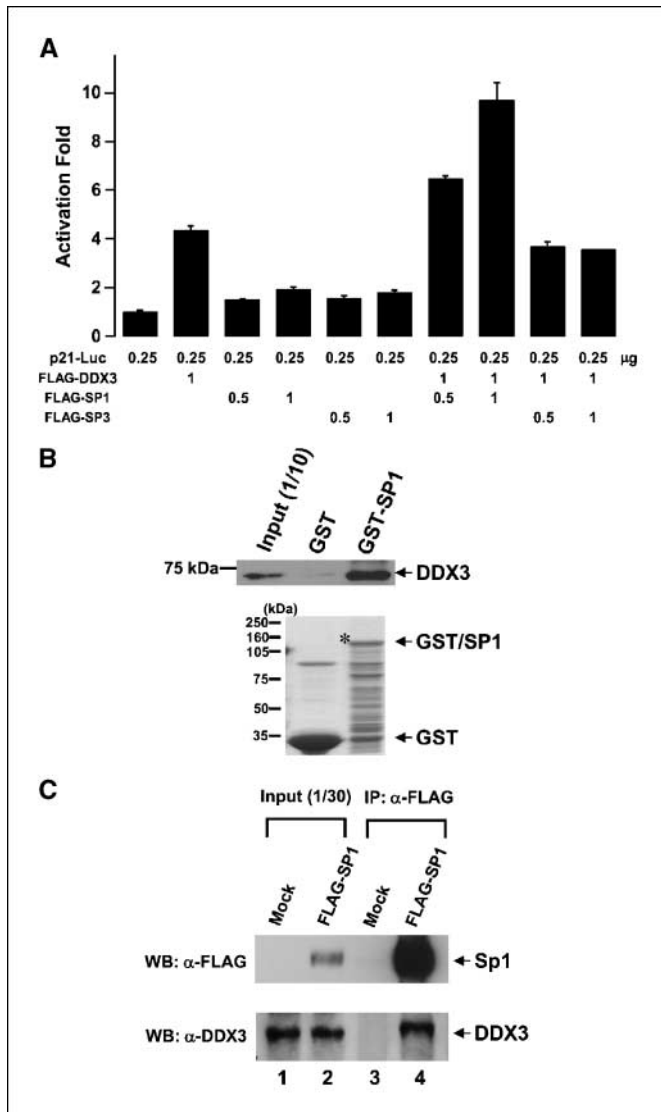


Figure 4. DDX3 interacts with Sp1 physically and functionally. **A**, functional interaction between DDX3 and Sp1 on *p21^{waf1/cip1}* promoter. HuH-7 cells were cotransfected with FLAG-DDX3, FLAG-Sp1, FLAG-Sp3 expression construct, and p21-Luc reporter as indicated. The total amount of plasmid DNA was kept constant (2.25 μg) by the addition of empty vector in each transfection. The luciferase assay was done at 48 hours posttransfection. The fold transactivation of the *p21^{waf1/cip1}* promoter-driven reporter elicited by DDX3 alone or together with Sp1 or Sp3 is presented as fold activation relative to the control transfection and is derived from at least three independent experiments done in triplicate. **B**, DDX3 interacts with Sp1 *in vitro*. GST and GST-Sp1 fusion proteins were purified and analyzed by SDS-PAGE and Coomassie blue staining (bottom). Asterisk, purified GST-Sp1 fusion protein. Total cell lysates of HuH-7 cells were incubated with GST or GST-Sp1 fusion protein–prebound glutathione-Sepharose 4B beads and the bound fractions were subjected to immunoblotting with antibody against DDX3 (top). Lane 1, input control (10% of cell extract without any treatment). **C**, Sp1 interacts with DDX3 *in vivo*. HuH-7 cells were transfected with 20 μg of FLAG-Sp1 expression construct (lanes 2 and 4). HuH-7 cell extracts (lane 3) or FLAG-Sp1-containing cell extracts (lane 4; 1.5 mg each) were immunoprecipitated with anti-FLAG antibody–conjugated beads and the immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG antibody or anti-DDX3 antibody. Lane 1, input control for mock transfection; lane 2, input control for FLAG-Sp1 expression construct transfected cells.

cases with *DDX3* and *p21^{waf1/cip1}* reduced status, 13 cases harbored wild-type *p53* and 7 had *p53* mutation (Fig. 5B). Thus, it seems that the decreased *DDX3* expression in HCC samples leads to a reduction of *p21^{waf1/cip1}* expression irrespective of *p53* status.

Next, the expression of *DDX3* in SCC patients was examined. Several cutaneous SCC and their matched normal skin specimens were stained with *DDX3* antibody. Our results revealed that among the *DDX3*-positive cells of normal epidermis, *DDX3* was localized mainly in the nuclei and less in the cytoplasm (Fig. 6A). In contrast, in SCC cells, the nuclear distribution of *DDX3* was significantly decreased or completely absent, and *DDX3* was predominantly detected in the cytosolic compartments instead (Fig. 6A). To further confirm this observation, the intensity of nuclear staining and the percentage of nuclear positive cells obtained from immunohistochemistry analysis of 17 normal epidermis and 34 SCC specimens were analyzed statistically. As shown in Fig. 6B, both the nuclear staining intensity of *DDX3* and the percentage of cells positive in *DDX3* nuclear staining were much higher in normal epidermis as compared with neoplastic squamous cells (normal versus SCC, 94% versus 18% cases for strong and moderate grade intensity; 94% versus 12% cases with >50% *DDX3* nuclear staining positive cells; $P < 0.001$). This observation indicates that the loss of *DDX3* nuclear localization is a frequent event in cutaneous SCC. Together, our results suggest that the decreased expression or loss of nuclear localization of *DDX3* may play a regulatory role in the formation of liver tumor and cutaneous SCC.

Discussion

DEAD box RNA helicases participate in disparate cellular functions (36). Here, we have shown by a colony formation assay that *DDX3* RNA helicase harbors a growth-suppressive property. Forced expression of *DDX3* leads to the suppression of colony formation activity of HCC, cervical carcinoma, colon cancer, and murine fibroblast cells (see Fig. 1A). Furthermore, the growth rate is delayed by ectopic expression of *DDX3* (see Fig. 1B). These results support the notion that *DDX3* plays a negative role in growth regulation.

In this study, we also identify *p21^{waf1/cip1}* as a target gene of *DDX3*, and the up-regulation of *p21^{waf1/cip1}* expression is linked to the growth-suppressive effect exerted by *DDX3* (see Fig. 2). *DDX3* enhances *p21^{waf1/cip1}* gene expression through up-regulation of promoter activity of *p21^{waf1/cip1}*. Because the activation of *p21^{waf1/cip1}* promoter activity was shown in various cell lines with diverse *p53* status (*p53* wild-type in HepG2, HCT116, and NIH 3T3; *p53* mutation in HuH-7; *p53* degraded by human papillomavirus E7 in HeLa; *p53* inactivated by SV40 large T antigen in 293T), this *DDX3*-mediated up-regulation on *p21^{waf1/cip1}* promoter seems to be *p53* independent. Supporting this notion is the fact that the *DDX3*-induced activation level of serial 5'-deleted *p21^{waf1/cip1}* promoter-directed reporters that lack two *p53* responsive elements (at the -2,301 and -1,394 nucleotide region upstream of the transcription start site; ref. 17) is comparable with the full-length *p21^{waf1/cip1}* promoter-driven reporter, p21-Luc (see Supplementary Fig. S2B). Moreover, in this work, the *DDX3*-responsive region is delineated to 60 bp (between -123 and -63) of the *p21^{waf1/cip1}* proximal promoter region (see Fig. 3B), and the integrity of multiple tandem Sp1 sites in this region is required for the activation induced by *DDX3* (see Fig. 3C). This requirement of multiple Sp1 sites to confer *DDX3*-mediated transactivation of the *p21^{waf1/cip1}* promoter is

different from other stimuli targeting to this GC-rich responsive region of the $p21^{waf1/cip1}$ promoter. For example, TGF- β and lovastatin target to the Sp1-3 site (17) whereas both of Sp1-3 and Sp1-4 sites are required for the action of the histone deacetylase inhibitor suberoylanilide hydroxamic acid (22). Most interestingly, our data also reveal that, like other $p21^{waf1/cip1}$ promoter-regulating factors acting on this GC-rich responsive region, such as Smad3 (24), KLF6 (37), and c-Myc (38), DDX3 up-regulates $p21^{waf1/cip1}$ promoter through physical interaction with Sp1 (see Fig. 4).

Recently, several RNA helicases involving in transcriptional regulation have been reported. They may exert a transcriptional function or act as comodulators for other transcription factors. However, whether the enzymatic activities of the RNA helicase

(ATP hydrolysis and RNA unwinding) are required or contribute to the transcriptional modulation depends on individual case (39–43). Moreover, although several studies addressed the involvement of enzyme activity in the transcriptional regulation activities of certain RNA helicases, most of them do not distinguish precisely which activity is involved. For example, an ATPase- and/or helicase-dependent mechanism is essential for RNA helicase A-mediated transactivation function (41) and the functional interaction with nuclear factor κ B (42). This requirement also exists in functional interaction between RNA helicase DP103 and SF-1 (40). In our case, we show that the ATPase activity, but not the helicase activity, contributes to the DDX3-mediated transactivation (see Supplementary Fig. S1). Thus, a dual mechanism including the

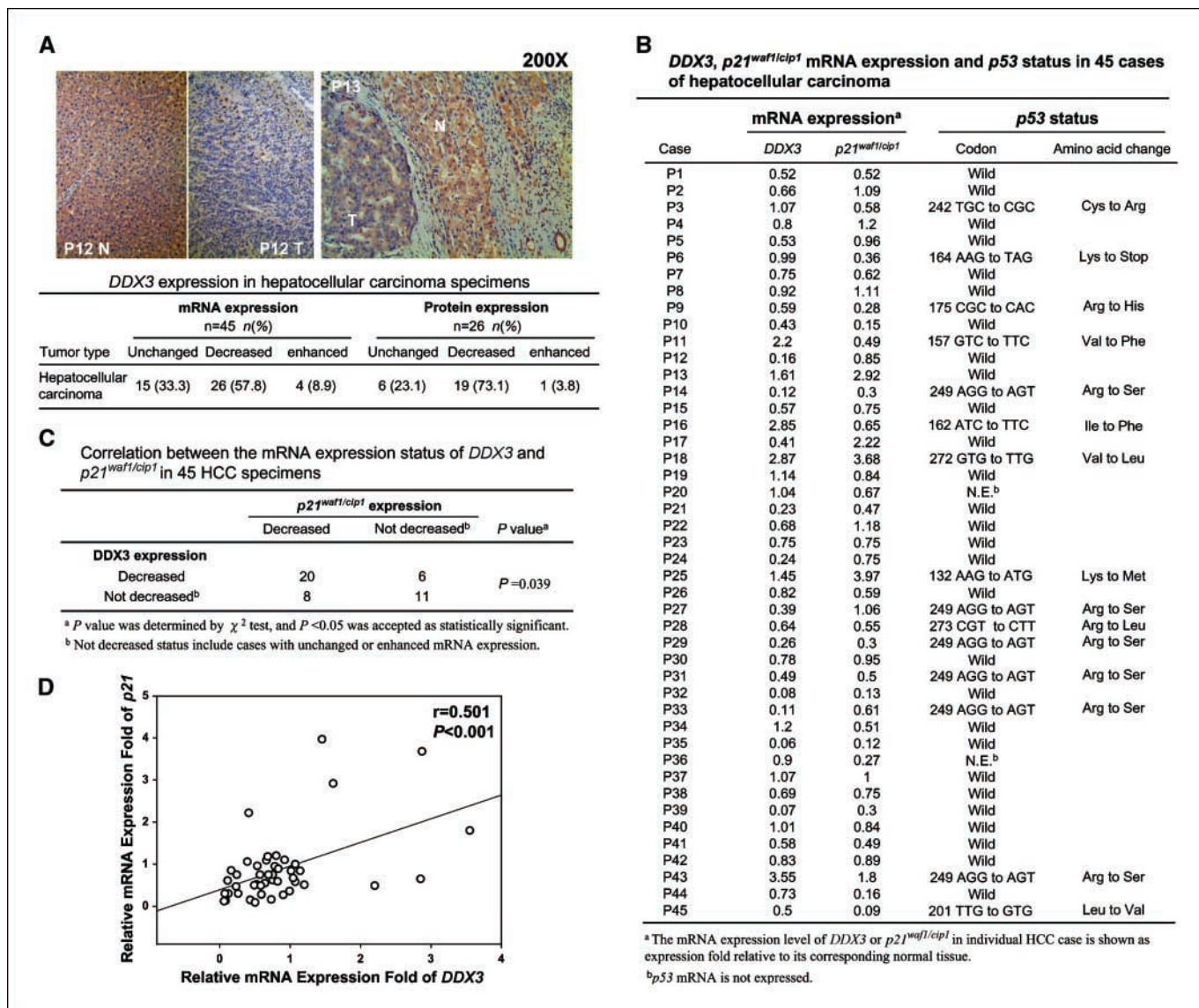
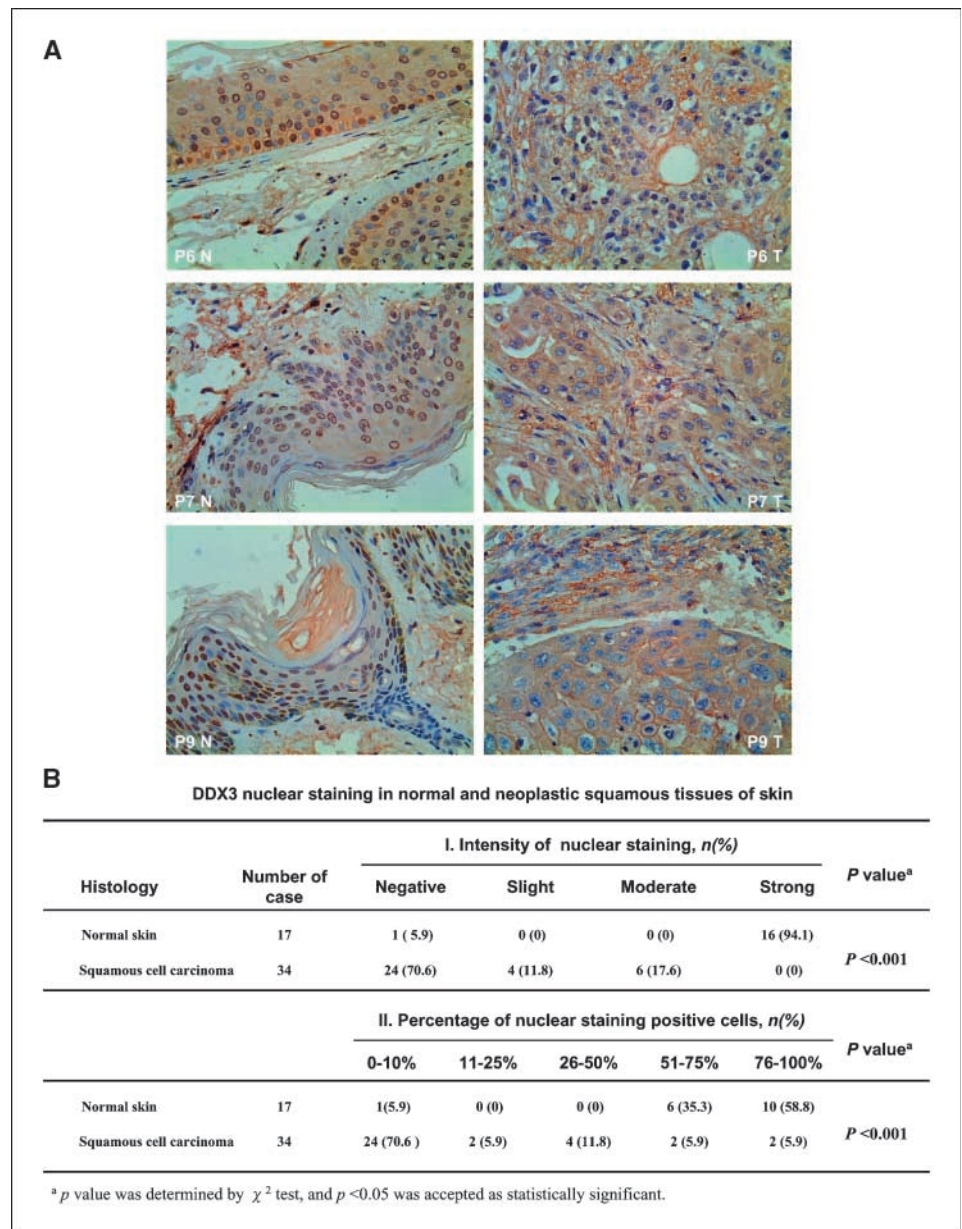


Figure 5. The expression of *DDX3* is altered in HCC. *A*, top, immunohistochemical staining of *DDX3* in HCC and paired adjacent normal liver tissues. *DDX3* was labeled with specific antibody (red stain) and nuclei were counterstained with hematoxylin (blue color). *N*, normal liver tissue; *T*, tumor. Bottom, *DDX3* expression in HCC specimens. Expression levels of *DDX3* mRNA in HCC samples as shown in (*B*) are classified into unchanged, decreased, and enhanced categories. At least 25% decrease or 2-fold increase in expression fold is considered as decreased or enhanced status, respectively. Protein expression levels of *DDX3* analyzed by immunohistochemical staining are also subjected to similar classification (see Materials and Methods). *B*, *DDX3*, $p21^{waf1/cip1}$ mRNA expression, and *p53* status in HCC. The mRNA expression profile of *DDX3* and $p21^{waf1/cip1}$ in 45 normal-tumor paired HCC samples was determined by quantitative real time-PCR (see Materials and Methods) and is shown as fold expression relative to its corresponding normal tissue. The *p53* status in HCC samples was determined as described in Materials and Methods. *C*, significant correlation between the mRNA expression status of *DDX3* and $p21^{waf1/cip1}$ in 45 HCC specimens. *D*, a positive correlation between the mRNA expression levels of *DDX3* and $p21^{waf1/cip1}$ in 45 HCC cases is found by linear regression analysis. $P < 0.05$ is considered significant.

Figure 6. The nuclear localization of DDX3 is altered in SCC. **A**, immunohistochemical staining of DDX3 in cutaneous SCC and paired normal skin. DDX3 was strongly immunolocalized in the nucleus and less intensively in the cytosol of squamous cells in normal skin, but the nuclear staining was lost or dramatically decreased in cutaneous SCC. *N*, normal skin; *T*, cutaneous SCC. **B**, DDX3 nuclear staining in normal and neoplastic squamous tissues of skin. The intensity of DDX3 nuclear staining and the percentage of DDX3 nuclear staining positive cells in normal skin and cutaneous SCC tissues are summarized.



involvement of ATP hydrolysis and recruitment of other transcription factors (e.g., Sp1) would seem to occur with DDX3-mediated transcriptional regulation.

Because DDX3 harbors growth-suppressive ability and transcriptional modulation activity of the *p21^{waf1/cip1}* promoter, in this work we further examined the deregulated expression of *DDX3* in tumors by a cancer profiling array and immunohistochemical staining analysis. Our results clearly indicate that the expression of *DDX3* is decreased in HCC and the subcellular localization of DDX3 is altered in cutaneous SCC (see Figs. 5 and 6). Cutaneous SCC is a clinically aggressive cancer, and the inactivation of *p21^{waf1/cip1}* has been shown to play a critical role in the formation of skin tumor in knockout mice models (14, 15). Because DDX3 harbors a dominant nuclear localization in normal squamous cells and exerts a transactivation function on *p21^{waf1/cip1}* promoter, presumably the inactivation of transcriptional modulation function of DDX3 through cytoplasmic mislocalization is

relevant to the deregulation of cell growth in cancerous squamous cells.

Recently, Huang et al. (44) have reported that the mRNA expression of *DDX3* is up-regulated in HCC, and they considered *DDX3* to be a promoter for hepatocarcinogenesis. However, according to our present study and recent report (45), both the mRNA and protein expressions of *DDX3* are down-regulated in 58% to 73% of HCC specimens (see Fig. 5A). Furthermore, a tendency that *DDX3* may regulate *p21^{waf1/cip1}* expression independently from p53 is also noted in HCC specimens (see Fig. 5B-D). This observation is consistent with our *in vitro* studies (see Figs. 2 and 3), which indicate that *DDX3* up-regulates *p21^{waf1/cip1}* promoter activity in a p53-independent manner. Taking into account that it acts as a positive regulator of *p21^{waf1/cip1}* expression and has growth-suppressive ability on liver cancer cells, presumably *DDX3* is a candidate tumor suppressor gene in liver. Support for this notion comes from several studies that have indicated the

involvement of the *p21^{waf1/cip1}* inactivation in hepatocellular carcinogenesis (46, 47).

During the multistep process of carcinogenesis, decreased expression of tumor suppressors is often caused by epigenetic modification or abnormal degradation (48, 49). Additionally, alterations of subcellular localization through aberrant splicing or genetic mutations also provide ways to inactivate tumor suppressor genes (50). In view of these, further work to delineate the deregulation of DDX3 expression or localization will shed light on diverse mechanisms for inactivation of DDX3 in different cancers. In conclusion, our study characterizes the biological function of *DDX3* as a putative candidate tumor suppressor gene, at least in HCC and cutaneous SCC. Most importantly, due to

the universal tumor growth-suppressive property of this gene in various kinds of neoplasm regardless of *p53* status, DDX3 is a potential therapeutic target for gene therapy.

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