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# Downregulation of Dkk3 activates β-catenin/TCF-4 signaling in lung cancer

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**Although the oncogenic role of the Wnt/β-catenin pathway is well defined, it remains unclear how this pathway is aberrantly activated in lung cancer. We found that Dickkopf (Dkk)-3, a member of Dkk family of Wnt antagonists, is frequently inactivated in lung cancer and plays a role in suppressing lung cancer cell growth through inhibition of β-catenin/T-cell factor (TCF)-4 signaling. *Dkk3* is the only *Dkk* family member abundantly expressed in normal lung, but silenced by promoter hypermethylation in a large fraction of lung cancer cell lines and lung tumors. Downregulation of Dkk3 was correlated with tumor progression and expression of nuclear β-catenin in lung tumors. Ectopic expression of Dkk3 in lung cancer cells with *Dkk3* hypermethylation induced apoptosis and inhibited TCF-4 activity as well as nuclear accumulation of β-catenin and expression of TCF-4 targets c-Myc and cyclin D1. Furthermore, small interference RNA knock down of *Dkk3* in cells lacking *Dkk3* hypermethylation was sufficient to promote cell proliferation, β-catenin nuclear translocation and expression of c-Myc. These observations suggested that epigenetic inactivation of *Dkk3* activates the Wnt/β-catenin pathway, thereby promoting the growth of lung cancer cells.**

## Introduction

The Wnt/β-catenin oncogenic pathway plays an important role in tumorigenesis (1–4). This pathway is almost invariably activated in colorectal cancer cells through inactivating mutations in *APC* tumor suppressor gene or activating mutations in *β-catenin* (5,6). Mutations and deletions in *β-catenin* coding sequence were identified in a variety of human tumors (1). β-catenin is normally kept in the cytoplasm where it is bound by APC and degraded through the ubiquitin/proteasome system (2). Upon binding of the Wnt ligands to coreceptors frizzled or low-density lipoprotein receptor-related protein 5/6, the degradation of β-catenin is inhibited, which allows β-catenin to translocate to the nucleus where it forms a transcriptional complex with the T-cell factor (TCF)/lymphoid enhancer factor family proteins, such as TCF-4 (7). Transactivation of several target genes, including *c-Myc* and *cyclin D1*, promotes cell proliferation and survival (8,9).

Wnt/β-catenin signaling has recently emerged to play a role in lung cancer, the most common form of cancer in the world (10). Lung cancer cells and lung tumors consistently express high levels of nuclear β-catenin, which cause activation of TCF-4 and overexpression of cyclin D1 (11,12). Overexpression of several other regulators of this pathway, such as Wnt and Dishevelled, was identified in non-small-cell lung cancer (NSCLC) cells (13,14). Activation of Wnt/β-catenin signaling can promote the survival and hyperproliferation of lung cancer cells (11,12,14). However, both *APC* and *β-catenin* mutations are very rare in lung cancer (15–17), suggesting different mechanisms underlying the aberrant activation of Wnt/β-catenin signaling in lung cancer.

Dickkopf (Dkk) family proteins, including Dkks1–4, are a group of secreted glycoproteins and antagonists of Wnt signaling (18,19). Dkk1, Dkk2 and Dkk4 interact with Wnt coreceptors low-density lipoprotein receptor-related protein 5/6, which disrupt their bindings to the Wnt ligands (18). Dkk family members, in particular Dkk3, were shown to be downregulated in a variety of tumor cells, such as those from hepatocellular carcinoma, lymphoblastic leukemia, prostate cancer, renal cell carcinoma and melanoma (19–25). Hypermethylation of *Dkk1* and *Dkk3* promoters was detected in several types of tumors (23,24,26–30). However, the physiological relevance of altered expression of Dkk family proteins in tumor formation remains unclear. Furthermore, the functional role of Dkk3 in Wnt/β-catenin signaling has been obscure. While earlier studies suggested that Dkk3 does not affect Wnt signaling (18,31,32), recent studies demonstrated that Dkk3 can reduce cytoplasmic accumulation of β-catenin in Saos-2 osteosarcoma cells (33) and inhibit TCF-4 activity in PC12 rat pheochromocytoma cells (34).

In this study, we identified *Dkk3* as one of the most significantly downregulated genes in lung cancer. Pathological and functional studies indicated that Dkk3 can suppress lung cancer cell growth, probably through inhibiting β-catenin/TCF-4 signaling by preventing β-catenin nuclear translocation.

## Materials and methods

### *Bioinformatic analysis*

Expression of *Dkk* family members was analyzed using the National Center for Biotechnology Information serial analysis of gene expression (SAGE) databases (http://cgap.nci.nih.gov/SAGE). A pool of SAGE libraries from lung cancer tissues were compared with that from normal lung tissues using SAGE Digital Gene Expression Displayer program. SAGE Anatomic Viewer program was used to analyze the *Dkk3* expression in different tissues. To identify CpG islands, 2 kb DNA sequence 5′ to the translation initiation site of *Dkk3* was analyzed using CpG Island Searcher (http://cpgislands.usc.edu) program and verified by CpG Island Plot (http://www.ebi.ac.uk/emboss/cpgplot) program.

### *Cell culture*

The lung cancer cell lines were from American Type Culture Collection (Manassas, VA), except for the 273T and 201T, which were from the University of Pittsburgh Cancer Institute lung cancer program. Cells were maintained at 37°C and 5% $CO_2$. All cell lines were cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% defined fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). For demethylation, cells were treated with 5 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St Louis, MO) for 6 days. Fresh medium was added after days 1, 2 and 3.

### *Western blotting*

Cell lysates were collected and western blotting was performed as described previously (35). The antibodies used for western blotting included the goat anti-Dkk3 antibodies and monoclonal antibodies against c-Myc (9E10), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3 (Stressgen Bioreagents, Ann Arbor, MI), caspase-9 (Cell Signaling Technology, Beverly, MA), α-tubulin (Calbiochem, San Diego, CA) and β-catenin (BD Biosciences, San Jose, CA).

### *Reverse transcriptase–polymerase chain reaction*

Total RNA was isolated from lung cancer cells with or without 5-aza-dC treatment using the RNAgents Total RNA Isolation System (Promega, Madison, WI). First-strand cDNA was synthesized from 10 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). The type a isoform of *Dkk3* transcript was amplified using primer pair 5′-GCGGGAGCGAGCAGATCCAG-3′/5′-GGAAGCTGGCAAACTGGCAG-3′, whereas the type b isoform was amplified using 5′-GGACAGAGTCTAGGTGAGCTG-3′/5′-CAGTCCTCGTCGATGATGCAC-3′. Real-time reverse transcriptase–polymerase chain reaction was performed in an MJ Mini Personal thermocycler (Bio-Rad, Hercules, CA) using the same *Dkk3* primers with

**Abbreviations:** BrdU, bromodeoxyuridine; Dkk, Dickkopf; MSP, methylation-specific polymerase chain reaction; NL, normal lymphocyte; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; SAGE, serial analysis of gene expression; siRNA, small interference RNA; TCF, T-cell factor; 5-aza-dC, 5-aza-2′-deoxycytidine.

glyceraldehyde-3-phosphate dehydrogenase as the internal control. The cycle conditions are available upon request.

*Isolation of genomic DNA and bisulfite modification*

Genomic DNA was isolated from lung cancer cell lines and tissues using QIAamp DNA Blood Mini Kit and QIAmp DNA Mini Kit (Qiagen, Valencia, CA), respectively. A mixture of 0.25 μg of genomic DNA along with 1.0 μg of carrier salmon sperm DNA (Promega) were used for bisulfite modification. DNA from lung cancer cell lines was modified using the previously described method (36). DNA from tissues was modified using the EZ DNA methylation-Gold Kit (Zymo Research, Orange, CA) as described (37).

*Bisulfite sequencing and methylation-specific PCR*

PCR reactions were performed in 20 μl final volume with 2 units of Platinum *Taq* DNA polymerase (Invitrogen) and one-twentieth of bisulfite-modified DNA as template. The conditions included 35 cycles of 95°C for 20 s, 55°C for 30 s and 72°C for 90 s. For bisulfite sequencing, two sets of primers (5′-AGATTAAGGTGGGATTGAGG3′/5′-TAAACCAAACACAAATCAACCC-3′ and 5′-GGTTGAGAGAGGTTTGAGGTG-3′/5′-ATCCTCCATCAATTC-CTCAAC-3′) were used to amplify the *Dkk3* promoter region. Gel-purified PCR products were subsequently sequenced using the same primers. For methylation-specific polymerase chain reaction (MSP), methylated *Dkk3* promoter was amplified using the primer pair 5′-GGCGTTAGGGGCGGGCGGC-3′/5′-GCTCTGCGCCCGCAACCGCCG-3′, whereas unmethylated *Dkk3* promoter was amplified using the primer pair 5′-GGGTGTTAGGGGTGGGTGGTG-3′/5′-CCACTCTACACCCACAACCACCA-3′. The MSP primers for *p16* were described previously (36). All MSP products were analyzed by electrophoresis on 2% agarose gels.

*Tissue samples*

The acquisition of the tissues was approved by the Institutional Review Board at the University of Pittsburgh. Frozen specimens, including 30 randomly selected NSCLC and their matched histologically normal lung parenchyma adjacent to the tumors (within a centimeter of the discrete tumor margin), and the normal lung parenchyma distal to the tumors (at least 4 cm away from the tumors) were obtained from the University of Pittsburgh Cancer Institute lung cancer program. The clinicopathologic characteristics information of the patients were summarized in supplementary Table 1 (available at *Carcinogenesis* Online). Tissues microarray slides containing 94 NSCLC and 46 normal lung samples, including 32 matched pairs, were purchased from US Biomax (Ijamsville, MD). The information of these samples was summarized in supplementary Table 2 (available at *Carcinogenesis* Online).

*Immunohistochemistry*

Tissue sections were deparaffinized by submerging the slides in xylene, rehydrated in decreasing concentrations of ethanol (100% twice followed by once each of 95% and 70%) and boiled twice with each for 5 min in 0.1 M citrate buffer antigen retrieval solution (pH 6.0). Staining was performed using goat anti-Dkk3 antibodies (Santa Cruz Biotechnology) or mouse anti-β-catenin antibody (BD Biosciences), followed by corresponding biotinylated rabbit anti-goat antibodies (Vector laboratories, Burlingame, CA) or goat anti-mouse antibodies (Pierce Biotechnology, Rockford, IL). The signals were detected using Vectastain Elite ABC kit (Vector Laboratories). Hematoxylin was used for counterstaining.

The staining distribution was scored based on the percentage of positive cells: 0 (0%), 1 (1–30%), 2 (31–60%) and 3 (61–100%). The signal intensity was scored using the criteria: 0 (no signal), 1 (weak), 2 (moderate) and 3 (marked). The staining was considered to be positive if the sum of distribution and intensity scores was $>2$.

*Dkk3 and β-catenin transfection*

The expression construct for *Dkk3* was generated by cloning a PCR-amplified full-length human *Dkk3* cDNA fragment into pCDNA3.1/V5-His vector (Invitrogen). The inserts were verified by restriction digestion and DNA sequencing. β-catenin construct was described previously (8). A549 and H1299 cells were transfected using Lipofectamine™ 2000 (Invitrogen). Cells were collected 48 h after transfection and apoptosis was analyzed by nuclear staining with Hoechst 33258 as described previously (38). Treatment with pan-caspase inhibitor z-VAD-fmk (20 μM; R&D Systems, Minneapolis, MN) was initiated 4 h prior to Dkk3 transfection. Colony formation assays were performed in six-well plates as described previously (39).

*TCF-4 reporter assay*

A549 and H1299 cells were cotransfected with *Dkk3* and the transfection control β-galactosidase reporter pCMVβ (Promega), along with TCF-4 reporter plasmid pTOPFlash or the control inactive reporter pFOPFlash (40). Cell lysates were collected and luciferase activities were measured as described previously (41). In some experiments, A549 and H1299 cells were cotransfected with *Dkk3* and wild-type *β-catenin* or mutant *β-catenin* with deletion of the first 45 amino acids (ΔN).

*β-catenin immunofluorescence*

SW480 colorectal cancer cells were transfected with *Dkk3* in chamber slides. Twenty-four hours later, cells were fixed and permeabilized by cold methanol for 10 min and blocked with 100% goat serum for 1 h at room temperature. After three washes in phosphate-buffered saline with 0.05% Tween 20, slides were incubated with anti-β-catenin antibody (BD Biosciences) in 10% goat serum/phosphate-buffered saline overnight. After four washes by phosphate-buffered saline with 0.05% Tween 20 for 5 min each, slides were incubated with Alexa$^{488}$-conjugated anti-mouse antibodies (Invitrogen) at 1:250 dilutions in phosphate-buffered saline with 0.05% Tween 20 for 30 min at room temperature and counterstained with 4′,6-diamidino-2-phenylindole (2 μg/ml). The mounted slides were subjected to microscopic analysis under a Nikon fluorescence microscope (TS800) equipped with a SPOT camera and imaging software.

*Knock down of Dkk3 by small interference RNA*

H1752 cells were transfected with two different ON-TARGETplus small interference RNA (siRNA) specific for *Dkk3* (J-018352-11 and -12; Dharmacon, Lafayette, CO) or the control scrambled siRNA by Lipofectamine™ 2000. After 36 h, cells were incubated with 10 μM bromodeoxyuridine (BrdU; Sigma) for 2 h, then fixed and permeabilized with cold methanol for 10 min. BrdU incorporation was visualized using monoclonal anti-BrdU Alexa-Fluor 594 antibody (Invitrogen) according to the manufacturer's instructions.

*Statistical analysis*

Statistical analysis was performed using GraphPad Prism IV software. $P$ values $<0.05$ were considered to be statistically significant. The means ± 1 SD were displayed in the figures.

## Results

*Downregulation of Dkk3 in lung cancer*

Through analysis of global gene expression patterns in lung cancer and normal lung tissues using National Center for Biotechnology Information SAGE databases (42,43), we identified *Dkk3* as one of the most significantly downregulated genes in lung cancer. Analysis of 159 059 transcripts from three lung adenocarcinomas and 159 917 transcripts from three normal lung specimens revealed that Dkk3 expression was decreased by 36-fold in lung cancer compared with normal lung tissues ($P < 0.05$) (Figure 1A). *Dkk3* is the only *Dkk* family member that is highly expressed in normal lung but markedly downregulated in lung cancer (Figure 1A). *Dkk3* downregulation did not appear to be a general phenomenon and was only observed in tumors of lung, prostate and ovary among 24 tissues of epithelial origin (data not shown). Previous studies have identified type a and type b alternatively spliced isoforms of *Dkk3* transcripts, which contain exon 1a and exon 1b as the first exon, respectively (26). Real-time reverse transcriptase–PCR analysis using primers specific for each isoform showed that both isoforms are abundantly expressed in normal lung tissues, but significantly downregulated in lung tumors and lung cancer cell lines (Figure 1B).

To test whether Dkk3 is downregulated in lung cancer via epigenetic mechanisms, six lung cancer cell lines were treated with 5-aza-dC, a pharmacological inhibitor of DNA methyltransferase. Both *Dkk3* messenger RNA and protein levels were found to be significantly elevated in several cell lines in response to 5-aza-dC treatment (Figure 1C). Immunohistochemical analysis confirmed this observation (Figure 1D).

*Dkk3 hypermethylation in lung cancer cell lines and tumors*

We then analyzed *Dkk3* CpG island sequences using bisulfite-modified genomic DNA from eight lung cancer cell lines, with DNA from normal lymphocytes as a control. Two CpG islands were identified in the *Dkk3* promoter region (Figure 2A). Although no methylated CpG sites were identified in the first CpG island, the second CpG island was found to be extensively methylated, with 90–100% of the CpG sites completely methylated in four of eight

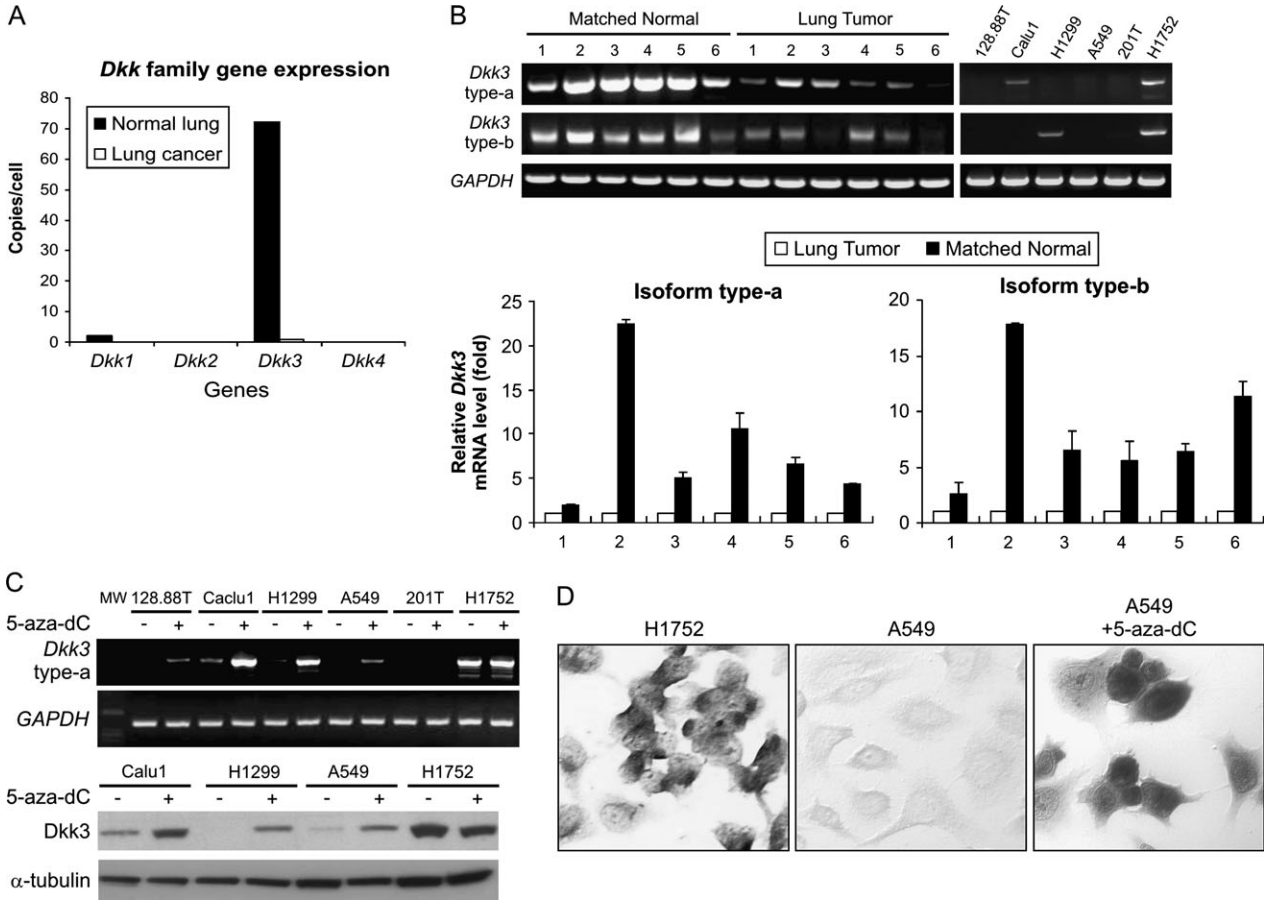


**Fig. 1.** Downregulation of *Dkk3* in lung cancer. (**A**) Expression of *Dkk* family members (*Dkks*1–4) in normal lung and lung cancer. Bioinformatic analysis of SAGE databases identified 36 *Dkk3* tags among 159 917 tags from three normal lung specimens and only one *Dkk3* tag among 159 059 tags from three lung adenocarcinomas. The expression (copies/cell) was normalized based on the estimation that there are ~300 000 transcripts in one cell (53). (**B**) Reverse transcriptase–PCR was used to analyze the expression of *Dkk3* isoforms type a and type b in six matched pairs of normal/tumor tissues and six lung cancer cell lines. Upper panel: PCR products were analyzed by gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Lower panel: Relative expression levels of *Dkk3* isoforms in lung tumors and matched normal tissues were determined by real-time reverse transcriptase–PCR. The expression levels in tumors were defined as one. (**C**) The expression of *Dkk3* in six lung cancer cell lines with or without 5-aza-dC treatment. Upper panel: *Dkk3* type a expression was determined by reverse transcriptase–PCR. Lower panel: Dkk3 protein expression was analyzed by western blotting. MW: molecular weight marker. (**D**) Dkk3 expression was analyzed by immunohistochemistry in H1752 cells and A549 cells with or without 5-aza-dC treatment.

lung cancer cell lines analyzed (Figure 2A and supplementary Figure S1, available at *Carcinogenesis* Online). The methylation patterns matched with *Dkk3* expression changes in response to 5-aza-dC treatment, suggesting that hypermethylation is responsible for its downregulation. To further study *Dkk3* hypermethylation, we developed a MSP assay that was sensitive enough to detect 5–10 copies of DNA with Dkk3 methylation among a large amount of unmethylated normal lymphocytes DNA (Figure 2B). MSP results demonstrated *Dkk3* hypermethylation in 15 of 22 (68.2%) lung cancer cell lines (Figure 2B). In comparison, hypermethylation of *p16*, a well-known target of epigenetic inactivation in lung cancer (44), was detected in 11 (50.0%) of these cell lines (Figure 2B).

Next, MSP was used to analyze *Dkk3* methylation status in matched tissue samples from 30 patients, including their lung tumors, histologically normal lung tissues adjacent to the tumors and histologically normal lung tissues distal to the tumors (supplementary Table 1, available at *Carcinogenesis* Online). The representative data were shown in Figure 2C. *Dkk3* was found to be methylated in 16 (53.3%) tumors, 3 (10%) adjacent normal and 1 (3.3%) distal normal samples (Figure 2C). Bisulfite sequencing was performed on six representative tumors with *Dkk3* hypermethylation identified by MSP. In each case, >90% of the CpG sites in the genomic region of *Dkk3* were found to be methylated in the tumors (data not shown). To determine whether hypermethylation was responsible for the silencing of Dkk3 in the tumors, Dkk3 expression was analyzed by immunohistochemistry (supplementary Figure S2A, available at *Carcinogenesis* Online). We found that all 16 tumors with *Dkk3* hypermethylation did not express Dkk3 (Figure 2C and D). Among the 30 tumors analyzed, 20 were found to lose Dkk3 expression compared with their matched normal lung tissues (Figure 2D). Twelve of these 20 cases had *Dkk3* hypermethylation in the tumors but not in the normal. In contrast, none of the six Dkk3-positive tumors contained *Dkk3* hypermethylation (Figure 2C and D). The correlation between loss of Dkk3 expression and promoter hypermethylation was statistically significant ($P < 0.05$, Fisher's exact test).

### *Downregulation of Dkk3 in NSCLC correlated with high tumor grade and nuclear β-catenin expression*

The 30 tumors analyzed by MSP were mostly in stage I (supplementary Table 1, available at *Carcinogenesis* Online), precluding a correlation between Dkk3 inactivation and clinicopathologic characteristics of the tumors. Therefore, another set of samples on a tissue microarray, including 94 NSCLC and 46 normal lung samples (including 32 matched tumor/normal pairs) (supplementary Table 2, available at *Carcinogenesis* Online), were analyzed by immunohistochemistry for Dkk3 expression. The representative results were shown in Figure 3A and supplementary Figure S2B (available at *Carcinogenesis* Online). While the majority (34/46, 73.9%) of normal lung

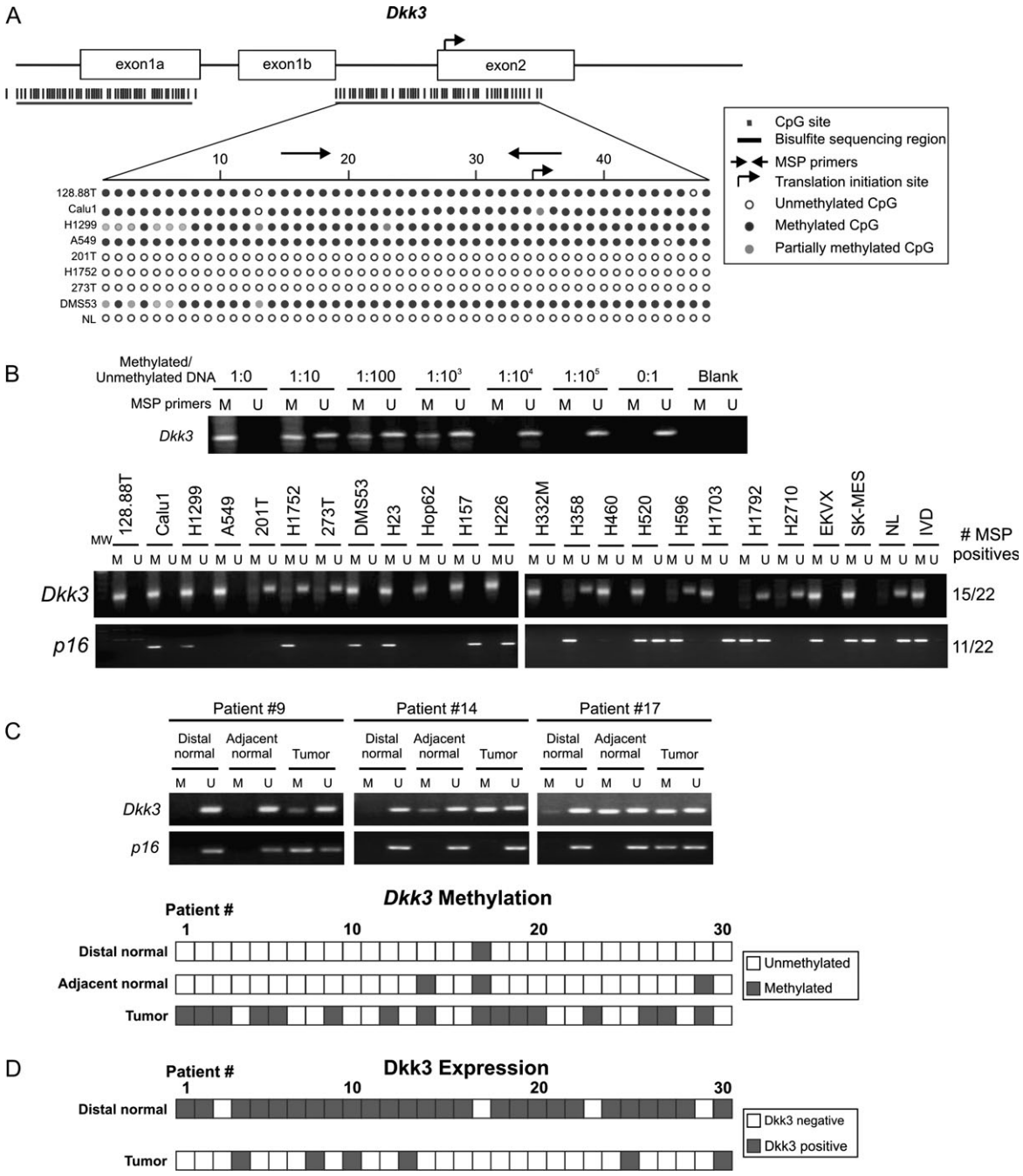


**Fig. 2.** Inactivation of *Dkk3* in lung cancer cell lines and lung tumors by promoter hypermethylation. (**A**) CpG site distribution in the promoter regions of *Dkk3* and the summary of bisulfite sequencing results for eight lung cancer cell lines and normal lymphocytes (NLs). (**B**) Analysis of *Dkk3* promoter methylation by MSP. Upper panel: bisulfite-modified genomic DNA from cells with and without *Dkk3* promoter hypermethylation were mixed at indicated ratios and analyzed by MSP (25 ng of input DNA per reaction). The ratio of 1:1000 was equivalent to 5–10 copies of methylated DNA mixed with 25 ng of unmethylated DNA. Lower panel: *Dkk3* and *p16* methylation status in 22 lung cancer cell lines. NL and *in vitro*-methylated DNA (IVD) were the negative and positive controls for MSP, respectively. MW: molecular weight marker. (**C**) MSP was used to determine the methylation status of *Dkk3* in 30 matched sets of samples (supplementary Table 1, available at *Carcinogenesis* Online), including primary lung tumors (Tumor), pathologically normal lung tissues adjacent to the tumors (Adjacent normal) and normal lung tissues distal to the tumors (distal normal). Upper panel: representative results from three sets of samples. Lower panel: summary of *Dkk3* methylation status in 30 matched sets of samples. (**D**) Summary of Dkk3 expression in distal normal and tumor samples analyzed by immunohistochemistry. M: PCR products amplified using primers specific for methylated DNA. U: PCR products amplified using primers specific for unmethylated DNA.

specimen were positive for Dkk3 expression, only 23.4% (22/94) NSCLC samples were positive for Dkk3 expression. The difference between tumor and normal was highly significant ($P < 0.001$, Fisher's exact test) (Figure 3B). Among 32 matched pairs, 19 were positive for Dkk3 staining in the normal tissues, but completely negative in the matched tumors, 6 were positive, whereas 7 were negative for both tumor and normal tissues (supplementary Table 2, available at *Carcinogenesis* Online). Importantly, loss of Dkk3 expression was found to be correlated with tumor grade, with Dkk3 immunostaining detected in 58.3% (7/12) of grade 1 tumors, but in only 23.5% (8/34) of grade 2 and 14.6% (7/48) of grade 3/4 tumors ($P < 0.01$, chi-square exact test) (Figure 3B and supplementary Table 2, available at

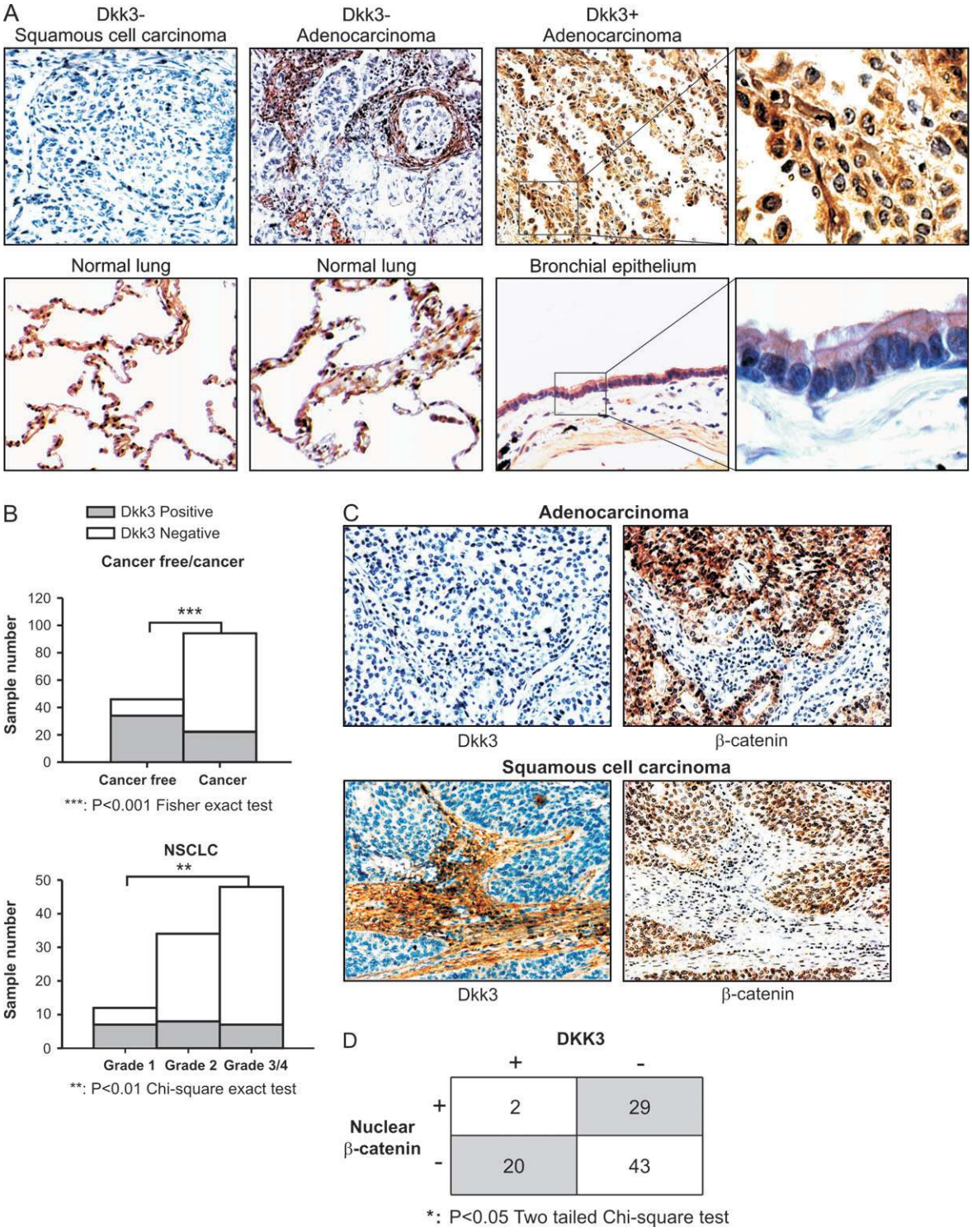


**Fig. 3.** Loss of Dkk3 expression was correlated with lung tumor progression and nuclear β-catenin expression. Dkk3 and β-catenin expression was analyzed by immunohistochemistry for samples on a tissue microarray, including 94 cores of histologically confirmed NSCLC samples and 46 cores of normal lung samples (including 32 matched pairs) (supplementary Table 2, available at *Carcinogenesis* Online). (**A**) Left: Dkk3 expression in example tumor and normal tissues (×400). Dkk3 is expressed in the normal lung but not in the tumor tissues. Right: example of an adenocarcinoma and bronchial epithelium with positive Dkk3 staining with the indicated fields enlarged. (**B**) Summary of Dkk3 staining results in lung tumors and normal tissues. The differences between cancer-free and cancer samples and between grade 1 and grade 3/4 tumors were statistically significant. (**C**) Examples of Dkk3 and nuclear β-catenin expression in lung tumors (×400). The Dkk3-negative tumors were positive for nuclear β-catenin staining, whereas the Dkk3-positive tumor was negative for nuclear β-catenin. (**D**) Summary of Dkk3 and nuclear β-catenin staining results in 94 NSCLC samples. *the inverse correlation between Dkk3 expression and nuclear β-catenin expression was significant ($P < 0.05$, two-tailed chi-square test).

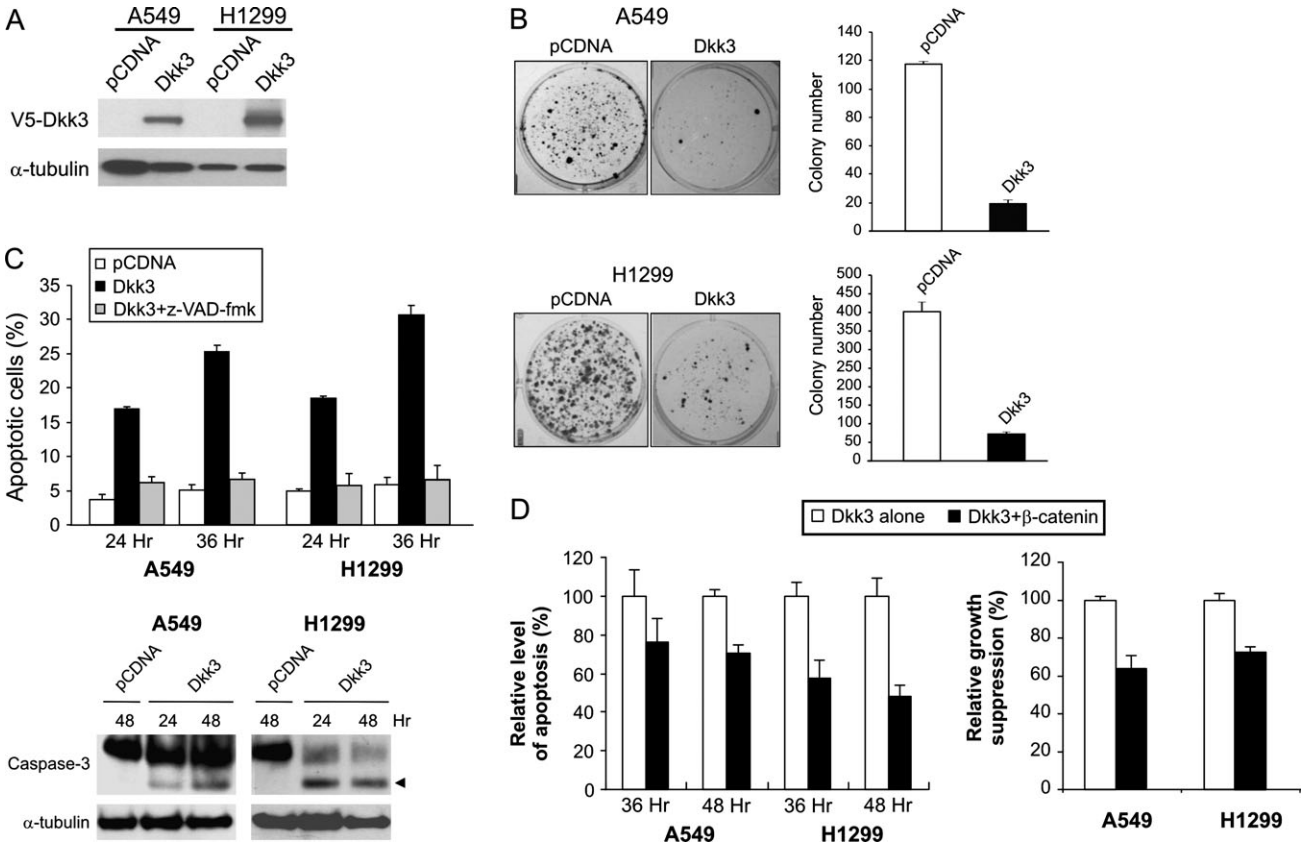


**Fig. 4.** Dkk3 suppressed proliferation and induced apoptosis in lung cancer cells. (**A**) V5-tagged Dkk3 or the control pCDNA vector was transfected into A549 and H1299 cells. The expression of Dkk3 was analyzed by western blotting 48 h after transfection. (**B**) Cells were plated out with G418 selection 48 h after Dkk3 transfection. Colonies were visualized by crystal violet staining after 11–14 days (left) and the colony numbers were plotted (right). (**C**) Cells with or without pretreatment with pan-caspase inhibitor z-VAD-fmk were transfected with Dkk3 as in (A). Apoptosis was analyzed by nuclear staining (upper panel) or caspase-3 activation using western blotting (lower panel) at the indicated time points after Dkk3 transfection. The arrow indicated active caspase-3 fragment. α-Tubulin was used as the loading control. (**D**) A549 and H1299 cells were transfected with Dkk3 alone or cotransfected with Dkk3 and β-catenin. Apoptosis was analyzed by nuclear staining at indicated time points (left). Growth suppression was determined by colony formation assay as in (B).

*Carcinogenesis* Online). In Dkk3-positive specimens, Dkk3 immunostaining was detected in the cytoplasm of normal bronchial epithelial and tumor cells (Figure 3A).

Since other Dkk family members have been implicated in the regulation of the Wnt/β-catenin pathway, we asked whether there is a relationship between Dkk3 downregulation and β-catenin expression. Among 94 NSCLC samples, 31 were found to express high levels of nuclear β-catenin (Figure 3C and supplementary Table 2, available at *Carcinogenesis* Online). Remarkably, 29 of these 31 tumors were Dkk3 negative, whereas only 2 of 22 Dkk3-positive tumors expressed nuclear β-catenin (Figure 3C and D). The inverse correlation between Dkk3 and nuclear β-catenin expression was statistically significant ($P < 0.05$, two-tailed chi-square test).

### *Dkk3 suppressed the growth and β-catenin/TCF-4 signaling in lung cancer cells*

To study the functional role of *Dkk3* in suppressing lung cancer cell growth, A549 and H1299 lung cancer cells were transfected with a Dkk3 expression construct. Dkk3 expression resulted in significant suppression of long-term cell growth by >80% in colony formation assays (Figure 4A and B). After Dkk3 transfection, a significant fraction of the cells were found to contain condensed chromatin and fragmented nuclei (Figure 4C and supplementary Figure S3, available at *Carcinogenesis* Online). Caspase-3 was also activated (Figure 4C). The effect of Dkk3 was blocked by the pan-caspase inhibitor z-VAD-fmk (Figure 4C). These data suggested that Dkk3 suppresses lung cancer cell proliferation by inducing apoptosis.

The inverse correlation between Dkk3 and nuclear β-catenin expression in lung tumors prompted us to investigate whether the effects of Dkk3 are mediated by β-catenin/TCF-4 signaling. We found that overexpression of β-catenin partially reversed the apoptotic and growth-suppressive effects of Dkk3 on both A549 and H1299 cells (Figure 4D). Transfection of *Dkk3*, but not that of the control vector, significantly inhibited the activity of TCF-4 reporter in A549 and H1299 cells (Figure 5A). Dkk3 also inhibited transactivation of TCF-4 reporter by the wild-type β-catenin, as well as that by the mutant β-catenin without the N-terminal phosphorylation sites required for its degradation (ΔN) (45) (Figure 5B). Furthermore, the expression of c-Myc and cyclin D1, two well-known TCF-4 downstream targets (8,9), was significantly suppressed by Dkk3 (Figure 5C). To test whether Dkk3 affects the subcellular localization of β-catenin, immunofluorescence was used to analyze β-catenin localization in SW480 colorectal cancer cells, which express an abundant level of endogenous β-catenin (5). β-catenin, which is normally localized in the nuclei, showed predominant cytoplasmic localization in a significant fraction of cells following Dkk3 transfection (Figure 5D). These results suggested that Dkk3 inhibited β-catenin/TCF-4 signaling by preventing the nuclear translocation of β-catenin.

### *Knock down of Dkk3 promoted cell proliferation and β-catenin/TCF-4 signaling*

To further study the role of Dkk3 in suppressing lung cancer cell proliferation, *Dkk3* was knocked down by siRNA in H1752 cells (Figure 6A), which lacked *Dkk3* methylation (Figure 2A) and

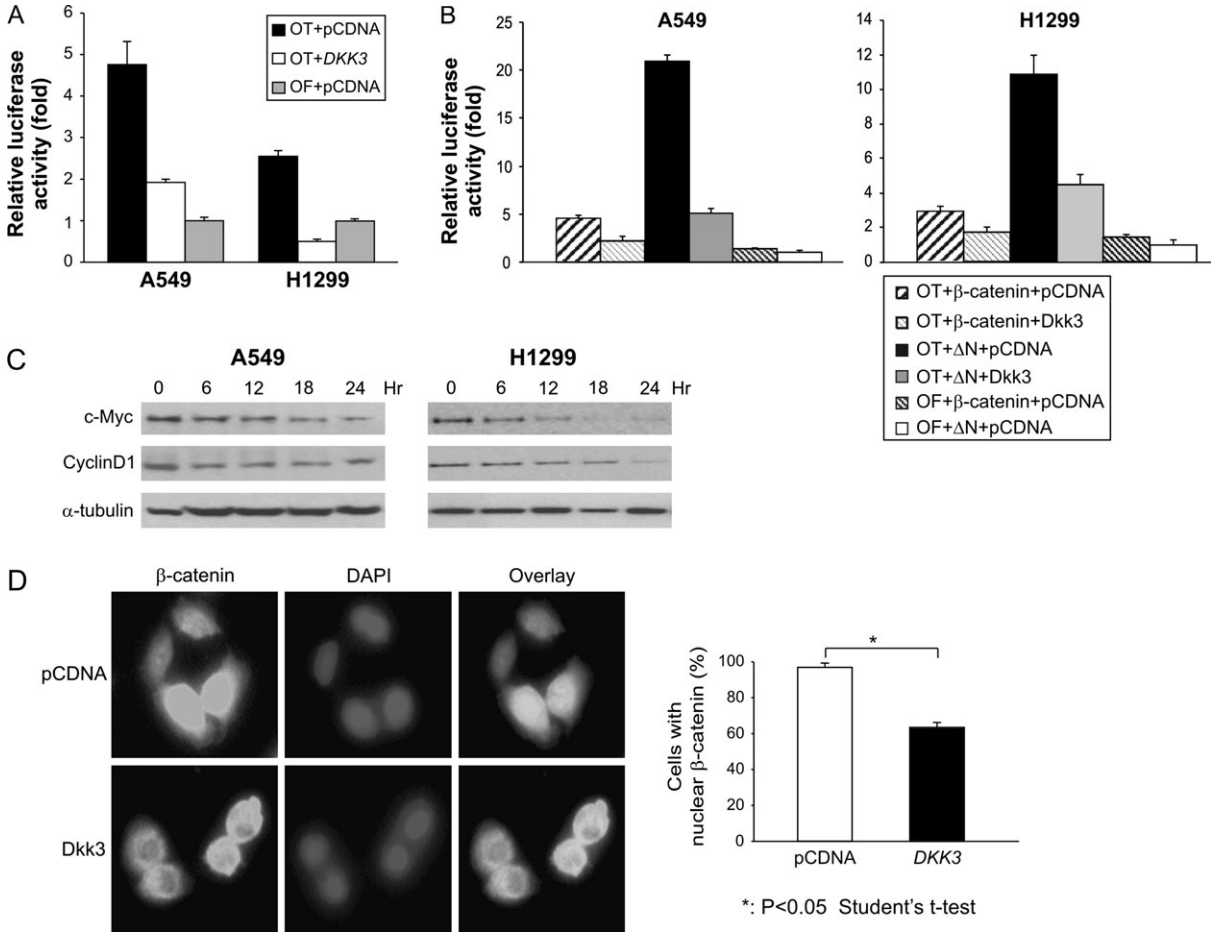


**Fig. 5.** Dkk3 inhibited β-catenin nuclear translocation and expression of TCF-4 targets c-Myc and cyclin D1. (**A**) Dkk3 inhibited TCF-4 reporter activity. A549 and H1299 cells were transfected with *Dkk3* along with the TCF-4 reporter pTOPFlash (OT) or the control inactive reporter pFOPFlash (OF). Normalized luciferase activity was determined 48 h after transfection. The activity of the OF was defined as 1. (**B**) The effects of Dkk3 on TCF-4 activities induced by wild-type or mutant β-catenin. ΔN: the mutant β-catenin with N-terminal 45 amino acids deleted. The reporter assays were performed as in (A). (**C**) c-Myc and cyclin D1 expression at the indicated time points after *Dkk3* transfection in A549 and H1299 cells was analyzed by western blotting. α-Tubulin was used as the loading control. (**D**) SW480 cells were transfected with *Dkk3* or the control empty vector. β-Catenin localization was analyzed by immunostaining 48 h after transfection. 4′,6-Diamidino-2-phenylindole (DAPI) (blue) was used to counterstain the nuclei.

expressed a normal level of Dkk3 protein (Figure 1C and D). We found that the fraction of BrdU-incorporating cells was significantly increased in cells transfected with *Dkk3* siRNA compared with those transfected with the control siRNA (Figure 6B). Analysis of β-catenin localization by immunofluorescence indicated that the fraction of cells with nuclear β-catenin was also increased following downregulation of Dkk3 (Figure 6C). Furthermore, c-Myc was also elevated in response to Dkk3 knockdown (Figure 6A), suggesting that suppression of Dkk3 is sufficient to stimulate cell proliferation and β-catenin/TCF-4 signaling, at least in some lung cancer cells.

## Discussion

Epigenetic inactivation, in particular aberrant DNA hypermethylation, is an important mechanism for tumor suppressor gene silencing in human cancer (46). In this study, we demonstrated that *Dkk3* is frequently silenced in lung cancer through promoter hypermethylation. In every cell line and tumor we analyzed, promoter hypermethylation was found to be associated with loss of Dkk3 expression. In contrast, hypermethylation was rarely found in histologically normal lung tissues. *Dkk3* messenger RNA and protein levels could also be restored by 5-aza-dC in cells with *Dkk3* hypermethylation. These observations and those from other groups provided convincing evidence that Dkk3 is silenced in human cancer primarily through epigenetic mechanisms (23,24,26–30,47). Interestingly, although the CpG island located between the non-coding exon 1b and exon 2 of *Dkk3* seems to be the major target of epigenetic inactivation in lung cancer, the CpG island upstream of exon 1a seems to be more frequently hypermethylated in other types of tumors (23,24). The basis of this tissue specificity remains to be determined. Promoter hypermethylation was found in 12 of 20 tumors that lose Dkk3 expression compared with matched normal tissues (Figure 2C and D), suggesting that other mechanisms are also involved in silencing of Dkk3. *Dkk3* is localized on 11p15, a region of frequent loss of heterozygosity in human cancer (48). Whether *Dkk3* is mutated or deleted in human tumors remains to be studied.

The MSP assay we developed could be useful for detecting *Dkk3* hypermethylation as a biomarker for lung cancer. *Dkk3* methylation was detected in 3 adjacent normal and 1 distal normal samples among the 30 patients analyzed. These methylation events might be found for two reasons. First, because all samples were collected from lung cancer patients with a smoking history, their histologically normal lung tissues were likely to be damaged by tobacco exposure and therefore were not 'true' normal. Alternatively, the detected methylation events in normal tissues might be due to an epigenetic field effect and/or infiltrating tumor cells as reported in other studies (49).

Our results suggested that inactivation of Dkk3 plays a role in lung tumorigenesis through aberrant activation of the Wnt/β-catenin pathway. Dkk3 and nuclear β-catenin expression was mutual exclusive in NSCLC. Dkk3 inhibited TCF-4 reporter activity as well as c-Myc and

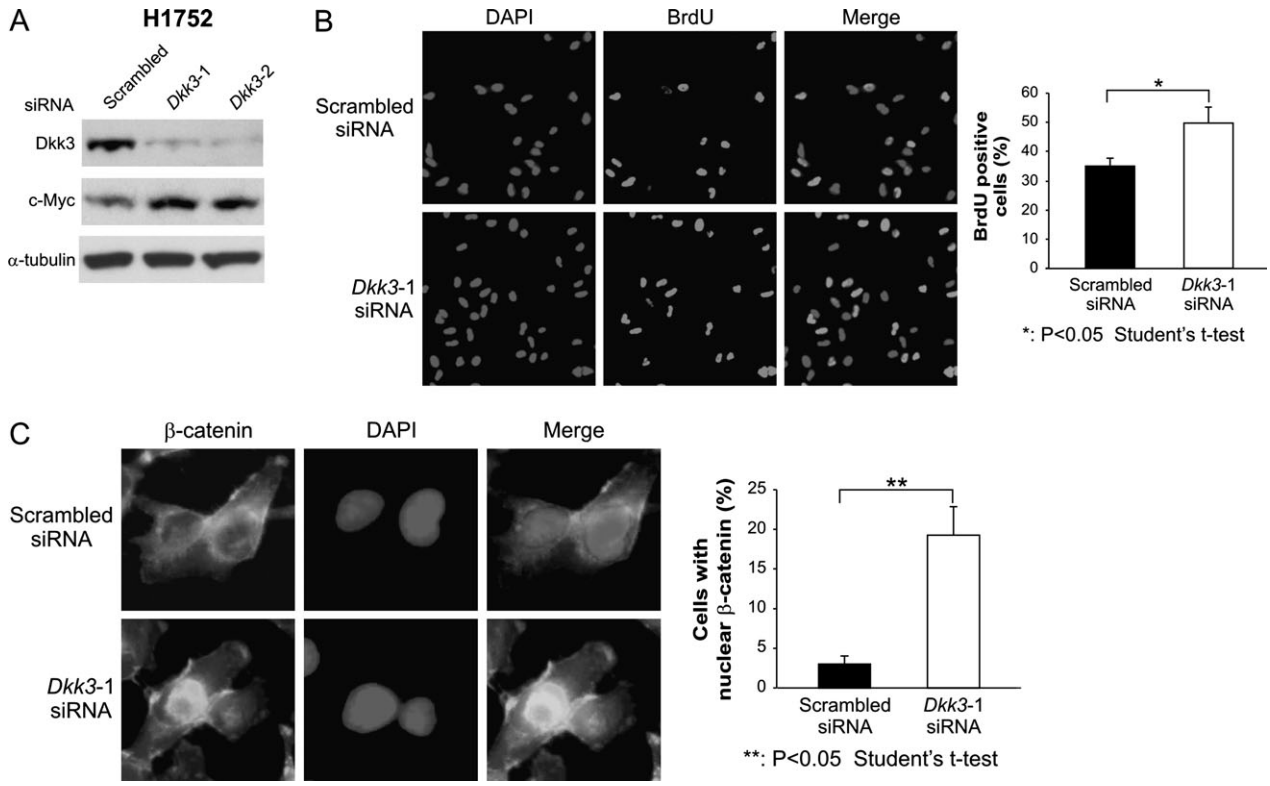


**Fig. 6.** Knock down of *Dkk3* by siRNA stimulated cell proliferation and β-catenin/TCF-4 signaling. (**A**) Two independent *Dkk3* siRNA or the control scrambled siRNA duplex was transfected into H1752 cells. Dkk3 and c-Myc expression was analyzed by western blotting 36 h after transfection. α-Tubulin was used as the loading control. (**B**) BrdU (red) incorporation was analyzed 36 h after transfection with *Dkk3*-1 siRNA. Cell nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) (blue). The results were quantitated (right). (**C**) β-Catenin localization was analyzed by immunostaining. 4′,6-diamidino-2-phenylindole (blue) was used to counterstain the nuclei. Three hundred cells were counted after each treatment and the average results of three experiments ±SD were shown (right).

cyclin D1 expression in lung cancer cells. These results are supported by a recent study demonstrating that activation of cyclin D1 by β-catenin promotes lung cancer cell proliferation (12). Unlike other Dkk family members that bind to Wnt coreceptors, Dkk3 seemed to inhibit Wnt/β-catenin signaling through preventing its nuclear translocation. Dkk3 did not appear to be primarily involved in β-catenin degradation as it inhibited the activities of both wild-type and mutant β-catenin without the N-terminal phosphorylation sites required for its degradation. However, it is possible that keeping β-catenin in the cytoplasm facilitates its interactions with other proteins and subsequent turnover. Consistent with our findings, recent studies showed that Dkk3 affected nuclear accumulation of β-catenin and inhibited TCF-4 activity in other cell systems (33,34).

We found that forced expression of Dkk3 in lung cancer cells without Dkk3 expression suppressed cell growth and caused apoptosis, a major tumor suppressing mechanism affected by aberrant Wnt/β-catenin signaling (50,51), whereas downregulation of Dkk3 was sufficient to promote cell proliferation, β-catenin/TCF-4 signaling and c-Myc expression. Furthermore, overexpression of β-catenin partially reversed the apoptotic and growth suppressive effects of Dkk3. These observations, along with the high frequency of Dkk3 downregulation, and the mutual exclusive relationship between Dkk3 and nuclear β-catenin expression suggest that inactivation of *Dkk3* through hypermethylation is an important mechanism underlying the aberrant activation of Wnt/β-catenin signaling in lung cancer. This may also explain why *APC* and *β-catenin* mutations are so rare in lung tumors (16,17). However, inactivation of Dkk3 alone is not sufficient for tumor initiation as knockout of *Dkk3* in mice did not seem to increase tumor incidence (52). Future studies are necessary to further delineate the mechanisms by which Dkk3 regulates Wnt/β-catenin signaling and its role in suppressing tumor progression *in vivo*.

## Supplementary material

Supplementary Tables 1 and 2 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/


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