DCUN1D3, a novel UVC-responsive gene that is involved in cell cycle progression and cell growth

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DCUN1D3 **(DCN1, defective in cullin neddylation 1, domain containing 3) was found during the process of high throughput screening of novel human genes associated with serum response element (SRE) pathway activation. The *DCUN1D3* gene is highly conserved among vertebrates. Human *DCUN1D3* complementary DNA (cDNA) encodes 304 amino acids with an apparent molecular mass of 34 kDa. However, there has been no report about the function of DCUN1D3. This study detected that *DCUN1D3* was broadly expressed in several tumor tissues and cultured cell lines; however, UVC irradiation of different doses significantly increased *DCUN1D3* expression level in these cancer cell lines. Over-expression of the DCUN1D3 inhibits cell growth in HeLa. When the *DCUN1D3* gene was silenced by siRNA in UVC-treated HeLa, the cell cycle in S phase was remarkably blocked; furthermore, the UVC-induced cell death was inhibited. In addition, DCUN1D3 localized mainly in the cytoplasm and perinuclear, but after UVC treatment, the DCUN1D3 gradually entered the nucleus. All the results above indicate that *DCUN1D3* is a novel UVC-response gene involved in cell cycle regulation and cell survival. (*Cancer Sci* 2008; 99: 2128–2135)**

The completion of sequencing of the human genome has resulted in the identification of thousands of novel genes, most of them with unknown or poorly understood functions by searching the human Refseq and EST databases in Genebank. High-throughput approaches in functional protein analysis are required to decipher these novel genes, Moreover, several high-throughput functional screening systems were established based on over-expression in human cell lines including the cell-based assays using reporter genes.[1] The serum response element (SRE) is present in the promoters of many immediate early genes such as c-fos[2] and can be induced by serum, activated oncogenes and proto-oncogenes as well as extracellular stimuli such as antioxidants and UV light. We have screened some novel genes correlated with the regulation of SRE by SRE reporter assay screening systems, and found the novel human gene *DCUN1D3* can significantly up-regulate SRE activity.

In the present study, we further found that the *DCUN1D3* gene is ubiquitously expressed in human cancer tissues and in cultured cell lines. However, when cancer cells were treated with UV light, the *DCUN1D3* expression level was significantly increased in a time-dependent manner. Over-expression of the DCUN1D3 can inhibit cell growth. When the *DCUN1D3* gene was silenced by siRNA in the UVC-treated HeLa, the cell cycle in S phase was remarkably blocked; furthermore, UVC-induced apoptosis was significantly inhibited. In addition, DCUN1D3 localized mainly in the cytoplasm and perinuclear under resting conditions, but after UVC treatment, it entered the nucleus gradually. These results indicate that this gene may be involved in the cell cycle progression and cell survival under DNA damage conditions.

Materials and Methods

Cells and culture conditions. HeLa cells (cervical carcinoma) were cultured in Roswell Park Memorial Institute (RMPI) 1640 medium with 10% fetal bovine serum (FBS). A549 cells (lung adenocarcinoma) were maintained in Dulbecco's modified Eagle's medium with 10% FBS. HCT116 colon carcinoma (p53 WT and $p53^{-/-}$; generously provided by Bert Vogelstein, The Johns Hopkins University Medical Institutions, Baltimore, MD, USA)[3] were maintained in McCoy's 5 A modified medium/ 10% FBS at 37°C in 5% CO_2.

Cloning of DCUN1D3 cDNA. Two-step polymerase chain reaction (PCR) was used to amplify the full-length complementary DNA (cDNA) of *DCUN1D3* in mixed tissue cDNA panels (Clontech, Palo Alto, CA, USA) with primers 5′-GCTCCTCTGGCTGATGGCAT-3′ (forward) and 5′-CTGAATCACCGAGACAGGGTCCT-3′ (reverse). The purified PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced using the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and subcloned into the EcoRI site of the mammalian expression vector pcDNA3.1/myc-His(–)B (pCDB) (Invitrogen). To generate green fluorescent protein (GFP) fusion construct, the entire coding region of *DCUN1D3* was amplified by PCR from pcDNA3.1-*DCUN1D3* using primer: 5′-AAGCTTATGGGCCAGTGTGTCACC-3′ (forward) and 5′-GGATCCCTAAGTCTGCTCCTCGGG-3′ (reverse). The PCR products were subcloned into the expression vector of the enhanced green fluorescent protein (pEGFP)-C3 expression vector (Clontech). The resultant construct was named pEGFP-*DCUN1D3*. For GST fusion protein construction, the entire coding region of the *DCUN1D3* was amplified by PCR using primers 5′-GGATCC ATGGGCCAGTGTGTCACC-3′ (forward) and 5′-CTCGAGCTAAGTCTGCTCCTCGGG-3′ (reverse). The PCR product was digested and cloned into the pGEX-4T-2 vector (Amersham Biosciences).

RNA extraction and reverse transcriptase–polymerase chain reaction (RT-PCR). RT-PCR was used to detect the expression profile of *DCUN1D3* in cancer tissues, seven cell lines and UVC-treated HeLa, A549 cells and HCT116 cell lines. RNA were collected at different timepoints and extracted by using TRIzol reagent (Invitrogen Life Technologies, USA) as the instructions described. Reverse transcription (RT) was performed by using the ThermoSCRIPT RT-PCR System (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Total RNA of 3 μg was used for the first strand cDNA synthesis using oligo (dT) 15 primer. The primers used for *DCUN1D3* were 5′-TGTGCAAATTCACCAGGAAG-3′ and 5′-TTCTCTTTTCCTTCGCTCCA-3′, corresponding to 702–722 and 1119–1139 bp of

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DCUN1D3 cDNA. The PCR was carried out at 94°C for 5 min, two cycles of 94°C for 1 min, 57°C for 30 s, 72°C for 30 s, two cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 30 s and 35 cycles of 94°C for 1 min, 53°C for 30 s, 72°C for 30 s followed by a one cycle extension at 72°C for 7 min. The expression of glyceraldehyde-3-phosphate dehydrogenase and β-actin was used as a control.

TaqMan real-time quantitative RT-PCR assays. These assays were performed following the manufacturer's specifications (PE Applied Biosystems). Primer pairs and TaqMan probes were designed by Applied Biosystems (assays on demand). Each sample was analyzed in triplicate.

Polyclonal antibody production. *In E.coli,* the open reading frame (ORF) of *DCUN1D3* cDNA was inserted into the BamHI and XhoI sites of the vector pGEX-4T-2 in-frame with a 5′-end sequence coding for glutathione S-transferase, and the primers were 5′-GGATCCATGGGCCAGTGTGTCACC-3′ and 5′-CTCGAGCTAAGTCTGCTCCTCGGG-3′. The protein was expressed in BL21 (DE3) pLyE induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. After purification, the protein was used to immunize rabbits. The specific antibodies were further affinity-purified with prokaryote expression protein coupled to cyanogen bromide (CNBr) activated Sepharose-4B (Amersham Biosciences).

Immunofluorescence staining and microcopy observation. To visualize the subcellular localization of DCUN1D3 protein, HeLa cells and A549 cells grown on coverslips were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde at room temperature for 30 min, then permeabilized with 0.1% Triton X-100 at room temperature for a further 10 min. After washing three times with PBS and blocking for 30 min in a blocking buffer (10% bovine serum albumin in PBS), the coverslips were incubated with primary antibody for 2 h at room temperature. The primary antibody used was affinity-purified rabbit anti-DCUN1D3-specific antibody: (1 :20) The cells were washed three times for 10 min each in PBS followed by a 1-h incubation at 37°C with the second antibody. The second antibody used was fluoroscein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulin G (IgG; antibodies were from ZhongShan Biotechnology). The cells were then washed three times with PBS and/or followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Samples were observed using Olympus laser-scanning confocal microscopy (Olympus Fluoview FV300). For GFP-DCUN1D3 localization, samples were observed directly under Inverted Fluorescence Microscopy (IX71-141, Olympus, Japan).

Colony formation assay. HeLa cells were transfected with pcDNA3.1-*DCUN1D3*, pcDNA3.1 (vector control) and pcDNA3.1-bax (positive control) by using electroporation method, and then the cells were plated in 6-cm dishes at 2×10^4 per cm^2 and cultured for 24 h at 37°C, and then harvested by trypsin, and replated in six-well plates at a density of 5×10^2/mL. After 2 weeks of G418 selection (800 μg/mL), colonies were fixed, stained with crystal violet and counted.

Cell cycle analysis. To prepare cells for fluorescence-activated cell sorter (FACS) analysis, $10^5 - 10^6$ HeLa cells were fixed in 70% ethanol overnight at 4°C. After washing with PBS, cells were incubated with RNase A (0.5 mg/mL) at 37°C for 30 min (Sigma). Finally, the cells were stained with propidium iodide (PI) (50 μg/mL) and analyzed by fluorescence-activated cell sorting (FACS) on a FACSCalibur instrument. DNA content per cell was measured using the CellQuest Pro program.

Protein preparation and Western blot analysis. Whole cell protein extracts were prepared in RIPA lysis buffer (RIPA buffer: 50 mM Tris-HCL pH 7.4; 150 mM NaCl; 1% deoxycholate Na; 1% NP-40; 0.1% sodium dodecylsulfate [SDS], protease inhibitor mixture, Roche Molecular Biochemicals, cocktail was freshly added). Equal amounts of cell lysates (80 μg) were resuspended in 5 × Tris-glycine SDS sample buffer, electrophoresed on SDS/15% polyacrylamide gels, and transferred to nitrocellulose membranes (Amersham Pharmacia, UK). Detection of proteins was done with anti-p53 Ab-6 antibody (1:1000, MBL), anti-DCNU1D3 antibody: (1:200) anti-actin Ab-1 antibody (1:5000, Oncogene Research Products), followed by corresponding IDRy second antibody. The blots were scanned by Odyssey Imaging System (LI-COR Bioscience, USA).

Cell viability assay. After transfection with DCUN1D3 siRNA and subsequent UVC irradiation, cell viability was determined by using both the Vi-CELL TM XR Cell Viability Analyzer (Beckman Coulter, USA). As for the Vi-CELL TM XR Cell Viability Analyzer, trypan blue positive cells were considered dead, and the number of viable cells was calculated by the Analyzer according to the manufacturer's instructions.

Results

Identification and characterization of the DCUN1D3 gene. The schematic overview of the high-throughput screening strategy of genes involved in SRE pathway activation is shown in Figure 1(a). A previously constructed cDNA library containing 575 genes was used as the source of screened genes, including *DCUN1D3*.[1] The full length sequence of *DCUN1D3* gene (GeneBank Accesion NP_775746) was obtained from mixed tissue cDNA panels (Clontech, Palo Alto, CA) by 2-step PCR and it is 3052 bp in length with an open reading frame encoding for a protein of 304 amino acids (Fig. 1b). The predicted molecular mass of this protein is 34 kDa with a theoretical isoelectric point 5.05. Bioinformatics analysis in the MnM (Minimotif Miner)[4] indicates that *DCUN1D3* harbors one putative pattern 4 nuclear localization signal (residues 279–283) (boxed in Fig. 1b). Analysis of the amino acid sequence of DCUN1D3 by Simple Modular Architecture Research Tool (smart.embl-heidelberg.de)[5,6] revealed an unknown conserved function domain DUF298, from 159 to 278 amino acids (underlined in Fig. 1b). Sequence analysis of the deduced protein indicated that this gene is highly conserved among vertebrates (Fig. 1c).

Transcripts of DCUN1D3 expression in several tissues and cell lines. RT-PCR was performed to investigate the transcriptional level of the *DCUN1D3* gene in various cancer tissues and cell lines. The primers used were designed to amplify the 437-bp fragment (702–1139 bp). The *DCUN1D3* transcripts were detected in seven different origins of cancer tissues (Fig. 2a) and seven cell lines (Fig. 2b); however, it was weakly expressed in HeLa and HT-29. All these differences were confirmed by the comparison with an RT-PCR of the housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase, as a reference. Furthermore, *DCUN1D3* mRNA was also detected in paired normal and tumor tissues (BD Clontech Matched Tumor/Normal cDNA Pairs) (Fig. 2c). There are higher expression levels in normal liver tissue, normal bladder tissue and normal renal tissue than their tumor tissue counterparts.

Characterization of DCUN1D3 protein expression. The encoding sequence of *DCUN1D3* was subcloned into pGEX4T-2 expression vector and was induced to express in *Escherichia coli*. Then, the purified DCUN1D3 was used to immunize rabbits and the specific antibody was further affinity-purified with the DCUN1D3 expressed by *Escherichia coli* (data not shown).

To identify the specificity of anti-DCUN1D3 antibody and the DCUN1D3 expression level in mammalian cells, the endogenous DCUN1D3 of HeLa cells, the over-expressed DCUN1D3 protein in HeLa cells and the DCUN1D3 expressed in *Escherichia coli* were analyzed by western blotting with anti-DCUN1D3 antibody. The results revealed that the over-expressing DCUN1D3 and endogenous DCUN1D3 in HeLa all yield the apparent predicted molecular masses of about 34 kDa (Fig. 2d), which is the same as the DCUN1D3 expression in *Escherichia coli* as the standard molecular weight control.

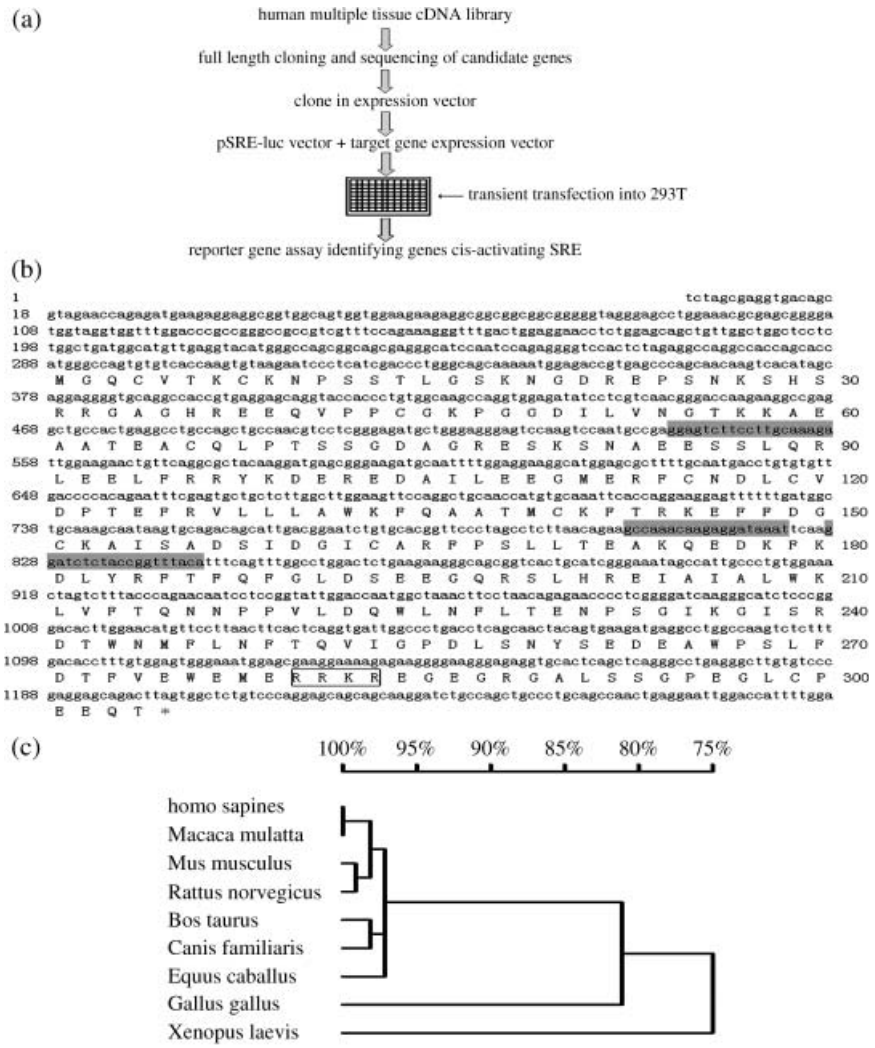

Fig. 1. The complete amino acid sequence of the DCUN1D3 and its high conservation among vertebrates. (a) The schematic overview of the high-throughput screening strategy of genes involved in serum response element (SRE) pathway activation. (b) The nuclear localization signal (NLS) sequence is boxed. Three fragments chosen as chemical small interference RNA (siRNA) binding sites are highlighted. (c) Comparisons with the expressed sequence tag (EST) databases indicated a high degree of identity among *Mus musculus*, *Macaca mulatta*, *Rattus norvegicus*, *Canis familiaris*, *Gallus gallus*, *Bos taurus*, *Xenopus laevis* and *Equus caballus*. Identities are shown as the homology tree.

UVC increases *DCUN1D3* gene expression in human cancer cell lines. Motif analysis in the MnM (Minimotif Miner, 3) database indicates that there is one motif at 273 amino acid of DCUN1D3 protein that recognizes unpaired thymidine in DNA.[7] It implies the DCUN1D3 may have a close relationship with DNA damage. So we chose UVC irradiation as the DNA damage factor in our experiments and then monitored the levels of *DCUN1D3* RNA in HeLa cells after UVC treatment by real-time PCR method. HeLa cells were UVC irradiated at 20 J/m^2, 30 J/m^2 and 80 J/m^2, respectively, and mRNA was monitored over 24 h. The results revealed that DCUN1D3 was induced efficiently but more smoothly at 20 J/m^2 and 30 J/m^2 than at 80 J/m^2 (Fig. 3a).

In addition, RT-PCR and western blot were used to check the DCUN1D3 expression profile after different doses of UVC. The results were similar to the real-time PCR results (Fig. 3b).

Also, to check if its UVC induction was p53-dependent, we treated the HCT116 p53$^{-/-}$ and the HCT116 p53$^{+/+}$ cell lines with 30 J/m^2 UVC. The results showed that the DCUN1D3 was induced on both mRNA and protein levels in the two cell lines (Fig. 3c).

Subcellular localization of DCUN1D3. To determine the subcellular localization of *DCUN1D3*, an indirect immunofluorescence assay was performed with the affinity-purified anti *DCUN1D3* antibodies in HeLa (Fig. 4a) and A549 (Fig. 4b) cells. The results showed that the DCUN1D3 localized mainly in cytoplasm and perinuclear, but after UVC treatment, the protein entered the nucleus gradually during 24 h. It suggests that *DCUN1D3* might play an active role in UVC response.

We further detected whether the over-expressed GFP-DCUN1D3 could also translocate from cytoplasm to nucleus in cancer cells after UVC treatment; the result showed that the DCUN1D3 protein localized mainly in cytoplasm without UVC irradiation, but it accumulated in the nucleus after UVC treatment (Fig. 4c).

Over-expression of DCUN1D3 inhibits cell growth in long-term observation. To study the function of DCUN1D3, we transiently transfected the pcDNA3.1-DCUN1D3 plasmid into HeLa cells. After DCUN1D3 was stably over-expressed in HeLa cells, the colonies were significantly fewer and smaller than the pCDB control group by colony formation assay (Fig. 5a). Above all, the results indicated that DCUN1D3 has a negative role in cell growth.

Over-expression of DCUN1D3 has minor effect on the S phase progression under UVC-treated condition. To explore the DCUN1D3 function on cell cycle progression, we transfected the pcDNA3.1-DCUN1D3 into the HeLa cells under UVC-treated or untreated conditions, respectively. The results showed that during 24 h after UVC irradiation, over-expression of *DCUN1D3* showed a 7 ± 2% decrease of S-phase cells compared with the vector control group (Fig. 5b).

Knockdown endogenous expression of DCUN1D3 by specific siRNA blocked the S phase progression after UVC irradiation. Then we studied the cell cycle change when the endogenous DCUN1D3 expression was inhibited. First, three candidate siRNA against

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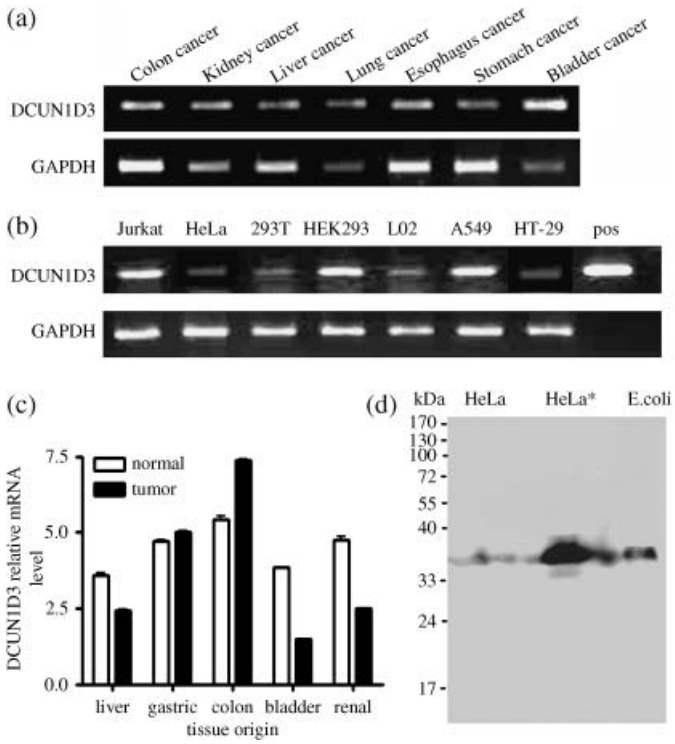

Fig. 2. Comparison of *DCUN1D3* mRNA transcription in various cancer tissues, cell lines, and protein expressed in prokaryotic and eukaryotic cells. (a) Human multiple cancer tissue complementary DNA (cDNA) panels were used to quantify the level of DCUN1D3 transcripts. The primers used were designed to amplify the 437-bp fragment (702–1139 bp). Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was examined as a control. Lane M shows the DNA marker. (b) Expression of *DCUN1D3* was analyzed in seven human cell lines by reverse transcriptase–polymerase chain reaction (RT-PCR). The GAPDH was used as control. (c) Quantitatve PCR analysis shows the mRNA expression of DCUN1D3 in paired normal and tumor tissues. The data represent the mean (± the standard deviation) of triplicate samples. (d) DCUN1D3 expressions in *E. coli* and HeLa cells were probed with affinity-purified anti DCUN1D3 antibodies. (HeLa* is the pcDNA3.1-DCUN1D3 over-expression in HeLa cells.)

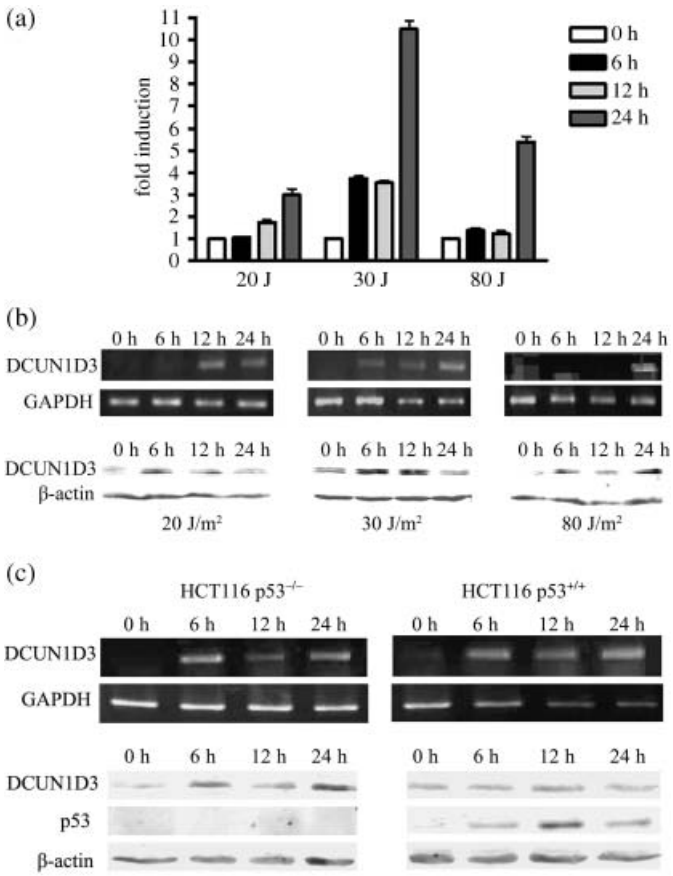

Fig. 3. UVC increases *DCUN1D3* gene expression in human cancer cell lines. (a) Quantitative polymerase chain reaction (PCR) analysis shows the mRNA expression of *DCUN1D3* after 20 J/m^2, 30 J/m^2 and 80 J/m^2 UVC. The data represent the mean (± the standard deviation) of three independent experiments. The relative fold increase of *DCUN1D3* RNA was determined by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (b) Reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blot detection of *DCUN1D3* mRNA and protein expression in HeLa cell line under 20 J/m^2, 30 J/m^2 and 80 J/m^2. (c) DCUN1D3's UVC induction pattern was analyzed in the HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cell lines. The cells were harvested at different time points for RT-PCR and Western blot detection.

DCUN1D3 were transfected into HeLa either alone or combined with a plasmid encoding pEGFP-DCUN1D3. 24 h after transfection, both DCUN1D3 mRNA and protein levels were significantly decreased in those cells that were transfected with si*DCUN1D3*-1 and si*DCUN1D3-3* as assessed by RT-PCR, fluorescence microscopy and western blot. So si*DCUN1D3*-3 was selected to be used for the following experiments (Fig. 6a).

Then, the si*DCUN1D3* siRNA was transfected into HeLa cell lines under UVC-treated or untreated condition, respectively, and the cells were harvested for DNA content analysis by FACS. The results indicated that there was no significant difference between the non-silencing and si*DCUN1D3* groups without UVC treatment, but under UVC irradiation condition, the si*DCUN1D3* transfected cells showed a 24 ± 5% increase of cells in S phase than the control cells 24 h after UVC irradiation (Fig. 6b). Therefore, the results suggest that S-phase progression after UVC in the siDCUN1D3 transfected group was blocked significantly. We also analyzed the molecular change of p53, which is an important cell cycle checkpoint regulator. The western blot result indicated that the p53 protein significantly decreased under UVC irradiation when the *DCUN1D3* siRNA was introduced (Fig. 6c). These results suggest that the *DCUN1D3* gene may participate in S phase checkpoint under UVC irradiation.

Inhibition of the endogenous DCUN1D3 expression protected cells from UVC-induced cell death. To test the HeLa cells' response to UVC-induced cell death after endogenous DCUN1D3 expression was silenced, the *DCUN1D3* specific siRNA were transfected into HeLa cells to inhibit its endogenous *DCUN1D3* expression. Trypan blue exclusion assay was used to examine the cells viability. The results indicated that after UVC treatment, there was a fewer percentage of apoptotic cells in the siRNA transfected cells compared with the controls (Fig. 7).

DCUN1D3 is not involved in γ-radiation-induced G2/M cell cycle arrest. Since IR generates double strand breat (DSB) damage and induces G2/M cell cycle arrest, we were curious about the behavior of DCUN1D3 and the modulation of DCUN1D3 dependent-checkpoint control after ionizing radiation. HeLa cells and HCT116 cells were irradiated at doses of 6 Gy and 12 Gy, respectively, according to the reference.[8,9] As a result, the DCUN1D3 protein could also be induced by ionizing radiation and its induction is also p53-independent (Fig. 8a), so DCUN1D3 expression is also ionizing radiation responsive.

Then the HeLa cells transfected with plasmid containing *DCUN1D3* and specific siRNA were irradiated with a dose of 6Gy γ-Radiation. However, DCUN1D3 might not be involved in γ-radiation-Induced G2/M cell cycle arrest (Fig. 8b). These results suggest that UVC and ionizing radiation cause different patterns of DNA damage and trigger different signal pathways.

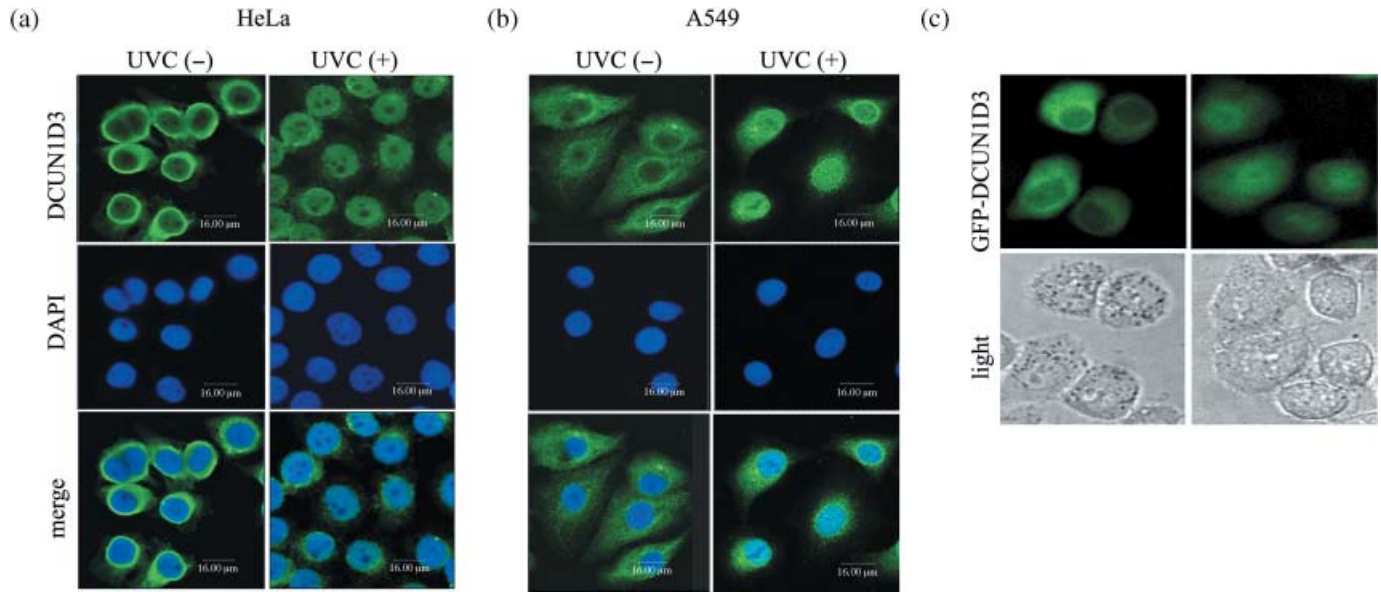

Fig. 4. The DCUN1D3 protein accumulated in the nuclear after UVC treatment. (a, b) HeLa and A549 cells were treated 80 J/m^2 and 30 J/m^2, respectively. The cells were fixed at 0 h, 24 h timepoints and processed to immunofluorescence staining using affinity purified rabbit anti DCUN1D3 antibody. (c) GFP-DCUN1D3 plasmid was transfected into the HeLa cells, and then the cells were UVC irradiated. And the images were captured at 0 min, 24 h.

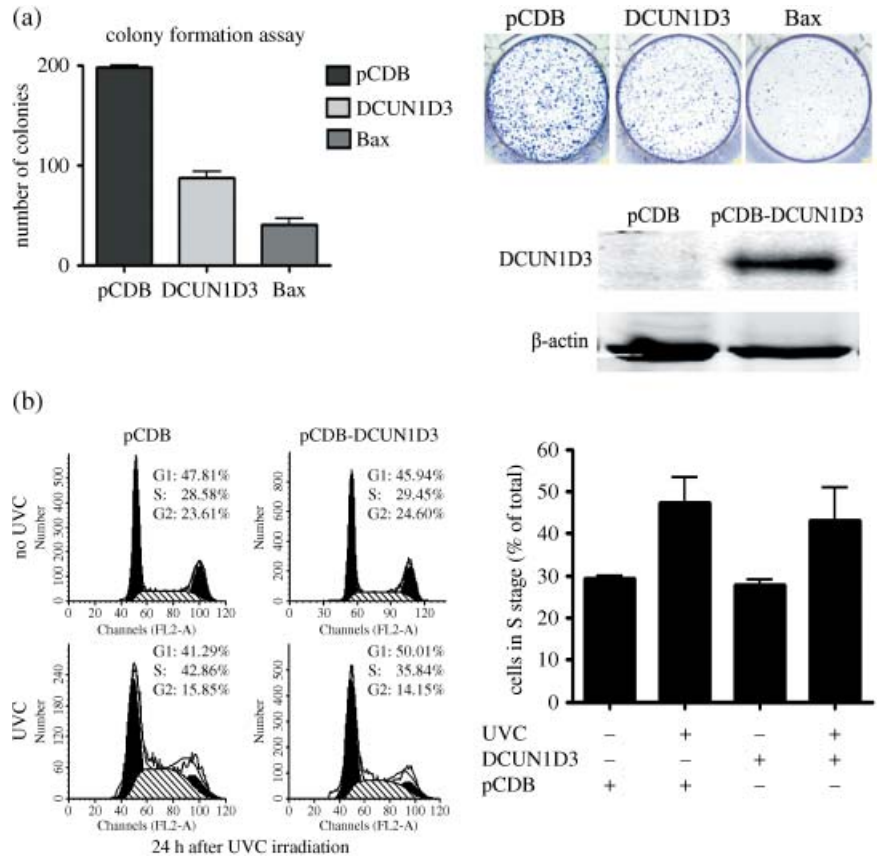

Fig. 5. Over-expression of DCUN1D3 inhibits colony formation of HeLa cells. And the effect of DCUN1D3 expression on the cell cycle progression of HeLa cells. (a) HeLa cells were transfected with pcDNA3.1-DCUN1D3, pcDNA3.1 (vector control) and pcDNA3.1-bax (positive control). The cells were then processed to colony formation assay. Finally the colonies were stained with crystal violet. Western detection of the pcDNA3.1-DCUN1D3 over-expressed protein using anti-DCUN1D3 specific antibody. (b) The effect of DCUN1D3 over-expression on the cell cycle profile of HeLa cells were transfected with a DCUN1D3-expressing vector. After the transfection, cells were treated with 80 J/m^2 UVC or no treatment; 24 h after treatment, cells were collected and analyzed for cell cycle profile. The percentage of cells in G1, S and G2 phases are shown (left panel). The cells in S stage (% of total) are shown in bar graph (right panel). Data with error bars are means ± standard errors of three independent experiments.

Discussion

In the present study, we identified a novel UVC-responsive protein DCUN1D3, which has a down-regulation activity for UVC-related cell cycle check point, cell growth and cell survival.

DCUN1D3 is a gene that we found during the process of high throughput screening of novel human genes associated with serum response element (SRE) pathway activation. In the screening process, mammalian cells were cotransfected with expression plasmids containing full-length ORF in combination with SRE *cis*-reporting system, including a SRE-driven luciferase reporter

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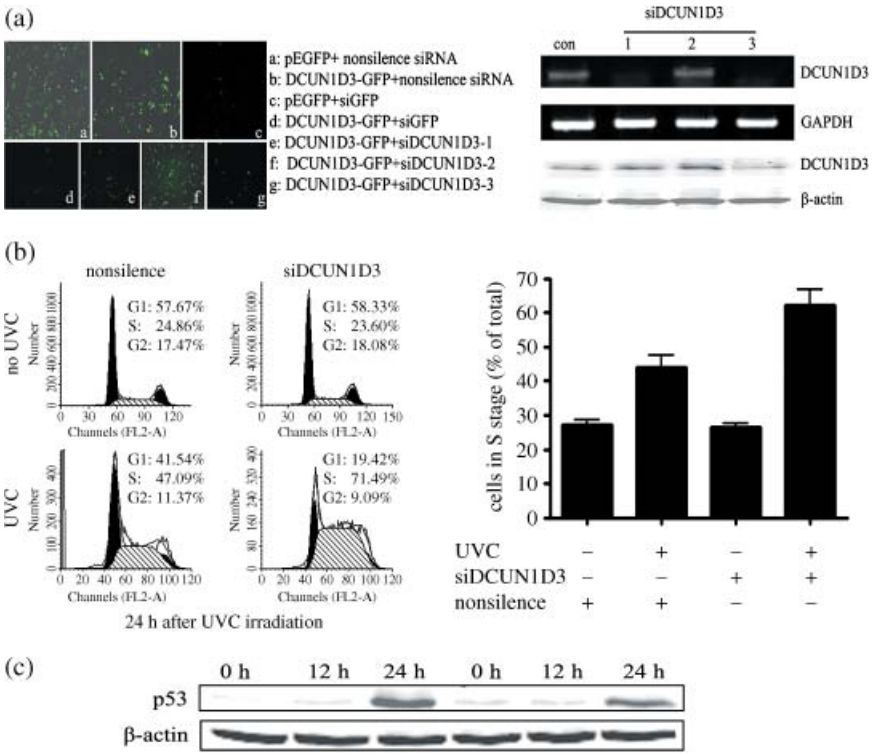

Fig. 6. Inhibition of DCUN1D3 expression on the cell cycle progression of HeLa cells under UVC irradiation. (a) screening of effective small interference RNA (siRNA). Three chemical-synthesized siRNA were transfected into the HeLa cells. The siRNA effects were determined by reverse transcriptase–polymerase chain reaction (RT-PCR) of mRNA expression, Western blot of protein expression and inhibition of the DCUN1D3-GFP over-expression. (b) The effect of DCUN1D3 silencing on the cell cycle profile of HeLa cells. HeLa cells were transfected with non-silence siRNA or DCUN1D3 specific siRNA. After the transfection, cells were treated with 80 J/m^2 UVC or no treatment; 24 h after treatment, cells were collected and analyzed for cell cycle profile. The percentage of cells in G1, S and G2 phases are shown (left panel). The cells in S stage (% of total) are shown in bar graph (right panel). Data with error bars are means ± standard errors of three independent experiments. (c) P53 expression decreased after the endogenous DCUN1D3 was knockdown. DCUN1D3 specific siRNA transfected HeLa cells were collected after UVC irradiation at 0 h, 12 h, and 24 h for p53 expression analysis by western blot.

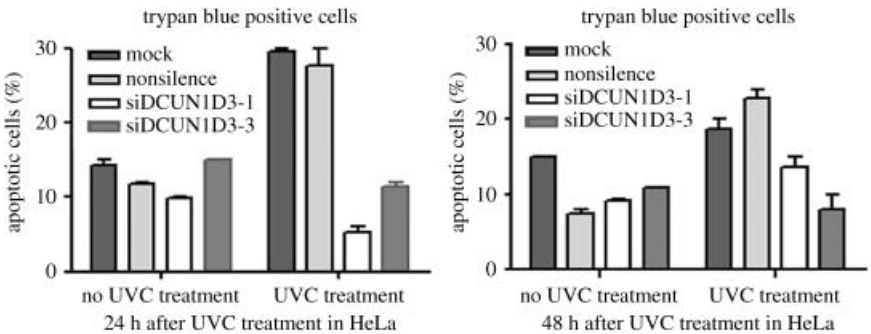

Fig. 7. Inhibition of the endogenous *DCUN1D3* expression protects cells from UVC induced apoptosis. *DCUN1D3* specific small interference RNA (siRNA) were introduced into HeLa cells, then the cells were irradiated with 80 J/m^2 UVC, 24 h and 48 h after UVC treatment, the cells were harvested and analyzed by Vi-CELL TM XR Cell Viability Analyzer (Beckman Coulter, USA). The apoptotic cell percentage for each timepoint was shown in the two figures. The experiment was repeated three times and similar results were got. The error bars represent the mean value of the three independent experiments.

and the thymidine kinase promoter *Renilla luciferase* reporter plasmid (pRL-TK) as an internal control. We have found the novel human gene *DCUN1D3* can significantly up-regulate SRE activity.

The SRE is present in the promoters of many immediate early genes such as c-fos, fosB, junB, egr-1 and –2, neuronal genes such as nurr1 and nur77 and muscle genes such as actins and myosins.[10] Many of them are involved in cellular response to growth factor stimulation and tissue injury. The SRE can be induced by serum, activated oncogenes and proto-oncogenes as well as extracellular stimuli such as antioxidants and UV light. As is well known, UV irradiation in sunlight is a ringleader in causing skin cancer.[11] UV irradiation produces DNA lesions that perturb DNA metabolism and then activate the p53 protein to exert a tumor-suppressing function by regulating cell-cycle arrest or by inducing apoptosis through its transcription factor activity, which binds to DNA in a sequence-specific manner to activate transcription of target genes such as those for p21/WAF1, MDM2, BAX and GADD45. But when the p53 or other protein described as above is mutated or inactived, the cells escape from apoptosis or growth inhibition, resulting in tumorgenesis after exposure to UV. In addition, UV can also activate multiple signal transductions[12] through growth factor receptors[13] and ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related) kinases.[14]

Motif analysis in the MnM (Minimotif Miner[3]) database indicates that there are two ATM kinase phosphorylation sites at the 214 250 amino acid and one motif at 273 amino acid that recognizes unpaired thymidine in DNA.[6] It implies the DCUN1D3 may have a close relationship with DNA damage. And we found UVC can increase its expression on both mRNA and protein level. We also observed its increasing mRNA level after doxorubicin treatment (data not shown) and increasing protein level after ionizing radiation, which is more correlated with the function of ATM.[15]

Besides its UVC-induced transcription expression, the unique localization of DCUN1D3 is one of the most exciting aspects that require further study. The predicted subcellular localization of DCUN1D3 by PSORT II[16] and NucPred[17] was to the nucleus. However, our immunostaining analysis with anti DCUN1D3-specific antibody and GFP fusion protein showed perinuclear or diffused cytoplasm localization. But after UVC treatment, the protein gradually accumulated in the nucleus. So the result demonstrates it is a UVC responsive gene again. From this we can

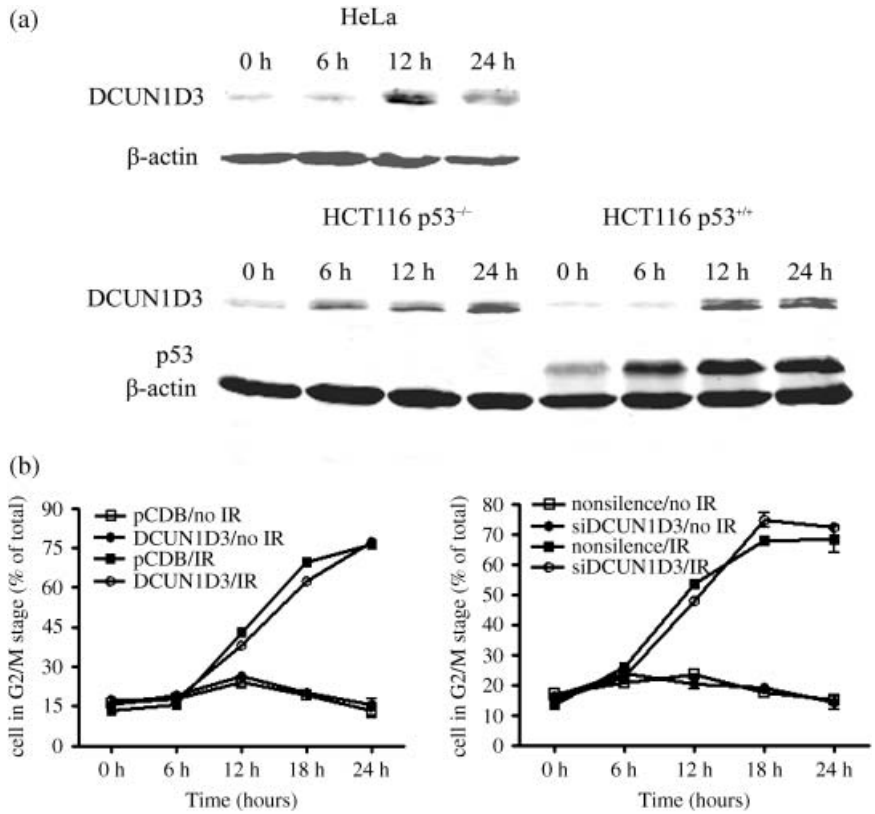

Fig. 8. DCUN1D3 is not necessary for γ-irradiation-induced G2 arrest. (a) HeLa cells and HCT116 cells (p53 wild type and deletion) were irradiated with 6Gy and 12Gy of γ radiation, respectively, and the cells were collected at 0 h, 6 h, 12 h, and 24 h. The DCUN1D3 protein expression was analyzed by western blot using its specific antibody. (b) HeLa cells were transfected DCUN1D3 expressing plasmid and DCUN1D3 small interference RNA (siRNA), respectively; 24 h after transfection, cells were irradiated with 6 Gy of γ radiation. Cells were then collected at the indicated time points, and the cycle profile was analyzed using propidium iodide (PI) staining and flow cytometry (left and right panels). Data with error bars are means ± standard errors of three independent experiments.

conclude that the nuclear localization signal at the C-terminal of the DCUN1D3 is vital to its nuclear accumulation after UVC treatment. Moreover, its nuclear accumulation may be indispensable for its function, such as cell cycle regulation, cell growth, DNA repair, etc. The UVC induction pattern of DCUN1D3 and its subcellular redistribution are similar to the reported DNA damage-related genes, such as p53, KIN17[18] and BRCA1.[19] In our experiments, we also found that over-expression of DCUN1D3 inhibited the colony formation in HeLa cells, and the siRNA knockdown of endogenous DCUN1D3 expression blocked the S phase progression after UVC treatment. Also, inhibition of the endogenous DCUN1D3 protein expression can protect cells from UVC induced apoptosis.

It is reported that there may be a p53 transcription factor binding site in the first intron of the DCUN1D3 gene by a robust approach that couples chromatin immunoprecipitation (ChIP) with the paired-end ditag (PET) sequencing strategy for mapping p53 targets in the human genome.[20] In our experiments, we did not find obvious difference of DCUN1D3 transcription between the Ad-p53 and Ad-GFP infected H1299 cells (data not shown). We also treated the HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells with UVC and ionizing radiation, but did not observe any significant difference in DCUN1D3 expression for either. These results suggest that p53 may play a less important role in the UVC and ionizing radiation induction of DCUN1D3.

Since IR generates DSB damage and induces G2/M cell cycle arrest, we are curious about the behavior of DCUN1D3 and the modulation of DCUN1D3 dependent-checkpoint control after ionizing radiation. However, in our experiments we did not find its involvement in radiation-induced G2/M cell cycle arrest. As a consequence, the relationships of DCUN1D3 with ATM or other DNA damage agents that cause DSB and trigger a different pathway from UVC warrant further study.

Bioinformatics analysis also provided useful hints about the function of DCUN1D3. In the MnM database, the search results predict that there is a motif for cell cycle arrest in SIV virus at 29 aa, two Cyclin A[21] motifs that binds cdk2 complexes at 126,198 aa, a Plk[22] phosphorylation site at 85 aa, two GSK3-ALPHA[23] consensus phosphorylation sites at 13,229 aa. In the ELM (The Eukaryotic Linear Motif resource for Functional Sites in Proteins) database, there are two RXXL motifs at 125–130 and 287–292 aa that binds to the Cdh1 and Cdc20 components of APC/C[24] thereby targeting the protein for destruction in a cell cycle dependent manner, a substrate recognition site that interacts with cyclin and thereby increases phosphorylation by cyclin/cdk complexes at 126–130 and 180–183 aa, and also the Plk phosphorylation site and GSK3 phosphorylation site. All above suggest that DCUN1D3 may have a close relationship with the cell cycle. However, the precise mechanisms involved in its function of cell cycle, cell survival and cell growth need further discussion.

With the accomplishment of human genome sequencing and developing high-throughput screening technologies, more novel genes will be found. Of course, it is also important to find novel DNA-damage related genes. Revealing the nature of their function in the cell cycle, apoptosis and DNA repair is essential for developing novel drugs targeted to reduce the threat of DNA damage agents in the environment. In our study, we describe a novel UVC-responsive gene that is correlated with cell cycle change and cell survival under UVC treatment, and also has a differential expression profile in some normal and tumor tissues. DCUN1D3 may be a potential suppressor gene sharing some similarities with the p53 suppressor gene.[25] It may also provide new clues for signal transduction of UVC and carcinogenesis caused by DNA damage agents.

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References

1 Lan W, Xia G, Peng G *et al*. Cell-based screening and validation of human novel genes associated with cell viability. *J. Biomol Screen* 2006; **11**: 369–76.
2 Treisman R. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. *Cell* 1985; **42**: 889–902.
3 Bunz F, Dutriaux A, Lengauer C *et al*. Requirement for p53 and p21 to Sustain G2 arrest after DNA damage. *Science* 1998; **282**: 1497–501.
4 Sudha B, Vishal T, Snigdha V *et al*. Minimotif miner: a tool for investigationg protein function. *Nat Methods* 2006; **3**: 175–7.
5 Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains Proceedings. *Natl Acad Sci USA* 1998; **95**: 5857–64.
6 Letunic I, Goodstadt L, Dickens NJ *et al*. Recent improvements to the SMART domain-based sequence annotation resource. *Nucleic Acids Res* 2002; **30**: 242–4.
7 Thomas MM, Christophe K, Oliver F. DNA mismatch repair and mutation avoidance pathways. *J Cell Physiol* 2002; **191**: 28–41.
8 Wang XF, Mcgowan CH, Zhao M *et al*. Involvement of the MKK6-p38g Cascade in γ-radiation-induced cell cycle arrest. *Mol Cell Biol* 2000; **July**: 4543–52.
9 Hong SY, Chen XM, Yang VW. Kruppel-like factor 4 mediates p53-dependent G1/S cell cycle arrest in response to DNA damage. *J Biol Chem* 2003; **278** (4): 2101–5.
10 Miano JM. Serum response factor: toggling between disparate programs of gene expression. *J Mol Cell Cardiol* 2003; **35**: 577–93.
11 Setlow RB. The wavelengths in sunlight effective in producing skin cancer: a theoretical analysis. *Proc Natl Acad Sci USA* 1974; **71**: 3363–6.
12 Ann MB, Zigang D. Mitogen-activated protein kinase activation in UV-induced signal transduction. *Sci STKE* 2003; **167**: 1–15.
13 Herrlich P, Ponta H, Rahmsdorf HJ. DNA damage-induced gene expression: Signal transduction and relation to growth factor signaling. *Rev Physiol Biochem Pharmacol* 1992; **119**: 187–223.
14 Durocher D, Jackson SP. DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr Opin Cell Biol* 2001; **13**: 225–31.
15 Martin FL, Domenico D, Luciana C. ATM and the DNA damage response. *EMBO Rep* 2006; **7**: 154–60.
16 Horton P, Park K-J, Obayashi T *et al*. 'WoLF PSORT: protein localization predictor', *Nucleic Acids Res* 2007; **35** (Web Server issue): W585–7.
17 Brameier M, Krings A, Maccallum RM. NucPred–predicting nuclear localization of proteins. *Bioinformatics* 2007; **23** (9): 1159–60.
18 Christel M, Farid M, Ghislaine P-L. Global genome repair is required to activate Kin17 a UVC-responsive gene involved in DNA replication. *PNAS* 2003; **100**: 616–21.
19 Zhang JR, Powell SN. The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Mol Cancer Res* 2005; **3** (10): 531–9.
20 Wei C-L, Wu Q, Vinsensius BV *et al*. A global map of p53 transcription-factor binding sites in the human genome. *Cell* 2006; **124**: 207–19.
21 Girard F, Strausfeld U, Fernandez A, Lamb NJC. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* 1991; **67**: 1169–79.
22 Francis AB, Herman HWS, Erich AN. Polo-like kinases and the orchestration of cell division. *Nat Rev Cell Biol* 2004; **5**: 429–41.
23 Sheelagh F, Philip C. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 2001; **359**: 1–16.
24 Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nature Rev Cancer* 2006; **6**: 369–81.
25 Liu YG, Molly K-M. p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific. *DNA Binding Carcinogenesis* 2001; **22** (6): 851–60.