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Tumor Suppressor Protein Pdcd4 Inhibits Translation of p53 mRNA*[S]

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Background: Pdcd4 is a tumor suppressor involved in translational regulation.
Results: Pdcd4 interacts with p53 mRNA and inhibits its translation via the 5′-UTR. Under stress conditions, the expression of Pdcd4 decreases.
Conclusion: Translation of p53 mRNA is suppressed by Pdcd4 under normal growth conditions.
Significance: Pdcd4 is a new translational regulator of p53.

The tumor suppressor protein Pdcd4 is thought to suppress translation of mRNAs containing structured 5′-UTRs by interacting with translation initiation factor eIF4A and inhibiting its helicase activity. However, natural target mRNAs regulated by Pdcd4 so far are mostly unknown. Here, we identified p53 mRNA as a translational target of Pdcd4. We found that Pdcd4 is associated with p53 mRNA and suppresses its translation. The inhibitory effect of Pdcd4 on the translation of p53 mRNA depends on the ability of Pdcd4 to interact with eIF4A and is mediated by the 5′-UTR of p53 mRNA, which is able to form a stable stem-loop structure. We show that treatment of cells with DNA-damaging agents decreases the expression of Pdcd4. This suggests that translational suppression by Pdcd4 plays a role in maintaining a low level of p53 in unstressed cells and that this suppression is abrogated due to low levels of Pdcd4 after DNA damage. Overall, our work demonstrates for the first time that Pdcd4 is directly involved in translational suppression of a natural mRNA with a 5′-structured UTR and provides novel insight into the translational control of p53 expression.

The tumor suppressor protein Pdcd4 (programmed cell death 4) is a multifunctional nuclear-cytoplasmic shuttling and RNA-binding protein that has been implicated in the development of a broad spectrum of human tumors. Pdcd4 is very highly conserved among vertebrates, with homologs being found also in evolutionarily divergent species, such as insects (*e.g. Drosophila melanogaster*), sponges (*e.g. Suberites domuncula*), and plants (Pfam Database). Pdcd4 was originally identified as a gene whose expression is increased during apoptosis (1) and subsequently identified as a tumor suppressor. Initial work showed that Pdcd4 is able to suppress tumor development in an *in vitro* mouse keratinocyte model of tumor promotion (2). Further work showed that decreased expression of Pdcd4 is associated with a large spectrum of tumors, including tumors of the lung, colon, liver, and breast (3–6). The decrease in Pdcd4 expression in many cases has been ascribed to the expression of microRNA miR-21, whose overexpression in certain cancer cells down-regulates Pdcd4 expression (7, 8). Pdcd4 is also regulated at the protein level. For example, it has been shown that $p70^{S6K}$ kinase-mediated phosphorylation of Pdcd4 triggers the ubiquitinylation of Pdcd4 by the E3 ubiquitin ligase complex $SCF^{\beta TRCP}$ and its subsequent degradation (9). Recent work has suggested that down-regulation of Pdcd4 expression might contribute to tumor development in several ways. siRNA-mediated knockdown of Pdcd4 in breast cancer cells has been shown to increase the mobility and metastatic potential of tumor cells (7, 10). This is in line with the observation that Pdcd4 suppresses AP-1-responsive promoters by inhibiting the activity of the transcription factor c-Jun (11, 12) and the fact that many of the AP-1 target genes are involved in cell motility and tumor cell invasion. In addition, accumulating evidence suggests that down-regulation of Pdcd4 might also contribute to tumor development by disturbing the cellular DNA damage response (13, 14).

A substantial body of evidence has suggested that Pdcd4 modulates the expression of other genes on two levels. Pdcd4 affects transcription of certain genes by inhibiting the activities of specific transcription factors, such as c-Jun (11, 12), Sp1 (10), and p53 (13). In addition, Pdcd4 is thought to act as a suppressor of translation. Pdcd4 interacts with and inhibits the eukaryotic translation initiation factor eIF4A, whose RNA helicase activity is required for the unwinding of mRNA secondary structures present in 5′-UTRs of certain mRNAs (15, 16). Pdcd4 harbors two so-called MA-3 domains, which mediate the binding of Pdcd4 to eIF4A (15, 16). The structure of the MA-3 domains and the complex formation with eIF4A have been analyzed in detail by NMR spectroscopy and x-ray crystallography (17–22). Together with biochemical evidence, this has led to a model in which binding of Pdcd4 to eIF4A inhibits the helicase activity of eIF4A (15, 16), resulting in the suppression of the cap-dependent translation of mRNAs with 5′-structured UTRs. However, as yet, physiological translational target mRNAs for Pdcd4 have not been unambiguously identified to validate this mechanism of translational suppression by Pdcd4.

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Here, we demonstrate that the tumor suppressor p53 mRNA is a physiological target of Pdcd4. We show that Pdcd4 is associated with p53 mRNA and suppresses its translation. The inhibitory effect of Pdcd4 depends on the ability to interact with eIF4A and is mediated by the 5′-UTR of p53 mRNA, which is known to form a stable stem-loop structure. We also show that Pdcd4 expression is diminished upon treatment with DNA-damaging agents. In summary, our work demonstrates for the first time that Pdcd4 is directly involved in translational suppression of a natural mRNA with a 5′-structured UTR. Furthermore, our work implicates Pdcd4 in restraining p53 expression in the absence of DNA damage, identifying it as a hitherto unknown regulator of the basal p53 expression level.

EXPERIMENTAL PROCEDURES

Cells—Cell lines were obtained from American Type Culture Collection. HeLa cell clones stably expressing Pdcd4-specific shRNAs have been described previously (13).

Expression Vectors—The p53 expression vector containing p53 UTRs and luciferase expression vectors containing p53 or Mdm2 UTRs were obtained from L. Xiong and L. Wu (23). The expression vector encoding GFP-p53 has been described (24). The human Pdcd4 expression vector pcDNA4-hPdcd4 (encoding full-length human Pdcd4) has been described (25). pcDNA4-hPdcd4-mut4 encodes a mutant of human Pdcd4 in which Glu-249, Asp-253, Asp-414 ,and Asp-418 were changed to Ala. The β-galactosidase expression vector pCVMβ was obtained from Invitrogen.

Antibodies—Pdcd4 was visualized with rabbit antiserum raised against the N terminus of human Pdcd4 (13). Monoclonal antibodies against p53 (DO-1) and β-actin (AC-15) were obtained from Sigma-Aldrich.

RNA Isolation—Total cellular RNA was isolated with the NucleoSpin® RNA II kit (Macherey-Nagel) following the instructions of the manufacturer. To isolate nuclear and cytoplasmic RNAs, cells were lysed in hypotonic buffer containing 10 mM Hepes (pH 7.5), 5 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40, and 1 mM PMSF supplemented with RNase inhibitor RNaseOUT™ (Invitrogen) and a protease inhibitor mixture (consisting of pepstatin A, leupeptin hemisulfate, and aprotinin) and pelleted at 14,000 rpm for 15 min at 4°C. RNA was isolated from the supernatant (cytoplasmic fraction) and the pellet (nuclear fraction) by TRIzol (Invitrogen) extraction and isopropyl alcohol precipitation.

cDNA Synthesis and Real-time PCR—First-strand cDNA synthesis was performed with a cDNA kit (Fermentas). The cDNAs were analyzed by quantitative real-time PCR with the following primers: p53, 5′-CAGGTAGCTGCTGGGCTC-3′ and 5′-GCTCGACGCTAGGATCTGAC-3′; Pdcd4, 5′-TGTAAACCCTGCAGATCCTGATAA-3′ and 5′-TGGAGGATGCTGAAATCCAA-3′; Mdm2, 5′-AAGCCTGGCTCTGTGTGTAA-3′ and 5′-CTGATCCAACCAATCACCTG-3′; β-actin, 5′-CGTCCACCGCAAATGCTT-3′ and 5′-GTTTTCTGCGCAAGTTAGGT-3′; luciferase, 5′-TTTGTGCCAGAGTCCTTCGAT-3′ and 5′-GAGAATCTCACGCAGGCAGTT-3′; and β-galactosidase, 5′-CTGGCTGGAGTGCGATCTTC-3′ and 5′-GGCGGATTGACCGTAATGG-3′. Real-time PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and a StepOnePlus real-time PCR machine (Applied Biosystems). p53, Pdcd4, and luciferase mRNAs in knockdown and transfection experiments were quantitated by the $\Delta\Delta C_T$ method. First, ΔC_T values for p53 (or Pdcd4) RNA were calculated by subtracting the C_T values obtained for this mRNA from those obtained for β-actin mRNA, thereby normalizing the amount of p53 and Pdcd4 mRNAs with respect to that of a housekeeping gene. Similarly, in transfection experiments, the amount of luciferase RNA was normalized with respect to the β-galactosidase RNA. $\Delta\Delta C_T$ values were calculated by substracting the ΔC_T values of the knockdown cells (or the Pdcd4-transfected cells) from the ΔC_T values of the control siRNA cells (or the control transfected cells). The normalized amounts were then calculated as $2^{-\Delta\Delta CT}$. For RNA immunoprecipitation (RIP)[3] experiments, $\Delta C_{T(\mathrm{RIP})}$ values were calculated by subtracting the ΔC_T values of input samples (after correcting them for dilution factors) from those of RIP samples. RNA amounts in RIP samples were then calculated as a percentage of input samples as $100 \times 2^{-\Delta CT(\mathrm{RIP})}$. For sucrose gradient experiments, C_T values obtained for p53 and Mdm2 mRNAs in pooled gradient fractions 4–7 and 8–11 were subtracted from the C_T values of pooled fractions 1–3, thereby normalizing to the amounts in the nonribosomal fractions. The obtained ΔC_T values were then used to calculate the relative amounts of these RNAs in fractions 4–7 and 8–11 as $2^{-\Delta CT}$.

Sucrose Density Gradients—Density gradient centrifugation and visualization of ribosomal RNAs were performed as described previously (25) except that the gradients were centrifuged in an SW 50.1 rotor (Beckman) at 45,000 rpm for 1 h at 4 °C. For RNA analysis by real-time PCR, gradient fractions were treated with proteinase K, and RNA was isolated by TRIzol extraction and isopropyl alcohol precipitation. cDNA synthesis and real-time PCR were performed as described above.

RIP—HepG2 cells were fixed with 0.5% formaldehyde in phosphate-buffered saline for 5 min at room temperature, followed by addition of 125 mM glycine for 5 min. Cells were lysed in hypotonic buffer. 10% of the supernatant (cytoplasmic fraction) was saved as the input sample, and the remaining material was used for immunoprecipitation for 2 h at 4 °C with rabbit antiserum raised against the N terminus of human Pdcd4 (13). The antibodies were prebound to protein A-Sepharose, which had been blocked with BSA and tRNA for 2 h at 4 °C, and washed several times with egg lysis buffer (50 mM Hepes (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM sodium PP_i, 0.5% Nonidet P-40, and 1 mM PMSF supplemented with protease inhibitor mixture and RNase inhibitor). The immunoprecipitates were washed several times with egg lysis buffer. After elution from the beads with 1% SDS, the samples were reverse cross-linked for 4 h at 65 °C and further treated with RNase-free DNase I for 15 min at room temperature, followed by proteinase K treatment for 1 h at 37 °C. RNA was purified by TRIzol extraction and isopropyl alcohol precipitation. cDNA synthesis and real-time PCR were performed as described above.

[3] The abbreviation used is: RIP, RNA immunoprecipitation.

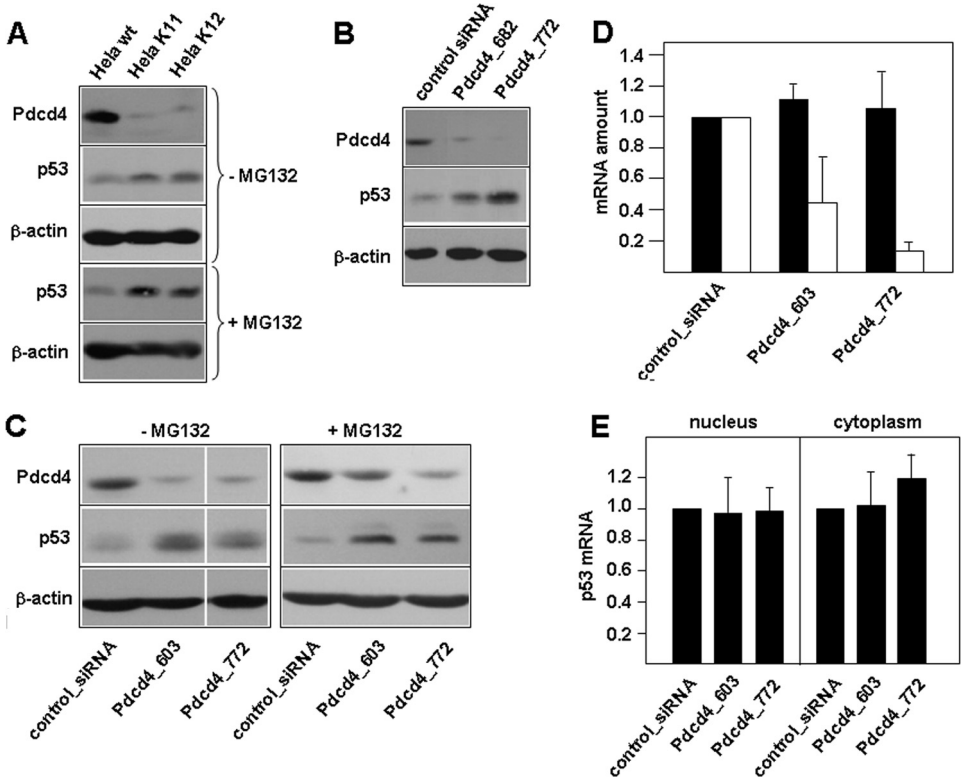

FIGURE 1. **siRNA-mediated knockdown of Pdcd4 up-regulates p53 expression.** *A*, Western blot analysis of HeLa cells (WT) and two stable Pdcd4 knockdown clones (K11 and K12). The Western blots were developed with antisera against Pdcd4, p53, and β-actin as indicated. Cells were grown in normal growth medium or in medium supplemented with 10 μM proteasome inhibitor MG132 for 4 h before harvesting. *B*, HeLa cells were transiently transfected with control siRNA or with two different Pdcd4-specific siRNAs. Cell extracts were analyzed by Western blotting for the expression of Pdcd4, p53, and β-actin. *C*, HepG2 cells were transiently transfected with control siRNA or with two different Pdcd4-specific siRNAs. Cells were treated with or without the proteasome inhibitor MG132 as indicated, and cell extracts were analyzed by Western blotting for the expression of Pdcd4, p53, and β-actin. *D*, total RNA isolated from siRNA-transfected HepG2 cells was analyzed by quantitative real-time PCR for the expression of p53 (*black bars*) and Pdcd4 (*white bars*) mRNAs. *E*, the nuclear-cytoplasmic distribution of p53 mRNA in HepG2 cells transfected with control or Pdcd4-specific siRNAs was analyzed by real-time PCR.

Transfections and Reporter Gene Assays—Transfections in the QT6 quail fibroblast line using the calcium phosphate coprecipitation method and luciferase and β-galactosidase reporter assays were performed as described previously (13).

RNA Interference—siRNA duplexes were obtained from Eurogentec (Liege, Belgium). The Pdcd4 siRNA target sequences were 5′-GUGUUGGCAGUAUCCUUAG-3′ (Pdcd4_603), 5′-GGAGAACUGUGUUUAUGAA-3′ (Pdcd4_682), and 5′-GCAUGGAGAUACUAAUGAA-3′ (Pdcd4_772). siRNA directed against *Renilla* luciferase (target sequence, 5′-AAACAUGCAGAAAAUGCUG-3′) was used as negative control. Approximately 2.5×10^5 cells were plated the day before transfection in 6-cm plates and received 1.5 ml of fresh growth medium prior to transfection. siRNA was transfected using Metafectene^{TM} Pro (Biontex Laboratories GmbH) or HiPerFect (Qiagen), according to the manufacturers' protocols. Cells were harvested 48 h later and processed further for Western blotting or RNA analysis as appropriate.

RESULTS

Knockdown of Pdcd4 Increases p53 Expression Levels—We have previously shown that siRNA-mediated knockdown of Pdcd4 stimulates the activity of p53, leading to increased transcription of certain p53 target genes, such as $p21^{Waf1/Cip1}$ (13). During this work, we noted that the knockdown of Pdcd4 resulted in a slight but reproducible increase in the amount of p53, suggesting that Pdcd4 influences not only the activity of p53 but also its expression. Fig. 1*A* compares the p53 expression levels of two clones of HeLa cells stably expressing Pdcd4-specific shRNA and of a control clone. The expression of Pdcd4 was substantially decreased in the knockdown clones, as expected, whereas the amount of p53 was increased in these clones. Because regulatory mechanisms that affect the expression of p53 in many cases alter the stability of p53 (26–28), we wondered whether the increased level of p53 after Pdcd4 knockdown was due to stabilization of p53. To address this, we compared the p53 protein levels between Pdcd4 knockdown and control clones after treating the cells with the proteasome inhibitor MG132. We reasoned that if the knockdown of Pdcd4 increased the half-life of p53, treatment with the proteasome inhibitor would override such a stabilizing effect. However, as shown in Fig. 1*A* (*lower panels*), this appeared not to be the case, as there was still a significant difference in the amount of p53 between the knockdown and control clones.

In addition to using the stable HeLa Pdcd4 knockdown clones, we performed transient transfections of HeLa cells with two different Pdcd4-specific siRNAs and with control siRNA. Fig. 1*B* shows that the expression of p53 was also elevated after transient knockdown of Pdcd4, indicating that the effects observed in the stable knockdown clones were not due to clonal variation. To demonstrate that the increased expression of p53 after Pdcd4 knockdown was not restricted to HeLa cells, we also

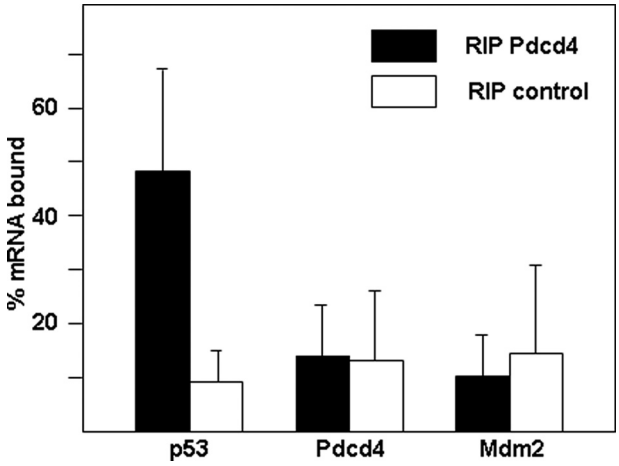

FIGURE 2. **Pdcd4 is associated with p53 mRNA *in vivo*.** Cytoplasmic extracts of HepG2 cells were subjected to RIP with Pdcd4-specific (*black bars*) or control (*white bars*) antibodies. *Bars* indicate the relative amounts of p53, Pdcd4, and β-actin RNAs in the immunoprecipitates as determined by quantitative real-time PCR.

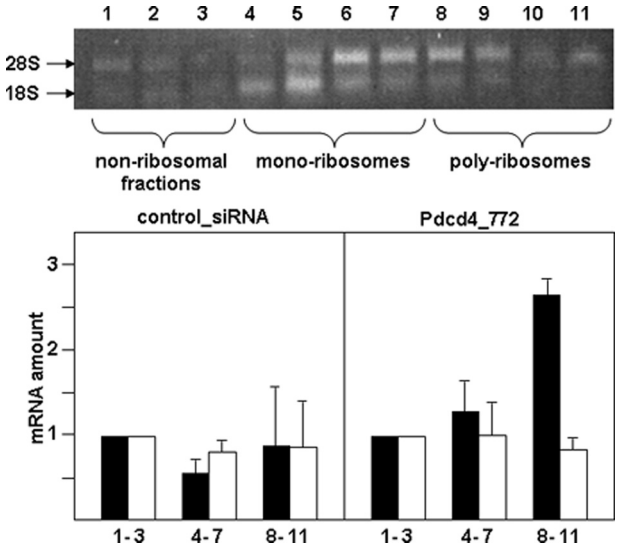

FIGURE 3. **Knockdown of Pdcd4 increases the polysomal association of p53 mRNA.** Cytoplasmic extracts of HepG2 cells transiently transfected with control or Pdcd4-specific siRNA were fractionated by sedimentation through 10–50% sucrose density gradients. Aliquots of the gradient fractions were analyzed by agarose gel electrophoresis to visualize the distribution of 18 S and 28 S ribosomal RNAs (*upper*). Subsequently, fractions 1–3, 4–7, and 8–11 were pooled as nonribosomal, monoribosomal, and polyribosomal fractions as indicated. Total RNA isolated from these pools was then analyzed by quantitative real-time PCR for p53 (*black bars*) and Mdm2 (*white bars*) mRNAs. The *bar height* reflects the amounts of p53 and Mdm2 RNAs normalized to the amount of the corresponding RNA in the nonribosomal pool.

analyzed p53 expression in HepG2 cells (which express wild-type p53) after transient transfection with Pdcd4-specific and control siRNAs. As shown in Fig. 1*C*, the p53 levels were also elevated in the cells transfected with Pdcd4-specific siRNA. This indicated that the increase in p53 expression after Pdcd4 knockdown was not a cell line-specific effect. Using HepG2 cells, we also confirmed that treatment with the proteasome inhibitor MG132 does not override the effect of the Pdcd4 knockdown. On the basis of these results, we concluded that Pdcd4 knockdown elevates the p53 expression level and that this is not due to increased stability of p53. To demonstrate that the stability of p53 was not affected by the Pdcd4 knockdown, we also compared the half-life of p53 in Pdcd4 control and knockdown cells (supplemental Fig. 1). These experiments did not reveal any significant differences in the stability of p53 after knockdown of Pdcd4.

To further understand how Pdcd4 affects the expression of p53, we asked whether Pdcd4 knockdown affects the level of p53 mRNA or its nuclear-cytoplasmic distribution. However, as shown in Fig. 1 (*D* and *E*), the reduced Pdcd4 expression did not significantly affect the amount of p53 mRNA or its distribution between the cytoplasm and nucleus. This demonstrated that Pdcd4 does not influence the transcription, stability, or nuclear export of p53 mRNA but rather suggested that Pdcd4 affects the translation of p53 mRNA.

Pdcd4 Binds to p53 mRNA and Suppresses Its Translation—To confirm that Pdcd4 affects the translation of p53 mRNA, we first asked whether Pdcd4 is associated with p53 mRNA *in vivo*. Previously, we have shown that Pdcd4 co-sediments with ribosomal preinitiation complexes in sucrose density gradients (25), suggesting that Pdcd4 directly targets certain mRNAs. To investigate whether Pdcd4 is associated with p53 mRNA, we performed RIP experiments. We prepared cytoplasmic extracts from formaldehyde-fixed HepG2 cells and immunoprecipitated them with Pdcd4-specific or control antiserum. RNA isolated from the immunoprecipitates was then analyzed by real-time PCR using gene-specific primers. Fig. 2 shows that p53 mRNA was enriched in the Pdcd4-specific immunoprecipitate compared with the control precipitate. By contrast, Pdcd4 and Mdm2 mRNAs, which were analyzed as controls, were not enriched in the Pdcd4-specific immunoprecipitate. This demonstrated that Pdcd4 is associated with p53 mRNA, consistent with the notion that Pdcd4 inhibits the translation of p53 mRNA.

To further explore the idea that p53 mRNA is a translational target of Pdcd4, we transfected HepG2 cells with Pdcd4-specific or control siRNAs and then fractionated cytoplasmic extracts of these cells by sucrose density gradient centrifugation. RNA was isolated from pooled nonribosomal, monoribosomal, and polyribosomal fractions, and the distribution of p53 mRNA between these fractions was determined by quantitative real-time PCR. This allowed us to determine whether the amount of p53 mRNA engaged in translation (*i.e.* associated with monoribosomes and polyribosomes) was affected by the Pdcd4 knockdown. We also analyzed the distribution of Mdm2 mRNA as a control. Fig. 3 shows that the relative amount of p53 mRNA in the polyribosomal fractions increased upon knockdown of Pdcd4, whereas the distribution of Mdm2 mRNA remained unaffected. This clearly demonstrated that knockdown of Pdcd4 results in increased translation of p53 mRNA. We therefore concluded that Pdcd4 associates with p53 mRNA and suppresses its translation.

Suppression of p53 Expression by Pdcd4 Is Mediated by the 5′-UTR of p53 mRNA—To address the mechanism of translational suppression of p53 mRNA by Pdcd4, we first examined the effect of Pdcd4 on the amount of p53 generated from a cotransfected p53 expression vector. Fig. 4*A* shows that Pdcd4 suppressed the expression of p53 from an expression vector containing the authentic p53 5′- and 3′-UTRs. This effect of

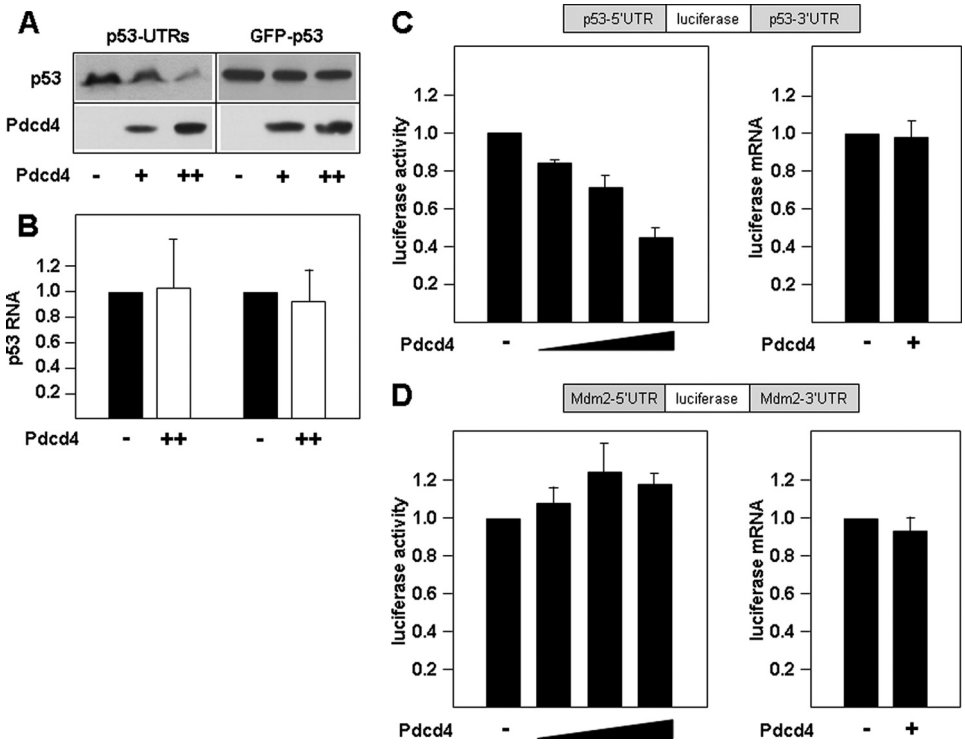

FIGURE 4. **Translational suppression by Pdcd4 is mediated by the UTRs of p53 mRNA.** *A*, QT6 fibroblasts were transfected with increasing amounts of expression vector for human Pdcd4 and with plasmids containing the p53 coding region and the authentic p53 UTRs or containing the coding region for p53 fused to GFP but lacking the p53 UTRs. Cells were additionally transfected with the β-galactosidase expression vector pCMVβ to normalize for transfection efficiencies. β-Galactosidase-normalized amounts of cell extracts prepared 24 h after transfection were then analyzed by Western blotting with antibodies against p53 and Pdcd4. *B*, total RNA of cells transfected as described for *A* was analyzed by real-time PCR for the expression of p53 mRNA. *C* and *D*, QT6 fibroblasts were transfected with increasing amounts of expression vector for human Pdcd4 and plasmids containing the luciferase coding region and both p53 or Mdm2 UTRs. Cells were additionally transfected with the β-galactosidase expression vector pCMVβ to normalize for transfection efficiencies. The *bars* in the *left panels* indicate the β-galactosidase-normalized luciferase activities of cell extracts prepared 24 h after transfection. The *right panels* show real-time PCR analyses of total RNA isolated from cells transfected with the same combinations of plasmids (using only the highest amount of Pdcd4 expression vector). The *bars* indicate the amounts of luciferase mRNA under the different conditions.

Pdcd4 was not due to inhibition of the amount of p53 mRNA generated from this vector, as shown by real-time PCR (Fig. 4*B*). Pdcd4 had virtually no effect on the amount of p53 when an expression vector for GFP-tagged p53 lacking the authentic p53 UTRs was used, suggesting that the inhibitory effect of Pdcd4 is mediated by the UTRs of p53 mRNA. To further explore this possibility, we used a luciferase expression vector in which the luciferase coding region was flanked by the p53 UTRs. Fig. 4*C* shows that the luciferase expression was suppressed by Pdcd4. By contrast, the luciferase activity generated by transfection of a similar expression vector containing the Mdm2 UTRs was not affected by Pdcd4 (Fig. 4*D*), confirming the specificity of the Pdcd4-mediated suppression. Real-time PCR analyses again confirmed that Pdcd4 did not affect the luciferase mRNA levels in both cases, consistent with the notion that Pdcd4 suppresses the translation of the luciferase RNA containing the p53 UTRs.

It has been shown previously that the p53 5′-UTR has the potential to form a stable hairpin structure (29). Disruption of such hairpins during translation initiation is thought to require the helicase activity of eIF4A (30). Because Pdcd4 is known to interact with eIF4A and to inhibit its helicase activity (15), we were interested to know whether the p53 5′-UTR is the target for the suppressive effect of Pdcd4. To investigate the role of the p53 5′-UTR in Pdcd4-dependent translational suppression, we used a luciferase construct containing only the p53 5′-UTR. Fig. 5*A* shows that the luciferase expression of this construct was

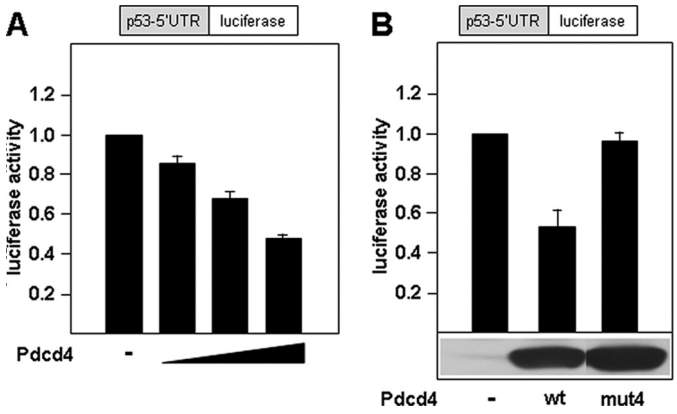

FIGURE 5. **Translational suppression by Pdcd4 is mediated by the p53 5′-UTR and is dependent on the ability of Pdcd4 to interact with eIF4A.** *A*, QT6 fibroblasts were transfected with increasing amounts of expression vector for human Pdcd4 and plasmids containing the luciferase coding region and the p53 5′-UTR. Cells were additionally transfected with the β-galactosidase expression vector pCMVβ to normalize the transfection efficiencies. The *bars* show the β-galactosidase-normalized luciferase activities of cell extracts prepared 24 h after transfection. *B*, cells were transfected and analyzed as described for *A* except that equal amounts of expression vector for WT human Pdcd4 or a Pdcd4 mutant not able to interact with eIF4A (Pdcd4-mut4) were transfected. Comparable expression of both Pdcd4 proteins was confirmed by the Western blot shown at the bottom.

suppressed by Pdcd4 as efficiently as the expression of the construct containing both UTRs shown in Fig. 4*C*. To further examine whether the suppressive effect of Pdcd4 is dependent

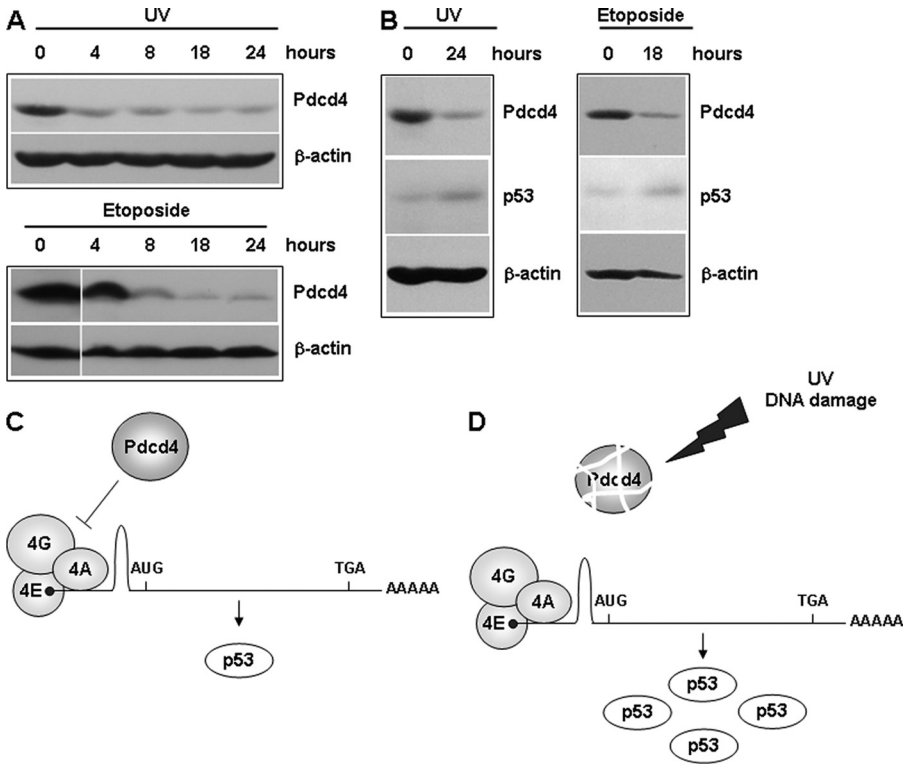

FIGURE 6. **Treatment with DNA-damaging agents down-regulates Pdcd4 expression.** *A*, HepG2 cells were irradiated with ~100 J/m^2 UV light or treated with 15 μg/ml etoposide, followed by further incubation for different times and Western blot analysis of total cellular protein extracts with antibodies against Pdcd4 and β-actin. *B*, HepG2 cells were treated and incubated for the indicated times as described for *A*, followed by Western blot analysis with antibodies against Pdcd4, p53, and β-actin. *C* and *D*, schematic models explaining the function of Pdcd4 in the suppression of p53 mRNA translation under normal growth conditions (*C*) and after induction of DNA damage (*D*). *4A*, *4E*, and *4G* refer to the translation initiation factors eIF4A, eIF4E, and eIF4G, respectively.

on the ability of Pdcd4 to interact with eIF4A, we used a mutant of Pdcd4 (referred to as Pdcd4-mut4) that carries several amino acid substitutions of residues known to interact with eIF4A and is thus unable to interact with eIF4A. Fig. 5*B* shows that the Pdcd4 mutant failed to suppress the luciferase expression of the construct containing the p53 5′-UTR, indicating that Pdcd4 suppresses translation by interfering with the function of eIF4A. We also investigated the effect of Pdcd4 on truncated versions of the p53 5′-UTR to see if the ability of Pdcd4 to inhibit translation correlates with the secondary structure-forming potential of the UTRs. The experiments shown in supplemental Fig. 2 demonstrate that this was the case, suggesting that the ability of Pdcd4 to suppress translation is dependent on the secondary structure-forming potential of the 5′-UTR. The p53 5′-UTR also contains an internal ribosomal entry site (31, 32). We generated a bicistronic reporter plasmid containing the p53 internal ribosomal entry site to see if Pdcd4 affects its activity; however, we found that Pdcd4 did not affect internal ribosomal entry site-dependent translation (data not shown). This further supports the notion that Pdcd4 affects the cap-dependent translation of p53 mRNA via binding to eIF4A.

Treatment with DNA-damaging Agents Down-regulates Pdcd4 Expression—Our data suggest a novel role of Pdcd4 in reducing the level of p53 expression in cells grown under normal growth conditions. We therefore wondered if and how the suppressive effect exerted by Pdcd4 is relieved upon DNA damage, when higher levels of p53 are required. To address this, we irradiated HepG2 cells with UV light. When the Pdcd4 expression level was analyzed by Western blotting, it was apparent that the amount of Pdcd4 was significantly decreased following UV irradiation, whereas the level of p53 was increased (Fig. 6). A similar observation was made with another cell line, HCT116 (supplemental Fig. 3), and when HepG2 cells were treated with the topoisomerase II inhibitor etoposide. These observations suggested that the suppressive effect of Pdcd4 is diminished under DNA-damaging conditions. Taken together, our data suggest the models depicted in Fig. 6 (*C* and *D*). In cells grown under normal growth conditions, Pdcd4 appears to suppress the translation of p53 mRNA, thereby contributing to the low steady-state level of p53. In cells stressed with DNA-damaging agents, Pdcd4 levels are reduced, abrogating the Pdcd4-dependent suppression of translation and permitting increased translation of p53 mRNA.

DISCUSSION

The p53 protein is a key regulator of cell survival and death, and its function is tightly controlled at multiple levels. In particular, regulation of proteasome-mediated degradation of p53 by the E3 ubiquitin ligase Mdm2 and of post-translational modification has been identified as a key regulatory mechanism to control the half-life and activity of p53 in response to DNA damage (26–28). There is strong evidence that p53 protein levels are also regulated at the level of translation of p53 mRNA (33). The p53 5′-UTR is able to form a stable stem-loop that is expected to affect the initiation of translation of p53 (29). Furthermore, an internal ribosomal entry site whose activity is

stimulated by DNA damage has been mapped in the 5′-UTR of p53 mRNA (31, 32). Several proteins bind the p53 UTRs. For example, it was shown that the binding of the ribosomal protein RPL26 to the p53 5′-UTR increases the translation of p53 mRNA under normal conditions and after DNA damage (34, 35). The p53 3′-UTR has also been implicated in translational regulation of p53 mRNA, mediated by the RNA-binding proteins HuR (36) and Wig-1 (37). Recently, a novel regulatory mechanism that involves complementary nucleotide sequences in the 5′- and 3′-UTRs has been described and implicated in translational regulation mediated by RPL26 (38). Finally, the p53 3′-UTR is targeted by several microRNAs, which affect the stability or translation of p53 mRNA (39, 40).

We have identified Pdcd4 as a novel regulator of p53 mRNA translation. siRNA-mediated silencing of Pdcd4 expression caused an increase in the p53 protein level, which was not due to increased p53 protein stability or increased mRNA transcription. Rather, our data show that the amount of p53 mRNA associated with polysomes was increased after Pdcd4 knockdown and that Pdcd4 was associated with p53 mRNA *in vivo*. Taken together, these observations identify p53 mRNA as a physiological target of Pdcd4 and implicate Pdcd4 as a novel factor involved in translational regulation of p53 mRNA.

Because Pdcd4 directly interacts with translation initiation factor eIF4A and inhibits the helicase activity of eIF4A (15, 16), Pdcd4 was proposed to act as a translational suppressor of mRNAs containing structured 5′-UTRs whose disruption during translation initiation requires the helicase activity of eIF4A. Although this idea gained preliminary support from studies showing that translation of artificial RNA constructs harboring stable stem-loop structures at their 5′-ends was decreased when Pdcd4 was overexpressed (15, 16), physiological mRNAs regulated by Pdcd4 via such a mechanism of translational control are not known so far. We recently identified proto-oncogene c-*myb* mRNA as a translational target of Pdcd4 and showed that Pdcd4 suppresses translation of c-*myb* RNA independently of its 5′-UTR but via a responsive element located in the c-*myb* coding region (41). The work described here identifies p53 mRNA as a novel target of Pdcd4 and clearly shows that Pdcd4 suppresses the translation of p53 mRNA in an eIF4A-dependent manner via the p53 5′-UTR. Our work therefore demonstrates for the first time that Pdcd4 is directly involved in translational suppression of a natural mRNA with a 5′-structured UTR.

Our work also provides new insight into the regulation of Pdcd4 itself. We showed that Pdcd4 protein levels decreased after treatment of cells with DNA-damaging agents, such as UV light or the topoisomerase inhibitor etoposide. This suggests a model in which Pdcd4 serves to suppress p53 translation in the absence of DNA damage when p53 protein levels are low, whereas translational suppression by Pdcd4 is abrogated due to the decrease in Pdcd4 in the presence of DNA damage when p53 levels increase. Thus, Pdcd4 appears to contribute to maintaining a low level of p53 expression that is crucial for the homeostasis of unstressed cells. Previously, we showed that Pdcd4 also inhibits the activity of p53 by interfering with the CBP (cAMP-responsive element-binding protein-binding protein)-dependent acetylation of p53 (13). Thus, Pdcd4 apparently affects p53 by two different mechanisms resulting in the suppression of the synthesis and activity of p53. The existence of a dual mechanism by which Pdcd4 controls p53 underlines the importance of the suppressive effects exerted by Pdcd4 on p53.

What are the consequences of p53 inhibition by Pdcd4? As pointed out above, p53 has well established roles in controlling the balance between death, senescence, and survival of DNA-damaged cells. By disturbing this balance, deregulation of Pdcd4 could result in decreased susceptibility to apoptosis and, therefore, in the survival of cells containing damaged DNA. This is in line with our previous observation that the knockdown of Pdcd4 suppresses apoptosis in UV-irradiated cells (13) in a p53-dependent manner (14). The survival of such cells might ultimately contribute to the development of tumors. Beyond its role in the response to acute genotoxic stress, p53 has been implicated in numerous aspects of cellular physiology in response to different kinds of stress as well as in unstressed cells. There is clear evidence for a role of p53 in the regulation of the cellular energy metabolism and antioxidant function, autophagy, invasion and motility, angiogenesis, differentiation, necrosis, and inflammation (42–46). By affecting the translation and activity of p53, Pdcd4 is likely to exert pleiotropic effects on these biological processes and thereby influence the cellular homeostasis. The identification of p53 mRNA as a translational target of Pdcd4 therefore provides new perspectives for future studies on the function of Pdcd4.

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