Tumor Suppressor PDCD4 Represses Internal Ribosome Entry Site-Mediated Translation of Antiapoptotic Proteins and Is Regulated by S6 Kinase 2

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Apoptosis can be regulated by extracellular signals that are communicated by peptides such as fibroblast growth factor 2 (FGF-2) that have important roles in tumor cell proliferation. The prosurvival effects of FGF-2 are transduced by the activation of the ribosomal protein S6 kinase 2 (S6K2), which increases the expression of the antiapoptotic proteins X chromosome-linked Inhibitor of Apoptosis (XIAP) and Bcl-x_L. We now show that the FGF-2–S6K2 prosurvival signaling is mediated by the tumor suppressor programmed cell death 4 (PDCD4). We demonstrate that PDCD4 specifically binds to the internal ribosome entry site (IRES) elements of both the XIAP and Bcl-x_L messenger RNAs and represses their translation by inhibiting the formation of the 48S translation initiation complex. Phosphorylation of PDCD4 by activated S6K2 leads to the degradation of PDCD4 and thus the subsequent derepression of XIAP and Bcl-x_L translation. Our results identify PDCD4 as a specific repressor of the IRES-dependent translation of cellular mRNAs (such as XIAP and Bcl-x_L) that mediate FGF-2–S6K2 prosurvival signaling and provide further insight into the role of PDCD4 in tumor suppression.

Apoptosis, or programmed cell death, is important for the normal development of organisms and maintaining tissue homeostasis. Misregulation of apoptosis may lead to neurodegenerative disorders when apoptosis is increased and tumor formation when apoptosis is inhibited; thus, the pathway is tightly controlled, with both pro- and antiapoptotic factors playing a role. Of particular interest are members of the inhibitor of apoptosis (IAP) and Bcl-2 families of proteins that intercept virtually all apoptotic signals in the cell.

X chromosome-linked inhibitor of apoptosis (XIAP) is the most potent member of the IAP family; it directly interacts with and inhibits caspases 3, 7, and 9 and is therefore a key regulator of apoptosis (20). In contrast, Bcl-x_L controls apoptosis by maintaining mitochondrial membrane homeostasis (14). Interestingly, both the XIAP and Bcl-x_L mRNAs contain an internal ribosome entry site (IRES) that allows them to be translated during cellular stress by a cap-independent mechanism when cap-dependent translation is inhibited, which is necessary for their protective roles in the cell (4, 11, 16, 24, 49). Under normal growth conditions, translation of cellular mRNAs occurs through a cap-dependent mechanism that requires interaction of specific initiation factors (such as eukaryotic initiation factor 4E [eIF4E]) with the 5' cap of the mRNA, followed by recruitment of ribosomal subunits, recognition of the AUG start codon, and commencement of polypeptide chain elongation (reviewed in reference 26). However, certain cellular stresses such as nutrient deprivation, hypoxia, or low-dose irradiation cause attenuation of cap-dependent translation, and yet, under these conditions, a sizeable proportion of cellular mRNAs, perhaps as much as 10%, have been shown to be translated by a cap-independent mechanism, such as through an IRES (23, 28, 39). IRES elements are located within the 5' untranslated region (UTR) of some cellular mRNAs and are believed to recruit the ribosome directly, thereby bypassing the requirement

for the mRNA 5' cap and eIF4E. Moreover, while IRES-dependent translation requires some canonical translation initiation factors, most (if not all) cellular IRES elements require the activity of auxiliary RNA binding proteins that function as IRES *trans*-acting factors (ITAFs) (reviewed in references 28 and 32). The mechanism by which ITAFs function is poorly understood, and since different IRESs require different sets of ITAFs, elucidating the identity of all known ITAFs has proven challenging. Furthermore, it has been shown that ITAFs can function as either positive or negative regulators of IRES-mediated translation. For example, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) was shown to act as a repressor of XIAP IRES-mediated translation (33), whereas La autoantigen (19), hnRNP1 and hnRNP2 (18), and HuR (11) have all been shown to have a stimulating effect on XIAP IRES-mediated translation.

We have previously demonstrated that treatment of small-cell lung cancer cells with fibroblast growth factor 2 (FGF-2) results in enhanced cell survival due to increased expression of several antiapoptotic proteins, including XIAP and Bcl-x_L (35, 36). Mechanistically, this is due to a complex consisting of B-Raf, protein kinase C ϵ (PKC ϵ), and S6K2, which forms in response to the presence of FGF-2 and leads to the phosphorylation and activation of

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S6K2 by PKC ϵ (37). Ribosomal protein S6 kinase 1 (S6K1) and S6K2 are two closely related members of the AGC group VI serine-threonine kinases that regulate several cellular processes, most notably protein synthesis, via the phosphorylation of rpS6 and eIF4B (reviewed in reference 13). Importantly, although S6K1 and S6K2 are usually assumed to regulate overlapping sets of cellular targets, we have shown that S6K1 cannot replace S6K2 in the complex with B-Raf and PKC ϵ following FGF-2 treatment, thus identifying a novel function for S6K2.

We were interested in determining if FGF-2-activated S6K2 modifies a specific ITAF(s) that is involved in the regulation of XIAP and Bcl-x_L translation. We discovered that the tumor suppressor programmed cell death 4 (PDCD4) interacts with and is phosphorylated by S6K2 both *in vitro* and *in vivo*. We demonstrate that activation of S6K2 and subsequent phosphorylation of PDCD4 results in a loss of PDCD4 protein, which leads to enhanced translation of XIAP and Bcl-x_L via their IRES elements. We further show that PDCD4 binds directly to XIAP and Bcl-x_L IRES RNA both *in vitro* and *in vivo* and prevents formation of a translationally competent 48S initiation complex on the IRES. Thus, our work identifies PDCD4 as a novel specific repressor of IRES-mediated translation of cellular IRESs. Our findings also indicate a novel role for S6K2 in the transmission of mitogenic signals (such as the FGF-2 signaling cascade) via PDCD4 and its effect on selective protein synthesis. Furthermore, we provide insight into the mechanism by which PDCD4 specifically regulates IRES-mediated translation. Given the tumor suppressor properties of PDCD4 and the key roles that XIAP and Bcl-x_L play in establishing apoptotic resistance of cancer cells, our work uncovers a new signaling axis that contributes to enhanced chemoresistance in cancer.

MATERIALS AND METHODS

Cell culture, expression constructs, and transfection. Tetracycline-inducible kinase-active S6K2 cells (TOKAS6K2) were described previously (37). TOKAS6K2 and human embryonic kidney (HEK293T) cells were maintained under standard conditions in serum- and antibiotic-supplemented Dulbecco's modified Eagle's medium (DMEM). The bicistronic DNA reporter plasmids pBgal/5'(-1007)/CAT (containing the XIAP IRES element), pBic-S (containing the XIAP shorter 5' UTR that does not contain an IRES), pBic (containing the 104-nucleotide [nt] linker region from pcDNA3), pBgal/Bcl-x_L/CAT (containing the Bcl-x_L IRES element), and hemagglutinin-S6K2 (HA-S6K2) and the pcDNA3-Flag-hnRNP A1(F1) expression plasmid were described previously (4, 21, 33, 37, 38). The pTripz-Kate plasmid was generated by replacing the small hairpin RNA (shRNA) cassette of pTripz (Open Biosystems) with mKate cDNA (Evrogen). PDCD4 mutant plasmids were generated by site-directed mutagenesis in which serines 67, 71, and 76 were mutated to alanine; His-PDCD4 was generated by cloning PDCD4 into the pTrcHis-B plasmid. The PDCD4^{RBD} deletion mutant contains the putative RNA binding domain (RBD) of PDCD4 (amino acids [aa] 1 to 156); the PDCD4^{ΔRBD} mutant lacks the RBD (aa 157 to 469). All the mutant constructs were verified by sequencing. Transient transfections were performed using LipofectAMINE 2000 according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of 2.5×10^5 cells per well in 6-well plates and were transfected 24 h later with 2 μ g of plasmid DNA, or *in vitro*-transcribed RNA, per well. Cells were collected for analysis 4 h (for RNA) or 24 h (for DNA) posttransfection. Small interfering RNA (siRNA) transfections were performed using Lipofectamine RNAiMax according to the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of 2.5×10^5 cells/well in 6-well plates and were transfected 24 h later in serum-free

DMEM with a 25 nM final concentration of PDCD4 siRNA (Dharmacon), S6K2 siRNA (Qiagen), or a nonsilencing control siRNA (Qiagen). Cells were collected for analysis 48 h or 72 h posttransfection.

***In vitro* RNA synthesis.** DNA templates for the synthesis of reporter RNAs were generated from the corresponding 5' UTR-containing monocistronic hairpin construct (38) by PCR, using primers that have an incorporated T7 promoter sequence at the 5' end to allow RNA transcription. Reverse primers included the 3' end of the chloramphenicol acetyltransferase (CAT) gene as well as 31 T residues, thus providing the resultant PCR product with a poly(T) tail. All PCR products were purified using agarose gel electrophoresis and an UltraClean 15 DNA purification kit (Mo Bio Laboratories). *In vitro* transcription and capping were performed using an mMessage mMachine kit (Ambion). Newly synthesized RNA was purified using a Megaclear column (Ambion). The concentration of the RNA was determined using an ND-1000 spectrophotometer (Thermo Scientific).

***In vitro* synthesis of ³²P-labeled RNA.** DNA templates were generated from the XIAP IRES sequence by PCR using primers that have an incorporated T7 promoter sequence at the 5' end to allow RNA transcription. RNA was generated using [α -³²P]UTP and a MAXIscript T7 kit (Ambion) per the protocol provided by the manufacturer. The RNA was gel purified from a 5% acrylamide–8 M urea denaturing gel.

RNA-protein complex immunoprecipitation. HEK293T cells were transiently transfected with pcDNA3-Flag, Flag-PDCD4, or Flag-hnRNP A1(F1) expression plasmid for 24 h. RNA-protein complexes were cross-linked *in vivo* with 1% formaldehyde for 30 min at room temperature followed by 0.2 M glycine for 5 min to stop cross-linking. After washing with phosphate-buffered saline (PBS), the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Igepal, 1 mM sodium orthovanadate [Na₃VO₄], 1 mM NaF, aprotinin, leupeptin, and pepstatin [1 μ g/ml each], RNase inhibitor [Promega; 40 U/ml]) for 30 min at 4°C. Lysates were sonicated and treated with DNase I for 30 min. Proteins were immunoprecipitated (IP) with anti-Flag M2 affinity gel (Sigma) for 2 h at 4°C, and RNA was extracted using phenol-chloroform purification and ethanol precipitation. cDNA was generated using qScript cDNA Super-Mix (Quanta Biosciences), and the products were PCR amplified using GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Invitrogen), XIAP (QuantiTect primer assay; Qiagen), and Bcl-x_L (QuantiTect primer assay; Qiagen) gene-specific primers. PCR products were visualized on a 0.8% agarose gel by ethidium bromide staining.

UV cross-linking of RNA-protein complexes. RNA-protein UV cross-linking experiments were performed as previously described (33). Briefly, [³²P]UTP-labeled RNA was incubated with increasing concentrations of recombinant His-PDCD4 (0.5 μ g, 1.0 μ g, or 2.0 μ g) or glutathione S-transferase (GST) (2 μ g) in RNA binding buffer (10 mM Tris-HCl [pH 7.4], 3 mM MgCl₂, 300 mM KCl, 1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], leupeptin [20 μ g/ml]) for 30 min at room temperature. The complexes were UV cross-linked at 250 mJ/ μ m² in a Stratilinker, followed by treatment with RNase T1 (1 U/ μ l), RNase A (10 μ g/ml), and heparin (5 mg/ml) for 10 min. The samples were mixed with 2 $\times$ Laemmli buffer, boiled, separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and exposed to X-ray film at –80°C overnight.

Nitrocellulose filter binding assay. *In vitro*-transcribed, ³²P-labeled RNA was gel purified and incubated with recombinant proteins at room temperature for 10 min in RNA binding buffer. The reaction was spotted on a nitrocellulose membrane and drawn through the membrane by the use of a vacuum manifold, washed twice with RNA binding buffer, and dried for 30 min, and the radioactivity was measured by a scintillation counter.

Kinase assay. HEK293T cells were transiently transfected with HA-S6K2 for 24 h, treated with FGF-2 (10 ng/ml) for 3 h, and lysed in coimmunoprecipitation (co-IP) buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA [pH 8.0], 0.5% Triton X-100, 5 mM beta-

glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4) with sonication. Anti-HA-agarose beads (Sigma) were incubated with the lysate for 1 h at 4°C. Beads were washed 3 times with 1 ml of lysis buffer followed by a wash in 1× kinase buffer (20 mM Tris-HCl [pH 7.5], 5 mM beta-glycerolphosphate, 0.2 mM Na_3VO_4 , 0.5 mM DTT). Kinase-bound beads or recombinant GST-S6K2 (Invitrogen) was incubated with substrate in 1× kinase buffer in the presence of ATP (300 μM ATP, 66 mM MgCl_2 , 33 mM MnCl_2) and 5 μCi of γ - ^{32}P -labeled ATP for 20 min at 30°C. Laemmli sample buffer was added, and samples were separated by SDS-PAGE, transferred to a PVDF membrane, and exposed to X-ray film. The membrane was subsequently analyzed by Western blotting.

β -Galactosidase and CAT analysis. Transiently transfected cells were washed in 1 ml of PBS and harvested in 300 μl of CAT enzyme-linked immunosorbent assay (ELISA) kit lysis buffer according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). β -Galactosidase (β -Gal) enzymatic activity was determined by a spectrophotometric assay using *o*-nitrophenyl- β -D-galactopyranoside as previously described (34). CAT levels were determined using a CAT ELISA kit according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). Relative IRES activity was calculated as the ratio of CAT to β -Gal.

S6K2 TAP. A S6K2 open reading frame was introduced into the protein G-streptavidin-tandem affinity purification (GS-TAP) vector (6) by the use of Gateway technology (Invitrogen) to express the protein G and streptavidin binding peptide (SBP) as an N-terminal TAP fusion protein. TAP-S6K2 was transiently expressed in HEK293T cells; for each purification, 10 15-cm-diameter plates were pooled at 80% confluence to obtain cell lysates. Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 125 mM NaCl, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl_2 , 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4 , 1 mM EDTA, protease inhibitors) and lysates cleared by centrifugation. Cleared cell lysates were then incubated with rabbit IgG agarose (Sigma) for 2 h at 4°C. Bound proteins were washed 3 times with the lysis buffer followed by twice with TEV cleavage buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 0.2% NP-40) and eluted by incubating the IgG beads with 100 U of TEV protease at 4°C for 2 h. TEV-cleaved lysates were incubated with streptavidin beads (Pierce) for 2 h at 4°C and washed 4 times with cleavage buffer (with protease and phosphatase inhibitors). Bound proteins were eluted by boiling in sample buffer and separated on a polyacrylamide gradient gel (Invitrogen) (4% to 15%). The protein lane was then excised in 1-mm-thick gel slices and digested with trypsin. Digested peptides were processed and analyzed using an orthogonal acceleration quadrupole time of flight mass spectrometer (Synapt HDMS; Waters, United Kingdom). Liquid chromatography-tandem mass spectrometry (LC/MS/MS) data were then searched against a nonredundant protein database (UniProt 12.4) using the Mascot search engine program (Matrix Science, United Kingdom) and analyzed using Scaffold software.

HA-tagged protein immunoprecipitation. HEK293T cells were transiently transfected with HA-S6K2 for 24 h followed by treatment with or without FGF-2 (10 ng/ml) for 3 h. Cells were lysed in co-IP buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA [pH 8.0], 0.5% Triton X-100, 5 mM beta-glycerolphosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4) with sonication. Anti-HA-agarose beads (Sigma) were incubated with the lysate for 2 h at 4°C followed by 3 washes with lysis buffer. Laemmli sample buffer was added, and the samples were analyzed by Western blotting.

Endogenous protein immunoprecipitation. HEK293T cells were treated with FGF-2 (10 ng/ml) for 15 min, lysed in NP-40 buffer (50 mM HEPES [pH 7.4], 120 mM NaCl, 0.5% NP-40, 2 mM EDTA, 5% glycerol), and rotated for 30 min at 4°C. Lysates were centrifuged at 12,000 $\times$ g for 10 min at 4°C. The supernatant was precleared with protein A/G agarose beads for 1 h at 4°C. Protein A/G agarose beads were conjugated to 5 μg of goat anti-S6K2 (Santa Cruz Biotechnology) or rabbit anti-PDCD4 (Millipore) antibody overnight at 4°C in PBS, washed three times in cold PBS, and used for IP. The IP was performed for 2 h at 4°C, followed by three

washes in cold lysis buffer. Laemmli sample buffer was added, and the samples were analyzed by Western blotting using TrueBlot anti-goat or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (eBioscience).

Western blot analysis. Cells were washed in 1 ml of PBS and lysed in 150 μl of RIPA buffer for 30 min at 4°C, followed by centrifugation at 12,000 $\times$ g for 10 min to pellet debris. Protein concentrations were assayed by a bicinchoninic acid (BCA) protein assay kit (Pierce), and equal amounts of protein extract were separated by 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Samples were analyzed by Western blotting using mouse anti-rpS6 (Cell Signaling Technology), rabbit anti-phospho-rpS6 (Cell Signaling Technology), anti-Bcl- x_L (Cell Signaling Technology), anti-XIAP (rabbit anti-GST-XIAP; AEgera), or anti-RIAP3 (22), mouse antinucleolin (Santa Cruz Biotechnology), mouse anti-GAPDH (Advanced Immunochemical Inc.), rabbit anti-PDCD4 (Rockland), rabbit anti-PDCD4(S67-P) (Millipore), rabbit anti-GST (Santa Cruz Biotechnology), or HRP-conjugated anti-HA (Roche) antibodies followed by species-specific HRP-conjugated secondary antibodies (Cell Signaling Technology). Antibody complexes were detected using an ECL or ECL Plus system (GE Biosciences) and were quantified using Odyssey densitometry software (Li-COR Biosciences).

RNA extraction and quantitative RT-PCR (qRT-PCR) analysis. Total RNA was isolated from transfected cells by the use of an Absolutely RNA miniprep kit according to the manufacturer's instructions (Stratagene). cDNA was generated using an oligo(dT)₁₈ primer and a bulk First-Strand Synthesis kit according to the protocol provided by the manufacturer (GE Biosciences). The synthesized cDNA was used as the template for quantitative PCR using PerfeCTa SYBR green FastMix (Quanta Biosciences) along with gene-specific primers for the XIAP coding region (QuantiTect primer assay; Qiagen), XIAP IRES- or non-IRES-containing mRNA variants (38), Bcl- x_L (QuantiTect Primer Assay, Qiagen), Apaf-1 (5'-CTTGAGCCCTGGAGTTTGAG and 5'-TGCATGAAGTCCCATGA AAT), vascular endothelial growth factor A (VEGF-A) (5'-CGCGGAGG CTTGGGGCA and 5'-GGTTTCGGAGGCCCGACC), cIAP1 (15), or GAPDH (11) and analyzed on a Mastercycler realplex (Eppendorf) real-time thermocycler using the associated realplex software. Relative expression levels were determined using the standard curve method. Controls lacking reverse transcriptase (RT) demonstrated no significant genomic DNA amplification (>10-cycle difference).

Polysome profiling. HEK293T cells from three 15-cm-diameter plates per set of conditions were lysed in cold polysome lysis buffer (15 mM Tris-HCl [pH 7.4], 15 mM MgCl_2 , 300 mM NaCl, 1% [vol/vol] Triton X-100, cycloheximide [0.1 mg/ml], RNasin [100 U/ml]). Quantities representing equal units of optical density at 254 nm (OD_{254}) were loaded onto linear sucrose gradients (10% to 50%) and centrifuged at 39,000 rpm for 90 min at 4°C. Gradients were fractionated from the top (Auto DensiFlo; Labconco), and RNA was monitored at 254 nm using a high-performance LC (HPLC) system (Åkta Explorer; GE Biosciences). Fractions (1 ml) were collected and frozen. RNA was isolated from individual fractions by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA by the use of oligo(dT) primers and a reverse transcription kit (First-Strand cDNA synthesis kit; GE Biosciences). PCR primers specific for XIAP isoforms, Bcl- x_L , Apaf-1, VEGF-A, cIAP1, or GAPDH were used to amplify messages by the use of quantitative PCR as described above. Weighted averages (F_w) were calculated for the distribution of each mRNA as described previously (8). ΔF_w represents the difference between small interfering control cells and siPDCD4-treated cells in mRNA polysome distribution.

Toeprinting assay. Toeprinting was performed as described previously (42). Briefly, rabbit reticulocyte lysates (RRL; Green Hectares) were supplemented with recombinant His-PDCD4 or GST, as indicated, at 37°C for 15 min. Subsequently, RRL was treated with RNasin (Promega) and GMP-PNP (1.7 mM) for 5 min at 37°C. Uncapped, poly(A)-tailed XIAP IRES RNA (800 ng) and ATP (1.82 mM) were added, and the reac-

TABLE 1 Identification of S6K2-interacting proteins in FGF-2-treated and untreated cells^a

Identified protein	Accession no.	Molecular mass (kDa)	% protein identification probability	
			FGF2 ⁺	FGF2 ⁻
Sodium/potassium-transporting ATPase subunit alpha-1 of <i>Homo sapiens</i>	AT1A1_HUMAN	113	100	0
Hair type II keratin intermediate filament protein of <i>Ovis aries</i>	Q28582_SHEEP	55	100	0
Keratin, type II microfilibrillar, component 5 of <i>Ovis aries</i>	K2M3_SHEEP	55	100	0
Keratin, type I cytoskeletal 16 of <i>Homo sapiens</i>	K1C16_HUMAN	51	100	0
Proteasome (prosome, macropain) 26S subunit, ATPase 2 of <i>Homo sapiens</i>	A4D0Q1_HUMAN	49	100	0
Putative uncharacterized protein of <i>Homo sapiens</i>	A0A5E4_HUMAN	25	92	0
Programmed cell death protein 4 of <i>Homo sapiens</i>	PDCD4_HUMAN	52	99	0
CDC45L protein of <i>Homo sapiens</i>	Q20WK8_HUMAN	66	100	0
Chitobiosyldiphosphodolichol beta-mannosyltransferase of <i>Homo sapiens</i>	ALG1_HUMAN	53	88	0
4F2 cell surface antigen heavy chain of <i>Homo sapiens</i>	4F2_HUMAN	58	86	0
Putative uncharacterized protein HSPC117 of <i>Homo sapiens</i>	B2R6A8_HUMAN	55	82	0
Poly(rC)-binding protein 1 of <i>Homo sapiens</i>	PCBP1_HUMAN	37	98	0
Translocon-associated protein subunit alpha of <i>Homo sapiens</i>	SSRA_HUMAN	32	97	0
Heat shock protein (105 kDa) of <i>Homo sapiens</i>	HS105_HUMAN	97	26	0
Small nuclear ribonucleoprotein polypeptide B'', isoform CRA_a of <i>Homo sapiens</i>	B2R7J3_HUMAN	25	93	0
RNase/angiogenin inhibitor of <i>Rattus norvegicus</i>	Q80YN6_RAT	50	89	0
26S protease regulatory subunit 6A of <i>Homo sapiens</i>	PRS6A_HUMAN	49	88	0
Histone-binding protein RBBP7 of <i>Homo sapiens</i>	RBBP7_HUMAN	48	88	0
Tubulin beta-6 chain of <i>Homo sapiens</i>	TBB6_HUMAN	50	88	0
DNAJB1 protein of <i>Homo sapiens</i>	Q6FI51_HUMAN	38	83	0
Protein LTV1 homolog of <i>Homo sapiens</i>	LTV1_HUMAN	55	77	0
Suppressor of G2 allele of SKP1 homolog of <i>Homo sapiens</i>	SUGT1_HUMAN	41	77	0

^a HEK293T cells were transiently transfected with TAP-S6K2 plasmid, and the identification of binding partners was performed as described in Materials and Methods. LC/MS/MS data were searched against a nonredundant protein database (UniProt 12.4) using the Mascot search engine program (Matrix Science, United Kingdom) to determine the protein identity (probability-based protein identification). Only proteins differentially identified in FGF-2-treated versus untreated cells are shown.

tion mixtures were incubated at 37°C for 5 min. The reaction volume was brought to 40 μ l by the addition of toeprinting buffer [20 mM Tris-HCl (pH 7.6), 100 mM KOAc, 2.5 mM Mg(OAc)₂, 5% (wt/vol) sucrose, 2 mM DTT, 0.5 mM spermidine] and incubated at 37°C for 3 min. Subsequently, 5 pmol of toeprinting primer (5'-CTCGATATGTGCATCTGTA [labeled at the 5' end with IRDye 800]) was added and the reaction mixture was incubated on ice for 10 min. Deoxynucleoside triphosphates (dNTPs; 1 mM), Mg(OAc)₂ (5 mM), and avian myeloblastosis virus reverse transcriptase (Promega) (1 μ l) were added to the reaction mixture, and the final volume was brought to 50 μ l with toeprinting buffer. Primer extension occurred for 45 min at 37°C. The cDNA products were purified by phenol-chloroform extraction and analyzed using a standard 6% sequencing gel and a model 4200 IR2 sequence analyzer (LI-COR, Lincoln, NE).

Statistical analysis. All data are expressed as means $\pm$ standard deviations (SD). Unless otherwise stated, all results were obtained through a minimum of three independent experimental replications. For reporter assays, independent replicates consisted of three biological triplicate experiments. An unpaired *t* test was performed to determine data significance using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

S6K2 directly interacts with and phosphorylates PDCD4. We previously determined that, upon FGF-2 stimulation, a complex forms among S6K2, B-Raf, and PKC ϵ , leading to the activation of S6K2. Importantly, this activation results in a translational up-regulation of the antiapoptotic XIAP and Bcl-x_L proteins, leading to enhanced cell survival (37). These findings prompted us to determine the mechanistic link between S6K2 and the upregulation of XIAP and Bcl-x_L expression.

To identify novel interactors of S6K2 that may participate in XIAP and Bcl-x_L translation, we performed tandem affinity purification (TAP), using cells expressing S6K2 as bait, in the presence and absence of FGF-2 stimulation. The cell lysates were prepared and purified according to a previously published TAP methodology (6), the eluates separated by SDS-PAGE, and proteins within each of the lanes identified by mass spectrometry. We were interested in binding partners that were enriched upon FGF-2 treatment and discovered several proteins that fulfilled that criterion (Table 1). Among these, we identified programmed cell death 4 (PDCD4), a known tumor suppressor, as an S6K2 binding partner. PDCD4 was previously implicated in translational control (9, 30, 47); therefore, we were interested in determining if PDCD4 had a specific effect on XIAP and Bcl-x_L translation. In order to validate the TAP data, we overexpressed HA-tagged S6K2 in HEK293T cells and performed immunoprecipitation (IP) using anti-HA agarose beads. We found that endogenous PDCD4 does coprecipitate with S6K2 and that the interaction is enhanced following FGF-2 treatment (Fig. 1A). To verify that this interaction is specific and indeed occurs in cells between native proteins, we also performed reciprocal co-IP experiments using endogenous S6K2 or PDCD4. Again, we identified an interaction between endogenous S6K2 and endogenous PDCD4 following FGF-2 stimulation (Fig. 1B).

To study this interaction further, we sought to determine if S6K2 can directly phosphorylate PDCD4. HA-tagged S6K2 was overexpressed in HEK293T cells, immunoprecipitated 24 h later, and used in an *in vitro* kinase assay with purified recombinant GST-PDCD4 or with ribosomal protein S6 peptide, a known S6K2

FIG 1 PDCD4 associates with and is phosphorylated by S6K2. (A) HEK293T cells were transiently transfected with HA-S6K2-expressing plasmid and treated with FGF-2 or left untreated, and the lysates were immunoprecipitated with anti-HA agarose beads; PDCD4 and S6K2 protein levels were monitored by anti-PDCD4 or anti-HA antibodies, respectively. (B) HEK293T cells were treated with FGF-2, and endogenous PDCD4 or S6K2 was immunoprecipitated using anti-PDCD4 or anti-S6K2 antibodies. The levels of coprecipitated PDCD4 or S6K2 were monitored by Western blot analysis. (C) Immunoprecipitated HA-S6K2 was incubated with purified recombinant GST, GST-S6 peptide, wild-type (WT) GST-PDCD4, or GST-PDCD4 S67/71/76A mutant proteins in the presence of [γ -³²P]ATP, and the reaction products were separated by SDS-PAGE and transferred to a PVDF membrane. Phosphorylation was detected by autoradiography, and protein levels were subjected to Western blot (WB) analysis using a mixture of anti-GST and anti-HA antibodies on the same membrane. (D) Recombinant GST-S6K2 was incubated with the GST-PDCD4 wild type (WT) or GST-PDCD4 S67/71/76A mutant and processed as described for panel C. (E) HEK293T cells were transfected with control siRNA (siCTRL) or S6K2-targeting siRNA (siS6K2) for 48 h and treated with FGF-2 for the indicated times, and cells were harvested and analyzed by Western blotting. siPDCD4 was used as a control for the phospho-specific antibody.

target. We found that S6K2 is in fact able to phosphorylate PDCD4 (Fig. 1C). In contrast, a mutant version of PDCD4, in which serines (S67, S71, and S76) within the putative S6K-consensus site were mutated to alanines, was not phosphorylated. In order to eliminate the possibility that another kinase coprecipitated with S6K2 and was thus responsible for PDCD4 phosphorylation, we performed the *in vitro* kinase assay using purified recombinant GST-S6K2 (Fig. 1D). We observed that S6K2 robustly phosphorylated wild-type but not mutant PDCD4, thus identifying PDCD4 as a novel substrate of S6K2. Furthermore, we investigated the effect of FGF2 stimulation on PDCD4 phosphorylation *in vivo*. We treated HEK293T cells with FGF2 after S6K2 knock-down and measured phosphorylation of PDCD4 at serine 67 by the use of a phospho-specific antibody. We observed that PDCD4 was rapidly phosphorylated after FGF2 treatment but that the phosphorylation was lost when levels of S6K2 were reduced by the presence of siRNA (Fig. 1E). Together, our data demonstrate that S6K2 is required for the phosphorylation of PDCD4 in response to FGF-2 stimulation.

S6K2 activation results in degradation of PDCD4 and a concomitant increase in XIAP and Bcl-x_L protein levels. We have

shown that S6K2 interacts with and phosphorylates PDCD4. We next investigated the consequences of PDCD4 phosphorylation by S6K2 in cells. In order to do so, we utilized a tetracycline-inducible system where kinase-active S6K2 (TOKAS6K2) is expressed upon stimulation with doxycycline (37). We observed that stimulation of cells with doxycycline (1 μ g/ml) for 24 h resulted in a marked loss of PDCD4 (Fig. 2A), which correlated with an increase in XIAP and Bcl-x_L levels as previously reported (35, 36) (Fig. 2A). In contrast, addition of doxycycline had no effect on PDCD4, XIAP, or Bcl-x_L levels in control pTripz-Kate-transfected cells (Fig. 2A). The loss of PDCD4 preceded the increase in XIAP and Bcl-x_L levels (Fig. 2B) and was mediated by the proteasome, since treatment of the cells with the proteasomal inhibitor MG132 completely prevented PDCD4 loss upon doxycycline treatment (Fig. 2C). Furthermore, when PDCD4 expression was rescued in TOKAS6K2 cells by transient transfection, the enhanced expression of XIAP and Bcl-x_L was blunted (Fig. 2D and E), further supporting the notion that the degradation of PDCD4 and the enhanced expression of both XIAP and Bcl-x_L are mechanistically linked.

PDCD4 is a repressor of XIAP and Bcl-x_L IRES-mediated translation. XIAP and Bcl-x_L belong to the small subset of cellular

FIG 2 Activation of S6K2 leads to PDCD4 degradation, with a concomitant increase in XIAP and Bcl-x_L protein levels. (A) TOKAS6K2-stable or control cells (transiently transfected with an inducible pTripz-Kate plasmid) were treated with doxycycline (Dox) (1 µg/ml) or DMSO for 24 h, and cell lysates were analyzed by Western blotting. KAS6K2 induction is indicated by an increase in the phosphorylated rpS6 level. (B) TOKAS6K2 cells were treated with doxycycline as described for panel A, harvested after the indicated time points, and analyzed by Western blotting. (C) TOKAS6K2 cells were treated with doxycycline (1 µg/ml) in the presence of the proteasome inhibitor MG132 (2 µM) for 18 h; PDCD4 and nucleolin levels were monitored by Western blot analysis. (D) TOKAS6K2 cells were transfected with a control (pcDNA3) or a PDCD4-expressing plasmid for 24 h followed by doxycycline treatment for 24 h. Protein levels were monitored by Western blot analysis. (E) The protein levels of XIAP and Bcl-x_L shown in panel D were quantified relative to nucleolin expression (**, $P < 0.01$).

mRNAs whose stress-induced translation is regulated by an IRES element found within their respective 5' UTRs (21, 49). In addition, XIAP is encoded by two distinct mRNAs that differ in their 5' UTRs; the major, shorter 5' UTR promotes a basal level of XIAP expression under normal growth conditions, while the less abundant, longer 5' UTR contains an IRES element and supports cap-independent translation during stress (38). We were therefore interested in determining if PDCD4 is a specific regulator of the IRES-mediated translation of XIAP and Bcl-x_L in response to S6K2 activation. HEK293T cells were transfected with either PDCD4-targeting or nonsilencing control siRNA, and the levels of XIAP and Bcl-x_L proteins were determined 48 h later by Western blot analysis. We observed that reducing the levels of PDCD4 by siRNA treatment resulted in a marked increase in XIAP and Bcl-x_L protein levels compared with the levels seen with a nonsilencing control (Fig. 3A) while having no effect on XIAP and Bcl-x_L total mRNA levels (Fig. 3B). To demonstrate that PDCD4 regulates the translation of endogenous XIAP and Bcl-x_L mRNAs, we performed polysome profiling to examine the association of XIAP and Bcl-x_L mRNAs with translating ribosomes in cells with reduced levels of PDCD4. We observed that reduction of PDCD4 by siRNA did not change the overall polysome profile (Fig. 3C, top panel), indicating that PDCD4 is not essential for general protein synthesis. This observation was somewhat surprising, since

PDCD4 has been reported to affect global rates of protein synthesis (46). However, monitoring the relative distributions of the XIAP IRES and Bcl-x_L mRNA within the polysome by qRT-PCR showed that both mRNAs were shifted into the heavier polysomes in cells with reduced PDCD4 levels, confirming that translation of the XIAP IRES and Bcl-x_L mRNAs is enhanced when PDCD4 levels are reduced. Importantly, the relative distribution of the XIAP mRNA splice variant that does not contain an IRES (XIAP non-IRES) remained unchanged in cells with reduced levels of PDCD4 (Fig. 3C). These data indicate that PDCD4 specifically represses translation of cellular IRES-containing mRNAs, suggesting that PDCD4 is a novel IRES *trans*-acting factor (ITAF). Additionally, we investigated the translational patterns of three other mRNAs, Apaf-1, cIAP1, and VEGF, which are all known to contain IRES elements in their 5' UTRs (1, 43, 45), to determine if PDCD4 has a global effect on IRES-mediated translation or if this effect is specific to a subset of IRES mRNAs. After analyzing the polysome distribution of these mRNAs by qRT-PCR, we observed that their translation is not affected by reduced levels of PDCD4, suggesting that PDCD4 has specific targets and is not a general inhibitor of IRES-mediated translation (Fig. 3D).

To further study the effect of PDCD4 on IRES-mediated translation of XIAP and Bcl-x_L, we utilized a previously characterized bicistronic reporter system that contains the XIAP (21) or Bcl-x_L

FIG 3 Loss of PDCD4 correlates with an increase in XIAP and Bcl-x_L translation through their respective IRES elements. (A) HEK293T cells were treated with PDCD4 siRNA or control (CTRL), nontargeting siRNA. Cell lysates were harvested and subjected to Western blot analysis. (B) Steady-state mRNA levels were measured by qRT-PCR in PDCD4 siRNA- or control (CTRL) siRNA-treated cells. (C) PDCD4 was knocked down as described for panel A, and cell lysates were subjected to polysome profiling. The polysome profiles are shown in the top panel; distributions of the XIAP IRES, XIAP non-IRES, and Bcl-x_L mRNAs relative to that of GAPDH are shown as percentages of total mRNA (% mRNA) in each fraction. The results of a representative experiment are shown. LMWP, low-molecular-weight polysome; HMWP, high-molecular-weight polysome. (D) The distribution of mRNAs in each polysome fraction as shown in panel C was calculated as a ΔF_W value (8). (E) Bicistronic DNA constructs containing XIAP or Bcl-x_L IRES elements were transfected into HEK293T cells after treatment with PDCD4 siRNA or control (CTRL) siRNA. IRES activity was measured as the ratio of CAT expression to β -gal expression. (F) *In vitro*-transcribed IRES reporter mRNAs were transfected into HEK293T cells after treatment with PDCD4 siRNA or control, nontargeting siRNA. IRES activity was quantified as the ratio of CAT protein to CAT mRNA (**, $P < 0.01$).

(49) IRES element. In this system, translation of the first cistron (β -Gal) is cap dependent, whereas translation of the second cistron (chloramphenicol acetyltransferase [CAT]) is driven by the IRES. By calculating the ratio of CAT expression to β -Gal expression, the relative IRES activity can be determined. HEK293T cells

were initially transfected with PDCD4-targeting siRNA or a non-silencing control siRNA. At 48 h later, the cells were transfected with the bicistronic reporter plasmids, and reporter protein levels were assayed after 24 h. We found that the activity of both the XIAP and Bcl-x_L IRES elements was enhanced approximately

2-fold when PDCD4 levels were reduced by siRNA treatment (Fig. 3E). In contrast, reduction in PDCD4 levels did not increase CAT expression from control plasmids (the XIAP non-IRES 5' UTR and a short unrelated sequence, pBic). Since the validity of data generated with bicistronic DNA constructs has been questioned recently (29, 44), we also used direct RNA transfection of *in vitro*-transcribed XIAP and Bcl-x_L reporter RNAs. These capped and polyadenylated reporter RNAs incorporate a strong hairpin structure at the 5' end to restrict their capacity for cap-dependent translation; thus, the expression of CAT is driven solely by the IRES (38). Following transfection of these synthetic transcripts into HEK293T cells in which the PDCD4 levels were reduced by siRNA, CAT protein levels were quantified. Similar to the data obtained with the bicistronic DNA constructs, we observed an increase in IRES activity for both XIAP and Bcl-x_L when PDCD4 levels were reduced (Fig. 3F). These observations confirm that PDCD4 is a specific ITAF for the XIAP and Bcl-x_L IRESs and that, under normal conditions, PDCD4 acts as a repressor of their activity.

PDCD4 binds directly to IRES-containing RNA. Our observations that PDCD4 represses IRES-mediated translation of XIAP and Bcl-x_L mRNAs suggest that PDCD4 associates with these mRNAs by binding to their IRES sequences. To determine if PDCD4 binds directly to IRES RNA, we performed a UV-cross-linking experiment using purified, recombinant His-PDCD4 and *in vitro*-transcribed ³²P-labeled RNAs. We found that both XIAP and Bcl-x_L IRES RNAs cross-linked to His-PDCD4 in a dose-dependent manner, whereas a nonspecific, cIAP1 3' UTR RNA (50) did not bind under the same conditions (Fig. 4A). Furthermore, we performed nitrocellulose binding assays to determine the relative binding affinities of PDCD4 for the individual IRES RNAs. We observed that PDCD4 bound the XIAP and Bcl-x_L IRESs with similar apparent *K_d* (dissociation constant) values (253 nM and 183 nM, respectively); however, there was no binding detected with the cIAP1 3' UTR RNA (Fig. 4B). These data confirm that PDCD4 does indeed bind directly and specifically to IRES RNA.

We were further interested in identifying the specific domain(s) of PDCD4 required for binding to the IRES, as well as the sequence or structure of the IRES RNA to which PDCD4 binds. PDCD4 contains a putative N-terminal RNA binding domain (RBD) followed by two MA-3 domains involved in protein-protein interactions (47). We therefore generated HIS-tagged mutants of PDCD4 by deleting the RBD, leaving only the MA-3 domains (PDCD4^{ΔRBD}; amino acids 157 to 469), or by deleting both MA-3 domains (PDCD4^{RBD}; amino acids 1 to 156). We measured the binding of these mutants with the XIAP IRES compared to wild-type PDCD4 binding by nitrocellulose filter binding assays. We observed that the RBD of PDCD4 was both necessary and sufficient for binding to the XIAP IRES, since the PDCD4^{RBD} mutant exhibited binding similar to that seen with full-length PDCD4, whereas the PDCD4^{ΔRBD} mutant showed no binding (Fig. 4C). Additionally, we were interested in determining more specifically where PDCD4 binds on the XIAP IRES. The structure of the XIAP IRES was previously determined to contain two stem-loops joined by a flexible linker region (Fig. 4D) (2); interestingly, two known negative regulators of XIAP IRES, PTB and hnRNP A1, were both shown to bind domain II of the IRES, suggesting that repression of the IRES function is accomplished by repressor protein(s) binding to the 3' end of the IRES, in the vicinity of the

initiation AUG codon (2, 33). We therefore hypothesized that PDCD4 would also bind to this region. We previously generated a mutant XIAP IRES RNA deleted of stem-loop II [XIAP 3'(-47) (21)] and used the RNA derived from this mutant to measure the binding of PDCD4 in nitrocellulose filter binding assays. We observed that, compared to the results seen with the full-length XIAP IRES, the binding of PDCD4 was abolished when stem-loop II was deleted (Fig. 4D).

We further wished to determine if the association between PDCD4 and XIAP and Bcl-x_L IRES RNAs occurs *in vivo*. HEK293T cells were thus transiently transfected with Flag-PDCD4, pCDNA3-Flag (negative control), or Flag-hnRNP A1(F1) (a known XIAP and Bcl-x_L ITAF [4, 33]), and the RNA-protein complexes were immunoprecipitated using anti-Flag agarose beads under conditions that preserved the RNA-protein complexes. Following isolation of the RNA from these immunoprecipitates, cDNA was generated by reverse transcription and PCR amplified using gene-specific primers (Fig. 4E). We were unable to amplify significant amounts of XIAP or Bcl-x_L mRNA in immunoprecipitates from Flag-transfected cells. However, we identified both XIAP and Bcl-x_L RNAs in lysates from cells transfected with either Flag-hnRNP A1(F1) or Flag-PDCD4. We were unable to amplify the high-abundance GAPDH transcript from any lysates, indicating that the immunoprecipitation of endogenous XIAP and Bcl-x_L mRNAs is specific. These data support the *in vitro* binding assay results and confirm that PDCD4 associates with both endogenous XIAP and Bcl-x_L mRNAs *in vivo* to repress IRES-mediated translation.

PDCD4 prevents formation of the 48S initiation complex on IRES. We recently established an *in vitro* toe-printing assay to characterize the formation of the translation-competent 48S initiation complex on XIAP IRES RNA (42). We therefore used this technique to investigate the mechanism by which PDCD4 represses IRES-mediated translation of XIAP. We first examined the levels of PDCD4 in rabbit reticulocyte lysates (RRL) and noted that, in contrast to the results seen with HEK293T cells, the levels of PDCD4 are substantially reduced in RRL (Fig. 5A). After XIAP IRES initiation complexes were allowed to form in RRL supplemented with increasing amounts of purified His-PDCD4, or GST as a negative control, we observed that the ability of the XIAP IRES to recruit ribosomes (as determined by a toeprint +17 to +19 nt downstream of AUG) was severely impaired in the presence of His-PDCD4 (Fig. 5B; compare the first and second lanes to the fourth and fifth lanes). In contrast, addition of GST had no impact on the formation of the XIAP 48S complex (compare the third lane to the fourth and fifth lanes).

Taken together, our data strongly suggest that the mechanism of PDCD4-mediated inhibition of IRES-dependent translation involves direct binding of PDCD4 to its target RNA and subsequent interference with the formation of a translation-competent 48S initiation complex.

DISCUSSION

Several cellular mRNAs have been identified as containing IRES elements that allow them to be translated in times of cellular stress when global translation is attenuated. The mechanism of IRES-mediated translation is still poorly understood, and the many proteins involved in regulating the process are still unknown.

The ribosomal kinases S6K1 and S6K2 have been identified as important in the response to mitogen signaling, regulating a vari-

FIG 4 PDCD4 specifically binds to XIAP and Bcl-x_L IRES RNA both *in vitro* and *in vivo*. (A) Recombinant His-PDCD4 was incubated in the presence of ³²P-labeled, *in vitro*-transcribed RNA and subjected to UV cross-linking. RNA-protein complexes were separated by SDS-PAGE and analyzed by autoradiography. GST was used as a negative control. (B) Increasing concentrations of His-PDCD4 were incubated with ³²P-labeled, *in vitro*-transcribed RNA, and nitrocellulose filter binding assays were performed and analyzed as described in Materials and Methods. Levels of filter-bound RNA are plotted as a function of protein concentration. (C) Increasing concentrations of PDCD4 wild-type or mutant proteins, PDCD4^{RBD} or PDCD4^{ΔRBD}, were incubated with ³²P-labeled, *in vitro*-transcribed XIAP IRES RNA and analyzed as described for panel B. Levels of filter-bound RNA are plotted as a function of protein concentrations. (D) The structure of the minimal XIAP IRES (2) is shown on the left. The XIAP IRES 3'(-47) deletion is indicated by an arrow. Wild-type His-PDCD4 was incubated with ³²P-labeled, *in vitro*-transcribed XIAP RNA or mutant XIAP IRES 3'(-47) RNA, and protein-RNA complexes were analyzed as described for panel B. (E) Flag-PDCD4, Flag-hnRNP A1(F1) (positive control), and pcDNA3-Flag (negative control) were transfected into HEK293T cells for 24 h and immunoprecipitated with anti-Flag agarose beads. RNA was isolated and detected by RT-PCR. GAPDH RNA was used as a negative control. RNA-only samples show no genomic DNA contamination.

ety of cellular functions, including cell growth and proliferation. We have identified a novel signaling pathway wherein FGF-2 stimulation causes formation of a complex consisting of B-Raf, PKCε, and S6K2, leading to S6K2 phosphorylation and activation (35–37). In the present work, we extend those observations and identify tumor suppressor PDCD4 as a target of S6K2. We show that activated S6K2 phosphorylates PDCD4 both *in vitro* and *in vivo*, leading to its proteasomal degradation. We further demonstrate that the reduction in PDCD4 levels relieves translational repression of XIAP and Bcl-x_L mRNAs, thus allowing their increased expression. FGF-2 signaling is an important factor in tumor formation, since cancer cells frequently acquire the ability to produce growth factors and increase the number of membrane

receptors for such factors, thus resulting in a stimulation in proliferation through autocrine signaling (reviewed in reference 17). Our data suggest that FGF-2 signaling and subsequent PDCD4 degradation are key factors that cause an increase in the levels of the antiapoptotic proteins XIAP and Bcl-x_L, which likely play a role in the chemoresistance of cancer cells.

PDCD4 has been shown to be phosphorylated by S6K1, leading to its proteasomal degradation (9). The phosphorylation sites on PDCD4 appear to be the same for S6K1 and S6K2, since mutations within the reported S6K1 phosphorylation sites also prevented phosphorylation of PDCD4 by S6K2. This suggests that PDCD4 acts as an effector molecule in either the S6K1- or S6K2-mediated signal transduction pathways, broadening the range of physiolog-

FIG 5 PDCD4 inhibits recruitment of the ribosome on the XIAP IRES. (A) The lack of PDCD4 in rabbit reticulocyte lysates (RRL) was revealed by Western blot analysis. (B) XIAP IRES initiation complexes were formed on the *in vitro*-transcribed RNA in untreated RRL and in RRL preincubated with His-PDCD4, GST, or toeprinting buffer. Subsequently, initiation complexes were analyzed by toeprinting and are indicated by the line corresponding to the "17 to 19 nt from AUG" label.

ical events that regulate cellular translation via PDCD4. In addition, we were able to recapitulate the derepression of XIAP and Bcl-x_L protein synthesis by either overexpression of a kinase-active S6K2 or by targeting PDCD4 with siRNA in the absence of any additional stimuli. These data indicate that lowering the abundance of PDCD4 is sufficient for translational derepression of XIAP and Bcl-x_L mRNAs and that no additional modification of PDCD4 by S6K2 is required for this activity. Our results highlight the importance of S6K2 for the apoptotic resistance of tumor cells; targeting S6K2 to reduce its activity could be used to inhibit the pro-survival effect of FGF-2 on cancer cells, since the levels of PDCD4 would remain high and thus the levels of antiapoptotic proteins, particularly XIAP and Bcl-x_L, would be reduced. This decrease in XIAP and Bcl-x_L protein levels is expected to increase the resistance of cancer cells to death, as we have shown previously (37).

PDCD4 is a known tumor suppressor whose levels are decreased in a variety of cancers, and a link has been identified be-

tween decreased levels of PDCD4 and increased invasiveness and tumors exhibiting greater aggression (7, 27, 48). Recent studies have been aimed at elucidating the mechanism of PDCD4 action. It has been shown that PDCD4 is able to bind to eIF4A and eIF4G (41, 46), thus inhibiting the helicase activity of eIF4A and preventing cap-dependent translation, which suggests that PDCD4 acts as a general repressor of translation. However, some studies have shown that mRNAs containing a highly structured 5' UTR are preferentially repressed by PDCD4, suggesting an alternative model whereby PDCD4 has specific targets and is not a general inhibitor of translation (46, 47). Further support for this model was provided recently when it was reported that PDCD4 contains a putative RNA binding domain within its N terminus capable of binding to RNA (5). In addition, Singh et al. identified the c-myc coding region as an RNA target of PDCD4 and demonstrated that increased protein levels of PDCD4 repressed translation of c-myc; however, no molecular mechanism of this inhibition was identified (40).

Our data demonstrate that PDCD4 specifically interacts with distinct cellular mRNAs and mediates repression of their translation by interfering with the assembly of the 48S initiation complex. We have identified both XIAP and Bcl-x_L mRNAs as novel targets of PDCD4-mediated translation repression. Binding of PDCD4 to *in vitro*-transcribed XIAP and Bcl-x_L 5' UTR RNAs, but not to cIAP1 3' UTR RNA, supports the notion that PDCD4 exhibits binding specificity for a particular RNA sequence or structure. To further support this observation, we identified stem-loop II of the XIAP IRES as the structure that is bound by PDCD4, since deletion of this stem-loop causes a loss in binding, as measured by nitrocellulose filter binding assays. Moreover, we were able to define the N-terminal putative RNA binding domain of PDCD4 as being both necessary and sufficient for binding to RNA. We further demonstrated that PDCD4 interacts with both the XIAP and Bcl-x_L 5' UTRs *in vivo* by using RNA-immunoprecipitation assays, indicating that the binding occurs in a cellular context. Most importantly, we showed that PDCD4 translationally regulates both XIAP and Bcl-x_L through the IRES elements located within their respective 5' UTRs. Using reporter constructs, we demonstrated that the IRES activity of both XIAP and Bcl-x_L increases upon the loss of PDCD4, which correlates well with increasing protein levels. It has been suggested that PDCD4 does not inhibit IRES-mediated translation (46). It should be noted, however, that the IRES element of only one virus, encephalomyocarditis virus (EMCV), was tested to support that conclusion. Viral and cellular IRES elements do not share sequence or structural homology (2, 3), and the mechanisms of viral and cellular IRES-mediated translation are thus suggested to proceed through differing mechanisms. For example, while the canonical initiation factors eIF4GI and p97/DAP5 were shown to stimulate translation mediated by some cellular IRES (i.e., c-myc, XIAP, cIAP1, and p97) (25, 31), several viral IRESs function independently of these factors (i.e., those of hepatitis C virus [HCV] and cricket paralysis virus [CrPV] [10]). Notably, we confirmed an increase in levels of both XIAP and Bcl-x_L translation by the use of polysome profiling after knockdown of PDCD4 expression, which is represented as a shift of XIAP and Bcl-x_L mRNA from monosomes or light polysomes into the heavier polysomes. We did not observe any change in the overall polysome profile of PDCD4-deficient cells, suggesting that PDCD4 knockdown does not significantly affect global translation but rather translation of a subset of mRNAs. Our data thus

corroborate published reports that challenge the notion that PDCD4 is “only” a general inhibitor of translation. Instead, the sequence and structural features of the 5′ UTR are important determinants of whether or not the mRNA is regulated by PDCD4 (12, 46, 47). Finally, our toeprinting data provide a molecular mechanism for PDCD4’s mode of action. We show that a functional translation-competent 48S initiation complex is unable to form on cellular IRES in the presence of increasing concentrations of PDCD4. This suggests that direct binding of PDCD4 to domain II of the IRES interferes with ribosome recruitment, thus preventing translation initiation. It is possible that other proteins are involved in this repression, and it would be interesting to determine if binding of PDCD4 to its target mRNAs is altered in the presence of other binding partners. We have identified PDCD4 as a novel ITAF of both XIAP and Bcl-x_L mRNAs. However, as observed for other ITAFs, it is likely that other mRNAs exist that are translationally regulated by PDCD4 through their IRES elements. Given the tumor-suppressing properties of PDCD4, we predict that this “PDCD4 operon” will be shown to be composed of mRNAs that encode cell growth and cell survival- or cell death-regulating proteins. Indeed, our previously reported data support this notion—we have observed that expression of Bcl-2, an antiapoptotic protein whose translation is also mediated by an IRES, was increased upon FGF-2 stimulation (35, 36). In a preliminary analysis, however, we found that mRNAs of VEGF, Apaf-1, and cIAP1 that are known to harbor an IRES are not targets of PDCD4. It would be interesting to further examine the effect of PDCD4 on additional mRNAs through a genome-wide analysis.

In summary, we have identified PDCD4 as a novel target of S6K2 upon FGF-2 stimulation, providing a novel role for S6K2 within the cell. We have also identified PDCD4 as an ITAF that represses translation mediated by both the XIAP IRES and the Bcl-x_L IRES by interfering with the formation of the 48S ribosome initiation complex. Our work is the first to identify a mechanism of action for PDCD4’s specific regulation of cellular IRES activity and thus increases our understanding of the regulation of IRES-mediated translation in general. IRES-mediated translation of cellular mRNAs has emerged as a key mechanism that contributes to the survival and enhanced apoptotic resistance of cancer cells; targeting multiple steps of the FGF-2 pathway (such as regulation of IRES activity by PDCD4) may thus provide therapeutic options for a variety of cancers.

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U.L., O.P., M.S., and M.H. conceived the experiments; U.L., N.T., L.E.J., S.M.L., and R.R. conducted the experiments; U.L. and M.H. wrote the paper.

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