Oncogene (2009) 28, 3758–3764

www.nature.com/onc

SHORT COMMUNICATION

Disruption of the *Pdcd4* tumor suppressor gene in chicken DT40 cells reveals its role in the DNA-damage response

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The *programmed cell death gene 4* (*Pdcd4*) gene has been implicated as a new tumor suppressor gene in the development of several types of human cancer. Pdcd4 interacts with the translation initiation factor, eIF4A, and is thought to act as a translation inhibitor. Here, we have used the chicken B-cell line DT40 to disrupt the *Pdcd4* gene by homologous recombination. Our study shows that cells lacking a functional *Pdcd4* gene are viable and have no obvious defects when cultivated under normal growth conditions. However, *Pdcd4* knockout cells show an increased sensitivity to agents that cause DNA damage, such as UV light, etoposide or ethyl-methanesulfonate. In summary, our findings show that Pdcd4 has an important function in the cellular response to DNA damage. Low Pdcd4 expression, which is frequently observed in tumor cells, might therefore contribute to tumorigenesis by disturbing the cellular DNA-damage response.
Oncogene (2009) **28,** 3758–3764; doi:10.1038/onc.2009.239; published online 17 August 2009

Keywords: Pdcd4; DT40; DNA damage; UV; tumor suppressor

Programmed cell death 4 (*Pdcd4*) was originally identified as a gene activated during apoptosis (Shibahara *et al*., 1995). Subsequent studies using an *in-vitro* mouse keratinocyte model of tumor promotion (Cmarik *et al*., 1999) and an *in-vivo* mouse model of skin carcinogenesis (Jansen *et al*., 2005) showed that *Pdcd4* functions as a tumor suppressor gene. Further analysis showed that *Pdcd4* expression is reduced in human lung-, renal- and glia-derived tumors, and this contributes to their progression (Chen *et al*., 2003; Jansen *et al*., 2005; Zhang *et al*., 2006; Mudduluru *et al*., 2007; Wang *et al*., 2008). Together, these observations have established *Pdcd4* as a new tumor suppressor gene.

Pdcd4 encodes a highly conserved 55 kDa nuclear protein that is able to shuttle to the cytoplasm (Böhm *et al*., 2003). Pdcd4 is comprised of two so-called MA-3 domains, which occupy the central and the C-terminal part of the protein, and an N-terminal domain which exhibits intrinsic RNA-binding activity. The steady-state level and subcellular localization of Pdcd4 are controlled through multiple mechanisms. *Pdcd4* mRNA is targeted by the oncogenic micro-RNA, miR-21, overexpression of which downregulates *Pdcd4* expression (Asangani *et al*., 2008; Frankel *et al*., 2008; Lu *et al*., 2008). On the protein level, S6K-mediated phosphorylation of Pdcd4 triggers its ubiquitinylation by the E3 ubiquitin ligase complex SCF (βTRCP) and its subsequent degradation (Dorello *et al*., 2006). The subcellular localization of Pdcd4 is controlled by a protein kinase Akt-mediated phosphorylation (Böhm *et al*., 2003; Palamarchuk *et al*., 2005). So far, multiple functions have been ascribed to Pdcd4. Pdcd4 interacts with the eukaryotic translation initiation factor eIF4A and inhibits the helicase activity of eIF4A (Göke *et al*., 2002: Yang *et al*., 2003a, 2004; LaRonde-Blanc *et al*., 2007; Waters *et al*., 2007). Pdcd4 binds to eIF4A through its MA-3 domains, structure and complex formation with eIF4A of which have been analyzed in great detail (LaRonde-Blanc *et al*., 2007; Waters *et al*., 2007; Suzuki *et al*., 2008; Chang *et al*., 2009; Loh *et al*., 2009). eIF4A catalyzes the unwinding of stable secondary structure in the 5′ untranslated region of mRNAs, thereby allowing the recruitment of the 40S ribosomal subunit to the 5′ cap of mRNA (Hershey and Merrick, 2000). Overall, these findings have suggested that Pdcd4 suppresses the translation of mRNAs with 5′ structured untranslated regions.

In addition to its role as a translation suppressor, Pdcd4 affects the transcription of genes, apparently by interfering with specific transcription factors, such as AP-1 (Yang *et al*., 2003b; Bitomsky *et al*., 2004), Sp1 (Leupold *et al*., 2007) and p53 (Bitomsky *et al*., 2008).

Here, we have used the DT40 cell line, a chicken B-cell line that shows exceptionally high frequencies of homologous recombination (Buerstedde and Takeda, 1991), as a model system to study the role of *Pdcd4* by gene disruption. Genomic clones of the chicken *Pdcd4* gene (Schlichter *et al*., 2001) were used to generate targeting constructs, in which, parts of the *Pdcd4* gene were replaced by histidinol or puromycin resistance cassettes (Figures 1a and b). DT40 cells transfected with the histidinol-targeting construct were selected in the presence of 0.5 mg/ml histidinol and resulting clones were analyzed by Southern blotting for disruption of one copy of the *Pdcd4* gene. A positive clone was identified by the appearance of an approximately 7-kb

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Received 24 March 2009; revised 30 June 2009; accepted 13 July 2009; published online 17 August 2009

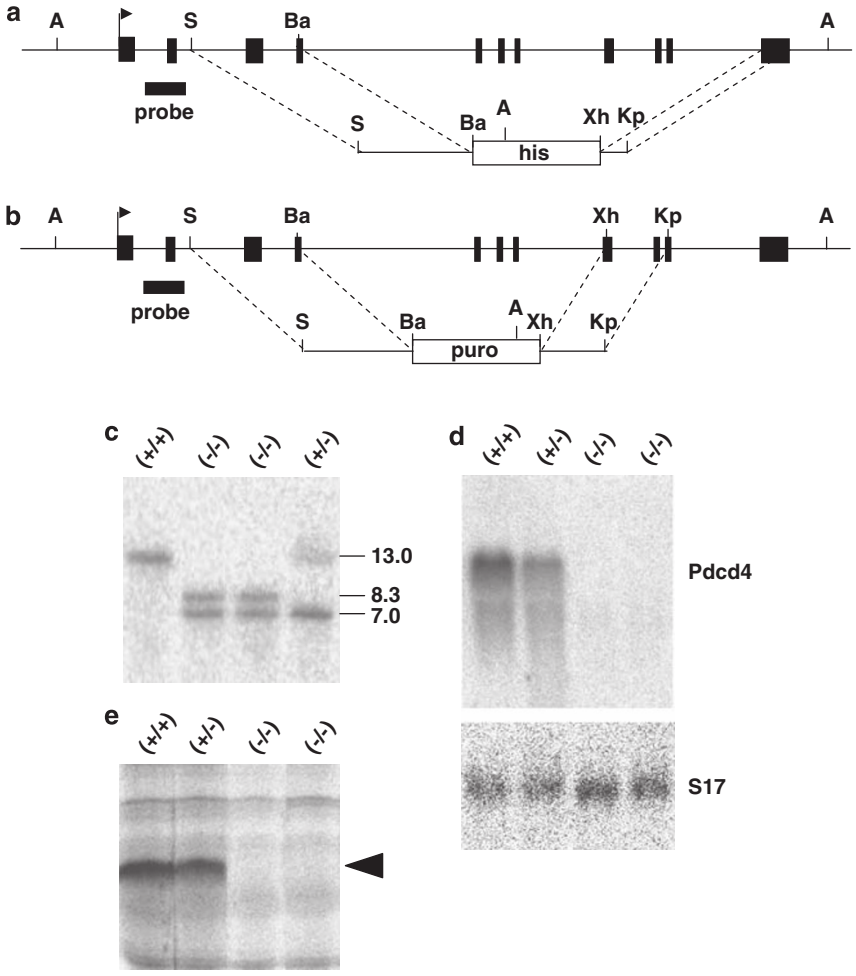

Figure 1 Generation of *Pdcd4* knockout cells. (**a**, **b**) Schematic representation of the *Pdcd4* locus (top) and targeting constructs (bottom) containing histidinol (his) and puromycin (puro) resistance cassettes. The targeting vectors were generated by first cloning a 2.7-kb *Sac*I/*Bam*HI fragment containing exons 3 and 4 as well as intron sequences for *Pdcd4*, and a *Bam*HI/*Xho*I fragment with a histidinol or puromycin resistance cassette containing the chicken β-actin promoter and the puromycin or histidinol resistance gene (Bezzubova *et al*., 1997) between the *Sac*I and *Xho*I sites of pbluescript. Subsequently, a fragment from the 3′ part of the gene was inserted between the *Xho*I and *Kpn*I sites of the plasmid. In case of the histidinol construct, a 0.6-kb fragment amplified by PCR from the last exon of the gene was used. In case of the puromycin-targeting construct a 2.2-kb fragment containing exons 8–10 and intervening sequences was inserted. Exons of *Pdcd4* are indicated by black boxes. Relevant *Apa*I (A), *Bam*HI (Ba), *Kpn*I (Kp), *Sac*I (S) and *Xho*I (Xh) restriction sites are shown. (**c**) Southern blot hybridization of *Apa*I-digested DNA of wild type (lane 1), heterozygous mutant (lane 4) and two homozygous mutant (lanes 2 and 3) clones using a 1.6-kb fragment from the 5′ side of the *Pdcd4* gene (indicated by a black bar in **a** and **b**) as probe. (**d**) mRNA from wild-type DT40 cells (+/+, lane 1), heterozygous mutant clone (+/−, lane 2) and two clones of homozygous mutant cells (−/−, lanes 3 and 4) were analyzed by northern blotting with probes specific for *Pdcd4* (top) and ribosomal protein *S17* mRNAs (bottom). (**e**) Whole-cell lysates of ^{35}S-methionine labelled wild type (+/+, lane 1), heterozygous mutant (+/−, lane 2) and two clones of homozygous mutant DT40 cells (−/−, lanes 3 and 4) were immunoprecipitated using antiserum against chicken Pdcd4, followed by SDS–PAGE and autoradiography. The Pdcd4 protein is marked by an arrow.

new *Apa*I restriction fragment (Figure 1c). This clone was then transfected with the puromycin-targeting construct and selected in the presence of 1 μg/ml puromycin. Doubly resistant clones were then analyzed by Southern blotting to identify cells in which both copies of *Pdcd4* had been disrupted. Targeting of the second copy of the *Pdcd4* gene led to the disappearance of the 13-kb parental *Apa*I fragment and the appearance of a new 8.3-kb *Apa*I fragment. The Southern blot shown in Figure 1c illustrates the successful targeting of both copies of the *Pdcd4* gene.

To confirm the absence of *Pdcd4* mRNA and protein in the knockout cells we analyzed wild-type (+/+), single (+/−) and two clones of double (−/−) knockout cells by northern blotting and immunoprecipitation. As shown in Figure 1d, *Pdcd4* mRNA was expressed at reduced levels in (+/−) cells and was undetectable in the (−/−) cells. Using an antiserum raised against bacterially expressed chicken Pdcd4, the endogenous protein was immunoprecipitated as an approximately 55 000-Mr polypeptide that was no longer detected in the (−/−) cells (Figure 1e). These data confirm that we have successfully disrupted both copies of *Pdcd4* by homologous recombination.

The finding that *Pdcd4* knockout cells are viable showed that *Pdcd4* is not essential for DT40 cells. DT40

(+/+), (+/−) and (−/−) cells grew with indistinguishable doubling times of approximately 12 h (Figure 2a), indicating that the loss of *Pdcd4* had no effect on the overall proliferation rate of the cells. We also determined the cell-cycle distribution of the (+/+), (+/−) and (−/−) cell populations using flow cytometry and found that the fractions of the cells residing in different cell-cycle phases were similar for each cell population (Figure 2b). Thus, the loss of *Pdcd4* did not result in proliferation defects or distortions of the cell cycle.

As we had previously observed that the knock down of *Pdcd4* in a human cell line alters the response of the cells to UV irradiation (Bitomsky *et al*., 2008), we were interested to investigate the effect of UV light on the DT40 *Pdcd4* (+/+) and (−/−) cells. We used two different assays to measure cell viability after exposure to UV light. Figure 3a illustrates the results of a soft-agar colony formation assay using DT40 wild-type cells

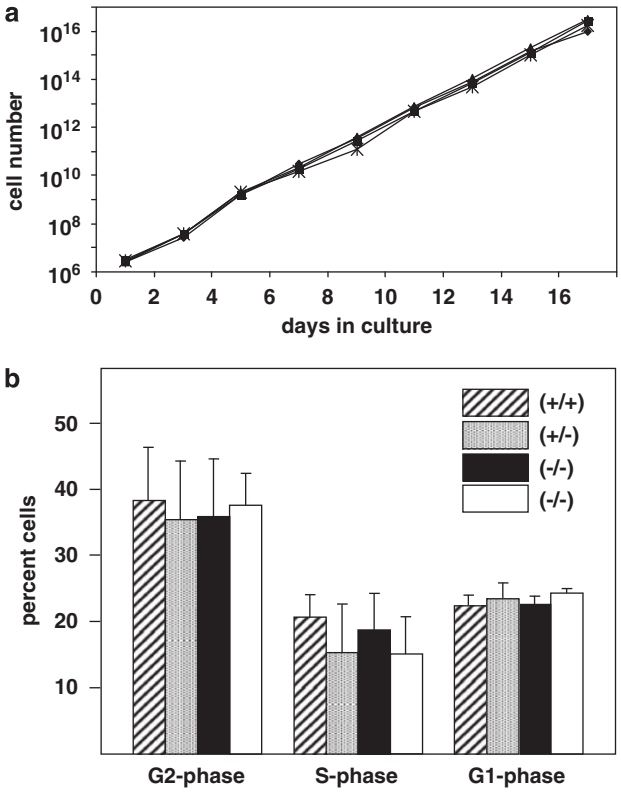

Figure 2 Growth characteristics of DT40 *Pdcd4* (+/+), (+/−) and (−/−) cells. (**a**) Wild-type DT40 (circles), *Pdcd4* single knockout (triangles) and two clones of *Pdcd4* double knockout (squares and crosses) cells were seeded in growth medium at a concentration of approximately 10^6 cells/ml and their growth was followed over several days. The number of viable cells was determined at the indicated time points and the cumulative cell number was plotted on a logarithmic scale against the time in culture. (**b**) Cell-cycle distribution of wild-type DT40 (hatched bars), Pdcd4 single knockout (stippled bars) and two clones of Pdcd4 double knockout (black and white bars) cells. Cells were stained with propidium iodide and analyzed by flow cytometry. The bars show the average percentage of cells of the total population that are in the indicated cell-cycle phases. Error bars are shown as thin lines.

as well as two different clones of *Pdcd4* (−/−) cells. The cells were irradiated with different doses of UV light, plated in semisolid medium and colonies were counted after several days. *Pdcd4*-deficient cells showed a much stronger decrease of the clonogenic potential than wild-type cells in response to UV irradiation. As a short-term assay of the proliferative capacity, we measured the ability of the cells to synthesize DNA. DT40 wild-type and *Pdcd4* (−/−) cells were subjected to different doses of UV irradiation, cultivated for 24 h and then labelled for one hour with ^{3}H-thymidine (Figure 3b). Analysis of the amount of radioactivity incorporated into DNA showed that the proliferative activity of the irradiated cells decreased strongly as the UV dose was increased. Consistent with the colony forming assay, *Pdcd4* (−/−) cells were much more sensitive to UV irradiation than wild-type cells. To exclude the possibility that the increased UV sensitivity of the *Pdcd4* knockout cells was due to clonal variation we re-expressed a hemagglutinin-tagged version of chicken *Pdcd4* in the knockout cells. The analysis of the resulting cells by ^{3}H-thymidine incorporation and colony formation showed that the Pdcd4 re-expressing cells were again more resistant to UV irradiation than the knockout cells (Figures 3c–e). This confirmed that the loss of *Pdcd4* expression was responsible for the increased sensitivity of the knockout cells.

To investigate whether *Pdcd4* (−/−) cells were also more sensitive than wild-type cells to other DNA-damaging agents, we compared the viability of *Pdcd4* (+/+) and (−/−) cells after treatment with a topoisomerase inhibitor, etoposide, or an alkylating reagent, ethyl-methanesulfonate. As shown in Figure 4, *Pdcd4*-deficient cells were more sensitive to both DNA-damaging agents than wild-type cells. Moreover, re-expression of Pdcd4 in the knockout cells again decreased their sensitivity towards EMS- or etoposide-induced damage, indicating that the observed differences were not due to clonal variation. Taken together, these experiments establish that the *Pdcd4* knockout cells show increased sensitivity towards different DNA-damaging agents and provide strong evidence that *Pdcd4* is involved in the cellular response to genotoxic stress.

Using siRNA-mediated knock down of *Pdcd4* in HeLa cells, we have recently observed that cells with decreased *Pdcd4* expression show increased survival after UV irradiation compared with control cells (Bitomsky *et al*., 2008). We also found that *Pdcd4* knockdown was accompanied by increased expression of the p53-regulated gene *p21* (*Waf1*/*Cip1*), suggesting that Pdcd4 exerts inhibitory effects on p53. Furthermore, we showed that these inhibitory effects were due to the inhibition of p300-mediated acetylation of p53 by Pdcd4 (Bitomsky *et al*., 2008). The upregulation of *p21* (*Waf1*/*Cip1*) after *Pdcd4* knockdown suppressed apoptosis and facilitated the survival of the cells after UV irradiation, consistent with the anti-apoptotic role of *p21* (*Waf1*/*Cip1*) (Gorospe *et al*., 1997; Seoane *et al*., 2002). Surprisingly, as we have shown here, the loss of *Pdcd4* in DT40 cells had the opposite effect on the survival of the cells after UV-induced DNA damage. As

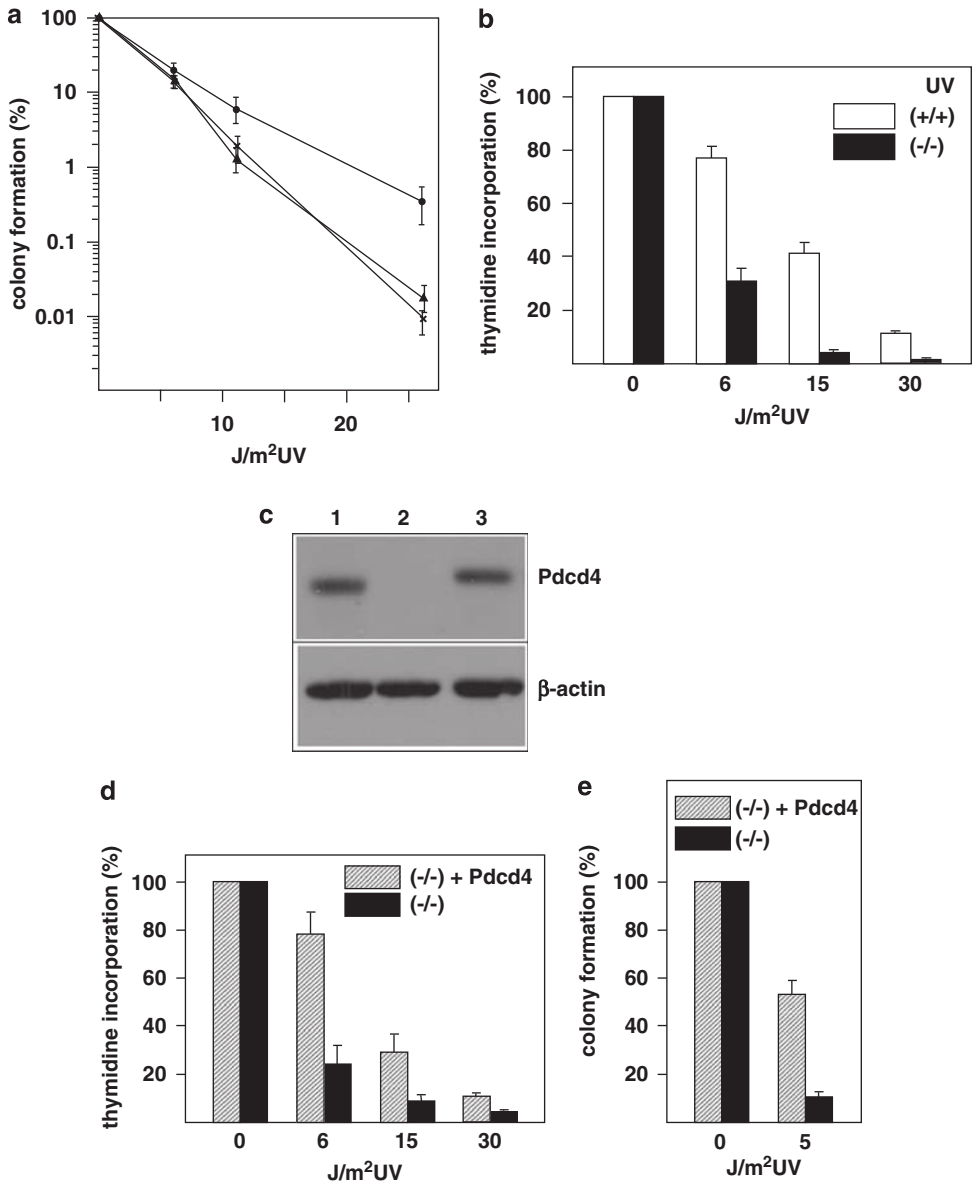

Figure 3 Response of *Pdcd4* (+/+) and (−/−) cells to UV irradiation. (**a**) DT40 wild-type cells (black circles) and two different clones of *Pdcd4* knockout cells (triangles and crosses) were subjected to different doses of UV irradiation and plated in soft agar. Colonies were counted after 7 days. Colony numbers (relative to non-irradiated controls) are plotted against the UV dose. Error bars are shown as thin lines. (**b**) Equal numbers of *Pdcd4* (+/+) and (−/−) cells were subjected to the indicated doses of UV irradiation. The proliferative activity was analyzed after 24 h by labelling the cells for 1 h with ^{3}H-thymidine and measuring the incorporation of radioactivity into trichloracetic acid (TCA)-precipitable material. The columns show the proliferative activity of irradiated cells relative to non-irradiated controls. *Pdcd4* (+/+) and (−/−) cells are represented by white and black columns, respectively. (**c**) Western blot analysis of total cell extracts of DT40 (+/+) (lane 1) and (−/−) (lane 2) cells and the same clone of DT40 (−/−) cells after stable transfection with an expression vector for hemagglutinin (HA)-tagged chicken Pdcd4 (lane 3). Blots were stained using antisera against human Pdcd4 (Rockland, Gilbertsville, PA, USA) and β-actin (Sigma, Munich, Germany). The small size difference between endogenous and exogenous Pdcd4 is due to the presence of the HA tag. (**d**) DT40 (−/−) (black columns) and (−/−) cells re-expressing Pdcd4 (hatched columns), were irradiated with different doses of UV light. The proliferative activity of the cells was analyzed as described in (**b**). (**e**) DT40 (−/−) (black columns) and (−/−) cells re-expressing Pdcd4 (hatched columns) were irradiated with a single dose of UV light (5 J/m^{2}) or left untreated. Cells were plated in soft agar and colonies were counted as described in (**a**).

DT40 cells do not express p53 (Takao *et al*., 1999), we considered the possibility that the p53 expression status of a cell might have a major impact on its response to DNA damage after Pdcd4 knockdown. To test this idea, we compared the effect of *Pdcd4* downregulation in HCT116 p53 wild-type and p53-deficient cells. Figure 5a shows that transfection with *Pdcd4*-specific siRNA caused a comparable knockdown of *Pdcd4* in both cell lines. Control siRNA- or *Pdcd4* siRNA-treated cells were then UV irradiated and plated in tissue culture dishes to determine their ability to form colonies. Consistent with our previous study (Bitomsky *et al*., 2008), p53-expressing cells formed more colonies after knockdown of *Pdcd4* (Figure 5b, left panels). By

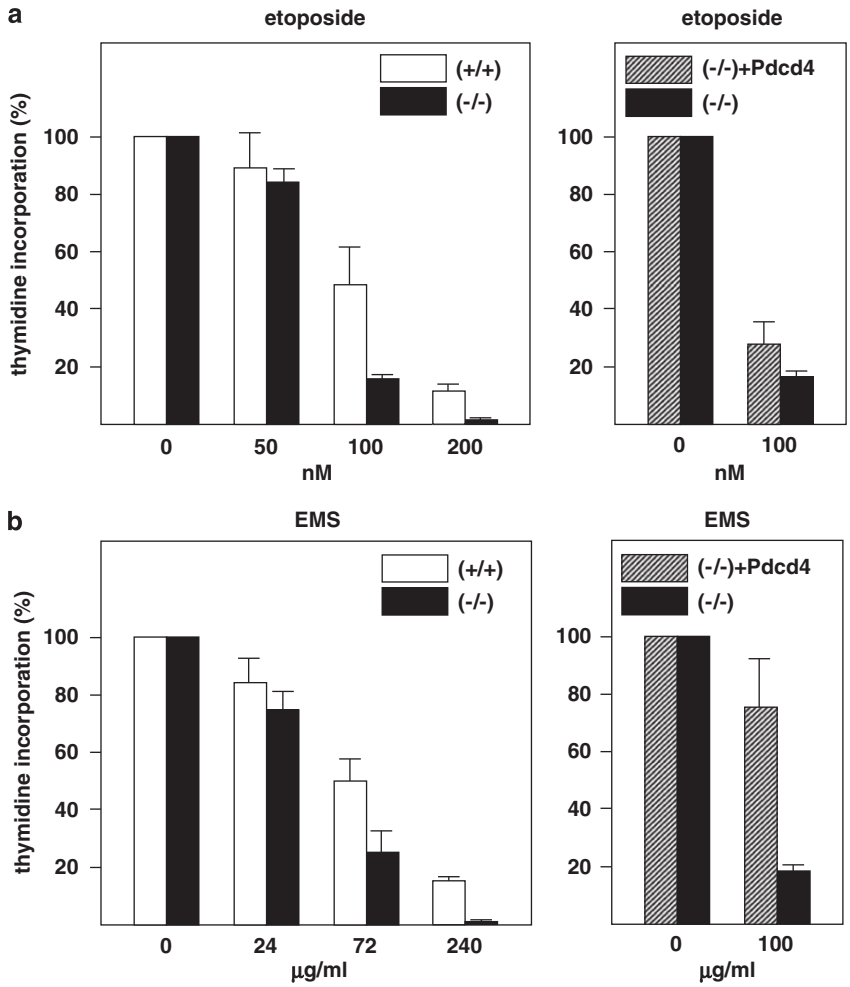

Figure 4 Response of *Pdcd4* (+/+) and (−/−) cells to etoposide and EMS. Equal numbers of *Pdcd4* (+/+) and (−/−) cells were grown for 2 days in the presence of the indicated concentrations of (**a**) etoposide or (**b**) ethyl-methanesulfonate (EMS). The proliferative activity of the cells was determined by measuring the incorporation of ^{3}H-thymidine into trichloracetic acid (TCA)-precipitable material as described in 3b. The left panels in (**a**) and (**b**) shows a comparison of *Pdcd4* (+/+, white bars) and (−/−, black bars) cells, and the right panels show comparisons between *Pdcd4* (−/−, black bars) cells and the same clone re-expressing Pdcd4 (hatched bars).

contrast, the response of the p53-deficient cells was reversed, that is, knock down of *Pdcd4* diminished their ability to survive and to form colonies (Figure 5b, right panels). This is consistent with the behaviour of the DT40 *Pdcd4*-deficient cells and strongly supports the idea that the p53 status has a major influence on the cellular DNA-damage response after a decrease in *Pdcd4* expression. In p53-positive cells, downregulation of *Pdcd4* increases p53 activity leading to an increased *p21* (*Waf1*/*Cip1*) expression and increased survival after UV irradiation, as shown before (Bitomsky *et al*., 2008). In the absence of p53, as exemplified here by the DT40 and HCT116 p53-deficient cells, the downregulation of *Pdcd4* exerts opposite effects on the survival of cells after UV-induced DNA damage. Our study, therefore, suggests that in addition to modulating p53 activity, Pdcd4 also affects the cellular response to DNA damage in a p53-independent manner.

Previous studies have shed light on the mechanism by which *Pdcd4* suppresses tumorigenesis. Several reports have suggested that decreased *Pdcd4* expression, which is frequently observed in cancer cells, increases the invasive properties of tumor cells. An overexpression of *Pdcd4* in a metastatic colon carcinoma cell line suppressed the invasiveness of the cells in a migration assay (Yang *et al*., 2006). Conversely, shRNA-mediated knockdown of *Pdcd4* expression in a non-invasive colon carcinoma cell line promoted invasive growth of the cells (Wang *et al*., 2008). In addition, overexpression of *Pdcd4* inhibits the expression of the urokinase-type plasminogen activator receptor, a cell surface protein that mediates the degradation of extracellular matrix components, and promotes tumor cells invasion and metastasis (Leupold *et al*., 2007). Our data highlight a new aspect of *Pdcd4* that is likely to be relevant for its role in tumor development. Our study suggests that the downregulation or absence of *Pdcd4* disturbs the cellular DNA-damage response, which might affect genomic stability and cause mutations in other genes. The exact mechanism by which *Pdcd4* acts at the molecular level to allow cells to cope with genotoxic stress remains to be elucidated. A likely possibility is that *Pdcd4* is involved

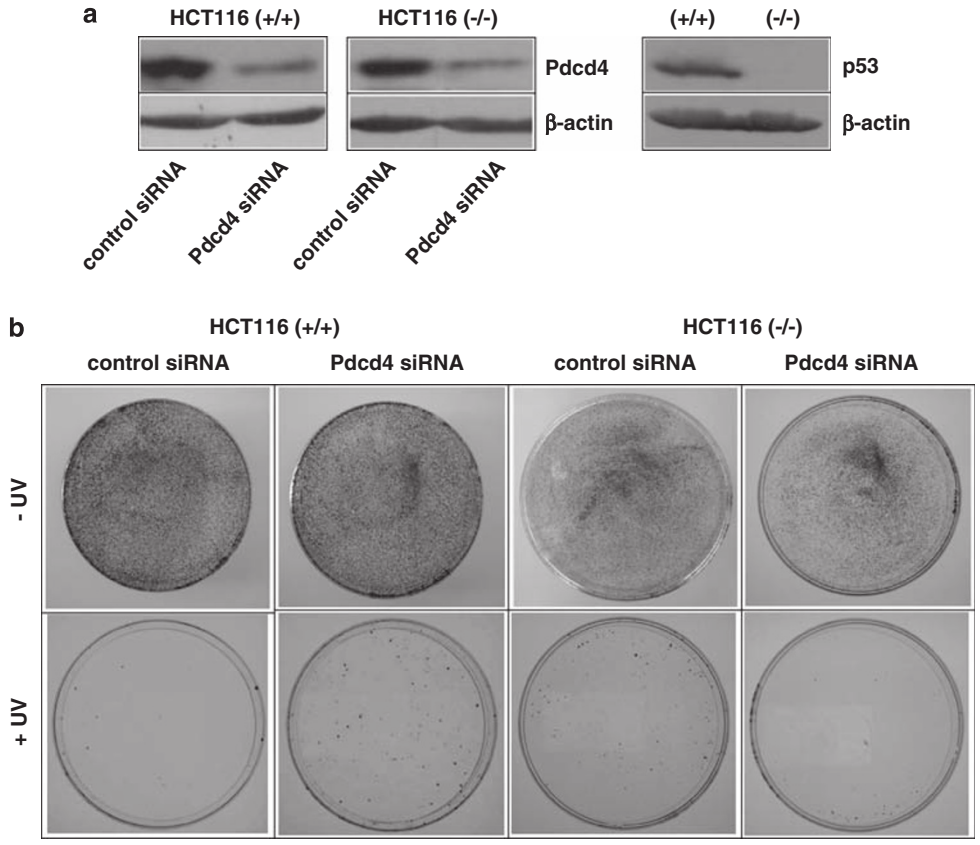

Figure 5 Knock down of Pdcd4 in HCT116 p53 wild-type and p53-deficient cells. (**a**) HCT116 *p53* (+/+) and (−/−) cells were treated with control siRNA or Pdcd4-specific siRNA followed by western blotting to monitor the Pdcd4 expression levels. The Pdcd4-specific siRNA was directed against the sequence 5′-GCATGGAGATACTAATGAA-3′ of human Pdcd4; the control siRNA was directed against the sequence 5′-AAACATGCAGAAAATGCTG-3′ of the *Renilla* luciferase gene. The rightmost panel shows a western blot of untransfected cells using antibodies against p53. Antibodies against β-actin were used in all panels as loading control. (**b**) HCT116 *p53* (+/+) and (−/−) cells were treated with control siRNA or siRNA specific for human Pdcd4. Equal numbers of viable cells were plated and UV irradiated (approximately 200 J/m^2) or left untreated. Cells were cultivated until colonies were visible, fixed and stained with crystal violet.

in the transcription or the translation of genes which themselves are involved in DNA-damage response.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank B Berkenfeld for excellent technical assistance. This study was supported by grants from the Deutsche Krebshilfe (10-1716) and the Wilhelm-Sander-Stiftung (2004.088.1). PS and RM were supported by fellowships from the Graduate School of Chemistry (GSC-MS) at the University of Münster.

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