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**ORIGINAL ARTICLE**

# A novel mechanism for the control of translation of specific mRNAs by tumor suppressor protein Pdcd4: inhibition of translation elongation

A Biyanee[1,2], J Ohnheiser[1], P Singh[1,3] and K-H Klempnauer[1]

The tumor suppressor gene *Pdcd4* (*programmed cell death gene 4*) has drawn considerable attention because its downregulation is involved in the development of several types of cancer. Because Pdcd4 interacts with the translation initiation factor eIF4A and inhibits its helicase activity, Pdcd4 has been implicated in the translational suppression of cellular mRNAs containing structured 5′-untranslated regions. However, Pdcd4's role in translation regulation is still poorly understood, because only very few physiological Pdcd4 target mRNAs are known. By using a Pdcd4-deficient clone of the chicken B-cell line DT40, we have discovered that the mRNA of the A-*myb* proto-oncogene is a novel Pdcd4 target RNA whose translation is suppressed by Pdcd4. Interestingly, the inhibitory effect of Pdcd4 is independent of the Pdcd4–eIF4A interaction, but is dependent on an RNA-binding domain at the N terminus of Pdcd4 and on sequences located within the coding region of A-*myb* mRNA, indicating that Pdcd4 suppresses A-*myb* translation by a novel mechanism. Our data show that the Pdcd4 RNA-binding domain preferentially recognizes an RNA secondary structure element formed by the part of the A-*myb* coding region that mediates Pdcd4-dependent suppression. Previously, we have shown that Pdcd4 also suppresses the translation of the c-*myb* mRNA by a similar mechanism involving binding of Pdcd4 to RNA secondary structure formed by the c-*myb* coding region. Surprisingly, our data show that Pdcd4 exerts its inhibitory activity only when the target region of Pdcd4 in A-*myb* and c-*myb* mRNA is itself translated, consistent with a mechanism in which Pdcd4 suppresses translation by interfering with translation elongation. Taken together, our work reveals a novel mechanism by which Pdcd4 affects the translational of cellular RNAs. Furthermore, as c-*myb* and A-*myb* are members of the Myb proto-oncogene family whose deregulation has been implicated in tumorigenesis, inhibiting their translation might contribute to the tumor-suppressive activity of Pdcd4.

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## INTRODUCTION

The *Pdcd4* (*programmed cell death 4*) gene was originally discovered as a gene whose expression is upregulated during apoptosis.[1] Subsequently, *Pdcd4* was shown to suppress tumor development in an *in vitro* keratinocyte model of tumor promotion[2] and in an *in vivo* model of skin carcinogenesis,[3] indicating that *Pdcd4* is a tumor suppressor gene. Decreased expression of *Pdcd4* has now been implicated in the development and progression of a variety of human cancers, including lung, colon, liver and breast cancer and glioblastoma,[4–8] further supporting its role as a tumor suppressor. Downregulation of *Pdcd4* expression is often associated with increased expression of oncogenic micro-RNA miR-21, which targets the 3′-untranslated region (UTR) of *Pdcd4* mRNA.[9–11] On the protein level, Pdcd4 is regulated by S6K-mediated phosphorylation, which triggers its ubiquitinylation via the E3 ubiquitin ligase complex SCF(betaTRCP) and its subsequent degradation.[12,13] Downregulation of Pdcd4 appears to contribute to tumor development in different ways: several studies have shown that decreased Pdcd4 expression increases the mobility and invasiveness of tumor cells.[8,11,14,15] In addition, decreased Pdcd4 expression has been shown to deregulate the cellular response to DNA damage.[16–19]

*Pdcd4* encodes a highly conserved, predominantly nuclear phosphoprotein that is able to shuttle between the nucleus and the cytoplasm.[20,21] The subcellular localization of Pdcd4 is controlled by protein kinase Akt-mediated phosphorylation.[21] Pdcd4 contains two so-called MA-3 domains located in the central and the C-terminal parts of the protein, and an N-terminal RNA-binding domain. Two main activities have been attributed to Pdcd4. Several studies have shown that Pdcd4 is involved in the control of transcription of specific genes by affecting the activities of specific transcription factors, such as c-Jun,[22,23] Sp1,[15] Twist1[24] and p53.[16,19] In addition, Pdcd4 affects the translation of specific mRNAs. Pdcd4 interacts with the eukaryotic translation initiation factor eIF4A, whose RNA helicase activity is required for unwinding secondary structures in 5′-UTRs of mRNAs.[25,26] The interaction of Pdcd4 and eIF4A is mediated by the MA-3 domains of Pdcd4[27–31] and inhibits the helicase activity of eIF4A.[25,26] It is therefore assumed that Pdcd4 acts as a translation suppressor of mRNAs with structured 5′-UTRs. This has been confirmed using artificial RNAs containing stable hairpin structures in their 5′-UTRs,[25,26] and, more recently, by the identification of p53 mRNA as a physiological Pdcd4 target mRNA.[18] However, the role of Pdcd4 in translation regulation is not well understood,

[1]Institute for Biochemistry, Westfälische-Wilhelms-Universität Münster, Münster, Germany and [2]Graduate School of Chemistry (GSC-MS), Westfälische-Wilhelms-Universität Münster, Münster, Germany. Correspondence: Dr K-H Klempnauer, Institute for Biochemistry, Westfälische-Wilhelms-Universität Münster, Wilhelm-klemm-Strasse 2, Münster 48149, Germany.
E-mail: klempna@uni-muenster.de
[3]Current address: Biozentrum, University of Basel, Basel, Switzerland.
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mainly because only very few target RNAs of Pdcd4 have been identified.

We have recently used Pdcd4-deficient clones of the chicken DT40 cell line to identify the mRNA of the c-*myb* proto-oncogene as a physiological target of Pdcd4.[32] Here we show that the translation of a second *myb* family member, A-*myb*, is also affected by Pdcd4. Interestingly, our data indicate that Pdcd4 suppresses the translation of A-*myb* and c-*myb* RNAs by a novel mechanism that is independent of eIF4A and involves a block of translation elongation.

## RESULTS

### Pdcd4 suppresses the expression of A-Myb

We have previously used Pdcd4-deficient chicken DT40 cells to identify the chicken c-*myb* mRNA as a *bona fide* translational target of Pdcd4.[32] During this work, we noted that Pdcd4 also affects the expression of another *myb* family member, A-*myb*. Figure 1a compares the steady-state levels of the A-Myb protein in DT40 wild-type cells, *Pdcd4* knockout cells and Pdcd4 knockout cells reconstituted by the introduction of a Pdcd4 expression vector. A-Myb appears as two bands in sodium dodecyl sulfate (SDS)–polyacryamide gels, presumably because of post-translational modification. It is apparent that the steady-state level of A-Myb was increased in the *Pdcd4* knockout cells and again decreased when Pdcd4 was re-expressed. Quantitative real-time polymerase chain reaction (PCR) analysis of the level of A-*myb* mRNA in these cells showed that the increased expression of A-Myb in the knockout cells was not due to increased transcription (Figure 1b); there was actually less A-*myb* mRNA in the knockout cells, although the amount of A-Myb protein was increased. Similarly, the decrease of A-Myb expression after re-expression of Pdcd4 was not caused by a decrease of A-*myb* mRNA, again indicating that the changes in A-Myb expression were not caused by corresponding changes of the A-*myb* mRNA levels.

We considered the possibility that the differences in A-Myb expression were caused by differences of the half-life of A-Myb in the presence or absence of Pdcd4. To investigate this possibility, we treated DT40 wild-type and *Pdcd4* knockout cells with the proteasome inhibitor MG132. If the lower expression of A-Myb in the wild-type cells was due to a shorter half-life of the protein, we would expect the levels of A-Myb in wild-type and knockout cells to be more similar in the presence of MG132. This was not the case if the difference in A-Myb levels between wild-type and Pdcd4 knockout cells was also observed in the presence of MG132 (Figure 1c). This suggested that the different expression levels of A-Myb were not caused by changes of the half-life of the protein. To corroborate this conclusion, we treated wild-type and *Pdcd4* knockout cells with cycloheximide to block new protein synthesis and determined the residual amount of A-Myb after different times. Figure 1d shows that A-Myb was not degraded faster in the wild-type than in the *Pdcd4* knockout cells, again demonstrating that the lower levels of A-Myb in the wild-type cells were not caused by increased turnover of the protein. We therefore concluded that the efficiency of translation of A-*myb* mRNA differs between the wild-type and Pdcd4-deficient cells.

### Pdcd4 affects A-Myb expression via its N-terminal RNA-binding domain

To further explore how Pdcd4 suppresses the translation of A-Myb mRNA, we investigated if Pdcd4 also suppresses A-Myb expression in a transfection experiment. We co-transfected expression vectors for chicken A-Myb and Pdcd4 and analyzed the amounts of both

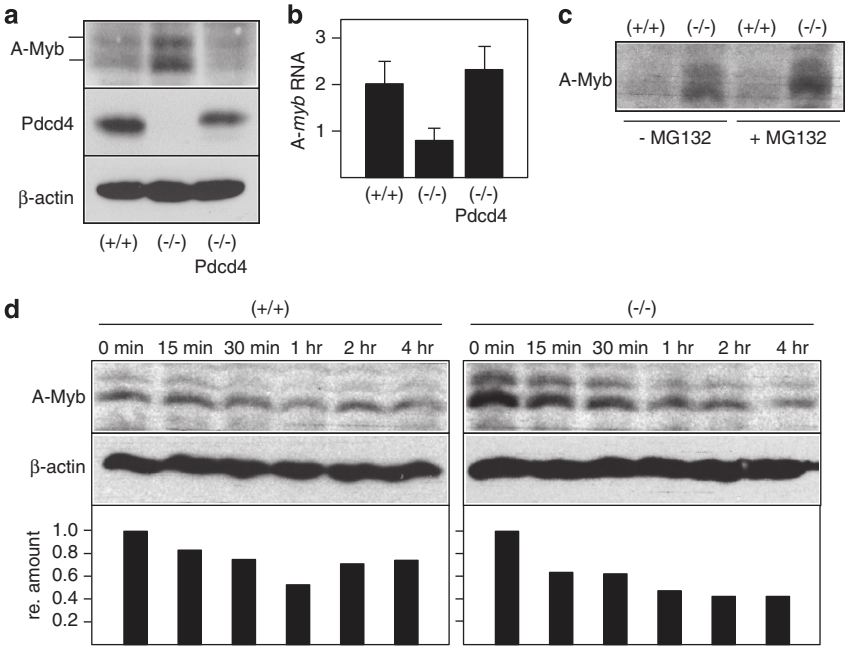


**Figure 1.** Analysis of A-Myb expression in wild-type and Pdcd4-deficient DT40 cells. (**a**) Wild-type DT40 cells (+/+), *Pdcd4* knockout cells (−/−) and Pdcd4 knockout cells re-expressing Pdcd4 (−/− Pdcd4) were analyzed by western blotting for expression of A-Myb (top), Pdcd4 (middle) and β-actin (bottom). A-Myb usually gives rise to two bands in SDS–polyacryamide gels, presumably because of post-translational modification. (**b**) mRNA from wild-type DT40 (+/+), *Pdcd4* knockout (−/−) and Pdcd4 knockout cells re-expressing Pdcd4 (−/− Pdcd4) was analyzed by real-time PCR for expression of A-*myb* mRNA. The columns indicate the amounts normalized to the expression of β-actin mRNA. (**c**) Extracts from wild-type DT40 (+/+) and *Pdcd4* knockout (−/−) cells were analyzed by western blotting with antibodies against A-Myb. Cells were treated with 10 μM of MG132 for 5 h before harvesting or left untreated. (**d**) Wild-type DT40 (+/+) and Pdcd4 knockout (−/−) cells were treated with 50 μg/ml cycloheximide. The cells were harvested after the indicated time periods and analyzed by western blotting for the expression of A-Myb and β-actin. The densitometric analysis of the western blots is shown at the bottom. The column below each lane indicates the ratio of A-Myb/β-actin normalized to 1 at the 0 min time-point.

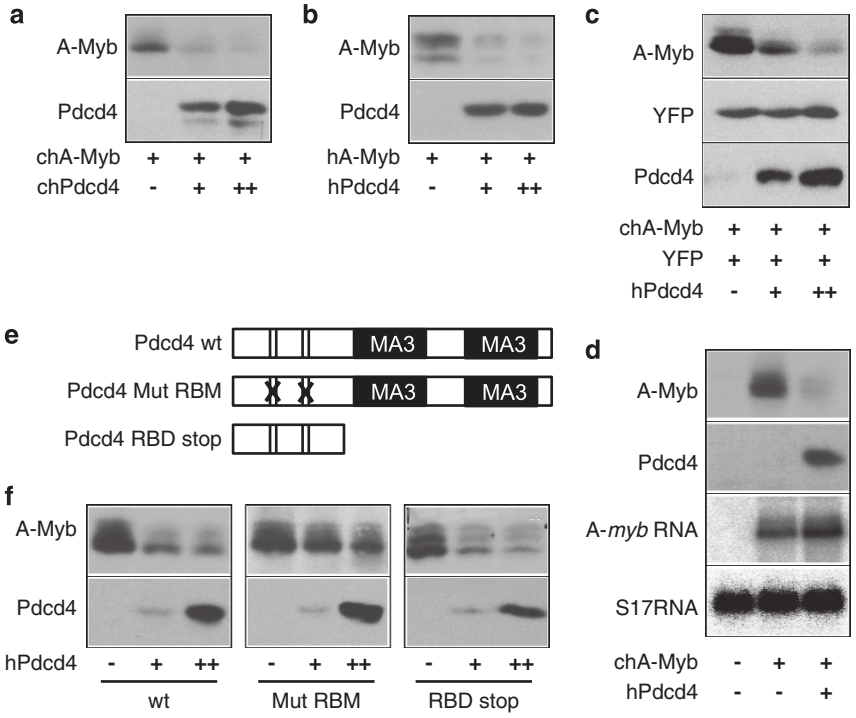


**Figure 2.** Pdcd4 suppresses translation of A-*myb* mRNA. (**a**) QT6 fibroblasts were transfected with expression vectors for chicken A-Myb and chicken Pdcd4, as indicated below the lanes. Total protein extracts were analyzed 24 h later by western blotting using antibodies against A-Myb or Pdcd4. (**b**) Similar experiment as shown in (**a**), except that expression vectors for human A-Myb and human Pdcd4 were used. (**c**) Similar to the experiment shown in (**a**), except that an expression vector for human Pdcd4 was used and an expression vector for YFP was included to assess the specificity of the inhibitory effect of Pdcd4. (**d**) QT6 cells were transfected with expression vectors for chicken A-Myb and human Pdcd4. After 24 h, total cell extracts were analyzed by western blotting as in (**a**) (upper panels). An aliquot of the transfected cells was used to isolate polyadenylated RNA, which was analyzed by northern blotting using radiolabeled probes for A-*myb* and ribosomal protein *S17* mRNA (bottom panels). (**e**) Schematic illustrations of Pdcd4 wt, mut RBM and RBD stop constructs. The crosses mark amino-acid replacements in two clusters of basic residues of the RNA-binding domain. (**f**) QT6 fibroblasts were transfected with expression vectors for chicken A-Myb and the indicated Pdcd4 constructs. Total cell extracts were then analyzed by western blotting for A-Myb (top panels) or Pdcd4 (bottom panels).

proteins. Figure 2a shows that Pdcd4 significantly suppressed the expression of A-Myb under these conditions, indicating that the inhibitory effect of Pdcd4 requires only the coding region of A-Myb. A similar experiment using human A-Myb and human Pdcd4 essentially led to the same result (Figure 2b). Figure 2c shows that Pdcd4 did not affect the expression of YFP, indicating that the effect of Pdcd4 was specific for A-Myb. We also confirmed that the decrease of A-Myb expression was not caused by a decrease of A-*myb* RNA (Figure 2d). In contrast to the endogenous A-Myb, which appeared in two bands, A-Myb expressed from an expression vector in most cases appeared as a single band. This difference could be due to the different cell types used (pre-B cells versus fibroblasts) or to the higher expression levels in transfected cells.

Next, we investigated which functional domains of Pdcd4 are involved in the suppression of A-Myb expression. Figures 2e and f show that a Pdcd4 mutant (Pdcd4 MutRBM), which lacks RNA-binding activity as a result of amino-acid replacements in the RNA-binding domain caused only a slight reduction of the amount of A-Myb, suggesting that the RNA-binding activity is involved in the inhibitory activity of Pdcd4. We also used a second mutant that consists only of the N-terminal part of Pdcd4, and therefore does not interact with eIF4A. Figure 2f shows that this extensively truncated form of Pdcd4 was able to suppress A-Myb expression. This indicated that the suppressive effect of Pdcd4 is not caused by an eIF4A-dependent mechanism but might be due to direct binding of Pdcd4 to A-*myb* mRNA.

To demonstrate that A-*myb* mRNA is a direct target of Pdcd4, we analyzed the interaction of Pdcd4 with A-*myb* RNA by RNA immunoprecipitation. DT40 wild-type and *Pdcd4* knockout cells were treated with formaldehyde to crosslink protein–RNA complexes. Cytoplasmic extracts were then immunoprecipitated with antibodies against Pdcd4, followed by reverse transcription of the co-precipitated RNA and PCR analysis. Figure 3 shows an enrichment of A-*myb* RNA relative to β-actin mRNA when the

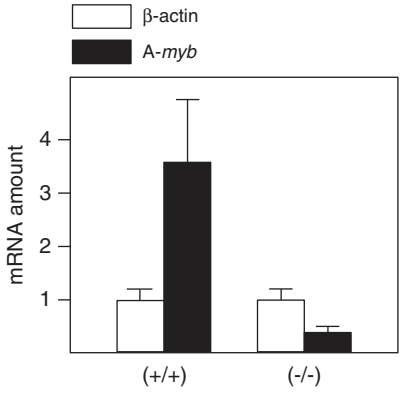


**Figure 3.** Pdcd4 is associated with A-*myb* mRNA *in vivo*. DT40-wt (+/+) and *Pdcd4*-deficient (−/−) cells were subjected to RNA immunoprecipitation using Pdcd4 specific antibodies. The relative amounts of A-*myb* (black columns) and β-actin (white columns) mRNAs present in the immunoprecipitates were measured by quantitative real-time PCR.

immunoprecipitation was carried out with extracts from DT40 wild-type cells. By contrast, when the immunoprecipitation was carried out with extracts from Pdcd4-deficient cells no enrichment ocurred. This demonstrated that Pdcd4 is associated with A-*myb* mRNA *in vivo*.

### Mapping of the 'Pdcd4 response region' in the coding region of A-*myb* RNA

The A-Myb expression vector used in Figure 2a contains only the coding region of A-*myb* mRNA but not its 5′- or 3′-UTRs, indicating that the A-*myb* coding region mediates the suppressive activity of Pdcd4. To investigate which part of the coding region is responsible for the inhibitory effect of Pdcd4, we analyzed the influence of Pdcd4 on the expression of deletion mutants of A-Myb. As shown in Figure 4, C-terminal truncation up to nucleotide 1743 did not significantly affect the ability of Pdcd4 to suppress the expression of A-Myb. When the truncation was extended up to nucleotide 1323, the expression of the resulting protein was only slightly suppressed by Pdcd4. Further truncation up to nucleotide 558 resulted in a protein whose expression was not affected by Pdcd4. We also examined the effect of Pdcd4 on several internal deletion mutants. These experiments showed that sequences up to nucleotide 1395 could be removed without abolishing the inhibitory effect of Pdcd4. In sum, these experiments indicated that the central part of the A-Myb coding region is responsible for the repressive effect of Pdcd4.

### Direct binding of Pdcd4 to the coding region of A-*myb* mRNA

To investigate if Pdcd4 directly binds to A-*myb* mRNA and to provide evidence for a role of RNA-binding in targeting Pdcd4 to A-*myb* mRNA, we performed RNA-binding experiments *with in vitro* synthesized RNA and bacterially expressed Pdcd4. Similar studies have previously demonstrated that Pdcd4 preferentially binds to an RNA secondary structure formed by the 'Pdcd4 response region' of c-*myb* mRNA.[32] We used several partially overlapping radiolabeled RNAs covering the 'Pdcd4 response region' of A-*myb* RNA for electrophoretic mobility shift experiments (Figure 5a). When analyzed in native polyacrylamide gels, each of the RNAs gave rise to fast and slow migrating forms; the latter of which disappeared when the samples were heat denatured before electrophoresis. This indicated that the slower migrating forms contain RNA secondary structures. (Figure 5b). We then performed electrophoretic mobility shift assays by incubating the RNAs in their native state, with increasing amounts of a bacterial glutathione *S*-transferase (GST)-Pdcd4 protein or identical amounts of a mutant protein that lacks RNA-binding activity (Figures 5c and d). As observed before, Pdcd4 does not form stable protein–RNA complexes under the conditions of electrophoresis.[32] Bound RNA therefore appears as a smear in the upper part of the gel. We noted that the band corresponding to the structured form of RNA no. 3 disappeared much faster than the band corresponding to the denatured form of the same RNA when the amount of GST-Pdcd4 was increased. This indicated that Pdcd4 preferentially binds to the structured form of RNA no. 3. Although all three RNAs formed secondary structures, preferential binding of Pdcd4 to the structured versus the denatured form of the RNA was not apparent in case of RNA no. 2. This suggests that preferential binding of Pdcd4 is mediated by a specific secondary structure formed by RNA no. 3 and not by the formation of secondary structure *per se*. In case of RNA no. 1, Pdcd4 appeared to bind slightly better to the structured than to the denatured form of the RNA. We performed RNA secondary structure prediction by M-fold (http://mfold.bioinfo.rpi.edu) to confirm that RNA A-*myb* no. 3 is indeed able to form a significant amount of secondary structure (Figure 5e). The structure shown is the energetically most favorable (i.e. having the most negative $\Delta G$ value) of the predicted structures. Taken together, these experiments indicated that Pdcd4 preferentially binds to a secondary structure formed by the 'Pdcd4 response region' of A-*myb* mRNA.

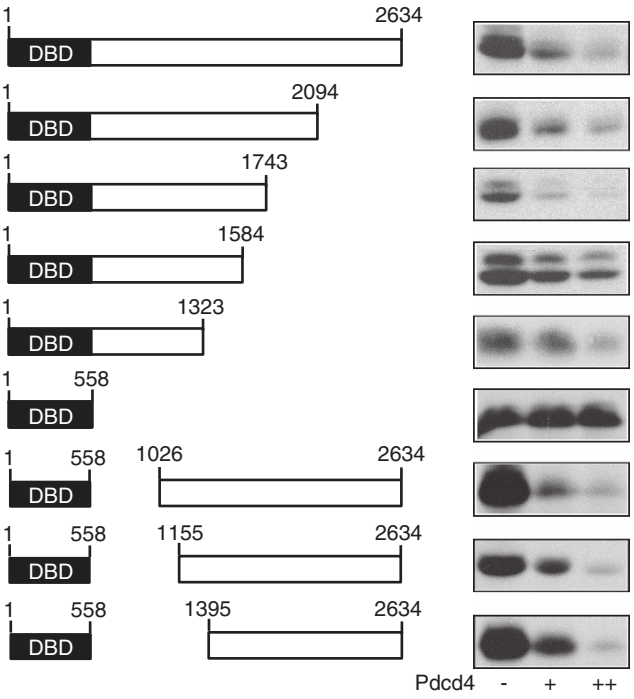


**Figure 4.** Mapping of the target region for Pdcd4-dependent suppression of A-Myb expression. A-Myb deletion constructs are shown schematically at the left. Numbers refer to nucleotides. QT6 fibroblasts were transfected with the indicated combinations of plasmids encoding full-length or truncated versions of A-Myb and increasing amounts of Pdcd4 expression vector, together with pCMVβ to control transfection efficiencies. After 24 h, total protein extracts were analyzed by western blotting with antibodies against A-Myb.

### Suppression of A-Myb expression by Pdcd4 is dependent on the translation of the 'Pdcd4 response region'

We were interested to know if the suppressive effect caused by the 'Pdcd4 response region' of A-*myb* mRNA could be conferred to a heterologous mRNA. To address this question, we constructed an expression vector for a fusion protein of YFP and the central part of A-Myb (nucleotides 1158–1743). Figure 6a shows that Pdcd4 suppressed the expression of the YFP/A-Myb protein, whereas the expression of YFP was not affected. This demonstrated that the ability to be suppressed by Pdcd4 can be transferred to a heterologous RNA via the central part of the A-*myb* coding region. This also demonstrated that this part of the A-Myb coding region is sufficient for the inhibitory effect of Pdcd4. To test whether the Pdcd4-responsive region also exerts its inhibitory effect as part of the 3′-UTR, we introduced a translational stop codon at the end of the YFP coding sequences. Interestingly, Pdcd4 failed to suppress the expression of YFP under these conditions. This suggested that the 'Pdcd4 response region' mediates the suppressive effect of Pdcd4 only when it is part of the coding region of the RNA. To extend this observation, we created a translational stop codon in the coding sequence of A-*myb* RNA 5′ end to the Pdcd4 response region, thereby truncating the translated part of the RNA. As shown in Figure 6b, the introduction of the stop codon almost completely abolished the inhibitory effect of Pdcd4. This confirmed that the presence of the Pdcd4 binding region alone is not sufficient for

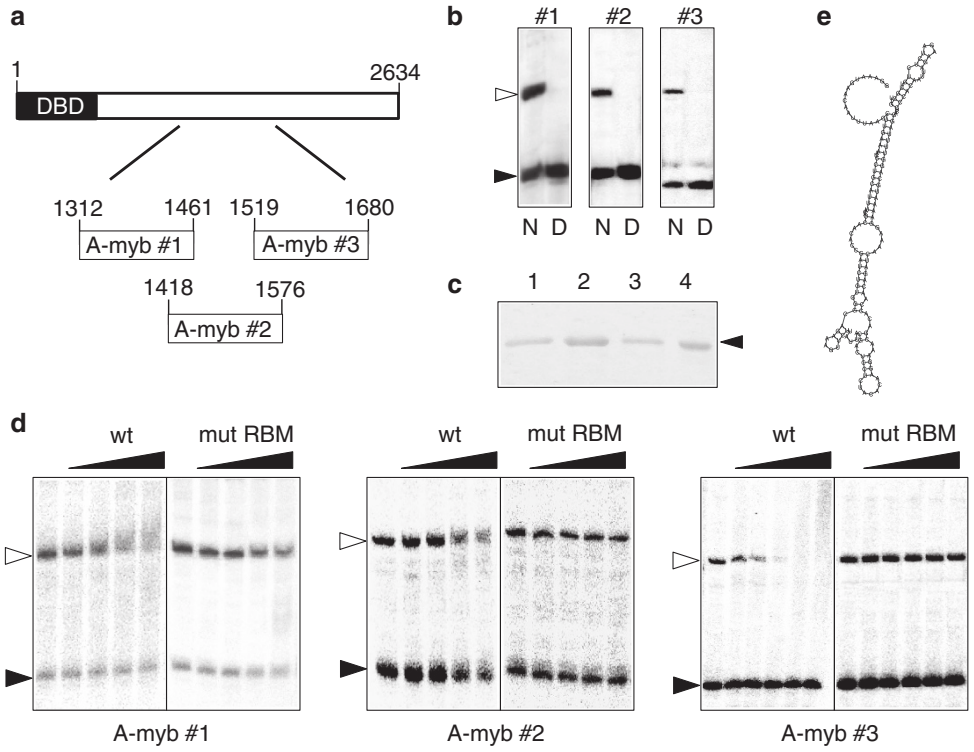


**Figure 5.** *In vitro* binding of Pdcd4 to RNAs derived from the Pdcd4 response region of A-*myb* mRNA. (**a**) *In vitro* transcribed RNAs from different parts of A-*myb* are shown schematically. The numbering refers to nucleotides of the A-*myb* coding region. (**b**) *In vitro* transcribed A-*myb* no. 1, no. 2 and no. 3 RNAs were analyzed by native polyacrylamide gel electrophoresis without (N) or after (D) heat denaturation. Black and white arrowheads mark fast and slow migrating forms of the RNAs. (**c**) Purified GST-Pdcd4-wt (lanes 1 and 2) and GST-Pdcd4-mut RBM (lanes 3 and 4) were analyzed by SDS–PAGE and stained with Coomassie brilliant blue. (**d**) A-*myb* RNAs were subjected to electrophoretic mobility shift assays with increasing amounts of the indicated GST-Pdcd4 proteins. The first lane of each panel shows the RNA without Pdcd4 protein. Black and white arrowheads mark fast and slow migrating forms of the RNAs. (**e**) RNA secondary structure prediction for A-*myb* no. 3 RNA using the M-fold web server (http://mfold.bioinfo.rpi.edu).

Pdcd4-mediated suppression, but that it must be located in the translated part of the RNA.

These findings raised the possibility that Pdcd4 might suppress the translation of A-*myb* RNA by interfering with the progression of ribosomes along the RNA during translation elongation. We have previously shown that the suppressive effect of Pdcd4 on the translation of c-*myb* mRNA is also dependent on sequences located within the c-*myb* coding region.[32] If Pdcd4 acts by suppressing translation elongation, this would provide a rationale as to why Pdcd4 binds to the coding region of these RNAs. To find out if the Pdcd4 response region of c-*myb* RNA must also be part of the coding region to be effective, we generated an expression vector for a YFP/c-Myb fusion protein (containing the Pdcd4-responsive part of c-*myb* RNA) and a similar vector that differed only by the presence of a stop codon at the end of the YFP coding sequences. Similar to the data shown in Figure 6a, the presence of the c-*myb* sequences was sufficient for Pdcd4 to suppress the expression of the YFP/c-Myb fusion protein, whereasile introduction of a stop codon abolished the inhibitory effect of Pdcd4 (Figure 7a). To substantiate this finding, we also created stop codons at two positions 5′ end to the Pdcd4 binding region of c-*myb* RNA. Figure 7b shows that this resulted in a complete loss of the ability of Pdcd4 to suppress the expression of the truncated c-Myb proteins. Taken together, these data demonstrate that the ability of Pdcd4 to suppress the translation of A-*myb* and c-*myb* mRNA is dependent on the translation of the part of the mRNA, which mediates the inhibitory effect of Pdcd4. On the basis of these findings, we propose a model in which Pdcd4 binds to a secondary structure element in the coding region of an mRNA and interferes with translation elongation.

## DISCUSSION

The tumor suppressor gene *Pdcd4* has attracted considerable attention because its expression is downregulated in many tumors by miR-21, an oncogenic micro-RNA that is frequently upregulated in cancer cells.[33] It is therefore thought that decreased expression of Pdcd4 is one important driving factor of the oncogenic activity of miR-21. In contrast to our understanding of the regulation of Pdcd4, there are still many open questions regarding its function, particularly with respect to its proposed role as a translation suppressor. Because Pdcd4 interacts with the translation initiation factor eIF4A and suppresses its helicase activity, it is thought that Pdcd4 suppresses the translation of RNAs containing structured 5′-UTRs. This has been confirmed using artificial mRNAs whose 5′-UTRs were designed to form stable stem-loop structures;[25,26] however, with the exception of p53 mRNA,[18] no naturally occurring mRNAs are known to be regulated by Pdcd4 through an eIF4A-dependent mechanism. Because only very few target mRNAs of Pdcd4 have been unequivocally identified, it is presently unclear whether Pdcd4 suppresses the translation of all RNAs containing structured 5′-UTRs or if there are mechanisms that allow Pdcd4 to target only specific RNAs. Furthermore, it is unclear whether Pdcd4 can also affect the translation of RNAs independent of its interaction with eIF4A.

The work presented here identifies the chicken A-*myb* mRNA as a novel *bona fide* target of Pdcd4. Our data show that Pdcd4 is associated with A-*myb* mRNA *in vivo* and suppresses its translation. Interestingly, unlike most other RNAs whose regulatory sequences usually are found in the 5′- or 3′-UTRs, our data show that the sequences that mediate the suppressive effect of Pdcd4 are located within the coding region of A-*myb* RNA. We

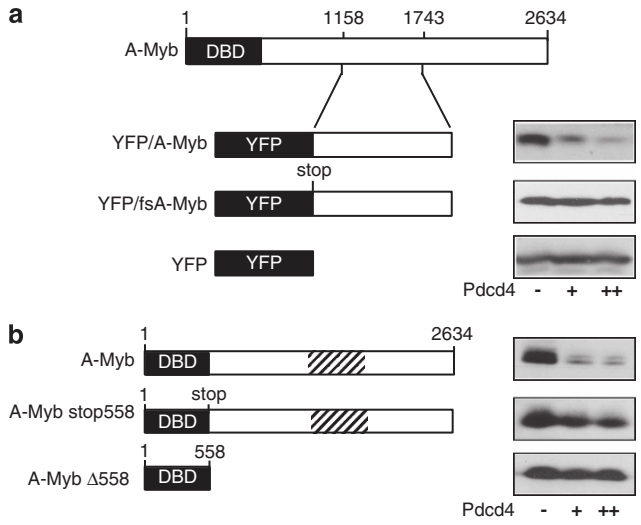


**Figure 6.** The Pdcd4 response region of A-*myb* RNA mediates Pdcd4-dependent suppression only as part of the coding region. (**a**) Expression vectors for YFP/A-Myb, YFP/fsA-Myb containing an in-frame stop codon upstream of the A-*myb* sequences and YFP were co-transfected with increasing amounts of Pdcd4 expression vector, as indicated. Total cell extracts were analyzed by western blotting with YFP-specific antibodies. (**b**) Expression vectors for full-length A-Myb and similar vectors that harbor in-frame stop codons at the 3′ end of the sequences encoding the DNA-binding domain (DBD) and contain or lack the A-*myb* sequences downstream of the stop codon were co-transfected with increasing amounts of Pdcd4 expression vector, as indicated. The proteins were detected by western blotting of total cell extracts with antibodies against the DBD of A-Myb.

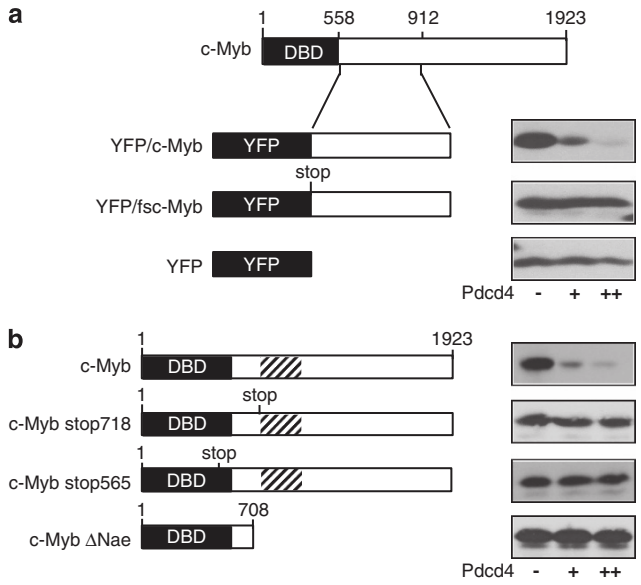


**Figure 7.** Pdcd4-dependent suppression of c-Myb expression is coupled to the translation of the Pdcd4 response region. (**a**) Similar analysis as described in Figure 6 except that the Pdcd4-responsive region from c-*myb* mRNA was fused to YFP without or with an in-frame stop codon. (**b**) Expression vectors for full-length c-Myb, similar vectors harboring in-frame stop codons at two positions or completely lacking c-*myb* sequences from the 3′ half of c-*myb* RNA were analyzed as described in Figure 6. DBD, DNA-binding domain.

have recently identified the chicken c-*myb* mRNA as a target RNA of Pdcd4.[32] Our work suggests that Pdcd4 affects the translation of A-*myb* and c-*myb* RNAs by similar mechanisms, as we have also mapped the Pdcd4-responsive sequences of c-*myb* mRNA to its coding region. However, this similarity is not due to the presence of conserved nucleotide sequences in both RNAs, as the part of the coding region, which mediates the suppressive effect of Pdcd4, is different in both cases and shows no nucleotide sequence homology. A-*myb* and c-*myb* are members of the *myb* proto-oncogene families whose deregulation has been implicated in the development of various tumors, such as leukemia,[34,35] breast and colon cancer,[36,37] adenoid cystic carcinoma[38] and glioma.[39,40] It is therefore conceivable that translational suppression of these genes by Pdcd4 contributes to the tumor suppressor function of Pdcd4. One interesting aspect of the regulation of Myb family members by Pdcd4 is the existence of an autoregulatory feedback loop between Pdcd4 and c-Myb. Previous work has shown that the promoter region of the *Pdcd4* gene contains several Myb binding sites[41] and that c-Myb and its retroviral homolog v-Myb stimulate the transcription of the *Pdcd4* gene.[42,43] As shown before[32] and confirmed here, Pdcd4 negatively affects c-Myb expression by suppressing the translation of c-myb mRNA, thereby establishing a feedback mechanism by which both proteins control their own expression levels. Whether a similar feedback mechanism also operates between Pdcd4 and A-Myb is not clear at present; however, because A-Myb is a potent transactivator, it appears possible that Pdcd4 is also a transcriptional target of A-Myb. Downregulation of Pdcd4, which has been observed in many tumor cells, will therefore disturb the balance between Myb and Pdcd4 and shift it toward higher Myb expression. Since c-Myb and A-Myb have both been implicated in tumorigenesis this will support their oncogenic potential.

We have previously shown that the highly conserved N-terminal domain of Pdcd4 acts as an RNA-binding domain that might allow Pdcd4 to target selectively specific RNAs by direct RNA binding.[20,44] In support of this idea, we have demonstrated that Pdcd4 binds preferentially to a secondary structure formed by the Pdcd4-responsive region of c-*myb* RNA.[32] The finding that Pdcd4 also binds preferentially to a secondary structure formed by the relevant part of A-*myb* mRNA further supports the notion that the ability of RNA-binding activity of Pdcd4 to recognize specific RNA secondary structures is involved in targeting Pdcd4 to specific mRNAs.

Our work also provides new insight into how Pdcd4 suppresses A-Myb expression. We have found that the N-terminal part of Pdcd4, which contains the RNA-binding domain, is sufficient to suppress A-Myb expression. Hence, the MA-3 domains, which mediate the interaction with eIF4A, are not involved in this case. This indicates that Pdcd4 is able to suppress translation also by an eIF4A-independent mechanism. How does Pdcd4 suppress translation in an eIF4A-independent manner? We have observed that the Pdcd4-responsive regions of A-*myb* and c-*myb* RNA mediate the inhibitory effects of Pdcd4 only when they are part of the translated region of the RNA. As shown in Figures 6 and 7, introduction of a single stop codon into the RNA upstream of the Pdcd4 binding region is sufficient to abrogate the inhibitory effect of Pdcd4. This observation is difficult to reconcile with a mechanism in which Pdcd4 suppresses translation initiation, but suggests a mechanism in which the binding of Pdcd4 to the RNA impedes translation elongation. We have previously observed that Pdcd4 fails to suppress c-Myb expression when the c-Myb coding region was placed under the control the herpes simplex virus-internal ribosome entry in a bicistronic RNA.[32] This observation apparently argues against a Pdcd4-dependent block of translation elongation; however, compared with cap-dependent translation, the level of internal ribosome entry-dependent translation was rather low in these experiments, which might diminish the inhibitory effect of Pdcd4 on translation elongation. We therefore propose that Pdcd4 controls translation elongation of certain mRNAs by binding to their coding regions. Sucrose density gradient fractionation experiments have previously shown

that increased expression of Pdcd4 leads to a shift of c-myb RNA from the polysomal fraction to lighter gradient fractions,[32] consistent with a model in which binding of Pdcd4 induces ribosome drop-off. Whether such a mechanism is specific for c-*myb* and A-*myb* mRNAs or more widespread will become clear as further Pdcd4 target RNAs are identified.

We are aware of only a few other examples of translational control taking place within the coding regions of mRNAs. These examples include the thymidylate synthase and dihydrofolate reductase, both of which bind to the coding regions of their own mRNAs,[45–47] the RNA-binding protein HuR, which interacts with the coding region of CD83 RNA,[48] and nucleolin, which binds to the coding region of spermidine/spermine-$N^1$-acetyltransferase and regulates its translation.[49] On the other hand, transcriptome-wide analyses of protein–RNA interactions have revealed surprisingly large numbers of interactions that take place in the coding regions of the specific mRNAs.[50–53] Regulation of translation from within the coding region, as exemplified here by Pdcd4, might therefore not be rare mechanism.

## MATERIALS AND METHODS

### Cells

Transient transfection of QT6 fibroblasts was performed as described previously.[23] In each transfection, the β-galactosidase plasmid CMVβ (Invitrogen, Karlsruhe, Germany) was included to monitor and normalize the transfection efficiency. DT40 Pdcd4 (−/−) cells and Pdcd4 (−/−) cells re-expressing hemagglutinin-tagged Pdcd4 have been described previously.[17]

### Expression vectors

pCDNA3chA-Myb encodes full-length chicken A-Myb and was obtained by subcloning the coding region of chicken A-Myb from plasmid pCRNC-chA-Myb[54] as an EcoRI/*Xba*I fragment into pCDNA3 (Invitrogen). pCDNA3chA-MybΔ2094, pCDNA3chA-MybΔ1743, pCDNA3chA-MybΔ1584, pCDNA3chA-MybΔ1323 and pCDNA3chA-MybΔ558 are derivatives encoding C-terminally truncated proteins (numbers refer to deletion end points in the nucleotide sequence, starting at the ATG). pCDNA3chA-MybΔ588–1026, pCDNA3chA-MybΔ588–1155 and pCDNA3chA-MybΔ588–1395 encode internally deleted proteins (numbers refer to the last and first nucleotide before and after the deletion). pCDNA3chA-Mybstop558 is a derivative of pCDNA3chA-Myb with a stop codon at the nucleotide 558 of the A-*myb* coding sequence. pCDNA3YFPchA-Myb(1158–1743) encodes a YFP/A-Myb fusion protein and was generated by cloning the coding region for EYFP (from plasmid pEYFP-C1; Invitrogen) and the A-*myb* coding sequences (nucleotides 1158–1743) in-frame between the *Xba*I and *Apa*I sites of pCDNA3. pCDNA3YFPfschA-Myb(1158–1743) is a similar plasmid containing an in-frame stop codon immediately 3′ end to the YFP coding sequences. An expression vector for full-length human A-Myb (pSG5-A-myb) was obtained from J Golay.[55] pCDNA3YFPchc-Myb(558–912) encodes a YFP/c-Myb fusion protein and was generated by cloning the coding region for EYFP and the c-*myb* coding sequences (nucleotides 558–912) in-frame between the *Xba*I and *Apa*I sites of pCDNA3. pCDNA3YFPfschc-Myb (558–912) is a similar plasmid containing an in-frame stop codon immediately 3′ end to the YFP coding sequences. pCDNA3chc-Myb and pCDNA3chc-Myb-ΔNae encode full-length and truncated chicken c-Myb and have been described previously.[32] pCDNA3chc-Mybstop565 and pCDNA3chc-Mybstop718 are derivatives of pCDNA3chc-Myb and were generated by creating a stop codon at nucleotides 565 and 718 of the c-*myb* coding sequence, respectively. Expression vectors for hemagglutinin-tagged chicken Pdcd4[55] and wild-type or mutant human Pdcd4 have been described.[44] pCDNA4-hPdcd4-mut RBM 1+2 encodes a mutant Pdcd4 protein with amino-acid substitutions of several basic amino acids in the N-terminal part of Pdcd4.[44] pCDNA4-hPdcd4 RBDstop carries a stop codon after amino acid 154 and encodes the N-terminal part of Pdcd4.

### Antibodies

A-Myb was detected with Myb-specific monoclonal antibodies 5E11[56] and CB100-18.[57] Pdcd4 was detected with a rabbit antiserum against the N terminus of human Pdcd4[16] or with a commercial antibody against the C terminus of Pdcd4 (Upstate Biomol, Hamburg, Germany). Antibodies against green fluorescent protein and β-actin were from Roche-Diagnostics (Mannheim, Germany) and Sigma-Aldrich (München, Germany), respectively.

### RNA analysis

Total RNA was isolated and reverse transcribed using commercial kits. The following primers were used for real-time PCR: β-actin mRNA, 5′-CGTCCACCGCAAATGCTT-3′ and 5′-GTTTTCTGCGCAAGTTAGGT-3′; chicken A-*myb* mRNA, 5′-GGCATAACCATCTCAACCCT-3′ and 5′-CTATTTCA GCCCATCGGTTT-3′.

### RNA immunoprecipitation

Approximately $10^8$ DT40 cells were fixed with 0.5% formaldehyde in the growth medium for 10 min at room temperature. Crosslinking was stopped by adding 125 mM glycine for 5 min. Cells were pelleted and lysed in 1.2 ml hypotonic buffer (10 mM HEPES (pH 7.5); 5 mM KCl; 2 mM $MgCl_2$; 0.5% NP-40). The supernatant (cytoplasmic fraction) was collected and was used for immunoprecipitation, which was performed at 4 °C overnight with a commercial Pdcd4 antiserum (Upstate Biomol). Immunoprecipitates were collected on protein-A sepharose for 2 h at 4 °C and washed several times with RIPA buffer (10 mM HEPES (pH 7.5); 50 mM NaCl; 0.5% NP-40; 0.1% SDS; 0.5% sodium deoxycholate; 1 mM phenylmethanesulfonyl fluoride; supplemented with protease cocktail and RNase inhibitor). Immune complexes were eluted from the beads with 1% SDS. Samples were reverse crosslinked for 4 h at 65 °C and treated with RNase-free DNase I for 15 min at 37 °C, followed by proteinase K treatment for 1 h at 42 °C. RNA was isolated by ribozol-OLS (Omni Life Sciences, Hamburg, Germany) extraction followed by ethanol precipitation. cDNA synthesis was performed with a commercial cDNA kit (Fermentas, St. Leon Roth, Germany). The cDNA was analyzed by quantitative real-time PCR using the primers described above (see previous paragraph).

### Electrophoretic mobility shift assay

Subregions corresponding to nucleotides 1312–1461 (A-myb no. 1), 1418–1576 (A-myb no. 2) and 1519–1680 (A-myb no. 3) of the A-*myb* coding region were amplified by PCR using primers with flanking *Hind*III and *Xba*I sites and cloned into pCDNA3. RNAs were transcribed from *Xba*I-digested template DNA in a volume of 20 µl, using 1 µg template DNA, 1 × T7 buffer (Invitrogen), 40 U/ml RNaseOUT (Invitrogen), 4 mM ribonucleotide triphosphatess, 8 mM $MgCl_2$, 25 µCi [α-$^{32}$P]UTP (3000 Ci/mmol) and 50 U T7 RNA polymerase (Invitrogen) for 1 h at 37 °C. The RNA was extracted with Trizol, ethanol-precipitated and dissolved in diethylpyrocarbonate-treated water. GST-Pdcd4-wt and GST-Pdcd4-mutRBM1+2 proteins[44] were purified by binding to GST sepharose in buffer containing 1 × phosphate-buffered saline, 1% NP-40, 2 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride and a protease inhibitor cocktail containing pepstatin A, leupeptin and aprotinin. To remove bacterial nucleic acids bound to the protein, the beads were then washed two times with binding buffer containing 300 mM NaCl and 150 mM KCl. The bound protein was eluted in 10 mM Tris–HCl (pH 7.5) supplemented with 15 mM reduced glutathione. RNA-binding reactions contained approximately $4 \times 10^6$ c.p.m. radioactive RNA, 5 mM Tris–HCl (pH 7.5); 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 2% (w/v) Ficoll and different amounts of bacterially expressed protein in a total volume of 20 µl. Binding reactions were incubated for 30 min on ice and analyzed by electrophoresis in 7.5% native polyacrylamide gels in 0.5 × TBE (Tris-borate-EDTA) buffer. Gels were dried and analyzed with a phosphor image analyzer (Fuji film, Duesseldorf, Germany).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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