

An evolutionarily conserved interaction of tumor suppressor protein Pdcd4 with the poly(A)-binding protein contributes to translation suppression by Pdcd4

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ABSTRACT

The tumor suppressor protein programmed cell death 4 (Pdcd4) has been implicated in the translational regulation of specific mRNAs, however, the identities of the natural Pdcd4 target mRNAs and the mechanisms by which Pdcd4 affects their translation are not well understood. Pdcd4 binds to the eukaryotic translation initiation factor eIF4A and inhibits its helicase activity, which has suggested that Pdcd4 suppresses translation initiation of mRNAs containing structured 5'-untranslated regions. Recent work has revealed a second inhibitory mechanism, which is eIF4A-independent and involves direct RNA-binding of Pdcd4 to the target mRNAs. We have now identified the poly(A)-binding protein (PABP) as a novel direct interaction partner of Pdcd4. The ability to interact with PABP is shared between human and *Drosophila* Pdcd4, indicating that it has been highly conserved during evolution. Mutants of Pdcd4 that have lost the ability to interact with PABP fail to stably associate with ribosomal complexes in sucrose density gradients and to suppress translation, as exemplified by *c-myc* mRNA. Overall, our work identifies PABP as a novel functionally relevant Pdcd4 interaction partner that contributes to the regulation of translation by Pdcd4.

INTRODUCTION

The tumor suppressor gene *Pdcd4* (programmed cell death 4) has been implicated in the development and progression of a multitude of human tumors, including cancer of the lung, colon, liver and breast and glioblastoma (1–6). The

expression of Pdcd4 is frequently downregulated in these tumors, often in conjunction with increased expression of oncogenic micro-RNA miR-21, which targets the 3' untranslated region (UTR) of *Pdcd4* mRNA (7–9). Downregulation of Pdcd4 protein expression 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 (5,6,9–11). In addition, decreased Pdcd4 expression appears to modulate the cellular response to DNA damage (12–15).

Pdcd4 is a highly conserved phosphoprotein that is able to shuttle between nucleus and cytoplasm (16,17). The subcellular localization of Pdcd4 is controlled by protein kinase Akt-mediated phosphorylation (17). Pdcd4 contains two so-called MA-3 domains, which are located in the central and the C-terminal parts of the protein, and an N-terminal domain involved in RNA binding. The MA-3 domains serve as binding regions for the translation initiation factor eIF4A (18–24), whereas the N-terminal domain interacts with specific RNA secondary structures as well as with certain proteins, such as the scaffold protein Daxx and the protein arginine methyltransferase 5 (15,16,25–28). Several studies have shown that Pdcd4 affects the activities of several transcription factors, such as c-Jun (29,30), Sp1 (11), Twist1 (31), p53 (12,15) and NF-κB (32) and thereby controls the transcription of specific genes. In addition, Pdcd4 acts as a translation suppressor of specific mRNAs. The interaction of Pdcd4 with eIF4A inhibits the RNA helicase activity of eIF4A, which is required to unwind secondary structures in 5' UTRs of mRNAs (18,33). It is therefore thought that Pdcd4 suppresses the translation initiation of mRNAs with structured 5' UTRs. This has been confirmed by using artificial RNAs containing stable hairpin structures in their 5' UTRs (18,33) and, more recently, by the identification of p53 mRNA as a physiological Pdcd4

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target mRNA with a highly structured 5' UTR (14). We have recently shown that Pdc4 also suppresses the translation of *c-myc* and *A-myc* mRNAs (27,28). The inhibitory mechanism in these cases appears not to involve the interaction with eIF4A but to depend on the binding of Pdc4 to secondary structure motifs located in the coding regions of these RNAs. Furthermore, this work has suggested that Pdc4 inhibits translation elongation rather than translation initiation in these cases (28).

We have now examined the role of Pdc4 as a translation suppressor in more detail and identified the poly(A)-binding protein (PABP) as a novel direct interaction partner of Pdc4. Our work shows that this interaction has been conserved during evolution and suggests that it plays an important role in tethering Pdc4 to target RNAs.

MATERIALS AND METHODS

Eukaryotic expression vectors

pCDNA3-chc-Myb is an expression vector for full-length chicken c-Myb (27). The expression vector for wild-type human Pdc4 has been described in (25). pCDNA4-hPdc4-V123A/W124A and pCDNA4-hPdc4-K114A/K115A encode mutant human Pdc4 proteins containing the indicated amino acid substitutions in the N-terminal part of Pdc4. pCDNA4-hPdc4-mut4 encodes a Pdc4 mutant in which E249, D253, D414 and D418 were replaced by alanine (14). pEGFP-eIF4G-Nt encodes a fusion protein of EGFP and amino acids 84–224 of human eIF4G and was generated by subcloning the corresponding part of the eIF4G coding region into plasmid pEGFP-C1. The expression vector for Myc-tagged human PABP was obtained from H. Okano (34). pCDNA3-6xMyc-PABP-RRM1+2, pCDNA3-6xMyc-PABP-RRM3+4 and pCDNA3-6xMyc-PABP-Ct are expression vectors for Myc-tagged subregions of human PABP. An expression vector for Flag-tagged Tiar was obtained from C. Gueydan.

Transient transfection of QT6 fibroblasts was performed by Calcium-phosphate coprecipitation as described in (30) or by lipofectamine, according to the manufacturer's protocol. The β -galactosidase plasmid CMV- β (Invitrogen) was included to monitor and normalize the transfection efficiencies.

Bacterial expression vectors

pGex-6P2-hPdc4wt encodes full-length human Pdc4 fused to GST (25). pGex-6P2-hPdc4-RBDstop is a derivative generated by introducing a translation stop codon at amino acid position 155 of Pdc4. pGex-6P2-hPdc4-mut4 encodes a GST-Pdc4 fusion protein in which Glu249, Asp253, Asp414 and Asp418 were mutated to Ala. pGex-6P2-hPdc4-mut1, pGex-6P2-hPdc4-mut2 and pGex-6P2-hPdc4-DM encode GST-Pdc4 fusion proteins carrying amino acid substitutions in the RNA-binding domain that disrupt the RNA-binding activity (25). pGex-6P2-hPdc4-R110A, pGex-6P2-hPdc4-R110K, pGex-6P2-hPdc4-K114A/K115A, pGex-6P2-hPdc4-K121A, pGex-6P2-hPdc4-V123A/W124A and pGex-6P2-hPdc4-A118E encode GST-Pdc4 fusion proteins

carrying the indicated amino acid substitutions. pGex-6P2- Δ RBD encodes a GST-Pdc4 fusion protein with an internal deletion of amino acids 66–150 of Pdc4. pGex-6P2-DmPdc4 encodes a *Drosophila melanogaster* Pdc4 GST fusion protein and was generated by amplifying the coding region of *Drosophila* CG10990 with the primers 5'-CGTGGAATCCCATGGAAGTGGGAATCGAAT-3' (forward) and 5'-ATATGCGGCCGCTTAATCACGCATGGT-3' (reverse) and cloning it between the EcoRI and NotI sites of pGex-6P2. pGEX-4T1-DmPdc4-RBDstop encodes a related fusion protein containing only the N-terminal domain of DmPdc4. To construct this plasmid the corresponding coding region was amplified using the primers 5'-GGCGAATTCATGGAAGTGGGAATCGAAT-3' and 5'-AGGCTCGAGTTACAGCTCCACATTACGATC-3' and cloning it between the EcoRI and XhoI sites of pGex-4T1. The bacterial expression vector for mouse His-PABP-RRM1–4 (amino acids 1–375) was a gift from Lorna Waters (Department of Biochemistry, Leicester, UK).

Antibodies

c-Myb was detected with Myb-specific monoclonal antibodies 5E11 (35). Pdc4 was detected with a rabbit antiserum against the N-terminus of human Pdc4 (12). Antibodies against GFP (Roche Diagnostics, Mannheim, Germany), eIF4A, eIF4G, eIF4E, PABP (Cell Signalling Technology), Y14 (Santa Cruz Biotechnology) and the Flag, Myc and His-tag were obtained from commercial suppliers. Antiserum against *Drosophila* PABP was kindly provided by E. Izaurralde (Tübingen, Germany).

GFP-Trap and co-immunoprecipitation

QT6 fibroblasts transfected with the desired expression vectors were lysed 24 h after transfection in egg lysis buffer (ELB) (120 mM sodium chloride, 50 mM Tris/HCl, pH 7.4, 20 mM sodium fluoride, 1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, 1 mM PMSF, 0.5% Nonidet P-40, 1 ng/ml of aprotinin, 0.2 ng/ml of leupeptin, 1 ng/ml of pepstatin) and centrifuged for 20 min at 14 000 $\times g$. 10% of the supernatant was retained for input control, the remaining 90% was incubated with GFP-trap beads (Chromotec, München) for 3 h at 4°C. Beads were washed three times with ELB buffer, boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed together with the input samples by SDS-(PAGE) polyacrylamide gel electrophoresis and western blotting. For co-immunoprecipitation, the desired antibodies were added to the total cell extract (TCE) and incubated for 2 h at 4°C. Immune complexes were then bound to protein A sepharose, washed and analyzed by western blotting.

GST pull-down assay

Glutathione S-transferase (GST) fusion protein expression was induced in cultures of transformed *Escherichia coli* BL21-pLysS bacteria by adding isopropyl-D-thiogalactopyranoside to a final concentration of 0.5 mM. After additional 3 h of growth at 37°C the bacteria

were harvested by centrifugation for 10 min at $5000 \times g$. Bacterial pellets were resuspended in GST lysis buffer (50 mM Tris-HCl; pH 8.0; 150 mM NaCl; 1% Triton X-100; 1 mM DTT; 0.1 mM Phenylmethylsulfonyl fluoride (PMSF)) and lysed by three freeze-thaw-cycles and sonication. An extract of soluble protein was prepared by ultracentrifugation for 1 h at $100\,000 \times g$. Extracts containing 5–10 μg of GST fusion protein were then mixed with 30 μl of glutathione-sepharose (GE Healthcare) and incubated at 4°C for 1 h. The sepharose beads were then washed three times with ELB buffer and used for GST pull-down assays as follows: QT6 cells transfected with the appropriate expression vectors were lysed in ELB buffer and aliquots of the lysate were then incubated under constant agitation for 1 h at 4°C with bacterially expressed GST fusion protein coupled to glutathione-sepharose. Alternatively, GST fusion protein-loaded sepharose beads were incubated with purified, bacterially expressed His-PABP-RRM1–4. Subsequently, beads were washed three times with ELB buffer. Bound proteins were eluted from the beads by boiling in SDS sample buffer and analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue or western blotting using appropriate antibodies.

Sucrose density gradient centrifugation of cytoplasmic extracts

Cells were treated with 50 $\mu\text{g}/\text{ml}$ cycloheximide for 30 min before they were washed with ice-cold PBS (containing 50 $\mu\text{g}/\text{ml}$ cycloheximide) and lysed for 20 min on ice in hypotonic buffer (10 mM Hepes, pH 7.5; 5 mM KCl; 2 mM MgCl_2 ; 1 mM DTT; 0.5% NP40; 1 mM PMSF; supplemented with 40 U/ml RNaseOUT (Invitrogen) and a protease inhibitor cocktail containing 5 ng/ml pepstatin A, 1 ng/ml leupeptin hemisulfate and 5 ng/ml aprotinin). The lysed cells were pelleted at 14 000 rpm for 15 min at 4°C . The supernatant was collected as cytoplasmic fraction and layered on top of a 10–50% sucrose density gradient made in hypotonic buffer. Gradients were centrifuged in a SW41 rotor at 36 000 rpm for 2 h at 4°C . The gradients were fractionated and aliquots of the fractions were analyzed by 10% SDS-PAGE and western blotting to determine the distribution of Pdc4, or treated with Proteinase K and 1% SDS and analyzed by agarose gel electrophoresis to determine the distribution of ribosomal RNAs.

RNA-binding assays

RNA-binding assays of GST-Pdc4 fusion proteins were performed as described before in (16). GST fusion proteins were fractionated by SDS-PAGE and blotted onto nitrocellulose. Radiolabeled RNA was synthesized *in vitro* using T7 RNA polymerase, [α - ^{32}P]-UTP and the linearized plasmid pCDNA3 c-myb#3 (27) as template. Blots were incubated first with 6 M urea; 0.2% NP40 for 10 min, washed four times 15 min each with RNA-binding buffer (10 mM Tris HCl, pH 7.5; 5 mM KCl; 2 mM MgCl_2) lacking RNA and then incubated for 15 min in RNA-binding buffer containing radiolabeled RNA. Subsequently, the blots were washed three times for 10 min with binding buffer lacking RNA and analyzed by autoradiography. All incubations were performed at room temperature.

Chromatography on poly(U)-agarose was performed as follows (all steps at 4°C). GST-Pdc4 fusion proteins were purified by binding to glutathione-sepharose and eluting them over night with 15 mM glutathione in 10 mM Tris-HCl, pH 7.5. Eluted proteins were then incubated for 1–2 h with poly(U)-agarose (Sigma-Aldrich) equilibrated in binding buffer (10 mM Hepes, pH 7.5; 5 mM KCl; 5 mM EDTA). The agarose beads were then transferred to a chromatography column, washed several times with binding buffer to remove unbound protein. Bound proteins were then eluted with binding buffer supplemented with increasing concentrations of NaCl. Aliquots of the input sample, the last wash fraction and the salt elution fractions were then analyzed by SDS-PAGE and western blotting.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with an oligo(A)₂₅ ribo-oligonucleotide end-labeled with [γ - ^{32}P]-ATP and T4 polynucleotide kinase. His-PABP-RRM1–4 was purified from bacterial extracts by binding to Ni-NTA beads followed by washing with purification buffer (50 mM NaH_2PO_4 , pH 8.0; 5 mM NaCl) and eluting the proteins in the same buffer supplemented with 200 mM imidazole as described above. Binding reactions were set up in a total volume of 20 ml of buffer (10 mM Tris-HCl, pH 7.8; 100 mM NaCl, 1 mM EDTA, 2% ficoll) containing the radiolabeled oligo(A)₂₅ and the desired proteins. Reactions were incubated for 30 min on ice and analyzed by electrophoresis in 7.5% native polyacrylamide gels in $0.5 \times$ TBE buffer. Gels were dried and analyzed with a phosphor image analyzer.

RESULTS

Pdc4 interacts with PABP *in vitro* and *in vivo*

We have shown previously that Pdc4 suppresses the translation of *c-myb* and *A-myb* mRNAs by binding to RNA secondary structures present in the coding regions of these mRNAs (27,28). Unexpectedly, the inhibitory effect exerted by Pdc4 on these RNAs was also observed when a mutant of Pdc4 was used that was not able to interact with the translation initiation factor eIF4A (28; and data not shown). This mutant (Pdc4-mut4) contained several amino acid substitutions in both MA3 domains that were previously shown to be essential for eIF4A binding (18–24). Therefore, we became interested to investigate whether Pdc4 interacts also with other translation factors in addition to eIF4A. *In vitro* binding experiments using GST-Pdc4 and extracts of human cells showed that Pdc4 was able to bind eIF4A, eIF4G, eIF4E and PABP present in the extract (Figure 1A). Comparison of the input and bound fractions of these proteins suggested that eIF4A and PABP were bound more efficiently than eIF4G and eIF4E. We therefore focused on the possibility that Pdc4 also binds to PABP in addition to its known interaction partner eIF4A. To investigate whether the binding of PABP by GST-Pdc4 was mediated by eIF4A, we performed a pull-down experiment with a Pdc4 mutant (Pdc4-mut4) that is unable to interact with eIF4A (18–24). This experiment clearly

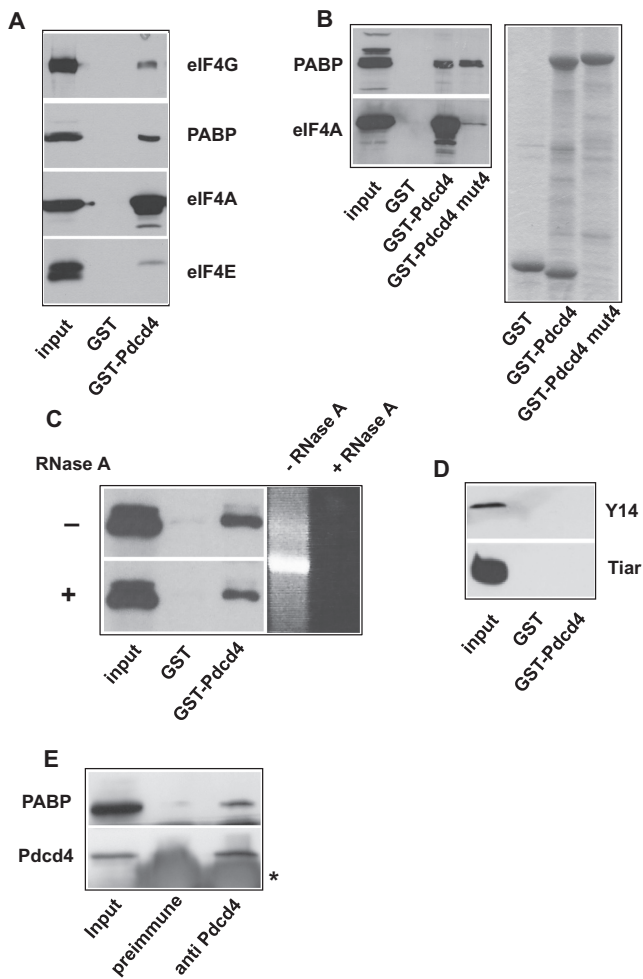


Figure 1. Pdc4 interacts with PABP. (A and B) GST, GST-Pdc4 or GST-Pdc4 mut4 proteins were immobilized on glutathione sepharose and incubated with HEK293 cell extract. Bound proteins and aliquots of the total extracts were analyzed by SDS-PAGE and western blotting with antibodies against eIF4A, eIF4E, eIF4G and PABP. A Coomassie blue stained gel demonstrating equal loading of the beads with GST proteins is shown on the right side of (B). (C) GST pull-down experiment showing the binding of PABP from extract of HEK293 cells. Binding reaction was performed in the absence or presence of 0.5 μ g/ml RNase A. The right side shows an agarose gel of *in vitro* translated RNA incubated without or with RNase A under the same conditions. (D) Extract of HEK293 cells (top) or of cells transfected with a Flag-Tiar expression vector (bottom) was analyzed for binding to GST and GST-Pdc4 as in (A). Proteins were detected with antibodies against Y14 and the Flag-tag. (E) Extract from HEK293 cells was precipitated with rabbit antiserum against Pdc4 or with pre-immune serum. Immunoprecipitates and an aliquot of the TCE (input) were analyzed by western blotting with antibodies against PABP and Pdc4. The asterisk marks an intense signal in the immunoprecipitate lanes, which is due to immunoglobulins from the antiserum.

showed that the binding of PABP to Pdc4 was independent of the Pdc4-eIF4A interaction and suggested that PABP might be a direct interaction partner of Pdc4 (Figure 1B). GST-Pdc4-mut4 also showed reduced binding of eIF4G and eIF4E suggesting that their interaction with Pdc4 was mediated by eIF4A (data not shown). Since PABP and Pdc4 are RNA-binding proteins, we were concerned that the binding of PABP to GST-Pdc4 might be due to RNA present in the cell extract. To address this pos-

sibility we performed an additional pull-down experiment to see if RNase A added during the binding reaction diminished the binding (left part of Figure 1C). A control reaction containing *in vitro* transcribed RNA and incubated under the same conditions confirmed that the amount of RNase A added to the binding reaction was sufficient to completely digest the RNA (right part of Figure 1C). Moreover, we also analyzed the binding of two unrelated RNA-binding proteins to GST-Pdc4. Figure 1C shows that the RNA-binding protein Tiar and the exon-junction complex protein Y14 were not bound by Pdc4, pointing toward a direct interaction between Pdc4 and PABP. Together, these experiments made it unlikely that the interaction of Pdc4 and PABP was mediated by unspecific binding to RNA present in the cell extract. To provide evidence that Pdc4 and PABP also interact as endogenous proteins, we performed co-immunoprecipitation experiments with extracts from untransfected HEK293 cells. Figure 1D shows that antiserum against Pdc4, but not the pre-immune serum used as control, was able to coprecipitate a significant fraction of PABP from cell extracts.

The interaction of Pdc4 and PABP is mediated by the RNA-binding domains of both proteins

To map the binding site for PABP within Pdc4, we performed *in vitro* binding experiments using wild-type and the mutant GST-Pdc4 proteins illustrated schematically in Figure 2A. The purified proteins are shown in Figure 2C. Figure 2B shows that a Pdc4 deletion construct consisting only of the N-terminal RNA-binding domain was able to bind PABP, whereas a deletion mutant lacking the RNA-binding domain but containing both MA3 domains failed to bind PABP. This showed that PABP interacts with the RNA-binding domain of Pdc4 and confirmed that binding of eIF4A and PABP are independent binding partners of Pdc4. We also tested the binding of PABP to Pdc4 proteins carrying amino acid replacements in one or both of two clusters of basic amino acids (referred to as RBD mut1, RBD mut2 and RBD-DM) that were found previously to be crucial for RNA binding by Pdc4 (25). Both single RBD mutants showed reduced binding of PABP and the double mutant did not bind to PABP at all (Figure 2B). This substantiated the conclusion that PABP interacts with the RNA-binding domain of Pdc4.

To map the binding site for Pdc4 within PABP, we performed *in vitro* pull-down experiments using partially deleted versions of PABP (Figure 2D). As shown in Figure 2E and F, only full-length PABP and the construct consisting of RRM motifs 1 and 2 was able to bind to Pdc4. We therefore concluded that the main interaction site for Pdc4 is located within RRM 1 and 2 of PABP. However, because the efficiency of binding of full-length PABP to GST-Pdc4 was higher than that of PABP-RRM1 + 2 it is possible that another part of PABP also contributes to the interaction with Pdc4.

Pdc4 and PABP are direct interaction partners

Because PABP was derived from extracts of transfected cells in the binding experiments shown in Figure 2, we could

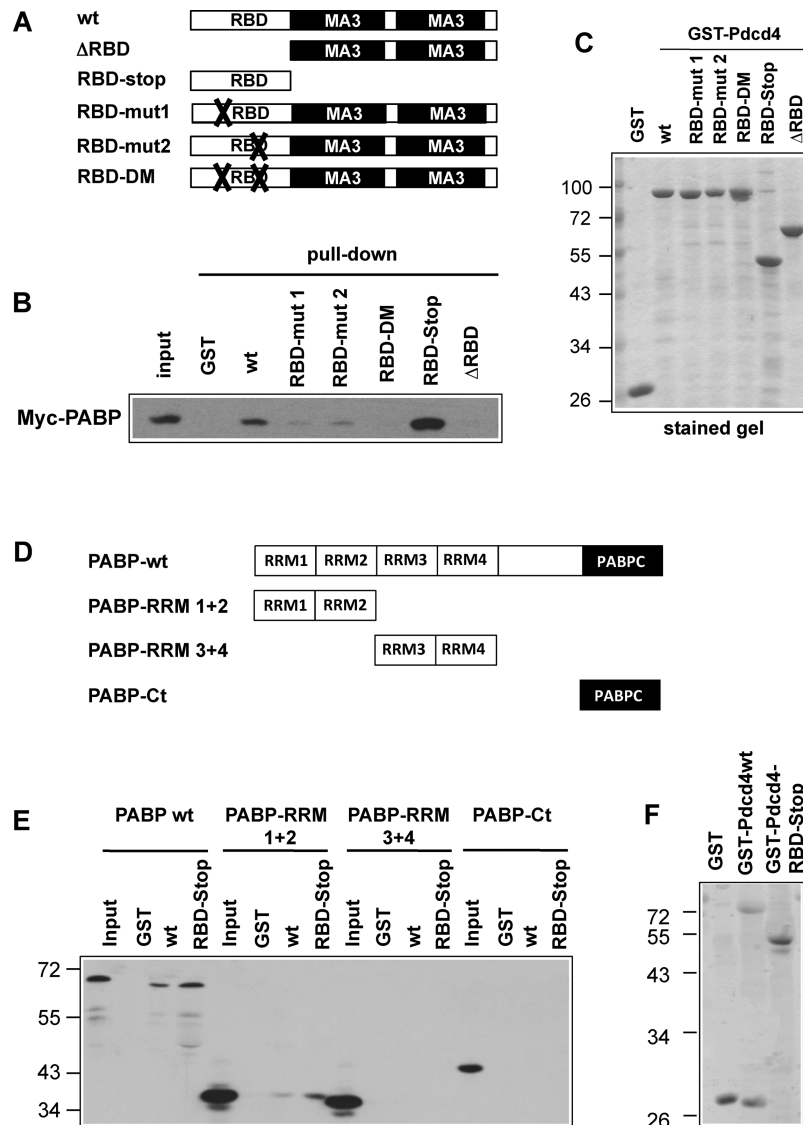


Figure 2. Mapping of the Pcd4- and PABP-binding regions. (A) Schematic structure of GST-Pcd4 proteins. RBD and MA3 refer to the RNA binding and MA3 domains. (B) Cell extract from QT6 fibroblasts transfected with an expression vector for Myc-PABP was analyzed for binding to GST-Pcd4 proteins as in Figure 1. Bound proteins and an aliquot of the input extract were analyzed by western blotting with antibodies against the Myc-tag. (C) Coomassie blue stained gel demonstrating equal loading of the sepharose beads with GST-Pcd4 proteins. (D) Schematic structure of Myc-PABP proteins. RRM1–4 and PABPC refer to the RNA-binding motifs and the C-terminal domain of PABP. (E) Cell extract from QT6 fibroblasts transfected with an expression vectors for Myc-PABP constructs was analyzed for binding to GST-Pcd4 proteins. Bound proteins and an aliquot of the input extracts were analyzed by western blotting with antibodies against the Myc-tag. (F) Equal loading of the sepharose beads with GST-Pcd4 proteins is shown by Coomassie blue staining.

not exclude the possibility that the interaction of Pcd4 and PABP was mediated by another protein present in the cell extract. We therefore expressed His-PABP-RRM1–4 in bacteria, purified it and subjected it to a pull-down experiment with bacterially expressed and purified GST-Pcd4. Figure 3 shows that bacterially expressed PABP-RRM1–4 bound efficiently to GST-Pcd4 and to a GST-Pcd4 protein containing only the RNA-binding domain of Pcd4. Since both purified proteins interacted in the absence of other eukaryotic proteins, we concluded that Pcd4 and PABP are directly binding to each other. We also confirmed by a binding reaction in the presence of RNase A that the

interaction was not mediated by unspecific binding of both proteins to RNA (Figure 3B).

The ability to bind to RNA and to interact with PABP is conserved between human and Drosophila Pcd4

Evolutionary conservation provides strong arguments for the functional importance of specific traits of proteins, such as their ability to undergo specific interactions. Since Pcd4 has been highly conserved during evolution, we were interested to know whether the ability to bind to RNA and to interact with PABP are conserved features of Pcd4. The *CG10990* gene of *Drosophila melanogaster* encodes a protein that is highly related to the mammalian Pcd4 pro-

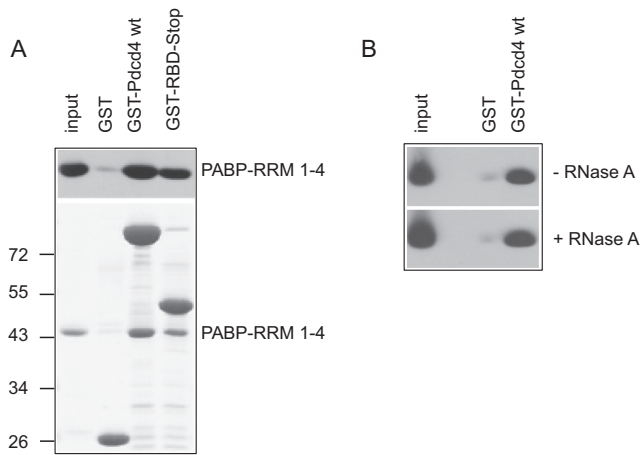


Figure 3. PABP and Pdc44 are direct interaction partners. (A) Bacterially expressed and purified PABP-RRM 1–4 was analyzed for binding to GST-Pdc44. Input and bound proteins were analyzed by western blotting with antibodies against PABP (top) or by staining with Coomassie blue (bottom). The input fraction corresponds to 2.5% of the bound fractions. (B) Binding of PABP-RRM 1–4 to the indicated GST-proteins was carried out in the absence or presence of 0.5 μ g/ml RNase A, followed by western blotting with antibodies against PABP.

tein. Figure 4A shows that the domain structures of human and *Drosophila* Pdc44 are very similar. To investigate if the *Drosophila* protein has RNA-binding activity, we employed a nitrocellulose blotting assay. Purified bacterially expressed human and *Drosophila* GST-Pdc44 proteins were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane or stained with Coomassie blue. The blots were then incubated with radiolabeled *in vitro* synthesized RNA, washed and analyzed by autoradiography. Figure 4B shows that human and *Drosophila* Pdc44 were able to bind RNA. As a control for specificity of the binding assay a mutant version of human Pdc44 (Pdc44-RBDmut) lacking RNA-binding activity (25) was included. The experiment also showed that the N-terminal domain of the *Drosophila* protein, like that of human Pdc44 (16,25), is sufficient for RNA binding. To investigate whether *Drosophila* Pdc44 also interacts with PABP we performed an *in vitro* binding experiment with TCE from *Drosophila* Schneider S2 cells. Figure 4C shows that full-length *Drosophila* Pdc44 as well as the N-terminal domain of the protein were able to bind PABP. Binding also occurred when RNase A was added to the cell extract, suggesting that it was not an artifact of unspecific binding of both proteins to RNA (Figure 4D). We therefore concluded that the N-terminal domain of *Drosophila* Pdc44 binds RNA as well as PABP, demonstrating that both binding activities are conserved during evolution.

Mutation of highly conserved amino acid residues within the Pdc44 RNA-binding domain abolishes its interaction with PABP

To further explore the role of the interaction of Pdc44 with PABP, we decided to generate Pdc44 mutants that disrupt the PABP-binding activity. Comparison of the Pdc44 amino acid sequence of widely divergent species revealed a very highly conserved region within the second half of the N-

terminal domain that has not been implicated in RNA binding (Figure 5A). To investigate if this region is involved in PABP binding, we mutated specific amino acid residues in this conserved region. These mutants were expressed as GST-Pdc44 fusion proteins and examined in pull-down experiments for their ability to interact with PABP. As shown in Figure 5B, two of the mutants (K114A/K115A and V123A/W124A) were unable to interact with PABP, demonstrating that the highly conserved part of the RNA-binding domain is involved in the Pdc44-PABP interaction. We also analyzed the RNA-binding activity of these mutants by a poly(U)-agarose-binding assay (Figure 5C). Wild-type Pdc44 and both mutant proteins were able to bind to poly(U)-agarose and were eluted at similar salt concentrations, demonstrating that both Pdc44 mutants had retained RNA-binding activity. Thus, the Pdc44 mutants K114A/K115A and V123A/W124A dissociate RNA- and PABP-binding activities of Pdc44 from each other, clearly demonstrating that these are independent activities of Pdc44.

Pdc44 mutants lacking PABP-binding activity do not suppress translation of *c-myb* mRNA

To explore the functional relevance of the interaction of Pdc44 and PABP, we first investigated if the Pdc44 mutants that failed to bind to PABP were still able to suppress the translation of *c-myb* mRNA. Figure 6A shows that wild-type Pdc44 suppressed the expression of c-Myb when co-transfected with a c-Myb expression vector, consistent with our previous work which has shown that the inhibitory effect of Pdc44 is due to suppression of translation of *c-myb* RNA (27). Interestingly, both Pdc44 mutants were unable to reduce c-Myb expression, suggesting that the interaction with PABP is necessary for the suppression of *c-myb* mRNA translation by Pdc44.

PABP is known to stimulate translation initiation by interacting with eIF4G bound at the 5' end of the mRNA, thereby causing formation of a closed loop structure of the mRNA (36,37). Several proteins have been shown to suppress translation by interfering with the circularization of the RNA. For example, the interaction with the PABP-interacting protein 2 (Paip2) displaces PABP from the poly(A)-tail of the mRNA and also inhibits its interaction with eIF4G (38,39). Similarly, the GW182 protein, which acts as a scaffold for the assembly of silencing complexes on specific mRNAs, is thought to disrupt PABP function also by blocking its interaction with eIF4G (40). To see if Pdc44 acts in similar manner, we investigated the effect of Pdc44 on the ability of PABP to bind to the poly(A)-tail and to interact with eIF4G. To study the influence of Pdc44 on RNA binding by PABP, we performed EMSAs with a radiolabeled oligo(A)₂₅ ribo-oligonucleotide and bacterially expressed, purified PABP-RRM1–4, GST-Pdc44 and GST-Pdc44- Δ RBD. Figure 6B shows that PABP bound strongly to the oligonucleotide, as evidenced by the mobility shift of the oligonucleotide, whereas Pdc44 itself did not bind. Addition of an excess of Pdc44 to the binding reaction containing PABP did not reduce the PABP-induced mobility shift. We therefore concluded that Pdc44 does not interfere with RNA-binding activity of PABP.

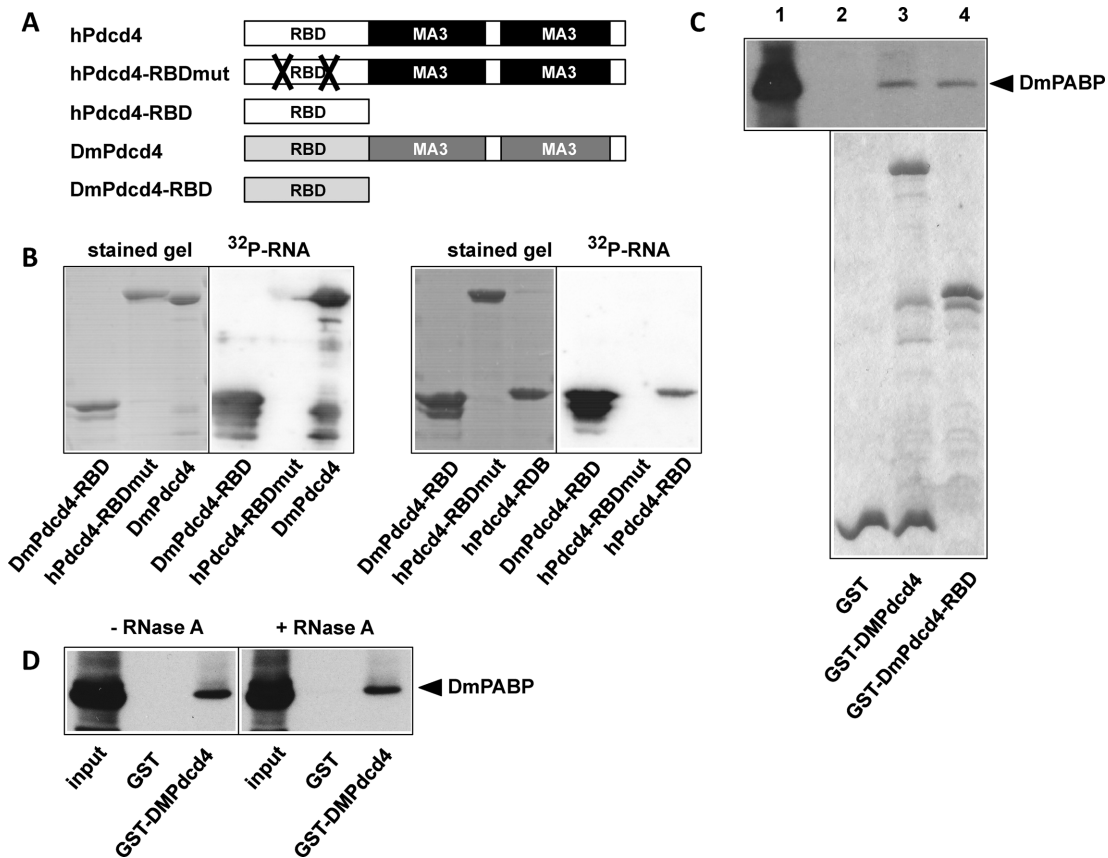


Figure 4. The ability of Pcd4 to bind to RNA and PABP is conserved between humans and *Drosophila melanogaster*. (A) Schematic structure of human and *Drosophila* Pcd4. (B) Bacterially expressed and purified human and *Drosophila* GST-Pcd4 proteins were fractionated by SDS-PAGE and stained with Coomassie blue or blotted onto nitrocellulose. The blots were incubated with radiolabeled RNA, washed and analyzed by autoradiography. (C) Extract from *Drosophila* Schneider S2 cells was incubated with the indicated GST proteins immobilized on glutathione sepharose beads. Bound proteins and an aliquot of the TCE was analyzed by western blotting with antibodies against *Drosophila* PABP (top). A stained gel demonstrating equal loading of the beads with GST proteins is shown below. (D) Extracts from *Drosophila* Schneider S2 cells were analyzed for binding to the indicated GST proteins as in (C). Binding reactions were performed in the absence or presence of 0.5 μ g/ml RNase A.

To address if Pcd4 interferes with the interaction between PABP and eIF4G, we transfected fibroblasts with different combinations of expression vectors for Pcd4, Myc-PABP and a GFP-eIF4G fusion protein that contains the binding site for PABP. TCEs were then analyzed by immunoprecipitation using sepharose beads carrying a covalently bound high-affinity GFP-binding protein. As expected, PABP was specifically precipitated via eIF4G, however, co-expression of Pcd4 had no effect on this interaction (Figure 6C). Taken together, these experiment suggested that Pcd4 does not suppress translation by interfering with the PABP-eIF4G interaction or with PABP RNA binding.

Pcd4 mutants lacking PABP-binding activity do not cosediment with ribosomal complexes

Fractionation of cytoplasmic extracts by sucrose density gradient centrifugation has previously shown that Pcd4 is associated with eIF4A-containing ribosomal complexes. These complexes sediment slightly faster than small ribosomal subunits but slower than complete ribosomes and are thought to be translation pre-initiation complexes (25). To investigate if the disruption of the Pcd4-PABP interaction

affects the association of Pcd4 with these complexes, we transfected expression vectors for the Flag-tagged Pcd4-K114A/K115A and V123A/W124A mutants into HeLa cells and fractionated cytoplasmic extracts of these cells in sucrose density gradients. Analysis of the gradient fractions showed that both mutant proteins had lost the ability to associate with ribosomal complexes and appeared only in the upper part of the gradient (Figure 7A and B). A control experiment using Flag-tagged wild-type Pcd4 confirmed that the Flag-tag by itself did not prevent interaction with ribosomal complexes (Figure 7C). Overall, these experiments indicated that the interaction with PABP is required for the association of Pcd4 with the ribosomal complexes.

DISCUSSION

The tumor suppressor gene *Pcd4* is attracting increasing attention because its expression is frequently downregulated in different types of human tumors. In many cases downregulation appears to be due to increased expression of micro-RNA miR-21, an onco-miR that is upregulated in many cancer cells (41). The function of Pcd4 is less well understood, particularly with respect to its role as a translation

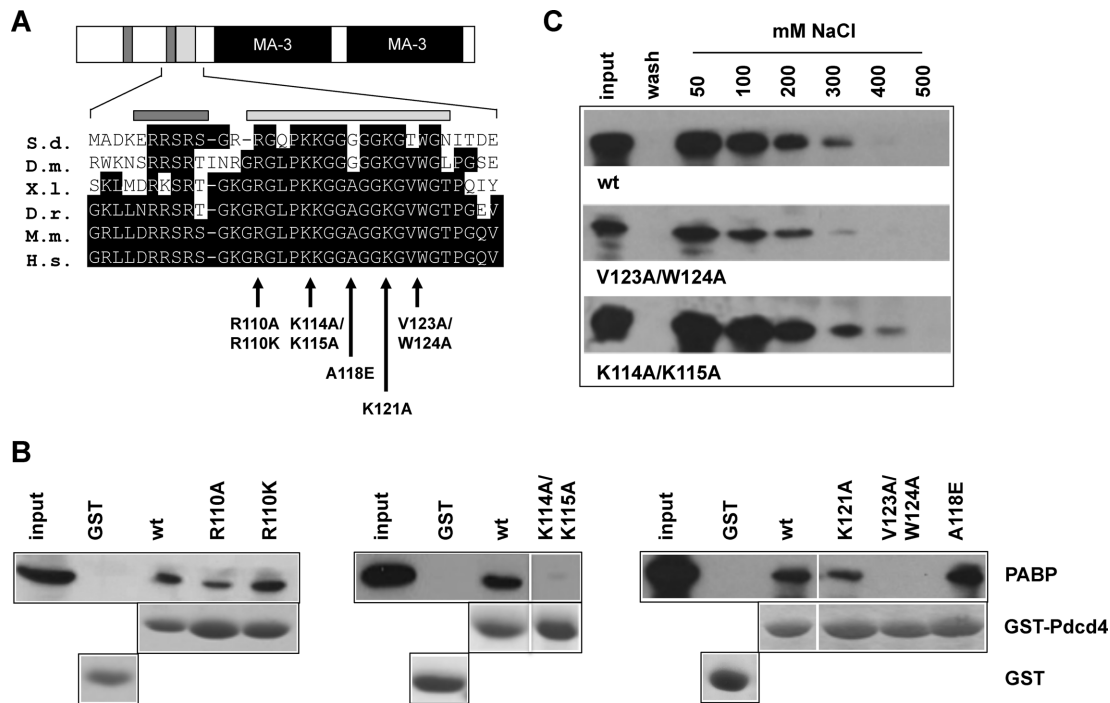


Figure 5. PABP- and RNA-binding are independent activities mediated by the N-terminal domain of Pdc4. (A) Amino acid sequences of part of the N-terminal domain of Pdc4 from different species. S.d., *Suberites domuncula*; D.m., *Drosophila melanogaster*; X.l., *Xenopus laevis*; D.r., *Danio rerio*; M.m., *Mus musculus*; H.s., *Homo sapiens*. Clusters of basic amino acids involved in RNA binding are marked by yellow boxes. Light and dark grey boxes mark the regions of highest conservation within the N-terminal domain and the clusters of basic amino acids implicated in RNA binding, respectively. (B) Extracts from cells expressing Myc-PABP were analyzed for binding to wild type and mutant GST-Pdc4 proteins. Input and bound Myc-PABP was detected by western blotting with anti-Myc antibodies. GST-proteins were detected by Coomassie blue staining. Only the bands corresponding to the respective GST proteins are shown. (C) GST-Pdc4-wt, GST-Pdc4-V123A/W124A and GST-Pdc4-K114A/K115A were analyzed by chromatography on poly(U)-agarose. Bound proteins were eluted from the column with binding buffer containing increasing amounts of NaCl. Samples of input, wash and salt-eluate fractions were analyzed by western blotting with Pdc4 antiserum.

suppressor. The finding that Pdc4 inhibits the helicase activity of translation initiation factor eIF4A has initially suggested that Pdc4 acts as a translation suppressor of mRNAs with structured 5' UTRs. That Pdc4 can suppress the translation of such RNAs has indeed been confirmed by analysis of the effect of Pdc4 on artificial RNA constructs that form stable stem-loop structures at their 5' ends (18,33). However, since natural Pdc4 target mRNAs were initially not known, several important questions have remained open: which mRNAs are regulated by Pdc4? Does Pdc4 suppress the translation of all mRNAs containing structured 5' UTRs? Are there alternative mechanisms of translational suppression by Pdc4? Are there mechanisms that allow Pdc4 to selectively target specific mRNAs?

We have recently identified several natural Pdc4 target RNAs, which has allowed us to address some of these questions and to investigate the mechanism of translational suppression by Pdc4 in more detail (14,27,28). By examining the effect of Pdc4 on p53 mRNA, a natural mRNA with a highly structured 5' UTR, we have confirmed the proposed mechanism of translational suppression by Pdc4. We have shown that the inhibition of p53 mRNA translation by Pdc4 is mediated by the 5' UTR and depends on the ability of Pdc4 to bind to eIF4A (14). Surprisingly, the analysis of the effect of Pdc4 on *c-myc* and *A-myc* mRNAs has indicated that there is an additional mechanism of translational suppression by Pdc4, which is indepen-

dent of the Pdc4/eIF4A-interaction, but instead requires the RNA-binding activity of Pdc4 to target Pdc4 to these mRNAs. We have shown that Pdc4 preferentially binds to RNA secondary structures formed by the coding regions of *c-myc* and *A-myc* mRNAs (27,28). As a clear indication that these binding regions indeed mediate the specific targeting of Pdc4 to these RNAs, we have shown that the fusion of the secondary structure regions to a heterologous RNA allowed Pdc4 to recognize these RNAs as targets for translational suppression. In the course of these experiments, we have observed that translation suppression by Pdc4 was abolished when a translation stop codon was introduced into the mRNA upstream of the Pdc4-binding region (28). This demonstrated that Pdc4 mediates translation suppression of these mRNAs only when it binds to the translated part of the RNAs, which suggested that Pdc4 suppresses the translation of these RNAs by inhibiting translation elongation. Taken together, these analyses have shown that there are at least two distinct mechanisms by which Pdc4 suppresses translation, namely inhibition of translation initiation via binding to eIF4A, and inhibition of translation elongation by direct binding of Pdc4 to the coding region of specific RNAs.

The identification of PABP as a Pdc4 interaction partner extends our picture of Pdc4's role as a translation suppressor and shows that the cooperation of Pdc4 with the translational machinery is more complex than previously

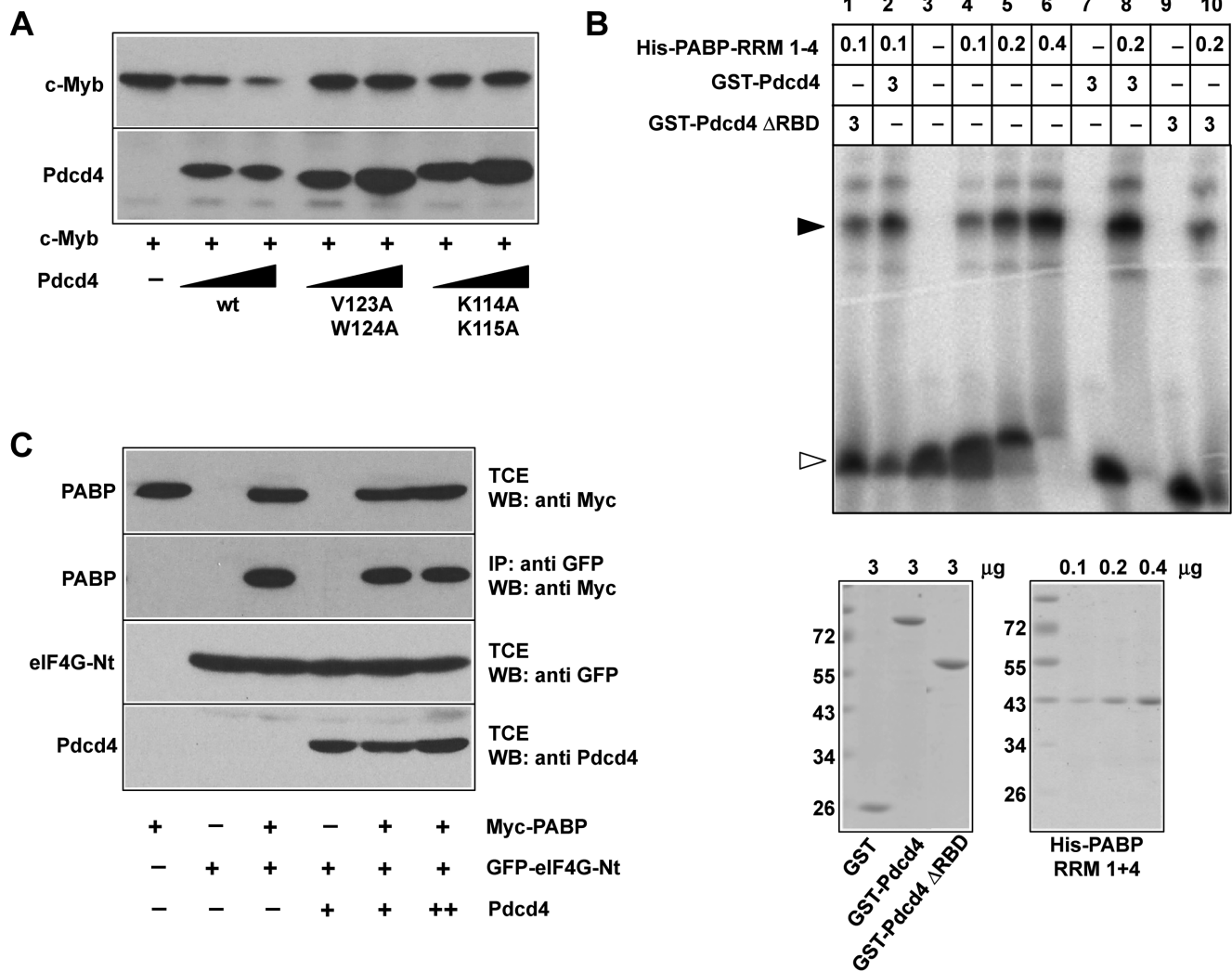


Figure 6. Pdc4 mutants lacking PABP-binding activity do not suppress translation of *c-myb* mRNA. (A) QT6 fibroblasts were transfected with expression vectors for c-Myb and wild-type or mutant Pdc4, as indicated. TCEs were analyzed by western blotting with antisera against c-Myb and Pdc4. (B) Bacterially expressed and purified His-PABP RRM1–4, GST-Pdc4 and GST-Pdc4 ΔRBD were subjected to electrophoretic mobility shift assays using a radiolabeled oligo(A)₂₅ ribo-oligonucleotide. Oligonucleotides were incubated with the indicated amounts (in μg) of the proteins and analyzed by electrophoresis in a nondenaturing polyacrylamide gel. The black and white arrowheads mark RNA-protein complexes and the free oligonucleotide, respectively. Coomassie-stained SDS-polyacrylamide gels demonstrating the purity of the proteins are shown below. (C) QT6 fibroblasts were transfected with expression vectors for Myc-PABP, Pdc4 and a GFP-eIF4G fusion protein encompassing the binding site for PABP. TCEs were analyzed by western blotting with the indicated antibodies. Cell extracts were also subjected to immunoprecipitation with antibodies against GFP, followed by western blotting of the immunoprecipitates with Myc-specific antibodies.

known. Our mapping experiments have shown that Pdc4 and PABP interact by direct protein–protein interactions via their RNA-binding domains. The part of Pdc4 implicated by our mutation analysis in the interaction with PABP shows the highest evolutionary conservation within the N-terminal domain of the protein, underling the importance of this region for the function of Pdc4. Indeed, the ability of Pdc4 to bind to RNA and to interact with PABP have been highly conserved during evolution, as demonstrated by the analysis of the *Drosophila* homolog of Pdc4.

PABP is an important component of the translation machinery but also plays key roles in other processes, such as the regulation of mRNA stability. PABP enhances the translation of capped and polyadenylated RNAs by inter-

acting with eIF4G and forming a closed loop structure of the mRNA (36,37). PABP also affects the formation of the translation initiation complex at subsequent steps, such as the joining of the ribosomal subunits (42–44). A variety of protein–protein interactions involving the RNA-binding and C-terminal domains of PABP have been shown to modulate the function of PABP in translation initiation, mRNA stability and micro-RNAs mediated silencing (40,45–47). How does the interaction with Pdc4 fit into this picture? Our *in vitro* RNA-binding experiments have shown that Pdc4 does not disrupt the binding of PABP to the poly(A) tail or interfere with the interaction of PABP with eIF4G. The role of Pdc4 is therefore clearly different from that of the Paip2, which also binds to the RNA-binding domain of

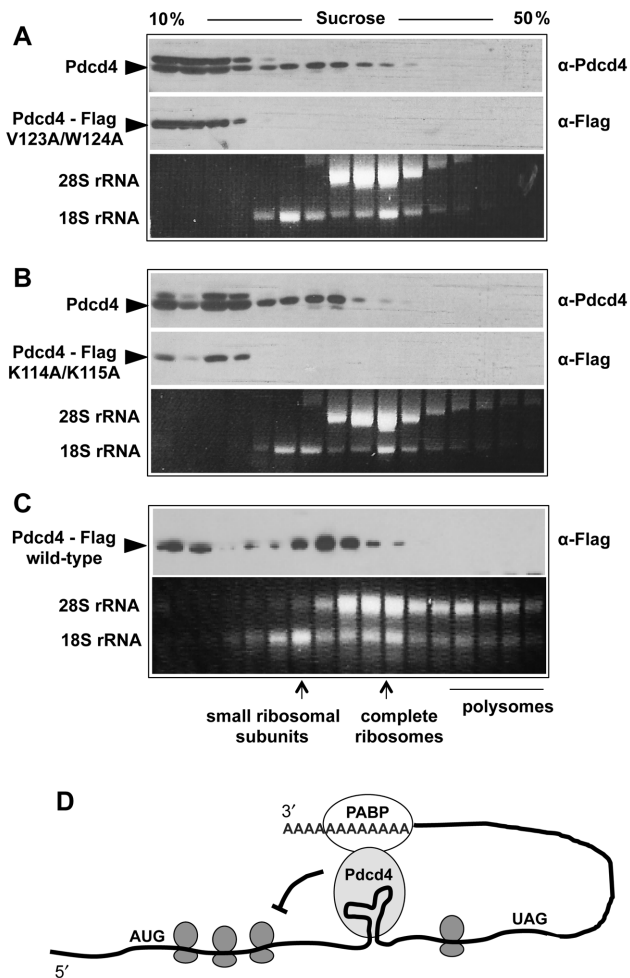


Figure 7. Ribosomal association of mutant Pdc4 proteins. HeLa cells were transfected with expression vectors for Flag-tagged Pdc4-V123A/W124A (A), Pdc4-K114A/K115A (B) or Flag-tagged wild-type Pdc4 (C). Cytoplasmic extracts were prepared 20 h after transfection and fractionated by sucrose density gradient sedimentation. Individual gradient fractions were analyzed by western blotting with antibodies against Pdc4 (top), the Flag-tag (middle) and by agarose gel electrophoresis to detect 18S and 28S ribosomal RNAs (bottom). In (C), western blotting were performed only with anti-Flag antibodies. The arrowheads mark endogenous and mutant Pdc4. Because of the Flag-tag the mutant proteins have a higher molecular weight than endogenous Pdc4 and are also visible in the top panels. (D) Tentative model of the regulation of *c-myb* mRNA translation by Pdc4. Pdc4 binds to a secondary structure formed by a part of the coding region of *c-myb* mRNA and inhibits translational elongation. The association of Pdc4 with the RNA is stabilized by interaction with PABP.

PABP but suppresses translation by disrupting PABP RNA binding and its interaction with eIF4G (38,39). We have shown that Pdc4 mutants that are defective for binding to PABP have lost the ability to stably associate with ribosomal complexes during sucrose density sedimentation. This suggests that the Pdc4-PABP interaction is required for tethering Pdc4 to these complexes. Consistent with the notion that the interaction with PABP stabilizes Pdc4 on its mRNA targets our data show that Pdc4 mutants that do not interact with PABP, fail to suppress the translation of *c-myb* mRNA. If Pdc4 in this case blocks translation elongation by directly binding to sequences located within

the coding region of the RNA, as suggested by our previous work, one might suspect that translating ribosomes will weaken or disrupt the Pdc4-RNA interaction. The ability of Pdc4 to bind PABP might therefore be important to stabilize Pdc4 on the RNA or allow it to rebind to the RNA in case it is displaced by an approaching ribosome. Thus, PABP appears to function as a cofactor helping Pdc4 to exert its suppressive function. Figure 7D depicts a tentative model for the regulation of *c-myb* mRNA translation by Pdc4. Some aspects of this model are reminiscent of the translation inhibition of *Drosophila msl-2* mRNA by the SXL-UNR co-repressor complex (44). The SXL-UNR complex binds PABP without interfering with the PABP-eIF4G interaction and closed-loop formation of the *msl-2* RNA, suggesting that the SXL-UNR complex requires PABP as a cofactor for translation suppression. It is worth pointing out that the Pdc4 RNA-binding domain is the target of post-translational modifications, such as phosphorylation of serine 67 by p70s6K or AKT kinases (17,48) or methylation of arginine 110 by PRMT5 (26). It is conceivable that these modifications also affect the binding of PABP, thereby linking translational suppression of specific mRNAs by Pdc4 to signal transduction pathways.

In summary, our work demonstrates that Pdc4 interacts with the PABP, a key player in the control of translation. This interaction has been highly conserved during evolution and contributes to the role of Pdc4 as translation suppressor. Given the multifunctional role of PABP in translation and RNA-metabolism our work also raises the possibility that Pdc4, by modulating the binding or function of other PABP-interacting proteins, might also affect other functions of PABP.

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REFERENCES

- Chen, Y., Knösel, T., Kristiansen, G., Pietas, A., Garber, M.E., Matsushashi, S., Ozaki, I. and Petersen, I. (2003) Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. *J. Pathol.*, **200**, 640–646.
- Afonja, O., Juste, D., Das, S., Matsushashi, S. and Samuels, H.H. (2004) Induction of PDCD4 tumor suppressor gene expression by RAR agonists, antiestrogen and HER-2/neu antagonist in breast cancer cells. Evidence for a role in apoptosis. *Oncogene*, **23**, 8135–8145.
- Zhang, H., Ozaki, I., Mizuta, T., Hamajima, H., Yasutake, T., Eguchi, Y., Ideguchi, H., Yamamoto, K. and Matsushashi, S. (2006) Involvement of programmed cell death 4 in transforming growth factor-beta1-induced apoptosis in human hepatocellular carcinoma. *Oncogene*, **25**, 6101–6112.
- Mudduluru, G., Medved, F., Grobholz, R., Jost, C., Gruber, A., Leupold, J.H., Post, S., Jansen, A., Colburn, N.H. and Allgayer, H.

- (2007) Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. *Cancer*, **110**, 1697–1707.
5. Wang, Q., Sun, Z. and Yang, H.-S. (2008) Downregulation of tumour suppressor Pcdcd4 promotes invasion and activates both β -catenin/TCF and AP-1-dependent transcription in colon carcinoma cells. *Oncogene*, **27**, 1527–1535.
 6. Park, Y.M., Hwang, S.J., Masuda, K., Choi, K.M., Jeong, M.R., Nam, D.H., Gorospe, M. and Kim, H.H. (2012) Heterogeneous nuclear Ribonucleoprotein C1/C2 controls the metastatic potential of glioblastoma by regulating Pcdcd4. *Mol. Cell. Biology*, **32**, 4237–4244.
 7. Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A. and Lund, A.H. (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J. Biol. Chem.*, **283**, 1026–1033.
 8. Lu, Z., Liu, M., Stribinskis, V., Klinge, C.M., Ramos, K.S. and Colburn, N.H. (2008) MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. *Oncogene*, **27**, 4373–4379.
 9. Asangani, I.A., Rasheed, S.A., Nikolova, D.A., Leupold, J.H., Colburn, N.H., Post, S. and Allgayer, H. (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pcdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene*, **27**, 2128–2136.
 10. Yang, H.S., Matthews, C.P., Clair, T., Wang, Q., Baker, A.R., Li, C.C., Tan, T.H. and Colburn, N.H. (2006) Tumorigenesis suppressor Pcdcd4 down-regulates mitogen-activated protein kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion. *Mol. Cell Biol.*, **26**, 1297–1306.
 11. Leupold, J.H., Yang, H.S., Colburn, N.H., Asangani, I., Post, S. and Allgayer, H. (2007) Tumor suppressor Pcdcd4 inhibits invasion/intravasation and regulates urokinase receptor (u-PAR) gene expression via Sp-transcription factors. *Oncogene*, **26**, 4550–4562.
 12. Bitomsky, N., Wethkamp, N., Marikkannu, R. and Klempnauer, K.-H. (2008) siRNA-mediated knock-down of Pcdcd4 expression causes up-regulation of p21(Waf1/Cip1) expression. *Oncogene*, **27**, 4820–4829.
 13. Singh, P., Marikkannu, R., Bitomsky, N. and Klempnauer, K.-H. (2009) Disruption of the Pcdcd4 tumor suppressor gene in chicken DT40 cells reveals its role in the DNA-damage response. *Oncogene*, **28**, 3758–3764.
 14. Wedeken, L., Singh, P. and Klempnauer, K.-H. (2011) Tumor suppressor protein Pcdcd4 inhibits translation of p53 mRNA. *J. Biol. Chem.*, **286**, 42855–42862.
 15. Kumar, N., Wethkamp, N., Waters, L.C., Carr, M.D. and Klempnauer, K.-H. (2013) Tumor suppressor proteins Pcdcd4 interacts with Daxx and modulates the stability of Daxx and the Hipk2-dependent phosphorylation of p53 at serin 46. *Oncogenesis*, **2**, e37.
 16. Böhm, M., Sawicka, K., Siebrasse, J.P., Brehmer-Fastnacht, A., Peters, R. and Klempnauer, K.-H. (2003) The transformation suppressor protein Pcdcd4 shuttles between nucleus and cytoplasm and binds RNA. *Oncogene*, **22**, 4905–4910.
 17. Palamarchuk, A., Efanov, A., Maximov, V., Aqeilan, R.I., Croce, C.M. and Pekarsky, Y. (2005) Akt phosphorylates and regulates Pcdcd4 tumor suppressor protein. *Cancer Res.*, **65**, 11282–11286.
 18. Yang, H.S., Jansen, A.P., Komar, A.A., Zheng, X., Merrick, W.C., Costes, S., Lockett, S.J., Sonenberg, N. and Colburn, N.H. (2003) The transformation suppressor Pcdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. *Mol. Cell Biol.*, **23**, 26–37.
 19. LaRonde-Leblanc, N., Santhanam, A.N., Baker, A.R., Wlodaver, A. and Colburn, N.H. (2007) Structural basis for inhibition of translation by the tumor suppressor Pcdcd4. *Mol. Cell Biol.*, **27**, 147–156.
 20. Waters, L.C., Veverka, V., Böhm, M., Schmedt, T., Choong, P.T., Muskett, F.W., Klempnauer, K.-H. and Carr, M.D. (2007) Structure of the C-terminal MA-3 domain of the tumour suppressor protein Pcdcd4 and characterization of its interaction with eIF4A. *Oncogene*, **26**, 4941–4950.
 21. Suzuki, C., Garces, R.G., Edmonds, K.A., Hiller, S., Hyberts, S.G. and Marintchev, A. (2008) PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 3274–3279.
 22. Chang, J.H., Cho, Y.H., Sohn, S.Y., Choi, J.M., Kim, A., Kim, Y.C., Jang, S.K. and Cho, Y. (2009) Crystal structure of the eIF4A-PDCD4 complex. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 3148–3153.
 23. Loh, P.G., Yang, H.S., Walsh, M.A., Wang, Q., Wang, X., Cheng, Z., Liu, D. and Song, H. (2009) Structural basis for the translational inhibition by the tumour suppressor Pcdcd4. *EMBO J.*, **28**, 274–285.
 24. Waters, L.C., Strong, S.L., Ferlemann, E., Oka, O., Muskett, F.W., Veverka, V., Banerjee, S., Schmedt, T., Henry, A.J., Klempnauer, K.-H. *et al.* (2011) Structure of the tandem MA-3 region of Pcdcd4 protein and characterization of its interactions with eIF4A and eIF4G. *J. Mol. Biol.*, **286**, 17270–17279.
 25. Wedeken, L., Ohnheiser, J., Hirschi, B., Wethkamp, N. and Klempnauer, K.-H. (2010) Association of tumor suppressor protein Pcdcd4 with ribosomes is mediated by protein-protein and protein-RNA interactions. *Genes Cancer*, **1**, 293–301.
 26. Powers, M.A., Fay, M.M., Factor, R.E., Welm, A.L. and Ulman, K.S. (2011) Protein arginine methyltransferase 5 accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death 4. *Cancer Res.*, **71**, 5579–5587.
 27. Singh, P., Wedeken, L., Waters, L.C., Carr, M.D. and Klempnauer, K.-H. (2011) Pcdcd4 directly binds the coding region of c-myc mRNA and suppresses its translation. *Oncogene*, **30**, 4864–4873.
 28. Biyanee, A., Ohnheiser, J., Singh, P. and Klempnauer, K.-H. (2014) A novel mechanism for the control of translation of specific mRNAs by tumor suppressor protein Pcdcd4: inhibition of translation elongation. *Oncogene*, doi:10.1038/onc.2014.83; [Epub ahead of print].
 29. Yang, H.S., Knies, J.L., Stark, C. and Colburn, N.H. (2003) Pcdcd4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation. *Oncogene*, **22**, 3712–3720.
 30. Bitomsky, N., Böhm, M. and Klempnauer, K.-H. (2004) Transformation suppressor protein Pcdcd4 interferes with JNK-mediated phosphorylation of c-Jun and recruitment of the coactivator p300 by c-Jun. *Oncogene*, **23**, 7484–7493.
 31. Shiota, M., Izumi, H., Tanimoto, A., Takahashi, M., Miyamoto, N., Kashiwagi, E., Kidani, A., Hirano, G., Masubuchi, D., Fukunaka, Y. *et al.* (2009) Programmed cell death protein 4 down-regulates Y-box binding protein-1 expression via a direct interaction with Twist1 to suppress cancer cell growth. *Cancer Res.*, **69**, 3148–3156.
 32. Hwang, S.K., Baker, A.R., Young, M.R. and Colburn, N.H. (2014) Tumor suppressor Pcdcd4 inhibits NF- κ -dependent transcription in human glioblastoma cells by direct interaction with p65. *Carcinogenesis*, **35**, 1469–1480.
 33. Yang, H.S., Cho, M.H., Zacowicz, H., Hegamyer, G., Sonenberg, N. and Colburn, N.H. (2004) A novel function of the MA-3 domains in transformation and translation suppressor Pcdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. *Mol. Cell Biol.*, **24**, 3894–3906.
 34. Kawahara, H., Imai, T., Imataka, H., Tsujimoto, M., Matsumoto, K. and Okano, H. (2008) Neural RNA-binding protein Musashi1 inhibits translation initiation by competing with eIF4G for PABP. *J. Cell Biol.*, **181**, 639–653.
 35. Sleeman, J.P. (1993) Xenopus A-myb is expressed during early spermatogenesis. *Oncogene*, **8**, 1931–1941.
 36. Tarun, S.Z. Jr and Sachs, A.B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. *EMBO J.*, **15**, 7168–7177.
 37. Imataka, H., Gradi, A. and Sonenberg, N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.*, **17**, 7480–7489.
 38. Kahalegpour, K., Svitkin, Y.V., Craig, A.W., DeMaria, C.T., Deo, R.C., Burley, S.K. and Sonenberg, N. (2001) Translational repression by a novel partner of human poly(A) binding protein, Paip2. *Mol. Cell*, **7**, 205–216.
 39. Karim, M.M., Svitkin, Y.V., Kahvejian, A., De Crescenzo, G., Costa-Mattioli, M. and Sonenberg, N. (2006) A mechanism of translational repression by competition of Paip2 with eIF4G for poly(A) binding protein (PABP) binding. *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 9494–9499.
 40. Tritschler, F., Huntzinger, E. and Izaurralde, E. (2010) Role of GW182 proteins and PABPC1 in the microRNA pathway: a sense of déjà vu. *Nat. Rev. Mol. Cell Biol.*, **11**, 379–384.

41. Kumarswamy,R., Volkmann,I. and Thum,T. (2011) Regulation and function of miRNA-21 in health and disease. *RNA Biol.*, **8**, 706–713.
42. Mangus,D.A., Evans,M.C. and Jacobson,A. (2003) Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biol.*, **4**, 223.
43. Kahvejian,A., Svitkin,Y.V., Sukarieh,R., M'Boutchou,M.N. and Sonenberg,N. (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev.*, **19**, 104–113.
44. Duncan,K.E., Strein,C. and Hentze,M.W. (2009) The SXL-UNR corepressor complex uses a PABP-mediated mechanism to inhibit ribosome recruitment to *mst-2* mRNA. *Mol. Cell*, **36**, 571–582.
45. Smith,R.W. and Gray,N.K. (2010) Poly(A)-binding protein (PABP): a common viral target. *Biochem. J.*, **426**, 1–12.
46. Brook,M. and Gray,N.K. (2012) The role of mammalian poly(A)-binding proteins in co-ordinating mRNA turnover. *Biochem. Soc. Transact.*, **40**, 856–864.
47. Fabian,M.R., Sonenberg,N. and Filipowicz,W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.*, **79**, 351–379.
48. Dorrello,N.V., Peschiaroli,A., Guardavaccaro,D., Colburn,N.H., Sherman,N.E. and Pagano,M. (2006) S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* **314**, 467–471.