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PUF proteins bind Pop2p to regulate messenger RNAs

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PUF proteins, a family of RNA-binding proteins, interact with the 3′ untranslated regions (UTRs) of specific mRNAs to control their translation and stability. PUF protein action is commonly correlated with removal of the poly(A) tail of target mRNAs. Here, we focus on how PUF proteins enhance deadenylation and mRNA decay. We show that a yeast PUF protein physically binds Pop2p, which is a component of the Ccr4p–Pop2p–Not deadenylase complex, and that Pop2p is required for PUF repression activity. By binding Pop2p, the PUF protein simultaneously recruits the Ccr4p deadenylase and two other enzymes involved in mRNA regulation, Dcp1p and Dhh1p. We reconstitute regulated deadenylation *in vitro* and demonstrate that the PUF-Pop2p interaction is conserved in yeast, worms and humans. We suggest that the PUF-Pop2p interaction underlies regulated deadenylation, mRNA decay and repression by PUF proteins.

Control of RNA stability and translation determines how much protein is produced from an mRNA[1,2]. Regulatory factors commonly bind control elements between the termination codon and poly(A) tail of target mRNAs, in the 3′ UTR[1,2]. The PUF protein family, found throughout eukaryotes, provides a model for these 3′ UTR–borne regulatory factors[3]. PUF proteins reduce expression by repressing translation or accelerating mRNA decay[3–6]. They have important biological effects: they control proliferation of diverse stem cells[3,7], regulate key steps in development[3] and are strongly implicated in synaptic plasticity and memory[8,9].

PUF repression activity correlates with shortening of the poly(A) tail of target mRNAs, though the mechanism of deadenylation and repression is unknown[3]. To examine the mechanism of PUF-mediated control, we studied PUF protein Mpt5p (also called Puf5p) from *Saccharomyces cerevisiae*. Like nearly all PUF proteins, Mpt5p has an RNA-binding domain (RBD) composed of eight repeated units (PUF repeats), which binds specifically to a short RNA sequence in the 3′ UTR of a target mRNA (refs. 3,10,11 and D.J.S., B.A.H., K. Evans and M.W., unpublished data). Mpt5p binds and regulates several mRNAs, including *HO* mRNA, which encodes a DNA endonuclease required for mating-type switching (refs. 11,12 and D.J.S., B.A.H., K. Evans and M.W., unpublished data). Mpt5p destabilizes *HO* mRNA to prevent aberrant switching[11]. We sought to understand how Mpt5p represses *HO* mRNA and triggers deadenylation.

RESULTS

Mpt5p-mediated repression of *HO* requires Pop2p

To assay Mpt5p repression activity, we used reporter genes (*ADE2* or *HIS3*) linked to the *HO* 3′ UTR (**Fig. 1a**,**b**). As shown previously, the *HO* 3′ UTR conferred regulation on *ADE2*, thereby preventing growth on selective media when Mpt5p was expressed from a plasmid (**Fig. 1a**)[11]. The Mpt5p RBD, overexpressed via the *ADH1* promoter, was sufficient for both repression (**Fig. 1a**) and RNA binding (see below). Missense mutations in two amino acid residues that disrupted RNA binding (data not shown) also disrupted repression (**Fig. 1a**, '*MPT5* mt' spots).

To identify genes that might be required for PUF-mediated control, we tested repression of the *HIS3-HO* 3′ UTR reporter in mutant yeast strains lacking single proteins involved in mRNA turnover (**Fig. 1b**). Deletion of the *POP2* gene partially prevented repression and permitted cells to grow on *HIS3*-selective media (**Fig. 1b**). Pop2p is a member of the CAF1 family of poly(A) nucleases[13] and a component of the Ccr4p–Pop2p–Not deadenylase complex[14,15]. The yeast Pop2p is required *in vivo* for deadenylation, and both yeast and mammalian Pop2 proteins have deadenylation activity *in vitro*[13,16–19]. Pop2p physically interacts with Ccr4p, the major cytoplasmic deadenylase in yeast[14,15]. In contrast to the *pop2* mutant, a *ccr4* mutant only marginally derepressed the reporter (**Fig. 1b**). Deletion of other components of the Ccr4p–Pop2p–Not complex, including *NOT3*, *NOT4*, *CAF40*, *CAF120* and *CAF130*, did not influence repression by Mpt5p (**Fig. 1c**). Similarly, repression was unaffected by deletion of *PAN2*, another deadenylase; of components of the exosome (*RRP6*, *SKI2*, *SKI3* or *SKI7*), a complex of 3′→5′ exoribonucleases; or of the cytoplasmic 5′→3′ exonuclease *XRN1*. Strains lacking decapping factors were not viable under the assay conditions and so were not assessed. Together, these data demonstrate that repression of *HO* mRNA by Mpt5p specifically requires the Pop2p protein and, to a lesser extent, Ccr4p.

Mpt5p stimulates degradation of *HO* mRNA

We analyzed the effect of Mpt5p on endogenous *HO* mRNA by comparing its half-life in wild-type versus *mpt5* deletion strains (**Fig. 2**). Total RNA was prepared at various times after addition of thiolutin, which inhibits RNA polymerase II[20]. Northern blotting

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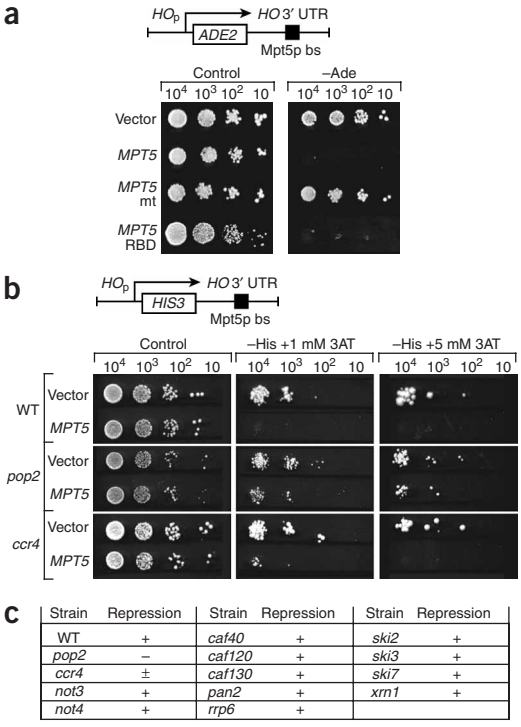

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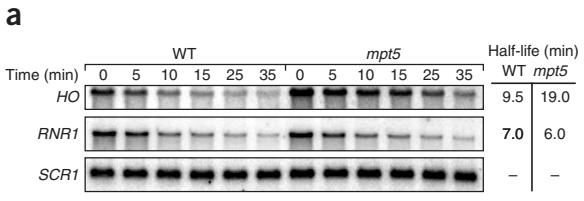
Strain	Repression	Strain	Repression	Strain	Repression
WT	+	*caf40*	+	*ski2*	+
pop2	–	*caf120*	+	*ski3*	+
ccr4	±	*caf130*	+	*ski7*	+
not3	+	*pan2*	+	*xrn1*	+
not4	+	*rrp6*	+		

Figure 1 Mpt5p repression is dependent on *POP2*. (**a**) The *ADE2-HO* 3′ UTR reporter (top) contains the *HO* promoter (*HO*p), *ADE2* coding sequence and *HO* 3′ UTR with the Mpt5p binding site (bs). Bottom, test spots. Test plasmids are indicated on the left; *MPT5* mt encodes Mpt5p mutant with disrupted RNA binding. Number of yeast cells spotted on media with (Control) or without adenine (–Ade) is indicated above spots. (**b**) The *HIS3-HO* 3′ UTR reporter (top) was tested for *MPT5* repression in wild-type (WT), *pop2* and *ccr4* yeast strains, as indicated on the left, on media with (Control) or without histidine (–His) and containing 3-aminotriazole (3AT). (**c**) Summary of *MPT5* repression of *HIS3-HO* reporter in gene-specific deletion strains. +, *MPT5* repression; –, no repression; ±, weakened repression.

revealed that, at steady state, two-fold more *HO* mRNA was present in *mpt5* mutants than in wild-type cells (**Fig. 2a**, 0 min). Consistent with these findings and those of ref. 11, *HO* mRNA half-life doubled from 9.5 min in wild-type cells to 19 min in the *mpt5* mutant (**Fig. 2a**). As controls, we analyzed mRNAs that do not interact with Mpt5p. The half-lives of *RNR1*, *PGK1*, *CTS1* and *CYC1* mRNAs were not altered by removal of *MPT5* (**Fig. 2a** and data not shown). Similarly, a noncoding RNA, *SCR1*, was unaffected by deletion of *MPT5* (**Fig. 2a**). These results demonstrate that Mpt5p specifically destabilizes *HO* mRNA.

To determine whether repression of *HO* by Mpt5p enhances deadenylation of *HO* mRNA, we analyzed the stability and poly(A) tail lengths of endogenous *HO* mRNAs in wild-type and *mpt5* mutant cells after transcriptional inhibition (**Fig. 2b**). To facilitate accurate measurement of poly(A) tail lengths, we truncated *HO* mRNA in preparations of yeast RNA using an antisense oligonucleotide and RNase H, then detected the 3′ fragment of *HO* mRNA, including the poly(A) tail, by high-resolution northern blotting (**Fig. 2b**). Deadenylation of *HO* mRNA after transcription inhibition occurred within minutes in wild-type cells. In contrast, polyadenylated *HO* mRNA was stabilized in the *mpt5* mutant cells and persisted to the end of the time course (35 minutes). *SCR1* RNA, analyzed on the same blot, was stable throughout the time course (**Fig. 2b**). Thus *MPT5* was required for the normal decay of *HO* mRNA, including its deadenylation.



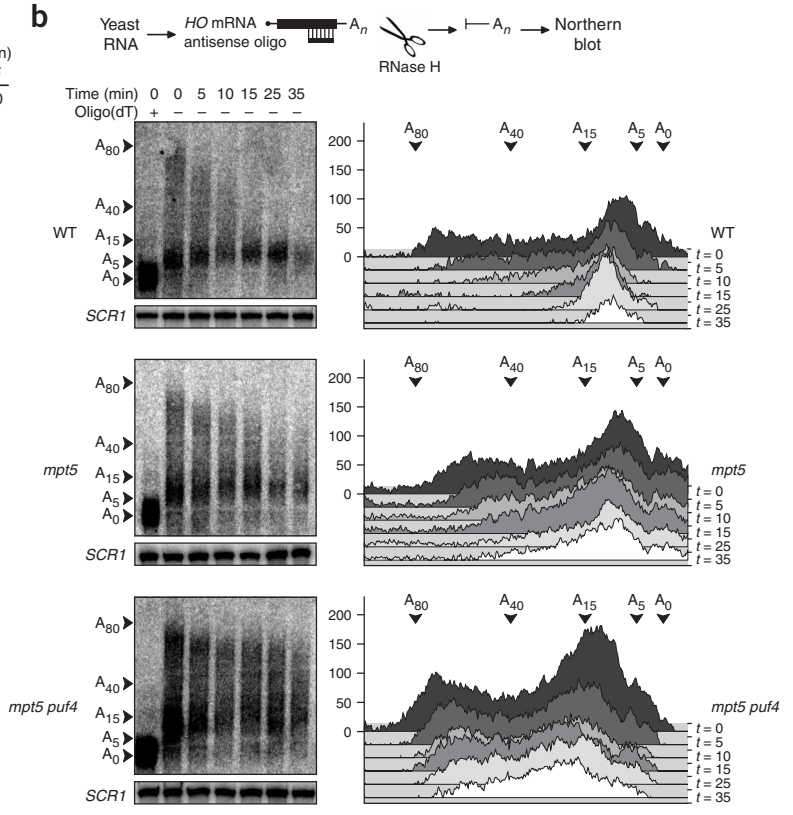


Figure 2 Mpt5p regulates decay of *HO* mRNA *in vivo*. (**a**) Northern blot analysis of *HO*, *RNR1* and *SCR1* RNA (as indicated on the left), in wild-type (WT) and *mpt5* deletion strains after transcriptional shutoff. Time points after shutoff are indicated above gel. Half-lives for each mRNA, in both strains, are indicated on the right. (**b**) High-resolution northern blot analysis of *HO* mRNA poly(A) tail length, indicated at left (in nt), in WT, *mpt5* and *mpt5 puf4* deletion strains. Diagram at top outlines experimental procedure for resolving poly(A) tail length. Time course after transcription shutoff and treatment of samples with oligo(dT) and RNase H to remove the poly(A) tail are indicated above gels. As a control, *SCR1* RNA was probed on the same northern blot. Graphs (right) of poly(A) tail length distribution were created from scans of signal density (in PhosphorImager units) for each time point from the wild type, *mpt5* and *mpt5 puf4* blots.

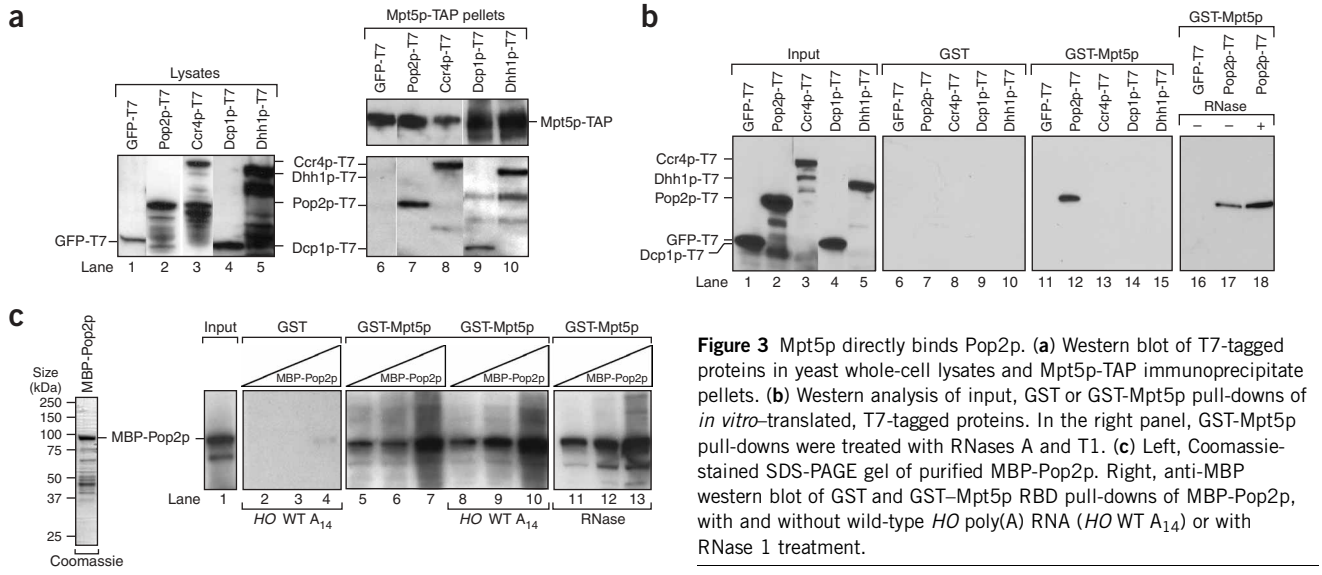


Figure 3 Mpt5p directly binds Pop2p. (**a**) Western blot of T7-tagged proteins in yeast whole-cell lysates and Mpt5p-TAP immunoprecipitate pellets. (**b**) Western analysis of input, GST or GST-Mpt5p pull-downs of *in vitro*–translated, T7-tagged proteins. In the right panel, GST-Mpt5p pull-downs were treated with RNases A and T1. (**c**) Left, Coomassie-stained SDS-PAGE gel of purified MBP-Pop2p. Right, anti-MBP western blot of GST and GST–Mpt5p RBD pull-downs of MBP-Pop2p, with and without wild-type *HO* poly(A) RNA (*HO* WT A_{14}) or with RNase 1 treatment.

To examine the PUF dependence of deadenylation in greater detail, we deleted a second PUF protein gene, *PUF4*, together with *MPT5*. We reasoned that the two genes might be redundant: Puf4p and Mpt5p proteins have similar RNA-binding domains, bind overlapping sets of mRNAs and have similar RNA-binding specificities[12]. Moreover, Puf4p is associated with *HO* mRNA in yeast lysates[12], and purified Puf4p binds its cognate site in the *HO* 3′ UTR with high affinity (data not shown). To test whether *PUF4* and *MPT5* act redundantly to affect poly(A) tail length, we analyzed *mpt5 puf4* double mutants. In these double mutants, the poly(A) tails of *HO* mRNAs were substantially stabilized relative to wild-type cells or *mpt5* single mutants (**Fig. 2b**). At every time point after transcription inhibition, the poly(A) tails on *HO* mRNAs were longer in *mpt5 puf4* mutants; for example, at 35 minutes, the poly(A) tails were less than 15 nucleotides (nt) long in wild-type cells but extended to more than 40 nt in the double mutants (**Fig. 2b**). We conclude that Mpt5p and Puf4p both regulate *HO* mRNA and enhance deadenylation.

Mpt5p associates with deadenylation and decapping factors

To test whether Mpt5p physically interacts with deadenylase or decapping proteins, we performed coimmunoprecipitation experiments from yeast extracts (**Fig. 3a**). Each yeast strain expressed tandem affinity purification (TAP)-tagged Mpt5p and a T7 epitope–tagged derivative of either a deadenylase subunit (Ccr4p or Pop2p) or a decapping factor (Dhh1p or Dcp1p). T7–green fluorescent protein (GFP) served as a control. TAP-Mpt5p was affinity-purified, and the immunoprecipitates were analyzed using antibodies directed against the T7 epitope. Pop2p and Ccr4p, as well as Dcp1p and Dhh1p, were physically associated with Mpt5p (**Fig. 3a**, lanes 7–10), whereas GFP (**Fig. 3a**, lane 6) and other proteins (A.C.G. and M.W. unpublished data) were not, even in longer exposures. The coimmunoprecipitation of Pop2p, Ccr4p, Dcp1p and Dhh1p with Mpt5p was due to protein-protein interactions rather than co-occupancy of RNA in the extract, as these proteins were still associated even after extensive treatment with RNases A and T1 or RNase 1 (data not shown).

Mpt5p directly binds Pop2p

To determine whether Mpt5p directly contacts Pop2p, Ccr4p, Dcp1p and Dhh1p, we first translated these T7-tagged proteins *in vitro*, along with GFP as a control (**Fig. 3b**, lanes 1–5), then incubated the lysates with purified recombinant Mpt5p RBD fused to glutathione *S*-transferase (GST) and attached to glutathione beads. Of the five proteins tested, only Pop2p bound the immobilized Mpt5p (**Fig. 3b**, lanes 11–15). Treatment with RNases A and T1 did not prevent the Mpt5-Pop2p interaction (**Fig. 3b**, lanes 17 and 18). None of the proteins bound beads carrying GST alone (**Fig. 3b**, lanes 6–10).

To confirm that Mpt5p and Pop2p bind one another, we purified Pop2p fused to maltose-binding protein (MBP-Pop2p) from bacteria (**Fig. 3c**). Immobilized GST-Mpt5p bound MBP-Pop2p (**Fig. 3c**, lanes 5–7). The addition of *HO* RNA, which binds Mpt5p, did not enhance this interaction (**Fig. 3c**, lanes 8–10), and treatment with RNase 1 did not prevent it (**Fig. 3c**, lanes 11–13). We conclude that the RNA-binding domain of Mpt5p directly binds Pop2p.

Mpt5p stimulates deadenylation *in vitro*

We next attempted to reconstitute sequence-specific deadenylation *in vitro* using purified Mpt5p recombinant protein and purified yeast Pop2p complexes (**Fig. 4**). First, recombinant Mpt5p fused to GST was purified from *Escherichia coli* (**Fig. 4a**) and tested for RNA-binding activity in an electrophoretic mobility shift assay. The substrate in this assay was a 5′ end–labeled RNA corresponding to the Mpt5p-binding site in the *HO* 3′ UTR, with 14 adenosine residues at the 3′ end (WT A_{14} in **Fig. 4b**). GST-Mpt5p bound the wild-type probe with an apparent K_d of 125 nM; mutation of the crucial UGU trinucleotide (mt A_{14} in **Fig. 4b**) abrogated the interaction.

Pop2p-containing complexes were purified from yeast extracts using TAP-tagged Pop2p. Extracts were incubated with IgG beads and washed extensively, and proteins associated with TAP-tagged Pop2p were specifically eluted using tobacco etch virus protease. Both Ccr4p and Pop2p were highly enriched in the purified preparations, as determined by silver staining and western blotting (**Fig. 4c**). To assay deadenylation activity of the Pop2p complex, we incubated 5′ end–labeled *HO* WT A_{14} RNA substrate for various times with three amounts of Pop2p complex (**Fig. 4d**, lanes 3–11). The products were then analyzed by electrophoresis. An identical RNA lacking an adenosine tail was used as a marker (**Fig. 4d**, lane 1). The Pop2p complex progressively removed the poly(A) tail in a concentration- and time-dependent fashion and was capable of completely

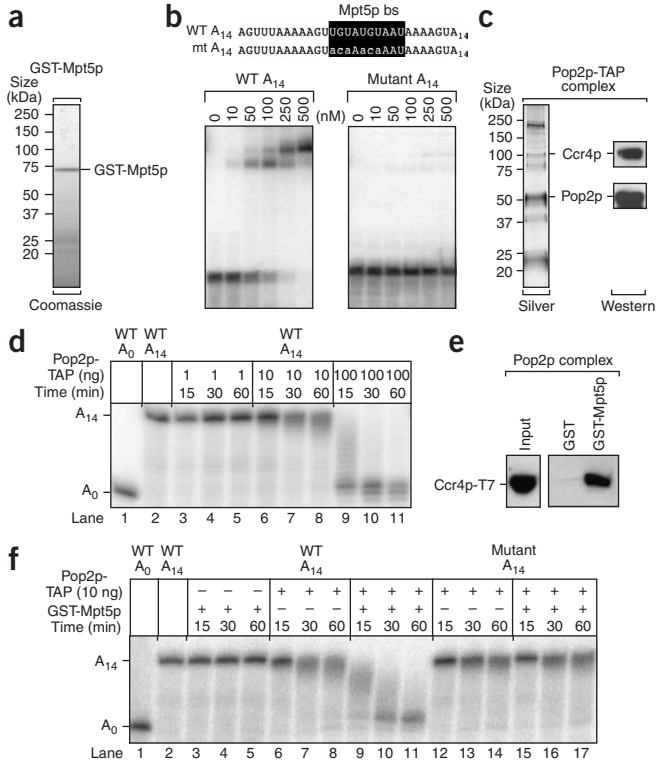


Figure 4 Mpt5p enhances deadenylation *in vitro*. (**a**) Coomassie-stained SDS-PAGE of purified recombinant GST-tagged Mpt5p RBD. (**b**) Top, sequences of wild-type (WT A_{14}) and mutant (mt A_{14}) *HO* substrate RNAs, with 14 adenosines at the 3′ end. Mpt5p binding site (bs) is highlighted. Bottom, electrophoretic mobility shift assays of GST-Mpt5p RBD with WT A_{14} and mt A_{14} *HO* mRNAs. Mpt5p protein concentrations are indicated above gels. (**c**) Left, silver-stained SDS-PAGE of purified Pop2p-TAP complex. Western blotting verified the presence of Pop2p and Ccr4p in the Pop2p complex. (**d**) *In vitro* deadenylation reactions were performed with *HO* WT A_{14} substrate RNA using amounts of Pop2p-TAP complex indicated above gel. Mobilities of RNA markers, without (A_0) or with (A_{14}) poly(A) tails, are indicated at left. (**e**) Western blot of T7-tagged Ccr4p in input (50 ng Pop2p complex) and pull-downs with GST or GST-Mpt5p of the TAP-purified Pop2p complex. (**f**) *In vitro* deadenylation reactions containing Pop2p-TAP complex and recombinant GST-Mpt5p RBD, as indicated above gel, were incubated for the indicated times. RNA substrates are indicated at top. RNA markers are indicated at left as in **d**.

deadenylating the substrate RNA at the highest concentration tested (**Fig. 4d**, lanes 3–11). Thus, the Pop2p complex had deadenylation activity.

We next tested whether the isolated Pop2p complexes were able to interact with Mpt5p. Immobilized GST-Mpt5p was incubated with the Pop2p complex. The beads were washed extensively and eluted, and the eluate was analyzed by Western blotting. To detect the Pop2p complex, we probed for the T7-tagged Ccr4p subunit. Ccr4p-T7 associated with Mpt5p and not GST (**Fig. 4e**). Therefore, the Pop2p complex was capable of binding to Mpt5p.

To determine whether Mpt5p stimulated the activity of the Pop2p complex, we tested whether Mpt5p enhanced deadenylation when a limiting concentration of Pop2p complex was used. We used an amount of Pop2p complex (10 ng) that yielded very little deadenylation on its own (**Fig. 4d**, lanes 6–8). As a control, we incubated Mpt5p alone with substrate RNA; it neither deadenylated nor destroyed the RNA (**Fig. 4f**, lanes 3–5). Similarly, the Pop2p complex alone removed only a few adenosines, even after a 60-min incubation (**Fig. 4f**, lanes 6–8). In contrast, the mixture of Mpt5p and Pop2p complexes resulted in rapid deadenylation and generated fully deadenylated RNA (**Fig. 4f**, lanes 9–11). This Mpt5p-dependent deadenylation was sequence specific: a mutation in the *HO* RNA substrate that abolished Mpt5p binding (**Fig. 4b**) eliminated enhanced deadenylation by the two mixed components (**Fig. 4f**, lanes 15–17). Mpt5p also stimulated the deadenylation of *HO* RNA substrates with longer poly(A) tails (data not shown). We conclude that Mpt5p specifically controls deadenylation of its target mRNA by directly recruiting the Pop2p deadenylase complex.

The PUF-Pop2p interaction is evolutionarily conserved

Both PUF proteins and Pop2p are conserved among eukaryotes[3,13]. We tested whether their interaction was also conserved using the GST pull-down assay with different, purified GST-PUF proteins (**Fig. 5a**) and *in vitro*–translated Pop2p homologs from various species. First, we tested whether *in vitro*–translated yeast Pop2p bound yeast Puf4p. Puf4p interacted with Pop2p, as did Mpt5p (**Fig. 5b**).

Notably, in addition to binding yeast Pop2p, Mpt5p also bound the *Caenorhabditis elegans* Pop2p homolog CCF-1 and one of the two human Pop2p homologs, CNOT8, but not human CNOT7 (**Fig. 5c**). A PUF protein from *C. elegans*, PUF-8, bound *C. elegans* CCF-1 and also the yeast Pop2p more weakly (**Fig. 5c**). A human PUF protein, PUM1, bound human CNOT8 protein and yeast Pop2p, but not human CNOT7 (**Fig. 5c**). We conclude that the PUF-Pop2p interaction is conserved.

DISCUSSION

Our observations may have broad implications for understanding mechanisms of 3′ UTR control. The direct contact between PUF proteins and Pop2p can explain the correlation between PUF activity and deadenylation, observed with multiple mRNAs and in many species[3]. Other regulatory proteins may work in the same way. For example, Smaug protein, which regulates *Hsp83* mRNA decay in

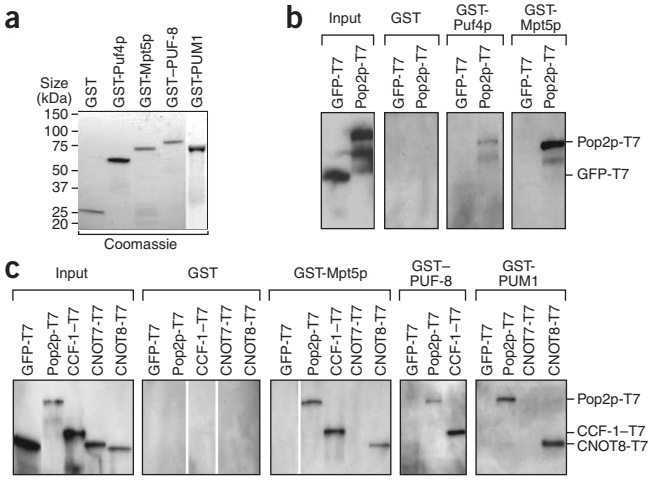


Figure 5 Conservation of PUF-Pop2p interaction. (**a**) Stained SDS-PAGE gels of purified GST-tagged PUF proteins from *S. cerevisiae* (Puf4p and Mpt5p), *C. elegans* (PUF-8) and *H. sapiens* (PUM1). (**b**) Western blot of T7-tagged, *in vitro*–translated yeast Pop2p or GFP in GST pull-down assays. (**c**) Western blot of T7-tagged Pop2p orthologs from *S. cerevisiae* (Pop2p), *C. elegans* (CCF-1) *and H. sapiens* (CNOT7 and CNOT8) in GST pull-downs with yeast Mpt5p, *C. elegans* PUF-8 and *H. sapiens* PUM1, or GST.

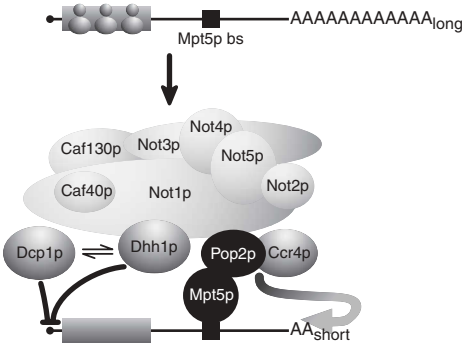


Figure 6 Model of Mpt5p-mediated repression. An actively translated target mRNA (top), containing an Mpt5p binding site (Mpt5p bs) in its 3′ UTR, is deadenylated and shown in a repressed state (bottom). Mpt5p directly binds Pop2p (both in black) and recruits the complex containing Ccr4p, Dhh1p and Dcp1p (dark gray). The presence of other components of the Pop2p–Ccr4p–Not complex (light gray) is inferred from previous studies[14,15]. Pop2p and Ccr4p cause poly(A) tail shortening of the mRNA, whereas Dcp1p and Dhh1p can cause decapping, translation repression or both.

Drosophila, and mammalian TTP, which regulates the decay of A+U-rich element–containing mRNAs, both coimmunoprecipitate CCR4 proteins in cell extracts[21,22]. It will be of interest to determine whether the dramatic roles of *Drosophila Twin* (also called *Ccr4*) in oogenesis[23], mouse Pop2p homolog in spermatogenesis[24,25] and *C. elegans* Pop2p homolog throughout early development[26] are mediated by proteins that specifically recruit Pop2p to mRNA targets.

Regulated deadenylation *in vitro* requires only Mpt5p and the Pop2p complex, suggesting that PUF proteins mediate repression through direct binding to the complex (**Fig. 6**). *In vivo*, deletion of *MPT5* reduces deadenylation of *HO* and stabilizes the mRNA, consistent with this model. Moreover, *mpt5* and *puf4* show additive effects on *HO* deadenylation (**Fig. 2b**), and both proteins bind Pop2p (**Fig. 5b**). Residual turnover of *HO* mRNA persists in the absence of Mpt5p and Puf4p; the mechanism may be the same as is observed in *ccr4* and *pop2* deletion strains, in which mRNA degradation is greatly reduced but not eliminated[18,19].

Ccr4p is believed to be the major cytoplasmic deadenylase in yeast, but Pop2p also has deadenylase activity *in vitro*[13,27,28]. Deletion of either gene reduces deadenylation of several mRNAs *in vivo*[18,19], but the relative roles of the individual enzyme activities are unclear. Yeast Pop2p has a variant active site relative to other members of the DEDD nuclease superfamily[13]. Mutations of the Pop2p active site eliminate its activity *in vitro* but do not affect deadenylation of the *GAL1* mRNA *in vivo*[13,16]. Our data demonstrate that Pop2p is crucial for repression by Mpt5p (**Fig. 1b**). The PUF protein could stimulate deadenylation by tethering Pop2p to its RNA substrate, by inducing a conformational change in Pop2p or by recruiting Ccr4p. In the absence of PUF activity, Ccr4p may catalyze a 'basal' rate of deadenylation. Alternatively, corecruitment of Dhh1p and Dcp1p via binding to Pop2p could be the crucial event (**Fig. 6**). Finally, we note that associations of other proteins in the Pop2p–Ccr4p–Not complex with PUF proteins (light gray proteins in **Fig. 6**) have not been examined, but are possible given the previously characterized composition of the free Pop2p–Ccr4p–Not complex[14,15]. Our genetic data (**Fig. 1c**) suggest that the complex recruited by Mpt5p may not be identical to this free complex. However, repression activity might differ quantitatively in mutant strains, yet escape phenotypic detection.

Two models have been proposed to explain the control of deadenylation by PUF proteins and 3′ UTR regulators more broadly[3,27]. In one, a change in the composition of the translated messenger ribonucleoprotein particle triggers translational repression, eliciting deadenylation, movement to specific cytoplasmic sites (P-bodies), decapping and decay. In the other, PUF proteins enhance deadenylation directly by recruiting the machinery involved. Our *in vitro* results show that regulated deadenylation can be reconstituted in a cell-free system using just the PUF protein and Pop2p complex. Yeast PUF proteins do not stably associate with P-bodies, but instead are localized throughout the cytoplasm; therefore, if PUF-mediated regulation occurs within P-bodies, their localization must be transient (refs. 12,29,30 and A.C.G. and M.W., unpublished data). Similarly, yeast Pop2p and Ccr4p are not present in P-bodies, and thus deadenylation is believed to occur outside these structures[29–31]. Our data demonstrate that PUF proteins can directly enhance removal of the poly(A) tail and recruit deadenylase and decapping factors (**Fig. 6**). They do not discount the notion that other changes may also be facilitated by the interaction between the PUF and Pop2p.

The simultaneous recruitment of Pop2p, Ccr4p, Dcp1p and Dhh1p provides robust opportunities for control of translation and stability of an mRNA. The composition and state of other proteins associated with Pop2p could determine whether the mRNA is repressed or degraded. Together, PUF-mediated deadenylation and decapping would not only expose a target mRNA to exonucleolytic attack, but might also silence it translationally. We suggest that these are the decisive steps in PUF repression, as deletions in mRNA decay genes that function downstream in the decay pathway did not affect PUF repression, implying that turnover *per se* is not essential. However, the 5′→3′ and 3′→5′ exonucleolytic pathways may be redundant in their action on *HO* mRNA, making it impossible to rule out a role for decay. Meanwhile, Dhh1p, which is recruited by Mpt5p, can cause translational repression[32] and bridges the decapping and deadenylation factors, providing a physical connection with Dcp1p[15,33] (**Fig. 6**). Likewise, interaction between the *Drosophila* PUF protein Pumilio and a fly Pop2p complex containing homologs of Dhh1p and Dcp1p could explain enigmatic findings on the regulation of *hunchback* mRNA. Pumilio binding to the 3′ UTR of that mRNA not only enhances its deadenylation, but can repress translation in the absence of a poly(A) tail[6,34]. The binding of PUF proteins to Pop2p can account for both translational repression and turnover through the simultaneous recruitment of proteins that remove the poly(A) tail, decap the mRNA and repress its translation.

METHODS

Yeast strains. Strain TTC59 was described[11]. Strain BY4742 and isogenic, gene-specific deletions of RNA decay factors were from Open Biosystems. These strains were created by PCR-mediated gene modification with the kanamycin-resistance (Kan^R) marker. The *MPT5*-TAP and *POP2*-TAP strains, also from Open Biosystems, were in the S288C strain background and contained C-terminal TAP tags integrated into the genomic coding sequence of the respective genes by PCR-mediated gene modification using a *HIS3* marker. The *mpt5* deletion strain was created in strain W303 (alpha mating type) by PCR-mediated gene modification using the Kan^R marker, thereby removing the *MPT5* open reading frame. The *PUF4* gene was then deleted in the *mpt5*::Kan^R strain by PCR-mediated gene modification with the *TRP1* marker to create the *mpt5*::Kan^R *puf4*::*TRP1* double deletion strain.

***MPT5* repression assays.** Assays in **Figure 1a** were performed as in ref. 11, using the strain TTC59 with a chromosomal *ADE2-HO* 3′ UTR reporter gene. Plasmid YEp195 *MPT5* contains the natural promoter, open reading frame and

3′ UTR of the *MPT5* gene[11]. The plasmid YEp195 *MPT5* mutant was created by site-directed mutagenesis (Stratagene) resulting in the mutations S454A and N455A in PUF repeat 7, using primers AG205 (5′-CTTGTTTGAA GTTCTCCGCCGCCGTTGTGGAAAAATTCATTAAAAAATTATTTAG-3′) and AG206 (5′-CTAAATAATTTTTTAATGAATTTTTCCACAACGGCGGCGGAGA ACTTCAAACAAG-3′). The Mpt5p RNA-binding domain (amino acid residues 126–626) was expressed via the *ADH1* promoter and 3′ UTR from the multicopy plasmid pACG1 NT. The *HIS3-HO* 3′ UTR reporter gene contains the *HIS3* coding sequence with 2 kilobases of the *HO* promoter upstream and 1 kilobase of the *HO* 3′ UTR downstream in plasmid YCp33. Wild-type yeast strain BY4742 or gene-specific deletion strains were transformed with the reporter gene plasmid and either empty vector YEp181 or the *MPT5* expression plasmid YEp181 *MPT5*, which was subcloned from YEp195 *MPT5*. Colonies were isolated and grown to mid-log phase at 30 °C and the indicated number of cells was plated on minimal media with or without histidine. The *HIS3* competitive inhibitor 3-aminotriazole was added to increase stringency.

Northern and mRNA decay analysis. Transcriptional-inhibition experiments were done using 20 μg ml^{-1} of thiolutin (Pfizer) as described[20]. RNA was purified by an acid-phenol-chloroform protocol at pH 4.6 and separated by 3.7% (v/v) formaldehyde, 0.8% (w/v) agarose gel electrophoresis. Transfer to nylon membrane (Ambion) was achieved by 10× SSC capillary action. Blots were probed with radiolabeled riboprobes specific to genes listed. The same experimental RNAs were cleaved with RNase H (Promega) and an antisense *HO* oligonucleotide (5′-GGACAGCATCAAACTGTAAGATTCCGCCAC-3′). When indicated, oligo(dT_{15}) (IDT) was added to the RNase H reactions. RNA was then analyzed by denaturing PAGE, transferred to nylon membranes and probed with an antisense *HO* 3′ UTR probe. The gels were scanned with a Typhoon PhosphorImager (Molecular Dynamics) and the data were analyzed with ImageQuant software and graphed with Microsoft Excel.

Immunoprecipitations. The TAP-tagged *MPT5* strain was from Open Biosystems. T7-tagged *POP2*, *CCR4*, *DHH1*, *DCP1* or GFP expression plasmids, in the vector pACG1-NT, were transformed into the *MPT5*-TAP strain and grown to an A_{660} of 1.0 in 0.5-l cultures. Cells were harvested and lysed by bead-bashing in TNEMN150 buffer (50 mM Tris-HCl (pH 8), 0.5% (v/v) Nonidet P40, 1 mM EDTA, 2 mM $MgCl_2$ and 150 mM NaCl). Lysates were clarified at 16,000*g* for 10 min. IgG agarose beads were added to the supernatant and bound for 2 h at 4 °C. Beads were washed four times with 100 volumes of TNEMN150. Mpt5p and associated proteins were eluted by tobacco etch virus protease (Invitrogen) cleavage overnight at 4 °C. Peroxidase/anti-peroxidase (PAP) antibodies (Sigma) and anti-T7 (Novagen) antibodies were used to detect TAP-tagged and T7-tagged proteins, respectively.

Deadenylation *in vitro*. Pop2p-TAP was purified from a 4-l culture at an A_{660} of 1.0 in the same manner as Mpt5p-TAP and analyzed by SDS-PAGE, silver staining and western blotting. The GST–Mpt5p RBD (residues 126–626) was purified from *E. coli* as described[10] and eluted with glutathione. Deadenylation reactions were carried out in a 20-μl volume with 50 mM Tris-HCl (pH 8), 20 mM NaCl, 0.1 mM $MgCl_2$, 10% (v/v) glycerol and 10 μM nonspecific competitor RNA (synthetic oligoribonucleotide with sequence 5′-UCUA AUCGGGGUACAAUUAUAUAAUAUA-3′). *HO* substrate RNAs (IDT) with sequences shown in **Figure 4b** were radioactively labeled with T4 polynucleotide kinase (Promega) at the 5′ end and added to the reactions at a final concentration of 10 nM. GST–Mpt5p RBD (250 nM) was added to reactions were indicated. This Mpt5p concentration is twice the measured K_d, thus approaching the maximum level of binding to the substrate RNA (**Fig. 4b**). Purified Pop2p-TAP complex (10 ng) was added to reactions, unless otherwise specified. We estimate the final concentration of Pop2p protein to be approximately 10 nM, but the uncertainty in the molecular weight of the complex makes this calculation approximate.

Electrophoretic mobility shift assays. RNA binding assays were performed as described[10] using the synthetic RNAs shown in **Figure 4b**.

GST pull-down experiments. GST-Puf4p (residues 536–888) and GST-Mpt5p (residues 126–626) from *S. cerevisiae*, GST–PUF-8 from *C. elegans* (residues 142–535) and GST-PUM1 from *Homo sapiens* (residues 828–1176) were bound to glutathione agarose beads (Amersham) at a concentration of 1 μg per 10 μl bed volume. T7-tagged test proteins were translated *in vitro* from PCR-generated templates using a rabbit reticulocyte–coupled T7 transcription and translation system (Promega). *POP2*, *CCR4*, *DHH1* and *DCP1* were cloned from the genomic DNA of *S. cerevisiae* strain S288C (Research Genetics). CCF-1 was cloned by reverse-transcription PCR from *C. elegans* total RNA. CNOT7 (GenBank accession number BC060852) and CNOT8 (GenBank accession number BC017366) were from an Open Biosystems complementary DNA clone collection. Translated lysate (10 μl) was mixed with GST fusions bound to beads with 5 bed volumes of TNEMN150. Binding was performed for 2 h at 4 °C. Beads were then pelleted and washed four times with 100 volumes of TNEMN150. Beads with bound protein were boiled in SDS loading dye and analyzed by SDS-PAGE and western blotting with monoclonal antibodies to T7 epitope (Novagen). Likewise, full-length Pop2p fused to maltose binding protein (MBP-Pop2p) was produced in *E. coli* and purified according to the manufacturer's protocol (NEB). For GST pull-down assays, MBP-Pop2p was mixed with GST or GST-Mpt5p RBD beads at final concentrations of 100, 200 and 400 nM in TNEMN150, washed and analyzed with a monoclonal antibody to MBP (NEB). RNase 1 (20 units; Promega) or a cocktail of RNases A (5 units) and T1 (200 units) (Ambion) were added to the reaction as indicated.

GST-Mpt5p pull-down of the TAP-purified Pop2p complex was accomplished by incubating GST-Mpt5p, immobilized on glutathione agarose beads, with 100 ng of Pop2p complex in deadenylation buffer for 2 h at 4 °C. The beads were collected and washed four times with 100 volumes of TNEMN150 with 0.05% (v/v) Nonidet P40. Bound protein (50% of total pellet) was then analyzed by SDS-PAGE and anti-T7 monoclonal western blotting to detect T7-tagged Ccr4p.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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